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Review Article

Theory of Coarse Suspension Formulation

By EVERETT N. HIESTAND

INTRODUCTION

A COMPLETE REVIEW of the theory of the physical stability of suspensions would include a survey of a large part of colloid science. However, by limiting the discussion to a descriptive treatment of coarse (particles larger than 0.1-μ diameter) suspensions,[1] a reasonably comprehensive outline of the basic concepts may be considered in this brief review.

The primary objective is to present information useful to the formulator. Current theories of suspension stability will be examined and the experimental evidence considered. Shelf-life[2] stability usually requires the use of flocculated particles when suspensions are formulated; therefore, most of this discussion will describe flocculated suspensions. The objective in most discussions of dispersions is to produce the stable (peptized) condition; hence, textbooks of colloid chemistry provide a much more thorough treatment of the deflocculated state.

FUNDAMENTALS

Wetting

The introduction of the particle into the vehicle is a critical step in the preparation of an elegant suspension. Usually, slightly lyophobic[3] (solvent fearing) powders present no difficulty. Strongly lyophobic powders may float because of the high interfacial energy of the vehicle-powder interface. Forced deaggregation of a nonwet powder may create some difficult problems because microscopic quantities of air stick to the particle surface. The particles tend to clump and float to the surface of the suspension. Usually, the objectionable air may be removed by high shearing action such as that obtained in a colloid mill; the presence of a wetting agent facilitates this operation. Either detergents or polyelectrolytes may be used as the surfactants. Detergents are superior in penetration because of their lower molecular weight.

If a good penetrating surfactant is present when the powder is added, the penetration of the liquid phase into the powder will be sufficiently rapid to displace the air from the particles. Also, the particles will separate at low shear forces and an elegant preparation is made more readily.

Contact Angle.—Equation 1 can be used in some cases to describe the thermodynamics of wetting in terms of the interfacial tension and the contact angle at the point of contact of the three phases.

$$\gamma_{SA} = \gamma_{LS} + \gamma_{LA} \cos \theta \qquad \text{(Eq. 1)}$$

γ_{SA}, γ_{LA}, and γ_{LS} are interfacial tensions at the air-solid, liquid-air, and liquid-solid interfaces, respectively. θ is the contact angle measured through the liquid.

For a discussion of this equation, nearly any general book on colloid chemistry may be con-

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[1] Throughout this review, suspension shall refer to use of particles >0.1 μ and dispersion to particles <0.1 μ diameter.

[2] Only the physical stability aspects are considered in this paper.

[3] Lyophilic particles are those that disperse spontaneously.

sulted, (*e.g.*, *References* 1, 2, and 3). Equation 1 describes an equilibrium situation and says nothing about the rate of the penetration. Of course, it does indicate when spontaneous spreading will not occur, *i.e.*, when $\theta > 0°$. At the other extreme of good wetting, the contact angle becomes zero at relatively low energies (4). The equation's primary value to the formulator is in helping him understand the wetting process. One seldom has the necessary data to use the equation; and obtaining the data is more time consuming than just trying a few surfactants.

Penetration Rate.—With the same objective, it is interesting to examine some of the kinetic studies that have been reported. Eley and Pepper (5) studied the displacement of one fluid by another from a capillary and also for displacement from a powder. They obtained the equation

$$\gamma_{12} \cos \theta_{12} \frac{rt}{4k^2} = \frac{(\eta_1 - \eta_2)x^2}{2} - \eta_2 lx \quad \text{(Eq. 2)}$$

where r is the average radius of the equivalent capillaries, k is the tortuosity factor and is obtained from bulk density and gas permeability data, γ_{12} is the interfacial tension between the two fluids, l is the length of the equivalent capillaries, x is the distance through which the meniscus has advanced in time, t, η_1 and η_2 are the viscosities of the two fluids, and θ_{12} is the contact angle. Equation 2 confirms one's intuitive expectations, however, these investigators found reproducibility only when the surfaces had been previously wet by the advancing liquid. Furthermore, the work of Kling and Lange (6) did not fit Eq. 2. Kawasaki (7) discussed some other factors considered in wetting, such as penetration of water molecules, change in orientation of polar groups in the solid surface, and the migration of polar groups of impurity molecules from the interior of the solid toward the surface. Bristow (8) reviews the theory of wetting and penetration in more detail. Obviously, much more work must be done before the rate of penetration of a fluid into a powder bed can be described quantitatively.

Supplemental reports of theoretical interest may be found in a group of papers on the "Interaction of Water and Porous Materials" (9) and on "Contact Angles, Spreading and Wetting" (10). Zisman and co-workers (11) have demonstrated the existence of a critical surface tension which may be considered as that value of the surface tension of a liquid below which liquids spread on the solid surface.

Selecting Surfactants.—In a more practical interest, a simple method can be used to screen wetting agents when penetration is a problem to the formulator. The author has used a narrow, very lyophobic trough several inches long (either paraffin coated or made of Teflon for aqueous systems). Powder is placed at one end of the trough; the other end is filled with a solution of the wetting agent. The relative rates of penetration of various agents may be observed directly. A more elaborate method of determining dispersibility is described by Zacek (12). He observes the fraction of the powder that passes through a standard gauze, which is wet by the solution, when a given amount of the dispersant solution is dropped onto it from a fixed point.

Based on the above procedure and knowledge, it shall be assumed that the formulator has found a satisfactory wetting agent and now has the powder immersed in the liquid phase.

Settling

If particles immersed in a liquid are not in true solution, usually the thermodynamic conditions favor aggregation. Lowering of the interfacial tension will reduce the associated free energy change, but will not remove it entirely. However, it is possible to shield particles from each other by surrounding them with an energy barrier, and thereby reduce the rate of aggregation to a negligible value.

Peptized Particles.—For peptized particles of colloidal size, ambient convection currents arising from slight thermal gradients will produce adequate mixing to overcome the very slow sedimentation. Consequently, colloidal particles may remain uniformly dispersed indefinitely. The significance of the very small size in producing a small sedimentation velocity is evident from Stokes'[1] law for the free fall of a sphere in a very large body of fluid

$$v = \frac{2 r^2 (\Delta\rho) g}{9 \eta} \quad \text{(Eq. 3)}$$

where v is the velocity, r the radius, $\Delta\rho$ the density difference between fluid and sphere, g the gravitational constant, and η is the viscosity of the Newtonian liquid.

Sometimes the concentration equalizing process of diffusion is combined with Eq. 3 to estimate how much settling must occur before the sedimentation rate becomes equal to the opposing diffusion rate. Perrin's equation includes these factors

$$\ln \frac{n_1}{n_2} = \frac{N}{RT} v (\Delta\rho) gh \quad \text{(Eq. 4)}$$

where n_1 and n_2 are the number of particles in each of the two layers spaced h distance apart, v is the volume of the particle, N is Avagadro's number, T is the absolute temperature, and R is the

gas constant. However, one should remember that a net transfer by diffusion occurs only when there is a concentration gradient—something one would like to avoid.

Sample calculations often are used to provide perspective for the appreciation of these equations (*Reference* 1, Chapter 3). When they are made, one is forced to conclude that single large particles in suspensions will sediment in the usually accepted shelf-life requirement of pharmaceutical products.

Flocculated Particles.—Subsidence is a term often used to describe the settling of flocculated suspensions. The subsidence rate usually refers to the settling rate measured by following the boundary between the sediment and the clear liquid overhead. This could not apply to peptized polydispersed suspensions because the boundary is not well defined. The large particles settle downward past the smaller ones; the smallest ones may fall very slowly. In a concentrated peptized suspension the particles do not fall completely independent of each other; the largest are said to undergo hindered settling and the smallest are accelerated over the Stokes' law value (13). In flocculated suspensions the particles are linked together into flocs and they settle initially at a rate determined by the floc size and the porosity of the aggregated mass. Later the rate is a compaction and rearrangement process. Because the smallest particles are a part of the flocs, they do not remain behind to form the cloudy layer on top. Probably intermediate cases are possible in which the particles are not all associated into flocs.

Both subsidence and hindered settling have been studied by various investigators. Oden (14) has summarized the early work. Better known are the works of Robinson (15), Egolf and McCabe (16), and Ward and Kammermeyer (17). Kynch (18) gave a very detailed theoretical treatment. Steinour (19) considered the settling of nonflocculated suspensions of spheres of uniform size under conditions that made Stokes' law applicable at infinite dilution. He found the empirical Eq. 5 represented all of his data for the settling rate, Q, of the top surface

$$Q = v_s \epsilon^2 10^{-1.82(1-\epsilon)} \qquad \text{(Eq. 5)}$$

where $10^{-1.82(1-\epsilon)}$ is the empirical term which represents both size and shape of flow space; v_s is the Stokes' velocity, and ϵ is the volume fraction of the liquid. Steinour modified this equation by using the hydraulic radius as a spacing factor and obtained an equation similar to those obtained by Higuchi (20) and others (21, 22), who have based their equations either on the Kozeny equation for permeability or on correction terms to Stokes' equation.

Steinour (23) considered concentrated flocculated suspensions in another communication. His equation (Eq. 6.) fits most of the data and includes an empirical dimensionless constant, w_i, and the specific surface, σ, calculated on an equivalent sedimenting sphere basis. The other terms in Eq. 6 have the same significance as before.

$$Q = \frac{0.246}{\sigma^2 (1 - w_i)^2} \cdot \frac{g\Delta\rho(\epsilon - w_i)^3}{\eta(1 - \epsilon)} \qquad \text{(Eq. 6)}$$

Starting with the equation of Richardson and Zaki (21), Michaels and Bolger (24) describe the settling rate and the sediment volume of aqueous flocculated suspensions as a function of concentration and container dimensions. Their model assumes that small flocs form and become the primary fundamental structural unit moving at the low shear of gravitational settling. Aggregates grow by collision of the flocs but may be broken by the shear force resulting from the weight of the particles above. This compacts the bottom layers. The settling rate, the sediment volume, and the aggregate size are expressed in terms of the floc volume concentration and the attractive force between the flocs.

Forces Between Particles

Even when the surface is covered with a good wetting agent, the interfacial tension may be finite. Therefore, two particles that make contact reduce the interfacial area and produce a lowering of the total free energy of the system. If this energy is small, comparable to the thermal energy, some spontaneous dispersion will result. However, van der Waals' forces of attraction must be considered also. Therefore, flocculation would be expected to occur. The deflocculated condition is a thermodynamically unstable one. However, it can be made to appear stable by surrounding the particles with ions so that the particles repel each other. This may reduce the rate of combination to a negligible rate. The stability of unprotected lyophobic colloid sols may be assigned to this mechanism.

Before proceeding further, a preferred definition of the terms lyophobic and lyophilic should be given. These terms are used ambiguously by some investigators. Sometimes they are considered to be synonomous with nonwetting and wetting. As noted in an earlier paragraph, the process of wetting involves the relative magnitude of three interfacial layers. Usually, the air-liquid and the air-solid interfaces as well as the liquid-solid interface are considered. The terms are more useful in the discussion of the stability of sus-

pensions or dispersions if lyophobic and lyophilic are considered as properties of only the liquid-solid interaction. A consequence of this definition is that some lyophobic particles are wet by the liquid—hence, the use of *slightly* and *strongly* lyophobic in the discussion of wetting.

Rebinder (25, 26) considers lyophilic materials to be those whose interfacial tension is less than some critical value, γ_m, which is fixed by the temperature, *i.e.*, γ_m is proportional to kT. A lyophobic material has an interfacial tension larger than γ_m. Lyophilic materials tend to disperse spontaneously because of the entropy factor. Glazman (27) disagrees and says that some materials that have interfacial tensions larger than γ_m are lyophilic. He distinguishes between the two by observing their behavior at various electrolyte concentrations. Lyophobic sols are very sensitive to the addition of electrolytes, and lyophilic sols are not. The test of sensitivity to electrolytes is used by Kruyt and Overbeek (28) also, but with the added test of reversibility. A lyophilic material that has been precipitated by electrolytes will redisperse upon dilution with the vehicle; a lyophobic substance will not. It is unnecessary to choose among these definitions in this discussion. By any of these standards, the subject discussed here is the theory of the formulation of suspensions of *lyophobic* materials.

The shelf-life stability of suspensions ideally requires that no settling occurs; but if some settling is allowed, it requires that the sediment be readily redispersed by mild shaking. Also, after shaking it must remain uniformly dispersed long enough for the withdrawal and administration of an accurately measured dose. Obviously, a peptized suspension that settles to form a clay does not meet these requirements. However, it is necessary to understand the theory of lyophobic colloid stability, which produces the peptized condition, in order to understand the unstable or flocculated condition. A brief review of these factors will be given.

Forces of Attraction.—van der Waals' equation for gases is well known. The constant for the attraction between molecules is usually given the symbol "a" in his equation. This constant does not designate the origin or the nature of the attractive forces. After Margenau (29) and de Boer (30), the definition of van der Waals' forces used here will include forces from dipolar and quadrupolar molecules as well as nonpolar forces. London (30, 31) discovered the close connection between nonpolar van der Waals' forces and optical dispersion. Consequently, these nonpolar forces are often called dispersion forces; they are also called London–van der Waals' forces to distinguish them from the other van der Waals' forces. London deduced on a theoretical basis that even for apolar atoms an attraction exists. This attraction arises because even neutral atoms consist of charged particles which form oscillating fields. These oscillating charges mutually interact to produce a net force of attraction. For atoms, the potential energy of interaction is inversely proportional to the sixth power of the distance, r, between the atoms. Deryagin, Abricosova, and Lifshitz (32) state that the interaction is through the magnetic fields. Because of the time required to transmit an electromagnetic wave, the inverse sixth power law does not hold at all distances. A detailed analysis of this subject is beyond the scope of this review.

Obviously, any interactions that depend on the inverse sixth power of the distance cannot act over a very long range. However, for colloids and larger particles, one is not considering isolated atoms, in which case an estimate of the London–van der Waals' energy may be obtained by summing the interactions between the atoms of the particles; the London energies are to a first approximation independent of the interaction with other atoms.

These calculations have been conducted by assuming that, except for close neighbors, the summation sign may be replaced by the integral. In this case, for flat plates, the interaction *energy* varies inversely as the third power of r, but for perfectly conducting plates it is as the fourth power. For two spheres this complex problem has been solved by Hamaker (33) to give, at small distances, a variation inversely proportional to r. One must again emphasize that these conclusions hold only for close distances compared to the size of the particle and the wavelength associated with the interaction; but, at least the forces may be considered active for distances approximating the particle radius. Overbeek points out that the ratio of the radius to the distance between the particles is the important factor, not the absolute size.

Deryagin and Abricosova (32, 34) were the first to measure accurately the London–van der Waals' forces by using an extremely well designed negative feed back balance. They measured the attraction between a quartz plane and a convex surface. They claim closer correlation to a theory they assign to Lifshitz (32) than to the earlier theories. Lifshitz's theory permits calculations without some of the assumptions in the method described above. However, most important, Deryagin and Abricosova obtained a correlation with the theoretical prediction of the power of the

term for distance between particles. They measured these forces at distances of 10^{-5} and 10^{-4} cm.; therefore, long range forces are observed. The quantitative aspects are beyond the scope of this review. The reader is referred to original references (32, 34–38) that pursue this subject.

The Diffuse Double Layer.—The theory of the double layer which is a primary factor in the stability of hydrophobic dispersions is equally as complicated as the London–van der Waals' force theory. However, in a qualitative sense it is easily understood. One's familiarity with the Debye-Huckel theory of electrolytes provides additional background.

As a result of either the preferential adsorption of ions from the surrounding liquid or the preferential loss of ions from the crystal surface, a net charge exists at the surface of most solids immersed in a liquid. Only by careful adjustment does one neutralize this charge. In the absence of thermal motion this charge would be neutralized by intimately associated ions of opposite charge. However, the ions of opposite charge have Brownian motion and tend to diffuse away from the surface. Consequently, they distribute themselves in the vicinity of the particle; the largest concentration of these counter ions is near the particle surface, and the concentration diminishes at greater distances because of the shielding effect of the ions nearer the surface.

Ions of the same charge as the particle will be repelled from the region near the particle, but thermal motion will tend to redistribute these ions also. Their concentration profile would be the mirror image of that of the counter ions, except that the screening effect is more limited by the solution concentration. The total result of the distribution of charges about the particle is to produce a diffuse region called the double layer that surrounds the particle. (*Reference* 1 develops these concepts in this way but in much more detail.)

Because the potential in this double layer diminishes gradually, the double layer cannot be assigned an absolute thickness that includes all of it. However, a reference thickness has been chosen as that thickness in which the potential decreases by a factor of $1/e$. An additional complication arises because the actual charge of the particle surface cannot be determined experimentally. A part of the double layer is immobilized and moves with the particle. Therefore, the charge determined by electrokinetic methods such as electrophoresis is the charge at this shear plane and is not the true charge at the particle surface. This charge is called the zeta potential and is often used as the best available estimate of the particle charge.

Combined Attraction and Repulsion Forces.—Deryagin (32) and independently Verwey and Overbeek (35) combined the potential energy resulting from the diffuse double layer with the London–van der Waals' energy of attraction to obtain the potential energy curves for model cases. They concluded that for sufficiently small or sufficiently large separation the potential energy is always negative, *i.e.*, attractive forces predominate. However, at intermediate distances the potential energy may become positive because of the repulsion from the double layers of the two surfaces. It is this positive potential energy region that provides in effect the "activation energy" to the process of the combining of two particles. Always, the probability is finite that the kinetic energy of two particles may exceed this barrier and the particles combine; but the rate of flocculation may be extremely small. The particles that do combine usually cannot be separated by thermal energy, for the potential energy minimum that exists when two particles are in contact is deep when compared to kT.

In calculating the potential energy curves for the interactions of particles, a smaller minimum, usually called the secondary minimum, is found at much greater distances from the particle surface than the larger minimum. The secondary minimum has significant magnitude only for particles larger than colloidal size. Schenkel and Kitchener (38) described a case of flocculation in this secondary minimum. They used surface sulfonated polystyrene beads of 10-μ diameter. Adding electrolytes, they observed flocculation when the energy barrier at close approach was estimated to be greater than 24 kT. They concluded that the flocculation must be in the secondary minimum. Flocculation in the secondary minimum should be readily reversed because the energy well is not deep. However, one should not conclude that whenever easily reversible flocculation is observed, it necessarily is a case of flocculation in the secondary minimum.

Because the repulsion between particles results from the diffuse double layer, the ionic strength and the kinds of ions on the surface and in the double layer will influence the total charge and/or the thickness of the double layer. Also, the effectiveness of the double layer in preventing the flocculation of particles will be related to both the total charge and the thickness of the double layer. It often has been demonstrated that particles may be flocculated by increasing the ionic strength and thereby reducing the thickness of the double

layer. Also, specific adsorption of a charged ion such as a detergent ion may, on the first addition, reduce the charge to the extent that it allows flocculation, while further additions reduce the charge to zero, reverse the charge, and finally, produce a large enough charge of opposite sign to form a peptized dispersion of opposite charge. Detergents are believed to adsorb in micellar or hemimicellar form when the concentration exceeds the critical micelle concentration (39, 40). This is easily detected by a discontinuity in the adsorption isotherm.

Pharmaceutical suspensions predominately are in aqueous vehicles. However, some products use lipophilic vehicles. Because of the large difference in the dielectric constant, there are significant differences in the stability characteristics of particles in the two types of vehicles. The potential between two charges is inversely proportional to the dielectric constant of the medium between them. Therefore, the distribution of ions in the double layer will depend on the dielectric constant of the vehicle. In low dielectric liquids such as oils, the double layer will be many times thicker than in liquids with a high dielectric constant such as water. Also, only a few ions in an oil will produce the same potential produced by many ions in water.

Albers and Overbeek (41) have concluded that for water-in-oil emulsions, except in extremely dilute systems, the thickness of the double layer is large compared to the distance between two particles. The potential energy of the particles is larger than when the particles are at infinite distance, *i.e.*, located outside each other's double layer regions. Therefore, the potential energy that must be overcome to bring two particles together is reduced by this amount. Consequently, in an oil vehicle the double layer around the particles often will not protect the particles against flocculation.

The only repulsion force usually considered to be acting between particles is based on the electric layer as described above. However, Voropaeva, Deryagin, and Kabanov (42) have obtained evidence of a repulsion force of undetermined origin. They measure the forces acting between two crossed platinum wires as they are brought closer and closer together. The wires have external electrical connections through which charges on the wires may be controlled. Present knowledge does not justify speculating on the possible role this nonelectrical repulsion force may play in suspension formulation.

Adsorbed Layers.—In the early literature, part of the stability of sols was assigned to the formation of lyospheres, *i.e.*, a thick layer of adsorbed vehicle covering the surface of the particle. These layers were considered somewhat diffuse. They provided protection against flocculation by reducing the interfacial tension to a negligible value and by preventing the particles from approaching sufficiently close to undergo strong interaction from the London–van der Waals' attraction. Proof of such thick films is lacking, although their existence has been invoked to explain numerous observations.

The confusion existing at the present time is illustrated in three papers that discuss the preferred contents of a course in colloid chemistry (25, 43, 44). Because only the Deryagin-Verwey-Overbeek theory of stability has yielded to quantitative interpretation, the relative importance of other factors has been a point of disagreement among various investigators. Deryagin has described the phenomena in term of a disjoining pressure consisting of two components: the electrical (P_e) and nonelectrical (P_o). Glazman (27) refers to this classification and concludes that for ideal lyophobic sols, P_o is much smaller than P_e. For lyophilic sols, P_e is nearly zero and P_o dominates. He concludes that in real cases, lyophobic sols are nearly always solvated so that P_o is a factor in nearly all stable systems.

Yampolskii and Shu-Chiu (45) studied the electrical conductivity of carbon-black suspensions in oil. They found that the conductivity increased with increase in temperature of their flocculated suspensions. This and other observations were consistent with the view that a very thin solvent layer is left between the dispersed particles. Rebinder (26) discusses these thin interlayers of the medium in his review article.

These concepts and the Deryagin-Verwey-Overbeek theory of lyophobic colloid stability will aid us in discussing qualitatively the theory of suspension formulations. The thick solvation layer concept does not appear to be valid or necessary to account for the shelf-life stability of suspensions.

The influence of adsorbed layers of nonsolvent molecules on the particle interactions has been considered in a theoretical treatment by Vold (46). She has derived a general formula for the interaction of spherical particles surrounded by adsorbed layers. Her calculations show that the London–van der Waals' attraction might be reduced by as much as 20% which is less than the possible reduction calculated for bound solvent layers. In both cases it is concluded that flocculation can be inhibited significantly only for very small particles or for very thick adsorbed layers.

Her calculations were not extended to layers more than 20 Å. thick. Mackor and van der Waals (47) treated the adsorption of rod-shaped molecules. They concluded that the free energy rises due to the interaction of two adsorbed layers and they give an expression for the free energy of repulsion.

More will be said about various factors in flocculation when it will be possible to show their function in influencing the shelf-life stability of a suspension. First, the kinetics of flocculation will be discussed.

Flocculation Rate

The rate of disappearance of primary particles has been treated by von Smoluchowski (48) as a collision rate calculation that is diffusion controlled. Every collision is assumed to result in the combination of two primary particles, and no repulsion forces are present. Many books discuss this subject in a reasonably concise manner (1, 49). The rate is given by

$$-\frac{dn}{dt} = 4\pi DRn^2 \qquad \text{(Eq. 7)}$$

where n is the total number of particles, t is the time, D is the diffusion coefficient, and R is the collision radius usually assumed to be equal to the particle diameter. If the diffusion coefficient is replaced by Einsteins's equation $D = kT/3\pi\eta R$, where k is the Boltzman constant, η is the viscosity, and T is the absolute temperature, it may be shown that the time, $t_{1/2}$, required to reduce the total number of particles to one-half their original number is

$$t_{1/2} = \frac{3\eta}{4\,kTn} \qquad \text{(Eq. 8)}$$

Any reasonable value for n and η substituted into this equation results in a small value for $t_{1/2}$.

If every collision does not result in a combination, then some protection exists. If the particle is assumed to be protected by an energy barrier, V, a rough approximation of the rate of disappearance of primary particles is obtained by multiplying Eq. 7 by $\exp(V/nkT)$. Obviously, the larger the protective energy barrier, the slower the rate of combination. The initial rate of coagulation has been observed experimentally and used to estimate the magnitude of V (50, 51). When V is very large, possibly 25 kT, the rate of flocculation is going to be negligible. von Smoluchowski's equations have been extended to describe other cases than spherical particles (52).

Gillespie (53) studied the flocculation of latex by methylcellulose and found that in this case there seemed to be a deflocculation rate also. An equilibrium existed between the rates of flocculation and of deflocculation. This is not expected in the usual case for electrolyte flocculation where the deflocculation rate will be negligible because of the deep potential energy minimum for two particles in contact. Flocculation produced by polymers forming "molecular bridges" between particles must be considered as a special case. They will be discussed in a later section.

When slow coagulation by electrolytes is considered, Bhattacharya and Bhattacharya (54) have demonstrated that in many cases the following relation holds

$$\frac{1}{c-a} = \frac{n}{m}t + \frac{1}{m} \qquad \text{(Eq. 9)}$$

where c is the electrolyte concentration, t is time of coagulation—or more accurately—the time required to reach a preselected degree of flocculation, and m, n, and a are constants. In this equation, plots of c *versus* $1/t$ have an intercept on the c axis at $1/t = 0$ or $t \to \infty$, so a is the concentration of electrolyte up to which the stability of the sol remains unaffected. When $1/t$ is very large, compared to n, *i.e.*, as $t \to 0$, n may be neglected so that $c - a \to m$ in the rapid coagulation zone; therefore, m represents the concentration of electrolyte in excess of a that will produce very rapid coagulation. It can be shown that n has the dimensions of $1/(\text{time})$. Note that n appears in the slope term of the linear equation. It can be shown that n represents the sensitivity of the sol to the electrolyte; this must be a cumulative effect of such things as shape, charge, dielectric constant, etc. Obviously, this type of information helps one to compare the flocculation of any systems to which it applies.

Another factor of interest in coarse suspensions is the comparison of the rate of collision produced by thermal energy and by mild laminar flow. Reich and Vold (55) discuss the analogy between temperature in a chemical reaction rate study and agitation in the flocculation rate of large particles. This analogy is possible because Brownian motion may be relatively ineffective in producing collisions of large particles when compared to the collision rate produced by agitation (48, 55). The collision of uniform spheres in sheared suspensions have been treated theoretically by von Smoluchowski (48) and by Manley and Mason (56). Reich and Vold give sample calculations for different diameter particles using a shear rate of 100 sec.^{-1} They assumed the weight fractions of solids constant; therefore, the total number of particles change. For 0.1-μ particles the probability of collision from Brownian motion is 100

times greater than from the agitation. However, for a 1-μ particle, the agitation-produced collision rate is ten times the Brownian motion rate. While these are calculated for a specific case, they illustrate the importance of particle size in determining which factor is significant as one goes from the colloidal size range to the microscopic size.

Reich and Vold conclude for unstabilized systems that even though the rate may be very small, the floc size will continue to grow in the absence of agitation. However, when they become large enough, they may be broken by the mild shearing forces produced by ambient thermal convection. Most important, however, is their observation that large particle systems deflocculated by agitation may appear nearly frozen in this deflocculated state for several hours. Since R appears in Eq. 8 indirectly because it determines n (when weight per cent is constant), either the floc size must be large or the volume fraction must be small before this apparent frozen state would be possible. Reich and Vold used low concentrations of solids in their studies.

Voet (57) has combined the methods of rheology, conductivity, and dielectric constant measurements to observe the flocculation breakdown during shear and also reformation of structure on the cessation of shear. In some cases, flocculation continued over a very long period of time. Both temperature and concentration effects were observed. For example, in a 2% dispersion the conductivity remained constant for about 1000 seconds after shearing was stopped; then the conductivity increased rapidly. This apparent induction period probably was the period of small agglomerate formation; it was followed by the formation of aggregates of sufficient size to bridge between the electrodes. Both higher temperatures and higher concentrations of solids reduced this induction period No induction period was observed at 8% or higher concentrations.

The probability of flocculation of large particles of different size during sedimentation has been considered by Andersson (13, 58). His theoretical treatment applies to very dilute systems. He concluded that hydrodynamic effects decrease the flocculation rate.

Hubley, Robertson, and Mason (59) used light scattering to observe flocculated suspensions subjected to laminar shear. Their apparatus resembled a Couette viscometer. Controlled shear rate was possible during the observations. They observed plug flow at very low shear rates. Frisch (60) studied coagulation in a turbulent flow system. He found that at low coagulation rates the von Smoluchowski equation could be used by replacing the normal diffusion coefficient with an eddy diffusivity constant.

Michaels and Bolger (61) considered the equilibrium between shear flocculation and deflocculation in a detailed mechanistic interpretation of the plastic flow behavior of kaolin suspensions. Smellie and LaMer (62) have developed a mathematical treatment of the competing flocculation and deflocculation processes produced in a polymer bridging process. Healy and LaMer (63) have extended the earlier model. The importance of agitation is indicated in their statement that if polymers are used to insure complete dispersion of a colloidal material, intense agitation should be used so that macro-flocs will be destroyed and complete adsorption, *i.e.*, total surface coverage, attained. This important work will be discussed in a later section.

Another important observation is that mild agitation may increase markedly the flocculation rate (64, 65). Freundlich and Juliusburger (66) discovered the analogous process in gels and gave the phenomenon the name *rheopexy*. The nature of flocculated suspension that seems to develop from all of these studies is not grossly different from that pictured by rheologists, previously. Specifically, it is one of the formation of small flocs which aggregate to form larger particle clusters. These aggregates form loose networks that extend throughout the suspension. These are broken by agitation—first into the aggregate at low shear, then into the flocs, and finally at high shear may be deflocculated. It is the problem of the formulator to control the nature of the flocculation in a manner that will produce a large sedimentation volume. Ideally, the individual flocs and aggregate size should be sufficiently small to produce a smooth appearance to the eye and to produce the large sedimentation volume, Neither the agitation in large tanks nor the filling of bottles provide sufficient shear to break the smallest flocs. Therefore, in practice, the flocculation and sedimentation processes do not start from the completely deflocculated state.

PREVENTING SEDIMENTATION AND CAKING

So far in this discussion, several major steps have been taken toward a basic understanding of the shelf-life stability of a suspension:

It has been recognized that for peptized suspensions, gravitational forces on large particles are sufficient to produce sedimentation.

A relatively low interfacial energy is preferred to

produce proper wetting when the particles go into the vehicle.

Flocculation of the suspension is possible, but the nature of the flocs may vary.

The initial flocculation rate may be fast, but the attainment of equilibrium may be very slow.

Therefore, to prevent sedimentation, one must either learn to control the flocculation process to produce the desired characteristics or find some other way of supporting the particles. One attractive concept is to trap the particles into a structure built of other materials. This will be called a structured vehicle since the structure is to be a part of the vehicle. Presumably, the particles will remain effectively deflocculated because they will be trapped into individual cells. It may help to peptize the particles so that they will stay apart long enough to permit the cells to form about them.

Structured Vehicles

Obviously, it will be necessary to construct the cells so that their holes are smaller than the particle to be trapped; otherwise, the particle will slip through. Therefore, the structure-former must have a smaller particle size than the particles to be suspended. One group of materials that might meet the requirements is soluble polymers. Thermal setting gels are not ideal because the suspensions might have to be heated before a dose could be withdrawn. Linear polymers usually do not form linkages of long durations, so they would permit the particles to drift downward as the Brownian motion of the polymer molecules released them. Of course, at very high concentrations they might function satisfactorily; but this forms a high apparent viscosity medium. It already has been concluded that acceptable fluidity is not compatible with the high viscosity needed to retard the sedimentation of the particles sufficiently.

McVean and Mattocks (67) report that Carbopol 934 solutions behave as an elastic body and do not relax when a small applied force is used. They conclude that this should be the ideal suspending agent. The author must agree with this conclusion but is unaware of definitive experiments that have been conducted to demonstrate that in the completed pharmaceutical suspension, Carbopol 934 produces a pure example of a structured vehicle. It would be necessary to demonstrate the absence of flocculation in the same system to do this. However, Meyer and Cohen (68) report the ability to suspend permanently marbles, golf balls, and sand. Certainly this is convincing evidence that structured vehicles are a possible approach to supporting large particles in a suspension.

Belugina, Zakieva, Konstantinova, and Rebinder (69) studied structured vehicles for suspending aluminum powder in a hydrocarbon medium. Aluminum soaps of oleic acid and naphthenic acids were used as typical surface-active structure producing agents, and polyisobutylene (mol. wt. 200,000) as an example of a surface-inactive structure producing agent. They did not obtain complete retardation of sedimentation in some of their suspensions. Consequently, they recommended that both adsorptional (protective colloid effect) and volume structuralization be used in the same system. Steiger-Trippi (70) has worked with aqueous systems and has concluded that structure in the vehicle supplemented by structure produced by flocculation of the suspended particles produces optimum results. Because the materials used to produce structure also adsorb on the particles, it will be advantageous to reconsider the combined effect after polymer flocculation has been discussed in more detail. Probably some suspending agents produce both structure in the vehicle and flocculation of the particles. The author has examined numerous elegant suspensions, some produced in our own laboratories and some by other pharmaceutical companies. The viscoelastic properties of the suspensions were observed; then the solids were removed by either filtration or centrifugation; the vehicle was reexamined. Usually no structure was detected in the vehicle. While the sensitivity of the method might not have been adequate to detect very weak structures, the evidence was sufficient to justify the conclusion that for these suspensions much of the structure was associated with the particles, *i.e.*, they were flocculated. Therefore, it appears that many elegant suspensions can be prepared in which structured vehicles are not the major "force" supporting the particles. This possibility will be explored in following sections.

Flocculated Suspensions

Let us consider the evidence that flocculation can produce the desired characteristics in a suspension. Later, possible mechanisms will be considered. One important aspect is the sedimentation volume which sometimes is defined as the final apparent volume the sediment occupies. Superior definitions will be considered also.

Sedimentation Volume.—By itself, the sedimentation volume is a meaningless number for lack of a reference value. To avoid this difficulty, Robinson (15) and Ward and Kammermeyer (17) used the ratio of the ultimate

settled height to the original height, $F = H_u/H_o$. H_u is the apparent height of solids after sedimentation, and H_o is the total height of the original suspension before settling. Dintenfass (71) used the same ratio in terms of volumes to describe sedimentation volume, $F = V_u/V_o$, but extends its usefulness to describe degree of flocculation designated as β.

A completely peptized suspension sediments to a close-packed structure of very small volume which he calls V_∞. $F_\infty = V_\infty/V_o$, so the ratio $\beta = F/F_\infty$ is the degree of flocculation. Dintenfass identifies three classes of sediments, *viz.*, cases where $F<1$, $F>1$, or $F = 1$. At first this is hard to accept since for $F>1$ (he claims values as high as 4 or 5), the settled volume must be larger than the total original volume. However, he points out that by dilution with the vehicle these suspensions will settle to occupy more than the original volume before dilution. These he calls compressed sediments when confined to the original suspension volume. The values for $F<1$ are obviously the usual ones, and he calls these expanded sediments. When $F = 1$, he says the system is in true equilibrium.

Dintenfass determines F_∞ either by observing a completely peptized suspension or by estimating it from rheological data. He states that log $\eta_\infty/\eta = F_\infty/1\text{-}F_\infty$, where η = viscosity of liquid vehicle, and η_∞ is viscosity of suspension at infinite shear-rate, *i.e.*, when all flocs are broken by the shearing action.

If sedimentation in the suspension is acceptable, a large sedimentation volume still is desirable because the particles are much less likely to form a hard cake. Therefore, the flocculated suspension is considered the preferred form and has sedimentational stability, especially when the sedimentation volume equals the total volume, *i.e.*, $F \geq 1$. Note that this is not a stable suspension in the usual colloid science sense. Chwala (72) described this situation as the "sedimentation paradox" (see Fig. 1).

Vold (73) has conducted some interesting theoretical studies that indicate how porous a sediment may be. She used a computer generated statistical model in which the probability of cohesion of two spherical particles upon contact varies from 0 to 1. According to these results, when the probability of cohesion is less than about 0.35, the sediment volume depends critically upon this probability. For higher probabilities of cohesion the sediment volume is not a sensitive measure of particle interactions. Also, when the probability equalled 1, her results indicated that for polydispersed systems the number of particle contacts may average 2 (74). While this model has some deficiencies in predicting lower volumes than may be observed, it emphasizes the point that a flocculated suspension may reasonably have a very porous structure. She concludes that the model needs to include both sedimentation and flocculation to give more representative results. In real cases still other factors may be important.

Nakagaki and Sunada (75) calculated the sedimentation volume of spherical particles attracted by cohesional forces. A simple relationship was found between the sedimentation volume and the ratio of range of attractive force to the radius of the sphere.

Another important consideration in any real suspension is the distribution of particle sizes. Vold (74) concluded that for the case she considered, the sediment structure was more sensi-

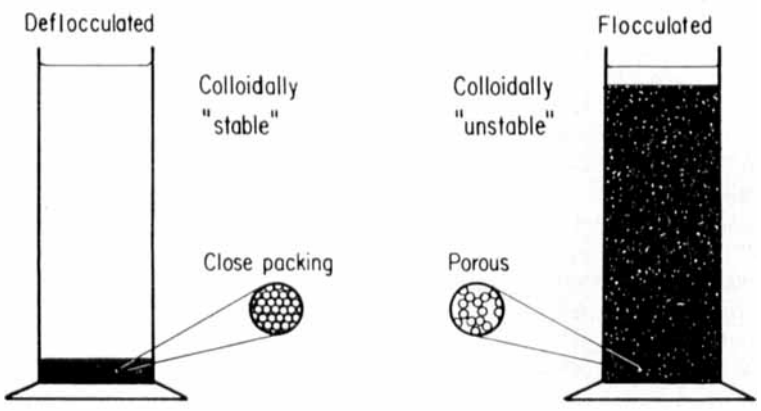


Fig. 1.—Most suspensions undergo sedimentation in the absence of structural support. Peptized suspensions (on left) form clays that are difficult to redisperse. Controlled flocculation can produce a large sediment volume. (From *Reference* 86).

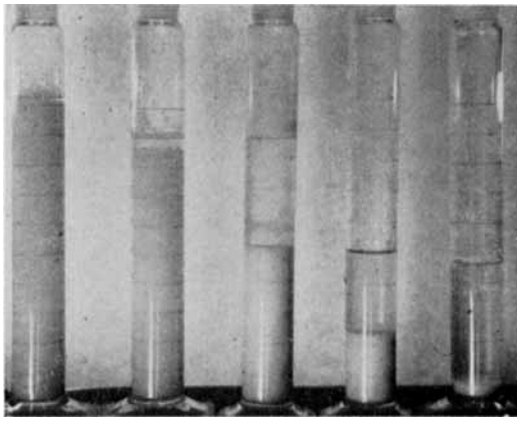
tive to size distribution than to the model used for interaction. There seems to have been no detailed experimental evaluation of this factor; however, Wolf and Kurtz (76, 77) observed that the smaller the particles, the greater the sediment volume. Wolf and Wolff (78) observed the effect of temperature on the sedimentation volume. Kuno (79) has determined the influence of vehicle viscosity and density. Except for the choice of vehicle, these factors are relatively unimportant, and are not variables that the formulator may use to control the sedimentation volume. Instead, the problem is to control the flocculation in a manner to obtain the desired characteristics in the vehicle when using particle sizes chosen on therapeutic criteria.

Figure 2 illustrates how much the sedimentation volume may vary. Note the gradual increase in sedimentation volume with increase in surfactant addition. Each graduate contains exactly the same weight of solid medicament. Often one adds a surfactant to produce deflocculation. But in this example the surfactant is producing the opposite effect. It is so effective that the sedimentation volume exceeded the total volume of the suspension. When enough surfactant was added, it became a rigid gel if confined in volume.

Redispersion.—When a flocculated suspension settles to occupy less than the total vehicle volume, there is an inherent benefit in the use of large particle size. Everyone is aware of the "Shake Well Before Using" label. Overbeek (80) considers the influence of particle size on the effectiveness of shaking in repeptizing a coarse suspension. He states, "Qualitatively this easy repeptization can be explained in the following way: By agitation a shearing motion of the liquid is excited. This causes a force trying to tear two neighboring particles apart, which force is proportional to the radius of the particles (Stokes' law) and to the distance between their centres (difference in the velocity of the liquid). So the separating force is proportional to the square of the radius of the particles. On the other hand, the attractive force is, at best, proportional to the first power of the radius of the particles (London force for spherical particles), and if the particles are not very regularly formed, the attractive force will soon be independent of the overall dimensions. . . . It may be remarked here that although a flocculated suspension is easily repeptized it is very difficult to redisperse the compact sediment of a stable suspension. . . . The sediment is so compact that the particles can only be attacked by hydrodynamic motions layer by layer which evidently is a very slow process." The difference between a peptized suspension and a flocculated one is shown diagrammatically in Fig. 1.

So far, this discussion has been presenting evidence that a flocculated suspension may be a preferred condition in avoiding caking in a suspension. The long history of the use of flocculation to produce a desirable suspension and the methods by which it has been obtained should be a convincing and interesting argument for it. Kinsman and Bowles (81) have reviewed the use of flocculation in pigment suspensions; Haines and Martin (82) used the sedimentation volume as a criterion of flocculation in their study of the prevention of caking. They related the flocculation characteristics to the zeta potential of the particles. One problem in discussing the results of studies of this kind is the difficulty of describing the real character of the flocs. Part of this difficulty arises because only a gross property, such as sedimentation volume, usually is observed. Unless this is expressed as a β value, the meaningful reference point is missing. Such phrases as partial flocculation or degree of flocculation are not very specific. They not only lack a quantitative interpretation, but also lack a clear connotation. For this reason, a terminology that seems to offer some advantages will be adopted in this review.

Sediment Structure.—When an interaction between two particles results in sufficient attraction to hold them together for a significant time, the interaction will be considered to have produced a *link* between the particles.



F>2 F = 1.7 F = 1.06 F = .52 F∞ = .1

Fig. 2.—The sedimentation volume of this suspension increased with increased surfactant concentration. Equilibrium vehicle was added to three containers on the left to provide space in which to sediment. Twenty-five mg./ml is normal dosage level. Center suspension would be a satisfactory product since at $F = 1$ sediment volume is equal to total volume desired. (From *Reference* 86).

Link is chosen in preference to bond to eliminate confusion between primary chemical bonds and the interactions producing flocculation. A particle may form links to more than one neighboring particle. The number of particles to which links are formed will be called the coordination number of the particle. This terminology would permit a reasonably clear description of the flocculated state if sufficient information were available; but it never is. However, it avoids possible confusion between the completeness of the process, the strength of the links, and the structure of the flocculated particles.

Two extreme cases are observed repeatedly (see Fig. 3). A compact floc consists of particles of very high coordination number. A loose floc has an open, scaffold-like, three-dimensional structure with many large void spaces, almost cellular in character. The average coordination number will be smaller in the loose flocs than in the compact ones. However, some of the scaffold building units may be small aggregates instead of individual particles. In this case a broad range of coordination numbers would exist. An adequate description of the structure would require a knowledge of the distribution of coordination numbers. Qualitatively, one would expect the high coordination number case to correspond to a low value of β, a broad distribution of coordination numbers to an intermediate value of β, and a low coordination number to a large value of β. For uniform spheres, the maximum number of nearest neighbors is 12, and the corresponding coordination number would be 12. For polydispersed systems, values larger than 12 would be possible for the large particles, but certainly the sediment containing particles whose average coordination number approaches 12 would not produce the large sediment volume of interest to the formulator. As previously mentioned, Vold (74) and also Nakagaki and Sunada (75) have explored the relationship between coordination number and sediment volume for idealized cases.

Rebinder (83) describes a most interesting case in which, apparently, the coordination number was controlled by the number of coagulation centers. This influenced the character of the flocculation produced. He states that where the area of these regions is a considerable fraction of the total particle surface, compact aggregates form. However, when the coagulation centers are localized at a few points, a network structure with thixotropic properties is formed. He adsorbed surfactants onto calcium stearate to control the number of coagulation centers. If one may generalize his observations, the procedure for preparing elegant suspensions is given direction. Perhaps this is a part of the explanation of the success of the flocculation study reported by Haines and Martin (82). However, their cationic coating agent did not reduce the coordination number sufficiently, so they added a suspending agent also. They observed that the suspending agent stabilized against sedimentation but did not report whether the suspending agent was acting also as a flocculating agent.

Probably as suggested above, thixotropy occurs only when there is a low coordination number for the particles. Elongated (flat or long) particles often produce thixotropic suspensions. One might expect the ends or edges to have different energies than the bulk of the surface. These could influence the effective number of coagulation centers. It has been demonstrated that the method of producing the flocculation may determine whether a gel is produced. For example, kaolinite flocculated with hydrochloric acid produces the scaffold-like "edge-to-face" flocculation; while sodium chloride flocculation produces "face-to-face" flocculation (84), a much more compact floc. Heller (85) has studied the flocculation of anisometric particles and has concluded that rapid flocculation by electrolytes produces structurally isotropic coagula, while slow flocculation by electrolytes and by stirring produces anisotropic coagula. Of course, his results cannot be extended to isometric particles.

Methods of Producing Flocculation.—The suspension may flocculate without the intentional addition to, or adjustment of, the vehicle to produce flocculation. Of course, the causes of flocculation in this case are identical to the intentionally induced flocculation. However, some points of interest are worth discussing.

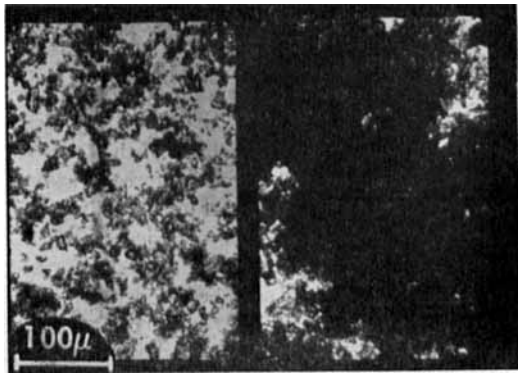


Fig. 3.—Comparison of flocculated sediments that produce a large sediment volume (left) and a small sediment volume (right). Both are aged slides to permit both flocculation and sedimentation to proceed. Initial suspensions had the same solids content.

In theory, any pure lyophobic material should flocculate when put into the pure vehicle unless a protective double layer forms. *Pure* is the important property to be considered here. Very few solids are pure, especially with respect to uncontaminated surfaces, and very few vehicles are pure enough to contain insufficient impurity to adsorb onto the particle surface. Consequently, it is not possible to predict *a priori* whether a flocculated or peptized suspension will result when a powder is put into a simple liquid. If a large sedimentation volume results, and if the particles are isometric, one may suspect that the surface is contaminated either initially or by adsorption from the vehicle. In some cases this can be demonstrated by noting a marked change in sediment volume when powders have been pretreated by placing them under vacuum for several hours or passing helium over them for several hours to remove impurities. Of course, the impurity has to be volatile for this to be effective. The technique is not selective to surface impurities since molecules of solvation might be removed by the same pretreatment. In one case observed in this laboratory, the crystals were extremely difficult to wet with water; most surfactants were ineffective. However, when the residual solvent was removed, the crystals wet readily without a surfactant. A formulation based on the purified material could have been entirely unsatisfactory if made using the contaminated powder. Subtle changes in the trace impurities may result from changes in the purification or crystallization procedure used in manufacturing the raw chemical. These changes may not be detected by usual control procedures; yet, they may produce marked changes in the characteristics of a simple suspension. It is feasible to use a control procedure based on the sedimentation volume in simple liquids to warn of such changes in the raw material.

Electrolyte Flocculation

The basic theory that applies to electrolyte flocculation has been discussed in an earlier section of this paper. The use of electrolyte flocculation does not provide a means of controlling the coordination number of the particles. In the absence of other controls, flocs produced by adding electrolytes would be expected to produce coarse, compact masses with a curdled appearance. Often, this is observed. The floc size probably is determined more by the kinetics of the process than by any other factor. Of course, this conclusion assumes that the flocculation is in the primary minimum. Flocculation in the secondary minimum might be different because of the ease of redispersion.

Detergent Flocculation

Detergent is used here in preference to surfactant to avoid confusion. Surfactant has already been used in a more general connotation than will be implied in this section. Detergents may be of the ionic type or nonionic type.

Ionic Detergents.—The influence of the ionic type on the double layer has been considered already. Neutralization and reversal of charge has been observed on numerous occasions (39, 40, 82). Figure 2 is an example of the control of the sedimentation volume using an ionic detergent. This figure is from a previous publication (86). Tomlinson (87) observed the sedimentation volume of Prussian blue as the adsorbent for cetyltrimethyl-ammonium bromide. The maximum sedimentation volume in water approximately coincided with the minimum sedimentation volume in benzene. The importance of the extent of the lyophilic character of the surface was demonstrated. As an explanation of the inefficiency of the cationic detergents as detergents at concentrations between 1 and 3%, Doscher (88) proposed that these materials are adsorbed as charged aggregates above their critical micelle concentration and that this leads to a more hydrophobic exterior. The hydrophobic surfaces interact to form links between particles.

Nonionic Detergents.—Nonionic detergents have not been studied as extensively as ionics Kuno and Abe (89) observed the adsorption of various polyoxyethylated nonylphenols on calcium carbonate. They detected no adsorption until after the critical micelle concentration was reached. However, on carbon black the adsorption occurred at lower concentrations. Mathai and Ottewill (90) observed the mobility of particles as a function of nonionic surfactant addition. The mobility decreased gradually as the concentration increased. This suggests that the surface charge density remained constant and the increased solvation carried by the nonionic detergent caused the electrokinetic plane of shear to move further into the liquid phase. The nonionic detergent produced some protection against flocculation by electrolytes. Flocculation seemed to result from a compression of the double layer and not by a bridging action. Koelman and Overbeek (91) concluded that some slight stablilization occurs with nonionics as a result of keeping the particles further apart.

Nonionic detergents would be expected to possess lower heats of adsorption than ionics.

Usually the energy involved would not be sufficient to orient the detergent molecule in a manner to produce a much more lyophobic surface. In cases where liquid nonionic detergents produce flocculation, the liquid bridge mechanism may account for their action. In our own laboratory, nonionic detergents have been used to increase the sedimentation volume of flocculated suspensions. Less than a monolayer of surfactant is necesssary; otherwise, the desired mosaic surface character with only a few coagulation centers would not be produced and the effective control of the coordination number would be lost. It is believed that the mosaic surface is effective only during the initial part of the flocculation process. Other controls must exist in the suspension if the slow compaction settling process is to be effectively arrested. Structured or high viscosity vehicles may be sufficient. Additional study is necessary before the above concept can be fully evaluated.

Detergents usually are considered to differ from polymers because often polymers have a very high molecular weight with many active centers spaced along the chain. A detergent usually has a much lower molecular weight and, at most, a few active centers on a single molecule. The importance of this will be emphasized in the section on polymer flocculation.

Liquids as Flocculating Agents

Water has been used for many years to prevent hard settling or clay formation of the pigments in oil base paints. In 1931, Rhodes and Jebens (92) attributed the function of water to the formation of structure within the paint. Fischer (93) describes the mechanism. When a hydrophilic solid is dispersed in oil, a small amount of water is collected by each particle. When two particles touch, the liquid film collects between the particles to form a liquid bridge.

Bloomquist and Shutt (94) observed the sedimentation volume of glass spheres in water and organic liquids. In some organic liquids, only when they are carefully dried, was the sedimentation volume as low as in water. In some cases the sedimentation volume was proportional to the water content; in others the liquid had to be nearly saturated before flocculation was observed. Zettlemoyer (95) reports that less than a monolayer of water may produce flocculation of some systems. It is not clear that such cases are liquid-bridging. Farnand, Smith, and Puddington (96) described the formation of compact aggregates in a liquid medium using liquid-bridge flocculation. The mechanism is essentially the same as that for the granulation of powders. Mild agitation is used to increase the number of collisions and thereby increase the opportunities for the particles to increase their coordination number. When adequate liquid is present to form the links, a compact aggregate results. Obviously, the interfacial tension in the regions of the link provide the forces acting to hold the particles together. Hydrophobic solids in water may be flocculated by adding hydrophobic liquids (97). The mechanism is the same.

In our own laboratories, liquid-bridge flocculation has been found useful. The assumption has been that for it to produce the desired properties, the maximum possible coordination number of the particles must be limited. This is accomplished by a partial coating of the lyophobic surface with a surface-active material. Then the liquid bridging agent is added to produce the flocculation. A product with a large sedimentation volume results. Again, protection against compaction on standing is not inherently present. However, the lower sediment volume suspension that results from compaction is readily redispersed.

Polymers as Flocculating Agents

By far the largest number of pharmaceutical suspensions contain lyophilic polymers as suspending agents. The success of the numerous hydrocolloids to suspend particles in aqueous vehicles is well known. They are used so frequently that it is hard to believe that the details of the mechanism of their action have not been resolved completely. The possible role of bridge flocculation in this suspending action will be explored.

Sensitization and Protection.—Classically, the action of hydrophilic colloids has been studied using particles of colloidal size. Zsigmondy (98) reported that gold sols were protected against coagulation by electrolytes when gelatin was present. Reinders and Bendien (99), using electrophoretic measurements, concluded that the particles were covered completely by the gelatin. Instead of protection, sensitization (less electrolyte required) was observed if the gelatin was present in small amounts. Zsigmondy and Joel (100) concluded that small amounts of gelatin caused the sensitization because the sol tried to envelope the gelatin. This last conclusion is not correct because the aggregates do not form until the shielding of the double layer is reduced by electrolyte addition. In modern terminology, the protection and sensitization are described in terms of the adsorption of the hydrocolloid onto the particles.

Heller and Pugh (101) have discussed the protective action of polymers in terms of steric protection. Only slight interpenetration would be

expected of polymer chains, which extend outward from neighboring particles. These protruding chains keep the particles separated sufficiently to avoid large interactions from dispersion forces. Rebinder (25, 26) describes the protective action as structural protection in the layer around the particle.

Sensitization results because polymer molecules can form bridges between two particles by adsorbing different portions of themselves onto each of two particles. Excess polymer reduces the uncoated area so that the probability of a link forming between two particles is reduced. This simple model has been used by Smellie and LaMer (62) to develop a quantitative theory for the flocculation of phosphate slimes. These investigators were interested in obtaining a high filtration rate. Consequently, the coordination number of the particles in the sediment they find to be optimum for filtration may be higher than desired for maximum sedimentation volume of the suspension. This illustrates the problem previously mentioned; *viz.*, the inability to describe adequately the flocs produced in a given process.

Polymer Adsorption.—It is desirable to consider the adsorption of polymers in more detail before continuing with the discussion of flocculation. Silberberg (102), in presenting a theoretical treatment of polymer adsorption, also reviewed the established facts of polymer adsorption that any theory must include. Some of these facts are of particular interest. The numerous references are not included here; the reader is referred to Silberberg's excellent paper which has an extensive bibliography. The amount of polymer that is adsorbed from solution is an unusually high proportion of the polymer molecules up to some limiting amount; thereafter, increases in polymer concentration in solution have little effect on the adsorption. In the regions of relatively low molecular weight, the amount adsorbed increases with molecular weight of polymer, but at higher molecular weights the amount adsorbed seems to be independent of the polymer molecular weight.

The polymer molecules may be considered as consisting of active centers spaced along the "backbone" of the molecule. The fraction of total segments adsorbed, *i.e.*, actually attached to the particle surface, is less than one-half of the total segments. The adsorption phenomenon is *relatively* insensitive to temperature changes, solvent changes, and even changes in adsorbent surface. (Little wonder that polymers have such universal application.)

The model that is most successful in explaining the observations is based on the adsorption of several adjacent segments of the polymer, and then several other adjacent segments forming a loop out into the vehicle, followed by the adsorption of another group of segments of this same molecule, etc. It had been assumed previously that many loose ends project into the vehicle; but Silberberg's theoretical treatment predicts that at equilibrium the ends are adsorbed and loops stick out into the vehicle. With molecules of this size, the equilibrium arrangement would be approached very slowly. (See the discussion at the end of *Reference* 102.) In a real case, there probably are ends available for adsorption onto bare portions of other particles. The effect of time of agitation was noted by Kraugh and Langston (103) with gelatin adsorbed on quartz. They observed an apparently irreversible effect in the direction of deflocculation. They postulated that when particles are kept apart long enough by agitation one may obtain a gradual rearrangement on the particle to adsorb more free chains onto the particle so that they are no longer available to form bridges.

That polymer bridge flocculation may be important in pharmaceutical suspensions has been suggested previously (86). Because hydrocolloids are macromolecules with numerous hydrophilic regions, it is not surprising that they may be used to flocculate particles. Because they are polyelectrolytes, the influence of pH and of ionic strength may not be negligible. Kraugh and Langston (103) observed an optimum pH for both settling rate and adsorption for gelatin systems.

At least part of the suspending action of hydrocolloids results from their adsorption and the associated flocculation. It is difficult to demonstrate the relative importance of various factors in a given suspension. However, in unpublished studies some insight has been obtained. Using different concentrations of the same suspending agent and the same solid material, a series of suspensions having different sedimentation volumes were prepared. Several techniques (to be described in the next section of this manuscript) were used to evaluate the suspensions. The observations lead us to postulate that the suspending agent is functioning both as a protective colloid and as a flocculating agent, just as in the filtration rate studies of Smellie and LaMer (62). It is performing these functions on different parts of the same particle. When most of the surface is covered, there is little free surface onto which many particles may attach to form polymer bridges. Therefore, the coordination number tends to be low. At lower surface coverage, the

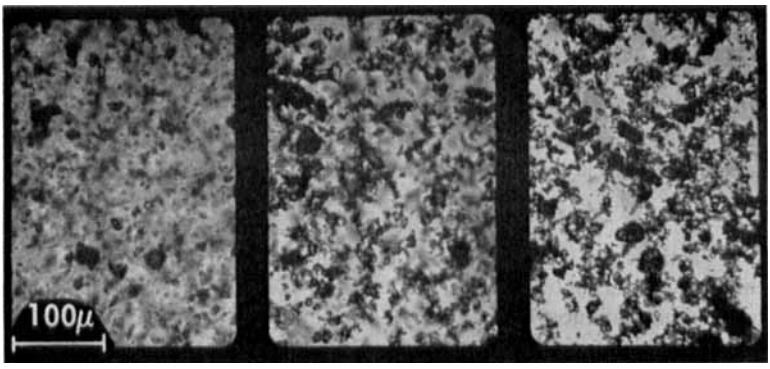


Fig. 4.—Time sequence photomicrographs of flocculation process. Case of a large sediment volume. Freshly prepared slide. (Left). Three and one-half hours after preparation. (Center). Forty-five and one-half hours after preparation. (Right). Freshly prepared slides may be misleading.

coordination number may be larger and the suspension appears more coarse. Here, the links need not be all polymer bridges.

Methods of Evaluating Suspensions

Techniques for evaluating the character of a suspension have been developed. Many of these have been reviewed by Kinsman and Bowles (81). Dielectric constant methods and electrical conductivity are useful only in nonconducting fluids. Conductivity methods have the further requirement that the particles must be conducting. Nevertheless, these methods have been most revealing of the nature of the flocculation process, see, for example, the work of Voet (57). Other methods, such as light scattering, are useful only in dilute suspensions. Less direct methods are often more easily applied and more suitable for the usual pharmaceutical suspension.

Low Shear Rate.—In our laboratories, it has been demonstrated that suspensions may be elegant products with reasonably long shelf-life stability even when the particles have not attained their maximum possible coordination number. This is demonstrated by agitating the suspension at an extremely low rate. The author uses a round bottle tilted at 45° from the vertical and rotated at 4 revolutions per hour. The bottle is not filled beyond the point where the contents have a triangular cross section if viewed in a plane through the axis of rotation. One pharmaceutically elegant suspension has been found that will "curdle" in less than 1 hour in this apparatus. Other good products withstand this treatment for several hours with no apparent change in their physical characteristics. A suspension that undergoes a significant change in the coordination number of its particles in this device might do so under the mild agitation it would receive in shipment. However, the conditions are not the same and further testing should be conducted.

The acceleration of flocculation at very low levels of agitation has been reported previously (64, 65). Also, some dispersions flocculate under high shear (81, 104). The author has not worked with any pharmaceutical suspensions that behave in this manner.

One experimental lot that contained a suspending agent was observed to "curdle" in less than 45 minutes when subjected to the rotation test. This suspension will be identified as suspension *A*. When it stood on the shelf in a carboy, within 2 months the solids had contracted so that a thin layer of clear liquid existed at the outside edge. Also, large cracks had developed. They extended into, but not through, the total structure of the solids. It is postulated that a slow rearrangement of the particles had occurred on standing; this resulted in a slight increase in the coordination number of the particles (syneresis phenomenon). In small containers the contraction was not obvious except by careful examination.

In discussing the adsorption of polymers, it was stated that the amount of polymer adsorbed is an unusually high fraction up to some limiting amount; thereafter, increases in polymer concentration in solution have little effect on the adsorption. Apparently, the surface becomes almost completely coated with polymer before a high concentration can exist in the equilibrium liquid. However, in the slow rotation experiment with suspension *A*, the suspending agent did not provide complete protection against flocculation; but enough of it must have been in the vehicle to provide sufficient structure to inhibit the flocculation process in the unagitated suspension. Adsorption isotherms can be made to determine the equilibrium concentrations.

Rheology.—Suspension *A* was studied further by using a Couette-type viscometer. The interaction energy was evaluated using the method of Gillespie (105). The Bingham yield values were nearly identical for the suspension and the equilibrium vehicle. Therefore, the presence of significant amounts of suspending agent in the vehicle was demonstrated clearly. Viscoelastic properties of both the suspension and the vehicle were

estimated using a device somewhat like that described by McVean and Mattocks (67). An important difference in technique is that the suspension is not disturbed to introduce a sphere beneath its surface. Instead, a probe is lowered into the aged suspension. The relaxation curve of the suspension demonstrated the existence of a structure that relaxed very slowly. The relaxation plot fit the logarithmic box distribution model. By comparison, the equilibrium vehicle exhibited a very weak structure. The apparatus used was not sensitive enough to permit characterization of the relaxation curve.

Microscopic Observations.—Microscopic observations of suspension *A* were not particularly useful because of the high (13%) solids content. When diluted with equilibrium vehicle to 2.6% solids, no significant flocculation was observed, even after standing for 2 weeks.

Suspension *A* seems to be an example of a suspension containing flocculated particles. However, the flocculation process has been arrested by either the structure and/or the high apparent viscosity of the vehicle. Therefore, the particles have not attained the maximum possible coordination number. Its shelf-life stability in small bottles was satisfactory to qualify as an elegant product.

Similar studies of other suspensions have demonstrated that other conditions also result in an elegant product. For example, a suspension that did not "curdle" in the slow rotation device produced very interesting photomicrographs. To obtain useable photomicrographs, it was necessary to dilute the suspension to one-fifth the original concentration. Both the dilution and the walls of the slide may influence the particle behavior; hence, one cannot be certain that the suspension looks like the photomicrograph. Figure 4 shows the results of this study. The zero time photomicrograph does not show flocculation. After 3.5 hours, noticeable flocculation has occurred. After 45.5 hours, the flocculation has produced the open-scaffold structure. Figure 3 shows the sediment of two suspensions of the same solid content. In one case very little suspending agent was used; both a small sediment volume and a large coordination number resulted. Increased amount of suspending agent resulted in the formation of the scaffold structure and a large sediment volume. The dilution step, necessary to obtain the photomicrographs, reduces the initial flocculation rate to approximately one-twenty-fifth of its rate in the undiluted suspension. Nevertheless, the results demonstrated the need to evaluate a microscope slide after waiting sufficient time for the flocculation to develop.

To obtain the photomicrographs in Figs. 3 and 4, each slide contained a well about 500 μ deep. This was filled with the freshly stirred suspension. The cover slip was carefully placed over the well and sealed. The slides were stored at 60° from the horizontal so that both sedimentation and flocculation could occur. Without the sedimentation, the high coordination number would not develop.

The observations discussed in the above paragraphs are consistent with the theoretical concepts presented. The basic principles involved in producing colloidally stable dispersions are not sufficient for shelf-life stability. Instead, it is necessary to use a structure produced by the particles themselves.

Phase Volume Ratio

Very little has been said directly about the phase volume ratio. Obviously, the particle population is important since a maximum theoretical limit must exist for the sedimentation volume of a dilute suspension and for the minimum volume of a highly concentrated suspension. The rheological properties will vary with the total solids. Chessick (106) has described methods to increase solid contents of nonaqueous dispersions without other effects. This involves the control of the interactions between particles. Both pediatric drops and parenteral dosages may require very high solids contents. The finished products will involve differences in the desired coordination number, etc., from more dilute suspensions. Particle size control may be of value (107), but the dissolution rate and therein the availability for adsorption may limit the choice of particle size.

It is obvious that there is no single answer to the question, "What conditions produce a stable (shelf-life) suspension?" Nevertheless, some guiding theories and useful techniques are available to the formulator. In an effort to present the above material in a manner as logical as possible, many of the complicating details have been neglected. At present, the body of knowledge available to the formulator provides only the guide lines. Each formulation presents unique problems. These relate to the chemical composition and physical properties of the suspended materials. Even chemically similar materials may exhibit different physical properties. This is especially true of surface properties where trace impurities are often the dominating factor. Consequently, guided empiricism shall continue to be the dominant course toward preparing an

elegant pharmaceutical product. However, as fundamental principles are unveiled, product formulation will shed its empirical character. It behooves the formulator to keep abreast of these developments. It is in this interest that this review has been prepared.

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Evaluation of Certain Hypotensive Agents V

Substituted Polymethylenediamines

By ROBERT P. HALLIDAY†, WILLIAM J. KINNARD, and JOSEPH P. BUCKLEY

The hypotensive activity of a series of substituted alkylenediamines was investigated in anesthetized rats and dogs. *N,N'*-Bis-[α-(2-pyridyl)ethyl]-ethylenediamine dimaleate (JB 5058) produced marked hypotensive effects of long duration in both species of animals. JB 5058 reversed the pressor response to epinephrine, 2 mcg./Kg., and decreased the pressor response to levarterenol, 2 mcg./Kg.; angiotensin II, 1 mcg./Kg.; and bilateral carotid occlusion. The oral administration of the compound to normotensive animals produced a mild transient depression of systolic blood pressure; however, 40 mg./Kg. administered orally to renal hypertensive dogs produced a marked decrease in systolic blood pressure which persisted from 34 to 57 hours. The data suggest that the primary mechanism of the hypotensive activity of JB 5058 is competitive blockade of the α adrenergic receptors.

SEVERAL ETHYLENEDIAMINE derivatives have been reported to produce hypotensive activity in experimental animals. Tonks (1) reported that a series of compounds structurally related to mepyramine, *N,N*-dimethyl-*N'*-(4-methoxybenzyl) - *N'* - (2 - pyridyl) - ethylenediamine, produced mild transient hypotension. The pressor response to epinephrine in the anesthetized dog and cat was potentiated by all of the compounds in this series except the *N*-ethyl-*N*-(2-naphthyl)-*N',N'*-dimethyl derivative which exhibited adrenolytic activity. Graham and Tonks (2) reported that 2 mg./Kg. of this compound reversed the pressor response to epinephrine and blocked the pressor response of levarterenol in the cat.

This report is concerned principally with the evaluation of the hypotensive activity of several substituted polymethylenediamines[1] in rats and dogs and the oral hypotensive activity in unanesthetized hypertensive dogs of the most promising compound of this series, JB 5058, *N,N'* - bis - [α - (2 - pyridyl)ethyl] - ethylenediamine dimaleate.

EXPERIMENTAL

Hypotensive Activity in Normotensive Rats.—The experimental compounds were screened for hypotensive activity in normotensive Wistar rats, anesthetized with 1.2 Gm./Kg., i.p., urethan as described by Bickerton, *et al.* (3). All compounds were administered in aqueous solution *via* a femoral vein in graded doses from 1–60 mg./Kg.; each animal received only a single dose of one of the compounds. In some instances, due to low water solubility or insufficient compound, the higher dosage range could not be administered.

Hypotensive Activity in Normotensive Dogs.—Certain compounds were further evaluated in the anesthetized normotensive dog. Mongrel dogs were anesthetized with pentobarbital sodium, 35 mg./Kg., i.v., and blood pressure recorded from a cannulated femoral artery *via* a Statham transducer onto a Grass polygraph. The effects of the experimental compounds on the pressor activity of the following were also recorded: epinephrine, 2 mcg./Kg.; levarterenol, 2 mcg./Kg.; bilateral carotid occlusion, 15 seconds; and synthetic angiotensin II, 1 mcg./Kg. All compounds were administered *via* the femoral vein.

Oral Hypotensive Activity in Unanesthetized Normotensive and Hypertensive Dogs.—Renal hypertension was produced in dogs utilizing the method described by Grollman (4). The blood pressures of the normotensive and hypertensive dogs were determined by the method of Prioli and Winbury (5). The dogs were restrained in a harness which limited movement but did not fully support the animals. The tail was shaved and a microphone of an Infraton unit secured to the tail beneath the coccygeal artery so that the pulse wave could be observed on the screen of an oscilloscope. A digital cuff (Beckman Instruments) was placed proximal to the microphone and the pressure increased in the cuff until pulse waves were no longer visible. The pressure was then reduced slowly until the pulse waves reappeared and the systolic pressure in mm. Hg recorded. JB 5058 was administered orally in capsule form to dogs fasted

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[1] Compounds supplied by Lakeside Laboratories, Milwaukee, Wis.

TABLE I.—EVALUATION OF CERTAIN SUBSTITUTED POLYMETHYLENEDIAMINES ON THE BLOOD PRESSURE OF ANESTHETIZED NORMOTENSIVE RATS

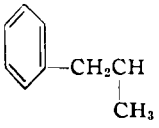
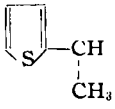
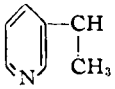
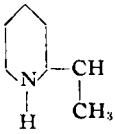
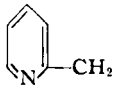
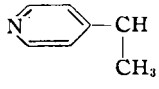
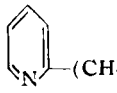
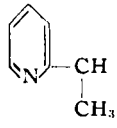
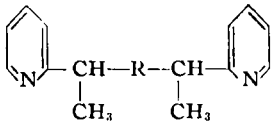
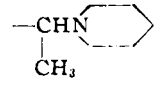
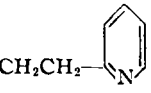
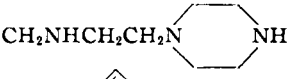
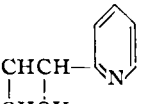
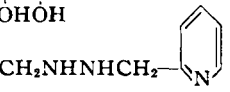
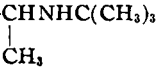
Compd.	R	Salt	Animals, No.	Dose, mg./Kg.	% Drop, b.p. $\bar{x} \pm$ S.D.	Duration, min. $\bar{x}$ ● S.D.
	Group I R—HN—CH_2—CH_2—NH—R					
EX 2837		Dimaleate	6	20	0	0
EX 3144		Dimaleate	15	40	0	0
EX 3460		4 HCl	6	60	0	0
EX 4420		Tetramaleate	6	20	0	0
EX 4467		Dimaleate	6	10	51 ± 4	190 to 490
EX 4468		Dimaleate	8	60	0	0
EX 4947		Dimaleate	6	60	42 ± 8	63 ± 20
JB 5058		Dimaleate	10	10	48 ± 10	150 to 840
	Group II 					
EX 3571	—$N(CH_3)(CH_2)_2N(CH_3)$	4 HCl	6	10	48 ± 5	140 to 360
EX 4525	····$NH(CH_2)_5NH$	Dimaleate	13	20	0	0
EX 10006	—$NH(CH_2)_3NH$	4 HCl	11	10	47 ± 6	270 to 490
	Group III 					
EX 4914	—$CH(CH_3)N(CH_3)_2$	CH_3Br	6	30	0	0
EX 4937		Fumarate	8	80	0	0

TABLE I.—(*Continued*)

Compd.	R	Salt	Animals, No.	Dose, mg./Kg.	% Drop, b.p. x ± S.D.	Duration, min. x ± S.D.
EX 4938		2 HCl	6	20	0	0
EX 4949	—CH_2NHCH_3	Maleate	7	60	0	0
EX 4950		4 HCl	6	60	40	45
EX 4955		2 HCl	5	60	0	0
EX 4959		Diacetate	6	40	0	0
EX 4963		Maleate	7	40	0	0
	Group IV					
EX 3132	$(CH_2)_3N(CH_3)_2$	Trimaleate	17	50	0	0
EX 3500	$(CH_2)_2N(C_2H_5)_2$	Trimaleate	6	50	0	0

12 to 18 hours and the systolic blood pressure obtained periodically.

RESULTS

Hypotensive Activity in Rats.—The hypotensive activity of the experimental compounds in anesthetized rats is summarized in Table I. Insertion of a needle into the rat femoral vein, or the physical characteristics of the solution injected, often produce transient hypotensive responses. To eliminate these possible artifacts, blood pressure recordings were measured 10 minutes after administration of the compound. This procedure, by necessity, also evaluated drug effects of less than 10 minutes duration as inactive. The compounds were divided into four structural groups, and doses listed in this table are either the HD_{50} (hypotensive dose$_{50}$, that dose which was estimated to produce approximately a 50% reduction in mean arterial pressure) or the highest dose tested. Three com-

TABLE II.—EFFECTS OF EXPERIMENTAL COMPOUNDS ON THE BLOOD PRESSURE AND SEVERAL VASCULAR RESPONSES IN THE ANESTHETIZED DOG

Compd.	Dose, mg./Kg.	Original Blood Pressure, mm. Hg	% Fall, b.p.	Duration, min.	% Control Responses BCO	EPI	L-ART	ANG
EX 2837	20	170	70	3	75	90	110	120
EX 3144	20	92	0	...	60	75	100	100
EX 3460	20	170	0	...	100	110	86	100
EX 4467	20	120	50	80+[c]	62	Reversal	...	160
EX 4468	25	140	0	...	100	100	...	100
EX 4525	20	150	0	...	100	170	160	130
EX 4914	20	150	33	1.5	90	80	86	100
EX 4937	40	140	28	1.0	63	180	112	120
EX 4938	40	136	+58	11	100	100	91	71
EX 4947	60	160	31	80+[a]	60	Reversal	40	67
EX 4949	20	145	+20	7	120	100	100	120
EX 4950	20	138	86	138	15	150	140	160
EX 4955	20	130	0	...	100	100	100	100
EX 4959	20	130	0	...	100	100	100	100
EX 4961	20	124	0	...	75	140	140	100
EX 4963	20	135	26	5	230	120	135	120
EX 10006	25	132	62	168	25	78	108	113
JB 5058	10	150	33	60	84	Reversal	...	...
...	20	96	40	80+[b]	50	Reversal	60	69
...	25	120	50	660	25	Reversal	...	...
...	40	140	49	1200+	28	Reversal	32	77

[a] Blood pressure at termination of experiment was 104 mm. Hg. [b] Blood pressure at termination of experiment was 65 mm. Hg. [c] Blood pressure at termination of experiment was 80 mm. Hg.

pounds in Group I exhibited hypotensive activity: EX 4947 at 60 mg./Kg. and JB 5058 and EX 4467 at 10 mg./Kg. EX 4947 produced a mean drop of 42% with a mean duration (time to return to pre-drug level) of 63 minutes. EX 4467 produced a mean drop in blood pressure of 51% and a duration of action ranging from 190 to 430 minutes. JB 5058 was the longest acting compound of this series since 10 mg./Kg. produced a mean fall in blood pressure of 48% persisting from 150 to 840 minutes. Two of the three compounds in Group II produced marked hypotensive effects. EX 3571 and EX 10006 produced hypotensive responses in excess of 4 hours. Group III contained one compound which was moderately active. EX 4950 at 60 mg./Kg. produced a 40% fall in blood pressure with a duration of 45 minutes. Group IV contained no active hypotensive agents when tested in the rat.

Modification of the basic structure of JB 5058 from the (2-pyridyl) to the (3-pyridyl) or (4-pyridyl) as seen in compounds EX 3460 and EX 4468 apparently eliminated the hypotensive activity. The conversion of both α-(2-pyridyl)ethyl groups to picolyl groups (EX 4467) produced a slight decrease in the duration of action. EX 10006, the trimethylenediamine homolog of JB 5058, was highly effective, whereas EX 4525, the pentamethylenediamine homolog, was inactive as was the hydrazine, EX 4959, which has no alkylene chain.

The data obtained from this series of compounds in anesthetized rats suggest that the following structural moieties should be present for optimal hypotensive activity: (*a*) the compound should contain two 2-pyridylmethyl groups joined by an alkylenediamine chain, and (*b*) the distance between the alkylenediamine nitrogens has a bearing on the hypotensive activity since all the active compounds contained a 2 or 3 carbon chain at this site.

Hypotensive Activity in Normotensive Dogs.—The effects of the experimental compounds on the blood pressure of anesthetized dogs and on the various pressor responses in these animals are summarized in Table II. All of the compounds which were active in rats were also active in the anesthetized dog. EX 4950 produced only moderate effects in anesthetized rats; however, 20 mg./Kg., i.v., produced a mean drop in blood pressure of 86% and a duration of action of 138 minutes. EX 3571 was not investigated in dogs because of the limited quantity of the compound available. EX 4947, JB 5058 (Fig. 1), and EX 4467 reversed the pressor response of exogenous epinephrine. EX 4947 and JB 5058 also depressed the levarterenol and angiotensin II pressor responses, and both compounds markedly depressed the pressor effect produced by bilateral carotid occlusion. JB 5058 did not alter the pressor response to central vagal stimulation but did produce atropine-like effects on the cardiac response to peripheral vagal stimulation.

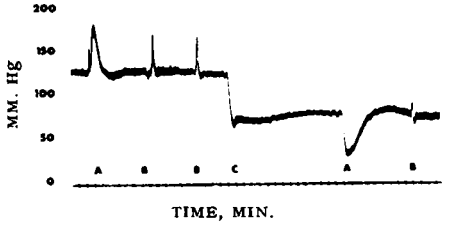


Fig. 1.—The effects of 40 mg./Kg., i.v., JB 5058 on epinephrine, 2 mcg./Kg., i.v., and bilateral carotid occlusion, 15 seconds, pressor responses. Key: A, epinephrine; B, carotid occlusion; C, JB 5058.

Oral Hypotensive Activity in Unanesthetized Normotensive and Hypertensive Dogs.—Single doses of JB 5058 were administered to two hypertensive dogs and one normotensive dog. Forty milligrams/Kg., orally, reduced the systolic pressure of the first hypertensive dog from 200 to 110 mm. Hg and was effective for approximately 34 hours (Fig. 2). This same dose lowered the systolic pressure of the

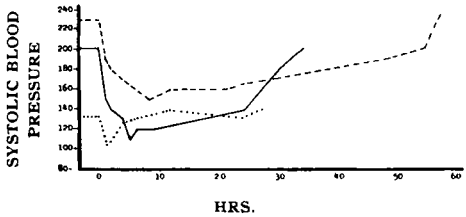


Fig. 2.—The effects of 40 mg./Kg. JB 5058, per os, on the blood pressure of unanesthetized normotensive and renal hypertensive dogs. Key: ---, hypertensive; ——, hypertensive; ..., normotensive.

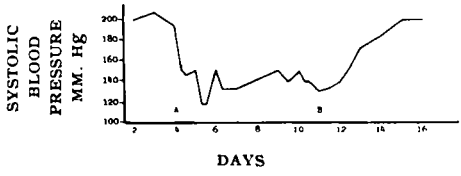


Fig. 3.—The effects of 20 mg./Kg. JB 5058, per os, daily in hypertensive dog No. 1. Key: A, initiation of JB 5058 administration; B, termination of treatment.

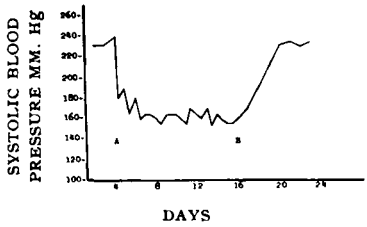


Fig. 4.—The effects of 20 mg./Kg. JB 5058, per os, daily in hypertensive dog. No. 2. Key: A, initiation of JB 5058 administration; B, termination of treatment.

second hypertensive dog from 230 to 150 mm. Hg within 8 hours with pressure returning to pretreatment levels in approximately 57 hours. The oral administration of 40 mg./Kg. of this compound to a normotensive dog produced a transient decrease in systolic blood pressure of 30 mm. Hg. The only observable effect of the compound in these animals was a relaxation of the nictitating membrane. JB 5058 was also administered once a day, 20 mg./Kg., per os, to two hypertensive dogs for periods of 7 and 12 days, respectively. The systolic pressure of the first dog decreased from 200 to 120 mm. Hg by the second day (Fig. 3). The blood pressure taken periodically each day

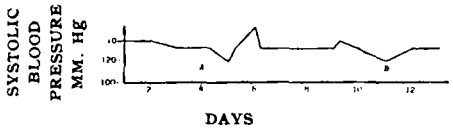


Fig. 5.—The effects of 20 mg./Kg. JB 5058, per os, daily in a normotensive dog. Key: A, initiation of JB 5058 administration; B, termination of treatment.

during the 7-day period varied from a low of 120 mm. Hg to a high of 150 mm. Hg. Treatment was then stopped, and the arterial pressure gradually returned to predrug levels in 4 days. The systolic blood pressure of the second hypertensive dog dropped from 240 to 155 mm. Hg over the 12-day period, returning to predrug levels 5 days after withdrawal of the compound (Fig. 4). JB 5058, 20 mg./Kg., administered orally each day to a normotensive dog did not significantly lower the systolic blood pressure of the animal over a 7-day period (Fig. 5).

DISCUSSION

JB 5058, *N,N'*-bis-[α-(2-pyridyl)ethyl]-ethylenediamine was the most active compound in a series of substituted polymethylenediamines. This compound produced marked hypotensive effects of long duration in anesthetized rats and dogs, reversed the pressor response to moderate doses of epinephrine, and decreased the pressor response to levarterenol. The reversal of epinephrine pressor response could be overcome by administering relatively large doses of epinephrine. The pressor response to bilateral carotid occlusion was decreased but not abolished, and the pressor response to single injections of angiotensin II was decreased approximately 25%. Pretreatment of anesthetized dogs with tripelannamine (sufficient to abolish the depressor response of 2.5 mcg./Kg. of histamine) and dichloroisoproterenol (up to 10 mg./Kg.) did not alter the hypotensive effect of this compound. These preliminary data suggest that JB 5058 blocks α adrenergic receptors (6) and that this action is most likely the primary mechanism of its hypotensive activity.

SUMMARY

Twenty-one substituted polymethylenediamines were investigated for their hypotensive activity. JB 5058, *N,N'*-bis-[α-(2-pyridyl)ethyl]-ethylenediamine dimaleate; EX 4467, *N,N'*-bis-[2-pyridylmethyl]-ethylenediamine dimaleate; EX 3571, *N,N'*-bis-[α-(2-pyridyl)ethyl]-*N,N'*-dimethylethylenediamine tetrahydrochloride; and EX 10006, *N,N'*-bis-[α-(2-pyridyl)ethyl]-trimethylenediamine tetrahydrochloride were the most active hypotensive compounds in the current series.

JB 5058 is a long-acting, orally effective hypotensive compound whose primary mechanism of action appears to be a blockade of α adrenergic receptors.

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Evaluation of Certain Hypotensive Agents VI

Tetramethylpiperidine Derivatives

By JOSEPH P. BUCKLEY, SIDNEY SHANOR, JOHN GLOSS, and WILLIAM J. KINNARD

The hypotensive activity of a series of 2,2,6,6-tetramethylpiperidine derivatives was investigated in anesthetized rats and dogs. The piperidine compounds were derivatives of pempidine (1,2,2,6,6-pentamethylpiperidine), an orally effective potent ganglionic blocking compound. *N*-Amino-*N*-[β-(2,2,6,6-tetramethylpiperidino-)-ethyl]-guanidine sulfate (EX 4510) appeared to be slightly more active than pempidine. All of the active hypotensive compounds in this series which were tested against pressor challengers in anesthetized dogs potentiated the epinephrine and angiotensin II pressor responses and depressed or blocked the bilateral carotid occlusion pressor response. Preliminary data suggest that the active compounds, like pempidine, are ganglionic blockers.

INVESTIGATORS (1-3) HAVE REPORTED that pempidine (1,2,2,6,6,-pentamethylpiperidine) is a long acting, orally effective ganglionic blocking compound. Smirk and Hodge (4) discussed the hypotensive activity of several compounds chemically related to pempidine and concluded that 2,2,6,6,-tetramethylpiperidine hydrochloride was slightly more potent than pempidine and was the most potent compound in this particular series. All of the related compounds appeared to be ganglionic blocking agents and produced side effects in hypertensive patients characteristic of this class of compounds. Several other investigators (5, 6) have also reported that pempidine is an orally effective ganglionic blocking compound in humans.

Twenty-six compounds,[1] chemically related to pempidine,[2] have been synthesized by Biel and his coworkers (7) and evaluated for hypotensive activity in rats and dogs (see Table I). This present report concerns the hypotensive activity of this series of compounds and pempidine.

EXPERIMENTAL

Hypotensive Activity in Normotensive Rats.—The compounds were evaluated for their hypotensive activity in anesthetized normotensive Wistar rats using the method described by Bickerton, *et al.* (8). Fresh aqueous solutions of the compounds were administered intravenously *via* a femoral vein and the blood pressure obtained by direct cannulation of a carotid artery and recorded on a slowly moving smoked kymograph. Blood pressure recordings were measured 10 minutes after administration of the compound. Compounds producing effects of less than 10 minutes' duration were classified as inactive.

Hypotensive Activity in Normotensive Dogs.—Certain of the compounds were further evaluated in anesthetized normotensive dogs. Mongrel dogs were anesthetized with pentobarbital sodium, 35 mg./Kg., i.v., and the blood pressure recorded from a femoral artery onto a Grass polygraph utilizing a Statham pressure transducer. The effects of the compounds on the pressor responses to 10-second bilateral carotid occlusion; epinephrine, 1 to 2 mcg./Kg., i.v.; and synthetic angiotensin II,[3] 1 mcg./Kg., i.v., were also investigated. In several preparations 1 to 2 mcg./Kg. levarterenol was also administered intravenously.

RESULTS

Hypotensive Activity in Normotensive Rats.—The hypotensive activity of the compounds studied in this current investigation is summarized in Table II. All of the compounds except EX 4608, EX 4493, EX 4399, and EX 4890 produced some degree of hypotensive activity. The most active compounds were pempidine; EX 4510, *N*-amino-*N*-[β-2,2,6,6-tetramethylpiperidino)-ethyl]-guanidine sulfate; EX 4602, 2,2,6,6-tetramethylpiperidine succinate, EX 4454, di-(2,2,6,6-tetramethylpiperidine) dihydrogenethylenediamine tetraacetate; EX 10020, *N*-methylamino-2,2,6,6-tetramethylpiperidine tartrate monohydrate; EX 10041, 4-nitrato-2,2,6,6-tetramethylpiperidine maleate; EX 4690, 2,2,6,6-tetramethyl piperidine salicylate; and EX 4707, 1,2,2,7,7-pentamethyl - 4 - (β -chloroethyl) -homo -piperazine dihydrochloride.

EX 4510 appeared to be slightly more active than pempidine, whereas EX 4602 appeared to be approximately equal in hypotensive action to pempidine. EX 4608, α-(2,6-dimethylphenoxy)-β-(2′,2′,6′,6′-tetramethylpiperidino)-ethane hydrochloride produced marked pressor effects in the anesthetized rat, and EX 4453, *N*-β-chloroethyl-2,2,6,6-tetramethylpiperidine hydrochloride, produced stimulatory effects.

Hypotensive Activity in Normotensive Dogs.—The effects of the compounds in anesthetized dogs are summarized in Table III. EX 4510 was also the most active compound when investigated in

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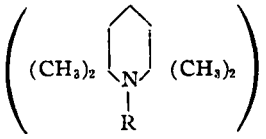
[1] Experimental compounds supplied by Lakeside Laboratories, Milwaukee, Wis.

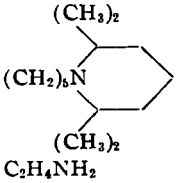
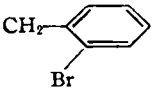
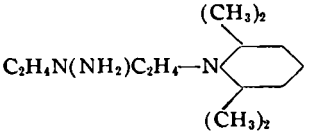
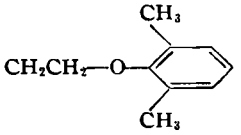
[2] Pempidine kindly supplied by May and Baker, Ltd., Dagenham, Essex, England.

[3] Kindly supplied as Hypertensin by Ciba Pharmaceutical Products, Inc., Summit, N. J.

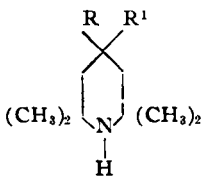
TABLE I.—CHEMICAL STRUCTURES OF COMPOUNDS INVESTIGATED

Group I



Compd.	R	X	R¹
Pempidine	CH_3	1	Bitartrate
EX 4392	$CH_2COOC_2H_5$	1	Maleate
EX 4399	CH_2COOH	1	
EX 4431	H	1	$(CSNHCH_2CO_2H)_2$
EX 4450	CH_2CH_2OH	1	Maleate
EX 4453	CH_2CH_2Cl	1	HCl
EX 4454	H	2	Ethylenediamine tetraacetate
EX 4458		1	2HBr
EX 4464	$C_2H_4NH_2$	1	2 HCl
EX 4469	$C_2H_4SCCH_3$ (O)	1	HCl
EX 4489	$C_2H_4NHCNH_2$ (NH)	2	H_2SO_4
EX 4493		1	HCl
EX 4510	$C_2H_4N(NH_2)C(=NH)NH_2$	2	H_2SO_4
EX 4513		2	3 HCl
EX 4531	$C_2H_4NH\ NH_2$	1	2 HCl
EX 4602	H	1	Succinate
EX 4608		1	HCl
EX 4690	H	1	Salicylate
EX 10020	$NHCH_3$	1	Tartrate monohydrate

Group II



·R²

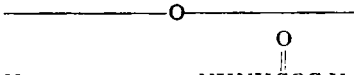
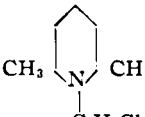
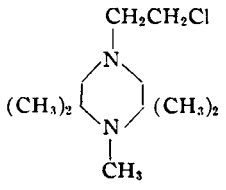
EX 4451			Maleate
EX 4601	H	$NHNHCOC_2H_5$ (O)	2 HCl
EX 4611	H	OH	HCl

TABLE I.—(*continued*)

Compd.	R	X	R¹
EX 4890	H	$NHNH_2$	Dimaleate
EX 10041	H	ONO_2	Maleate
		Group III	
EX 4634			·HCl
EX 4707			·2 HCl

anesthetized dogs. EX 4608, a compound which produced hypertensive effects in rats, failed to alter the blood pressure of an anesthetized dog. EX 4489, a compound which was lethal in rats in doses over 2.5 mg./Kg. was also lethal at the 20 mg./Kg. dosage in anesthetized dogs. In general, there was good correlation between the data obtained in anesthetized rats and that obtained in the anesthetized dog. All of the active pempidine derivatives produced responses characteristic of ganglionic blocking agents in that they potentiated the epinephrine pressor response, depressed or blocked the pressor response induced by bilateral carotid occlusion, and potentiated the anglotensin II and levarterenol pressor responses. The preliminary data obtained in this study suggest that the active compounds, like pempidine, produced their hypotensive effects through blockade of autonomic ganglia.

DISCUSSION

The following structure-activity relationship of the compounds reported in this communication was obtained utilizing the data found in Table II. The replacement of the methyl group in the number 1 position by an *N*-amino-*N*-ethylguanidine group (EX 4510) appeared to enhance the hypotensive activity of the piperidine structure, whereas replacement by a hydrogen group (EX 4602) produced a compound which was approximately equally potent to pempi-

TABLE II.—EVALUATION OF CERTAIN HYPOTENSIVE COMPOUNDS ON THE BLOOD PRESSURE OF NORMOTENSIVE RATS

Compd. No.	Dose, mg./Kg.	Animals, No.	Drop in b.p. % ±S.D.	Mean Time to Return to Predrug Levels, min. ±S.D.	Rating and Comments
Pempidine	5.0	8	36.7 ± 11.3	250 +	++++
EX 4392	10.0	5	35 ± 7	13 (2–34)	+
EX 4399	80.0	7	...	...	Inactive
EX 4431	20.0	6	26 ± 9	203 ± 78	++++
EX 4450	10.0	8	48 ± 16	77 ± 52.4	++
EX 4451	25.0	4	44 ± 6	104 ± 61.2	+++
EX 4453	40.0	5	11 to 53	...	Analeptic, convulsions
EX 4454	5.0	6	39 ± 5.7	184 ± 93	++++
EX 4458	10.0	6	35 ± 18	113 ± 58	+++
EX 4464	40.0	2	44.5	70.5	++
EX 4469	15.0	6	36 ± 12.8	59 ± 29	++
EX 4489	2.5	2	46.5	21	+
EX 4493	40.0	9	...	...	Primary pressor effect
EX 4510	5.0	6	44.5 ± 8.0	350+	++++
EX 4513	5.0	2	0	0	0
EX 4513	40.0	2	0	0	0
EX 4531	10.0	8	30.4 ± 7.2	53 ± 29	++
EX 4601	40.0	6	36.5 ± 5.9	84 ± 8	+++
EX 4602	5.0	6	39.2 ± 5.7	250+	++++
EX 4608	20.0	10	...	...	Pressor
EX 4611	25.0	10	43 ± 10	69 ± 30	++
EX 4634	40.0	9	28 to 43	10 to 36	+
EX 4690	10.0	8	42 ± 5	287 ± 9	++++
EX 4707	5.0	6	51.8 ± 7	141 ± 45	++++
EX 4890	40.0	6	...	...	Inactive
EX 10020	25.0	6	36 ± 9	354 ± 144	++++
EX 10041	25.0	6	42 ± 26	237 ± 22	++++

TABLE III.—EFFECTS OF EXPERIMENTAL COMPOUNDS ON THE BLOOD PRESSURE AND SEVERAL PRESSOR RESPONSES IN THE ANESTHETIZED[a] DOG

Compd. No.	Dose, mg./Kg.	Dogs, No.	% Drop in b.p.	Time to Return to Predrug Levels, min.	% Control Responses			
					BCO[b]	Epi[c]	Ang[d]	L-art[e]
Pempidine	5	1	51	720+	17	171	132	...
EX 4392	10	1	0	0	...	...	...	...
EX 4431	5	1	41	155+	0	114	156	...
EX 4451	2.5	1	68	190+	0	294	289	...
EX 4451	10	2	47	150+	57	215	117	...
EX 4458	10	1	50	200+	0	206	214	...
EX 4469	10	1	29	200+	34	190	120	...
EX 4489	20	1	Lethal	...	...	...	...	...
EX 4493	10	1	69	80	100	191	90	...
EX 4510	5	2	46	720+	24	150	139	...
EX 4513	10	2	8	9	...	...	...	...
EX 4531	10	2	44	415	23	172	136	...
EX 4601	20	2	28	170	31	251	177	...
EX 4602	5	1	32	600	25	174	192	...
EX 4608	5	1	0	0	84	75	100	...
EX 4611	20	2	35	233+	8	174	161	174
EX 4634	30	1	53	86	0	131	100	...
EX 4690	10	1	33	114+	0	285	261	173
EX 4707	2.5	1	27	100	55	132	117	162
EX 4707	5	1	34	93	21	273	137	184
EX 4890	10	1	26	105	72	91	114	...
EX 10020	15	1	43	160+	0	168	178	...
EX 10041	20	1	58	163+	0	211	90	176

[a] Anesthetic, pentobarbital sodium, 35 mg./Kg., i.v. [b] BCO = 10-second bilateral carotid occlusion. [c] Epi = epinephrine [d] Ang = synthetic angiotensin II. [e] L-art = levarterenol.

dine. The ethylhydrazine substitution in the 1 position (EX 4531) greatly reduced activity. Carbethoxyhydrazine substitution in the 4 position of 2,2,6,6-tetramethylpiperidine (EX 4601) markedly reduced hypotensive activity. β-Chloroethyl substitution at the 1 position (EX 4453) produced analeptic effects, while pressor activity was produced by ethylphenoxy (EX 4608) or *o*-bromobenzyl (EX 4493) substitution at the 1 position.

Smirk and Hodge (4) have reported that the *N*-methylamino substitution at the 1 position in place of a methyl group did not alter hypotensive activity in humans. In this current study, the *N*-methylamino substituted compound (EX 10020) was found to be approximately one-fifth as potent as pempidine on a dose-dose relationship. Although the 2,2,6,6-tetramethylpiperidine structure (EX 4602, EX 4690, and EX 4431) was highly active, it did not appear to be more potent than the parent pempidine structure when administered intravenously to rats.

SUMMARY

The hypotensive activity of a series of tetramethylpiperidine derivatives, chemically related to pempidine, was investigated.

N - Amino - *N* - [β - (2,2,6,6 - tetramethylpiperidino)-ethyl]-guanidine sulfate (EX 4510) appeared to be a slightly more active hypotensive compound than pempidine. The data reported in this current study suggest that the active compounds, like pempidine, produced their hypotensive effects through blockade of autonomic ganglia.

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Interaction of Weak Organic Acids with Insoluble Polyamides II

Study of Sorption of Selected Weak Organic Acids by Nylon 610

By A. J. KAPADIA, W. L. GUESS, and J. AUTIAN

Sorption studies were conducted at several different temperatures for a group of compounds having or behaving as weak organic acids using nylon 610 as the substrate. Six compounds were included in the investigation—benzoic acid, *o*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, methyl *p*-hydroxybenzoate, and propyl *p*-hydroxybenzoate. The collected data permitted the evaluation for each compound of (*a*) the saturation value of solute in the nylon, (*b*) the affinity of the solute toward the plastic, (*c*) the standard heat of sorption, (*d*) the apparent diffusion coefficient and (*e*) the activation energy of diffusion. With the Rotinyan and Drozdov equation, it was possible to confirm that the diffusion of the solute in the plastic was the rate-determining step in the sorption process.

GREATER USE is being found for insoluble polymeric materials in pharmacy and medicine. Such diverse uses as packaging material, administration devices, prosthetic devices, and vehicles for prolonged action medication emphasize the versatility of plastic materials and suggest even greater applications in the future for plastics in the various facets of pharmacy and medicine.

One particular phase of plastics which deserves special attention is the possible interaction of drugs with plastics. A knowledge of such reactions may be helpful to the industrial pharmacist and other investigators interested in solute-polymer interactions.

A previous paper (1) presented results from a sorption study of salicylic acid by nylon 66 and showed the influence of concentration, solvent system, temperature, and pH on the uptake of the drug by the nylon. It was assumed that the salicylic acid was adsorbed on the surface of the nylon followed by penetration and diffusion of the drug into the plastic. The rate-determining step, it was deduced, was the diffusion process.

This paper reports a sorption study of six agents by nylon 610. From the data it has been possible to derive some thermodynamic constants which may be used to compare the six agents. Kinetic treatment of the data also makes possible the calculation of apparent diffusion coefficients. Finally, the data may be treated in a manner to justify the assumption that the rate controlling step in the sorption process is one of diffusion in the plastic.

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EXPERIMENTAL

Equipment and Supplies.—Beckman DB spectrophotometer, Fullerton, Calif.; pH meter, Leeds and Northrup Co., Philadelphia, Pa.; benzoic acid, A.R., J. T. Baker Chemical Co., Phillipsburg, N. J.; *o*-hydroxybenzoic acid, A. R., Mallinckrodt Chemical Works, St. Louis, Mo.; *m*-hydroxybenzoic acid, A. R., K & K Laboratories, Inc., Jamaica, N. Y.; *p*-hydroxybenzoic acid, A. R., Eastman Organic Chemicals, Rochester, N. Y.; methyl *p*-hydroxybenzoate, A. R., Eastman Organic Chemicals, Rochester, N. Y.; propyl *p*-hydroxybenzoate, A. R., Eastman Organic Chemicals, Rochester, N. Y.; Nylon 610, (Zytel Fe-2419 NC-10) E. I. du Pont de Nemours & Co., Wilmington, Del., were employed.

Treatment of Nylon 610 Samples.—It was necessary to devise a washing procedure which would ensure the purity of samples needed for the experiments to remove contaminates or other components from the samples of nylon used throughout this investigation. The exact method of cleaning is described below.

Strips of nylon, measuring 1 × 13 in. were cut using a paper cutter and placed (approximately 200 strips) into a glass jar with 3500 ml. of solvent ether. The jar was capped and set aside (with intermittent shaking) for 2 hours at which time the solvent was decanted and acetone (3500 ml.) added. After 2 hours this solvent was decanted and the strips rinsed with distilled water, followed by the addition of 50% (v/v) ethyl alcohol. After 12 hours of contact, the alcoholic eluate was decanted and once again the strips rinsed with distilled water. The final step of washing consisted of exposing the strips to absolute ethyl alcohol for 2 hours, followed by repeated rinsings with distilled water. The strips were placed into a constant temperature oven at 65 ±1.0° for 8 hours to dry, then transferred into a desiccator for further use.

General Procedure for Studying Equilibrium Sorption Isotherms.—Exactly 150 ml. of each solution, prepared to a known concentration, was pipeted into the specially prepared glass tubes.

These tubes were closed by using ground glass stoppers and placed into a constant temperature water bath at a temperature slightly above the temperature which was to be used in the experiment. After an equilibrium temperature was reached inside the tube, the tubes were withdrawn, the stoppers removed, and a single strip of nylon (prepared as described earlier), previously weighed,[1] was immersed into the solution. The ground-glass stoppers were securely inserted to prevent leakage and the tubes placed back into the water bath at a specified temperature for a time period sufficient to ensure that equilibrium had been reached. Each experiment was done in duplicate. A control solution of each agent, without the nylon, was also included in each experiment.

Aliquots were drawn from each tube after equilibrium had been reached and the concentration of compound remaining in the solution determined. The difference between the amount of compound in the control solution and the amount left in the solution was considered as the quantity of agent sorbed by the nylon.

Method of Analysis.—In the experiments to be reported, six agents were employed. The assay for each agent was based upon a spectrophotometric method, using a Beckman spectrophotometer employing matched 1-cm. silica absorption cells. To determine the wavelength of maximum absorption, ultraviolet absorption spectra for methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate were carried out using distilled water as the solvent, while benzoic acid, *o*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid were run in a 0.1 *N* hydrochloric acid solution. Appropriate blanks were used in all cases. From the absorption curves of each agent, the maximum wavelengths found were: benzoic acid, 229 mμ; *o*-hydroxybenzoic acid, 303 mμ; *m*-hydroxybenzoic acid, 236 mμ; *p*-hydroxybenzoic acid, 255 mμ; methyl *p*-hydroxybenzoate, 255 mμ; and propyl *p*-hydroxybenzoate, 255 mμ. Each of the solutions obeyed the Beer-Lambert relationship in the concentration range employed.

During the actual experiments, concentrations of the agents in each solution were determined by removing an aliquot sample from the test solution, bringing it to room temperature, diluting with the appropriate solvent or solution until the concentration was in a range suitable for determination of the optical density in the spectrophotometer. In each instance, methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate were diluted with distilled water while the other four compounds were diluted with 0.1 *N* hydrochloric acid. Suitable blanks were run simultaneously with each compound. The optical density was converted to an equivalent concentration unit based upon standard solutions of each drug.

Effect of Concentration on Sorption.—An experiment was conducted at a temperature of 50±0.02° to determine the equilibrium concentration of each agent sorbed by nylon 610 at different original concentrations. The data was then fitted to a Langmuir relationship

$$\frac{1}{q} = \frac{1}{KSC} + \frac{1}{S} \qquad \text{(Eq. 1)}$$

where q is the amount of solute sorbed at equilibrium in moles per kilogram, K is a constant, S the saturation concentration when all sites in the nylon are occupied, and C is the equilibrium concentration of the agent in the solution in moles per liter. When $1/q$ is plotted againt $1/C$, a linear relationship exists. From the intercept $(1/S)$, it is possible to calculate S or the saturation value for each agent.

Table I summarizes $1/q$ and $1/C$ values for all of the six agents and the calculated S values. Figure 1 represents a Langmuir plot of one of the agents. The intercepts for the six compounds were computed by least square methods, using only those points which fell on the linear portion of the line.

Affinity and Heat of Sorption.—To accumulate useful data on the interaction of solute molecules with polymeric materials, it was necessary to find a way of describing the magnitude of the interaction in the form of an affinity value. The standard molar free energy or standard chemical potential ($-\Delta\mu°$) has been used in dyeing as a means of quantitizing the interaction of a dye with a particular fiber at steady state conditions, and this same value ($-\Delta\mu°$) has been called the "standard affinity" of the solute for the polymeric material. If it is assumed that the solute molecules are in the unionized state in both the liquid phase and the solid phase, an appropriate equation may be written for the affinity as

$$-\Delta\mu° = RT \ln a_s/a_l \qquad \text{(Eq. 2)}$$

where $-\Delta\mu°$ is the difference in standard chemical potential between the solute in the liquid phase and the solute in the solid phase, a_s is the activity of the solute in the solid phase, a_l the activity of the solute in the liquid phase, and RT the usual gas constant and absolute temperature, respectively. In dilute solution, the activities may be replaced by concentration terms or

$$-\Delta\mu° = RT \ln C_s/C_l \qquad \text{(Eq. 3)}$$

where C_s is in moles/Kg. in plastic and C_l is the concentration of the solute in the liquid phase expressed in the same units (moles/Kg.)

A series of experiments, whereby the concentration of the solute in the liquid phase and the solid phase were determined after equilibrium had been reached, were conducted at several temperatures. Calculated values for $-\Delta\mu°$ are recorded in Table II and have been rounded off to three significant places since the accuracy of these constants are probably no greater than to the third significant figure.

The standard affinity and the standard heat of sorption ($\Delta H°$) are related by

$$\frac{-\Delta\mu°}{T} = \frac{\Delta H°}{T} + C \qquad \text{(Eq. 4)}$$

where $-\Delta\mu°$ and $-\Delta H°$ are as already defined, T the absolute temperature, and C a constant. A plot of $-\Delta\mu°/T$ *versus* $1/T$ will give a linear relationship with a slope equal to $\Delta H°$ A representative plot for methyl *p*-hydroxybenzoate is shown in Fig. 2; the $\Delta H°$ values for all the agents are included in Table II. The negative sign indicates that the sorption process is an exothermic reaction.

Diffusion and Activation Energy of Diffusion.—Experiments were conducted to evaluate the apparent diffusion coefficient of each of the six

[1] Average weight of strips of nylon was approximately 2.0 Gm.

TABLE I.—EFFECT OF CONCENTRATION ON SORPTION OF SIX AGENTS BY NYLON 610 AT 50 ± 0.02° C.

Benzoic Acid		*o*-Hydroxybenzoic Acid		*m*-Hydroxybenzoic Acid	
1/C[b]	1/q[a]	1/C	1/q	1/C	1/q
961.5[d]	35.36[d]	1191.0	27.05	1191.0[d]	27.01[d]
386.5	13.77	587.9	14.15	586.2[d]	14.31[d]
289.1	10.50	391.5	9.4607	385.1	10.09
109.6	7.463	294.0	7.072	288.4	7.609
142.7	5.637	234.5[d]	5.718[d]	229.3	6.241
113.5	4.682	...	...	188.6	5.495
94.07	4.045	...	...	140.3	4.270
...	...	...	...	110.1	3.740
S^c = 1.05 moles/Kg.		S = 1.25 moles/Kg.		S = 0.934 moles/Kg.	
p-Hydroxybenzoic Acid		Methyl *p*-Hydroxybenzoate		Propyl *p*-Hydroxybenzoate	
1/C	1/q	1/C	1/q	1/C	1/q
1115.0[d]	25.65[d]	1609.0[d]	33.36[d]	2057.0	9.285
591.37[d]	13.87[d]	592.4[d]	14.87[d]	1634.0	7.557
350.9[d]	8.658[d]	394.8	8.850	1372.0	6.116
287.9	7.645	260.2	6.135	1168.0	5.383
228.6	6.315	194.0	4.700	811.0	3.865
189.4	5.394	153.0	3.965	...	...
140.1	4.300	...	...	...	...
110.4	3.708	...	...	...	...
S = 0.837 moles/Kg.		S = 1.22 moles/Kg.		S = 3.81 moles/Kg.	

[a] q = Amount of solute sorbed at equilibrium in moles/Kg. [b] C = Equilibrium concentration of solute in solution (moles/L.). [c] Saturation concentration of solute in nylon. [d] Points which did not fall on the linear portion of curve.

agents in nylon 610 at various temperatures. Solutions and samples were prepared as described earlier and the uptake of solute by the plastic determined at different time periods until equilibrium was reached. The apparent diffusion coefficient was calculated for each solute at each temperature by using Berthier's method as reported in a previous paper (1). In general, this procedure involves the plotting of two curves: (*a*) the experimental data is plotted as fractional uptake (M_t/M_∞) of the solute by plastic *versus* the square root of time, and (*b*) a theoretical curve based upon Berthier's work where fractional uptake (at a final per cent uptake) is plotted against the square root of a constant, K. The constant K is equal to Dt/l^2, D being the apparent diffusion coefficient (in cm.²/sec.), t the time at any fractional uptake, and l the thickness of the plastic. Since the solution has contact with both surfaces of the plastic, the thickness is divided by 2. It can be seen that a comparison of one curve with the other will permit the calculation of D. An experimental curve of fractional uptake (M_t/M_∞) *versus* square root of time is shown in Fig. 3 for one compound. Table III presents the apparent diffusion coefficient for each of the agents at each temperature.

By the use of the Arrhenius relationship

$$\log D = \log D_0 - \Delta E/2.303\,RT \quad \text{(Eq. 5)}$$

the activation energy of diffusion ($-\Delta E$) may be computed from the slope of the line when log D is plotted against $1/T$. Figure 4 demonstrates one such plot for one agent. Table III contains the calculated activation energies of the six agents studied.

Diffusion versus Rate of Interaction.—It has been assumed that the rate-determining step in the sorption of solute molecules by nylon is the diffusion process in the plastic in the experiments so far described. Much information from the dye industry lends support to this thesis, but it was felt that added proof of this fact with the six agents used in this investigation with nylon 610 would serve a useful purpose.

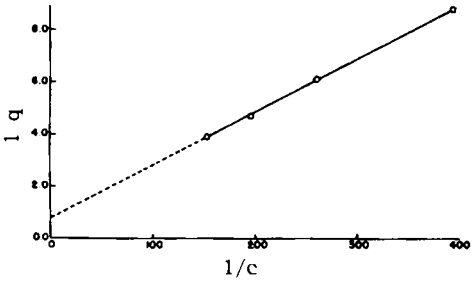


Fig. 1.—Sorption of methyl *p*-hydroxybenzoate by nylon 610 as a Langmuir plot at 50 ± 0.02°C.

Rotinyan and Drozdov (2) developed a basic mathematical formula which could be used to evaluate both the diffusion coefficient and the rate constant for a chemical reaction (first order) which could be applied to such diverse processes as roasting, leaching, washing, and cementation. The equation may be stated

$$\frac{1}{t} \ln \frac{a}{a - x} - \beta \frac{x}{t} = M \quad \text{(Eq. 6)}$$

where a is the initial concentration of solute in the liquid phase, x is the concentration of the solute reacting in time, t, and β and M are constants further defined as

$$\beta = \frac{vk}{kva + DSB\mu} \quad \text{(Eq. 7)}$$

$$M = \frac{kDSB\mu}{kva + DSB\mu} \quad \text{(Eq. 8)}$$

where v is the volume of solution in contact with the solid phase, k is the first-order rate constant, D is the diffusion coefficient, S is the surface area of the solid phase, and μ is a coefficient allowing for the transition from surface to volume concentration. The term β in the above two equations is equal to $xv/S\lambda$, where x and v are as previously defined, and λ is the thickness of the growing diffusion zone in the solid.

Equation 6 appears to be a very general equation

TABLE II.—AFFINITIES AND HEATS OF SORPTION OF SIX AGENTS BY NYLON 610

Benzoic Acid		*o*-Hydroxybenzoic Acid		*m*-Hydroxybenzoic Acid	
Temp., °C.	$-\Delta\mu^{\circ}$[b]	Temp., °C.	$-\Delta\mu^{\circ}$	Temp., °C.	$-\Delta\mu^{\circ}$
65.0	1.96	65.0	2.17	67.0	2.20
60.0	2.04	55.0	2.20	55.0	2.22
55.2	2.07	50.0	2.22	50.0	2.22
50.0	2.08	...	...	45.0	2.24
45.0	2.10	...	...	...	...
ΔH°[c] = −3.19		ΔH° = −3.39		ΔH° = −2.78	
(75 mg./ 150 ml.)[a]		(225 mg./ 150 ml.)		(75 mg./150 ml.)	

p-Hydroxybenzoic Acid		Methyl *p*-Hydroxybenzoate		Propyl *p*-Hydroxybenzoate	
Temp., °C.	$-\Delta\mu^{\circ}$	Temp., °C.	$-\Delta\mu^{\circ}$	Temp., °C.	$-\Delta\mu^{\circ}$
65.0	2.24	69.7	2.16	65.0	3.06
60.0	2.25	65.0	2.19	60.0	3.04
55.0	2.27	55.0	2.23	55.2	3.04
50.0	2.24	50.0	2.26	50.0	3.04
45.0	2.28	45.0	2.28	45.0	3.03
ΔH° = −2.75		ΔH° = −3.63		ΔH° = −2.60	
(75 mg./ 150 ml.)		(150 mg./ 150 ml.)		(45 mg./150 ml.)	

[a] Original solution concentration. [b] $-\Delta\mu^{\circ}$ = Difference in standard chemical potential between the solute in the liquid phase and the solute in the solid phase (in Kcal./mole) or *affinity*. [c] $-\Delta H^{\circ}$ = Standard heat of sorption (in Kcal./mole).

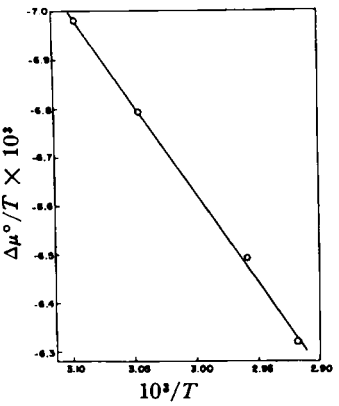


Fig. 2.—Relationship between standard affinity of methyl *p*-hydroxybenzoate toward nylon 610 as a function of temperature.

which may be applied to a process where molecules from one phase react at the surface of a second phase, then penetrate and diffuse into the second phase interacting at various sites as diffusion continues. If $1/t \ln(a/a - x)$ is plotted against x/t, a straight line should result with a slope of β and an intercept of M. To test the validity of Eq. 6, a plot was made of all six agents sorbed by the nylon; in each instance a linear relationship was noted. Figure 5 shows one such plot for methyl *p*-hydroxybenzoate. The values for β and M (by least squares) are shown in Table IV for each of the six agents at each temperature.

If we now assume that the diffusion process is the determining step in the sorption, then

$$DSB\mu \ll kva;\ \beta \rightarrow 1/a;\ M \rightarrow DSB\mu/va$$

Table IV shows that the calculated values for $1/a$ do approach the β values, hence the assumption of diffusion being the rate-determining step is justified. If $1/a$ were exactly equal to β, the diffusion coefficient would be zero, and the reaction would take place only at the surface of the plastic.

Effect of pH on Sorption.—To determine the effect of pH on the sorption of certain acidic compounds, benzoic acid, *o*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid were prepared as aqueous solutions at different pH values using hydrochloric acid at very low pH values and sodium hydroxide at the higher pH values. The other two agents (methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate) were not included in this experiment since degradation was taking place in the alkaline pH range. These solutions were placed in contact with samples of nylon 610 in glass tubes, stoppered, and stored in a constant temperature water bath at a temperature of 50 ± 0.02° for a period of time sufficient for equilibrium to have been reached. The amount of agent sorbed by the nylon for each compound at each pH was determined. At the same time the pH of each solution was measured. Plots of uptake *versus* final pH for each of the agents were made and in each instance appeared as shown for benzoic acid in Fig. 6.

DISCUSSION

Nylon 610 is a condensation polymer prepared from hexamethylenediamine and sebacic acid. In general, these polyamides have a number average molecular weight of around 25,000. The polymer, as with all nylons, has three polar sites in the chain (3): (*a*) terminal carboxylic end groups, (*b*) terminal amino groups (at other end), and (*c*) repeated amide groups. It is believed that interactions of certain ionic compounds are with the end amino groups, while those unionized compounds having acidic hydrogens will interact primarily at the amide linkages (3–5). In the sorption process solute molecules will penetrate into the nylon and diffuse through the amorphous zone. The crystalline regions in the nylon prevent the passage of solute molecules due to the extremely high degree of polymer chain orientation.

Samples of nylon 610 used in the experiments reported here were taken from the same lot of nylon to

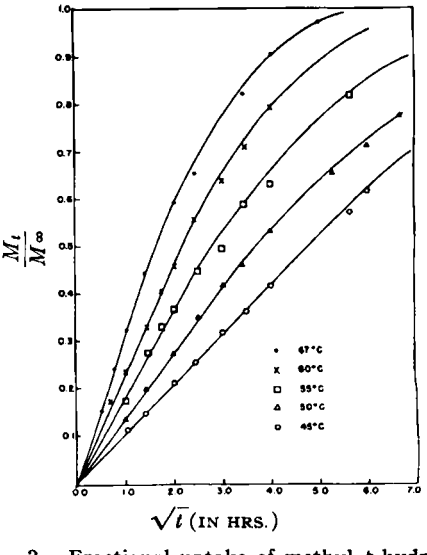


Fig. 3.—Fractional uptake of methyl *p*-hydroxybenzoate by nylon 610 *vs.* square root of time at a number of temperatures.

TABLE III.—APPARENT DIFFUSION COEFFICIENTS OF SIX AGENTS IN NYLON 610 AND ACTIVATION ENERGIES OF DIFFUSION

Compd. Name	Temp., °C.	Diffusion Coefficient, cm.²/sec.	Activation Energy, Kcal./mole
Benzoic acid (75 mg./150 ml.)[a]	65.0	3.32×10^{-9}	12.9
	60.0	1.46	
	55.2	1.04	
	50.0	0.693	
	45.0	0.305	
o-Hydroxybenzoic acid (225 mg./150 ml.)	65.0	3.06×10^{-9}	19.9
	60.0	1.99	
	55.0	1.35	
	50.0	0.770	
m-Hydroxybenzoic acid (75 mg./150 ml.)	60.0	9.15×10^{-10}	18.9
	55.0	5.61	
	50.0	3.93	
	45.0	2.34	
p-Hydroxybenzoic acid (75 mg./150 ml.)	65.0	9.40×10^{-10}	17.9
	60.0	7.44	
	55.0	4.74	
	50.0	3.07	
Methyl *p*-hydroxybenzoate (150 mg./150 ml.)	67.0	3.54×10^{-9}	20.9
	60.0	1.81	
	55.2	1.16	
	50.0	0.720	
	45.0	0.372	
Propyl *p*-hydroxybenzoate (45 mg./150 ml.)	65.0	3.93×10^{-9}	14.5
	60.0	2.98	
	55.2	1.73	
	45.0	1.01	

[a] Original concentration in solution.

ensure a uniform sample. In our own laboratory it has been found that there may be significant differences in certain physical properties of nylon when samples are taken from different lots. In these experiments, as well as others, it was necessary to wash the nylon thoroughly with selected solvents to remove any contaminates. Inadequate or improper washing led to erratic results.

Effect of Concentration.—An increase in the original concentration for each compound resulted in an increase in the equilibrium sorption of the compound by the nylon at isothermal conditions. A Langmuir plot (Fig. 1) made possible the calculation of the theoretical amount of solute which would be sorbed by the nylon if all the sites in the nylon were filled. Table I presents these values. In general the S values approached 1.0 moles/Kg. In the hydroxybenzoic acid group, the S value decreased as the hydroxyl group moved from the *ortho* to the *meta* to the *para* position. The highest value for S was seen with propyl *p*-hydroxybenzoate.

One must be careful in taking these saturation values as more than they are—approximate values. Slight changes in the slope of the line in the Langmuir plot (Fig. 1) will give different intercepts which can lead to significant changes in S values. Even though theoretically it is assumed that the extrapolated portion of the curve is to continue in a linear fashion, this may not actually be the case. Certain solute molecules (*i.e.*, phenol), when they reach a critical concentration in the solution, may alter the morphology of the nylon, creating more sites for interactions. In other words, for compounds of the phenolic type, data used from dilute solutions will give an S value which will be much less than S values taken from the more concentrated solutions. With the compounds used in these studies in the concentration range employed, no dramatic break was noted in the curves, even though five of the agents were phenolic in nature. However, there may have been a break in the curve if a much broader concentration range had been used.

The S value for propyl *p*-hydroxybenzoate was much higher than for the other compounds. The reason for this result may be attributed to the increase in hydrophobic character of the propyl *p*-hydroxybenzoate, which would tend to push this compound from the aqueous phase into the solid phase (plastic).

The saturation value may be used to compare different compounds sorbed by nylon and may be helpful in predicting if changes have occurred in nylon when stored under various conditions. For example, if the saturation value changes appreciably for the same nylon after storage, it should be evident that the plastic has been altered in some way.

Affinity and Heat of Sorption.—The affinity values for each of the compounds (except one in Table II)

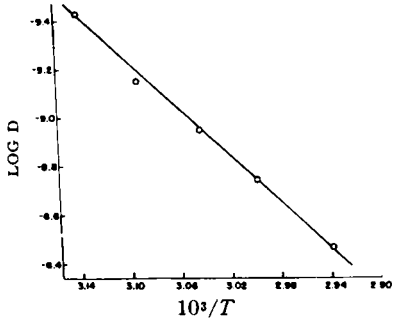


Fig. 4.—An Arrhenius plot of methyl *p*-hydroxybenzoate as log D *vs.* $1/T$.

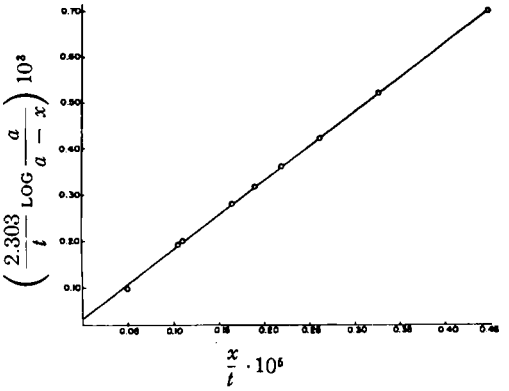


Fig. 5.—A plot of Rotinyan and Drozdov equation from kinetic data for methyl *p*-hydroxybenzoate. (See text for explanation).

TABLE IV.—VALUES OF SLOPE (β) AND INTERCEPT (M) FROM PLOTS OF KINETIC DATA USING THE ROTINYAN AND DROZDOV EQUATION

Compd. Name	Temp., °C.	$\beta \cdot 10^{-2}$	$M \cdot 10^{3}$	Theoretical[a] $1/a \cdot 10^{-2}$
Benzoic acid	65.0	2.498	0.07219	2.442
	60.0	2.487	0.05382	
	55.2	2.386	0.04370	
	50.0	2.462	0.03104	
o-Hydroxybenzoic acid	65.0	0.9211	0.1291	0.9207
	60.0	0.9175	0.09138	
	55.0	0.9305	0.06259	
	50.0	0.9216	0.04496	
m-Hydroxybenzoic acid	60.0	2.754	0.04874	2.762
	55.0	2.748	0.03258	
	50.0	2.784	0.01933	
	45.0	2.722	0.01577	
p-Hydroxybenzoic acid	65.0	2.881	0.04524	2.762
	60.0	2.842	0.03672	
	55.0	2.781	0.02605	
	50.0	2.819	0.01629	
Methyl *p*-hydroxybenzoate	60.0	1.580	0.07427	1.521
	55.2	1.585	0.05374	
	50.0	1.556	0.03981	
	45.0	1.539	0.02966	
Propyl *p*-hydroxybenzoate	65.0	6.708	0.4428	6.006
	60.0	6.580	0.3880	
	50.0	6.606	0.2497	

[a] a = Initial concentration of solution.

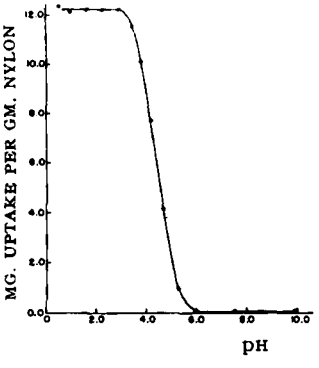


Fig. 6.—The effect of pH on sorption of benzoic acid by nylon 610.

reveal that as the temperature is decreased the affinity increases. This is expected since the distribution of solute molecules between the plastic and the solution will tend to move toward the solid phase with a decrease in temperature. No suitable explanation could be given for the reverse of this tendency in regard to propyl *p*-hydroxybenzoate. As may be noted from the table, at the same temperature, the $-\Delta\mu^\circ$ values for most of the compounds fell into a relatively narrow range. For example, for all of the compounds except propyl *p*-hydroxybenzoate at 50°, $-\Delta\mu^\circ$ was around 2.2 Kcal./mole, indicating that the affinity for these compounds toward nylon was approximately equal. The standard chemical potential ($-\Delta\mu^\circ$) of the propyl *p*-hydroxybenzoate at the same temperature (50°) was slightly above 3.0 Kcal./mole, indicating that this compound has the greatest affinity for the nylon 610 in the series of compounds studied.

Heats of sorption for the six compounds shown in Table II range from a value of −2.60 Kcal./mole for propyl *p*-hydroxybenzoate to −3.63 Kcal./ mole for methyl *p*-hydroxybenzoate. Even though heats of sorption values should not be equated directly to intermolecular forces existing between solute molecules and the nylon, some generalization may be made. Low values of ΔH° for the six compounds suggest that these compounds are bound to the nylon by weak molecular forces, probably of the van der Waals' type. Browne and Steele (6) have found that heat of sorption for benzoic acid by nylon 610 is approximately −2.2 Kcal./mole and have interpreted their experimental results on a number of organic acids sorbed by nylon as essentially a physical mixture where the intermolecular forces approach van der Waals' forces. Chipalkatti, Giles, and Vallance (3) have reported that molecules of the type studied in this investigation are probably bound to the nylon by hydrogen bonding and report that phenol has a heat of sorption of around −4.5 Kcal./mole. For large molecules, such as dyes, Meggy (7) and Derbyshire and Peters (8) have found even for those molecules having polar functional groups, the interaction forces can be dependent on the hydrophobic portion of the molecule. Even though the compounds studied here fell into a heat of sorption range below that which might be considered as due to hydrogen bonding, physical forces alone cannot account for the bonding energy between these solute molecules and the nylon. If this were the case, pyridine (a molecule of approximately the same size as phenol) would be sorbed. The absence of an acidic hydrogen in pyridine prevents the interaction, while with phenol and similar compounds, acidic hydrogens confer binding ability to the amide linkages. On the other hand, hydrogen bonding is not sufficient to hold the solute molecules to the nylon as may be surmised if one considers the overwhelming number of water molecules, which on the basis of the law of mass action, would compete for the same sites, dislodging the bound solute molecules. The intermolecular forces involved in the interaction of the solute molecules with the nylon reported here must be due to both hydrogen bonding which attracts the solute molecules and to van der Waals' forces which stabilize the interaction.

Diffusion and Activation Energy.—The data shown in Table III indicate that diffusion coefficients

for all of the compounds in the temperature range studied were of the order of magnitude of 10^{-9} to 10^{-10} cm.^{2}/sec. It can be further seen that as the temperature increased, the diffusion coefficient increased, as would be expected from theory. The activation energy for diffusion varied from a low of 12.9 Kcal./mole (for benzoic acid) to a high of 20.9 Kcal./mole (for methyl *p*-hydroxybenzoic acid). In a previous paper on nylon 66, the activation energy for diffusion for *o*-hydroxybenzoic acid was found to be 20.5 Kcal./mole. This compares to 19.9 Kcal./mole for the same compound in nylon 610 found in this study.

Activation energies for diffusion do indicate that structural changes in the molecule can alter the rate of diffusion. For example, the addition of a hydroxy group *ortho* to the carboxylic acid in benzoic acid increases the activation energy by 7 Kcal./mole. Unfortunately, since diffusion was not studied at the same original concentration for all of the compounds, it would be difficult to postulate exact effects of structure on the diffusion rate. It would also seem that many more compounds would have to be studied to get a general picture concerning the importance of structure on the diffusion rate.

Diffusion versus Rate of Interaction.—The use of the Rotinyan and Drozdov equation (Eq. 6) helped to support the accepted theory that solute molecules penetrate and diffuse through a plastic material and that the rate-determining step is the diffusion process. For all the compounds investigated in this paper, β approached $1/a$ which verified that the diffusion in the plastic was the determining step in the sorption process.

pH and Sorption.—Figure 6 shows that above pH 6.0 there is very little sorption taking place, but from pH 6.0 down to 3.8 a sudden increase in sorption takes place, leveling off after the pH is reduced below 3.8. This indicates that the four acidic compounds included in this particular experiment are sorbed in their unionized form.

SUMMARY

1. Sorption experiments were conducted on six compounds having similar functional groups. These compounds were benzoic acid, *o*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, methyl *p*-hydroxybenzoate, and propyl *p*-hydroxybenzoate. Nylon 610 was employed as the substrate.

2. Experiments were conducted at both steady state conditions and nonsteady state conditions and at a number of temperatures. From the accumulated data, the following constants were evaluated for each compound: saturation value (S), standard affinity ($-\Delta\mu^\circ$), standard heat of sorption (ΔH°), apparent diffusion coefficient (D), and activation energy of diffusion (ΔE).

3. The results suggested that the compounds employed in the study were bound to the nylon 610 by hydrogen bonding with secondary valence forces stabilizing the interaction. In this series of compounds, propyl *p*-hydroxybenzoate had the greatest affinity for the nylon which was explained on the basis of the greater hydrophobic character of the molecule.

4. Apparent diffusion coefficients for all of the compounds in nylon 610 were found to be of the order of 10^{-9} to 10^{-10} cm.^{2}/sec. The diffusion coefficients were related to temperature by the usual Arrhenius equation from which activation energies were calculated. Application of the Rotinyan and Drozdov equation to the kinetic data confirmed that the diffusion process in the nylon was the rate-determining step.

5. Changes in hydrogen ion concentration in the solutions revealed that the four acidic compounds were sorbed in the unionized state.

6. Methods employed in this study and the treatment of data help to generate information on drug-plastic interactions of a more fundamental nature. As more drugs or compounds are studied with one or more substrates, valuable information will become available concerning the most suitable plastic material to use in a specific pharmaceutical or medical application.

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Interaction of Sucrose and Propoxylated Sucrose Esters with Some Pharmaceuticals

By SEYMOUR M. BLAUG and DONALD S. EBERSMAN†

The interaction between either fatty acid mono or diesters of sucrose or fatty acid monoesters of propoxylated sucrose and benzoic acid or a series of hydroxy and amino substituted benzoic acids is reported. The sugar esters, because of their lack of long polyether chains, have been proposed for use in formulations in which association between a nonionic emulsifier and preservative is a problem. A dialysis method was used to study the interactions. The degree of interaction appears to be dependent upon the type of functional group present and its position in the complexed molecule. In the case of the surfactant the esters of the propoxylated sucrose appear to associate more than those of sucrose. The fatty acid moiety influences the extent of interaction; of the series studied, the laurate esters generally undergo interaction to the greatest extent, while the stearate esters exhibit only slight interaction. The results obtained differ to some degree from work previously reported with other nonionic surfactants. A possible mechanism of interaction based upon the binding of drug within the palisade layer of the micelles of the surfactant solution is proposed.

THE LAST DECADE has witnessed the appearance of a multitude of surface-active agents. These agents can roughly be classified into groups which describe the charge of the major portion of the molecule upon dissociation in water, *i.e.*, anionics, cationics, amphoterics, and those which do not dissociate—the nonionics.

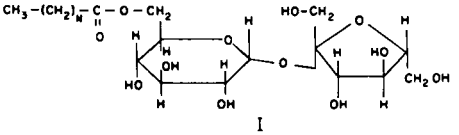
A group of nonionic surfactants were introduced in 1956, the fatty acid esters I and II of sucrose (1, 2). Members of this group have been proposed for use (3–5) in the petroleum industry, the textile industry, and for agricultural use. In England, the sugar esters have been given wide attention for possible use as laundry detergents (6, 7). Pollution of rivers and streams (from which drinking water is taken) by synthetic detergents is becoming a widespread problem. Sucrose esters do not constitute a threat to the purity of drinking water since they can be metabolized by microorganisms.

In the pharmaceutical and cosmetic fields the sucrose esters can be widely used in forming emulsion bases and as dispersing agents in suspensions. They have the advantage of being tasteless and odorless, a factor which gives them a decided advantage over competitive products. Some of the esters are solids, resulting in easier handling than with the liquid surfactants.

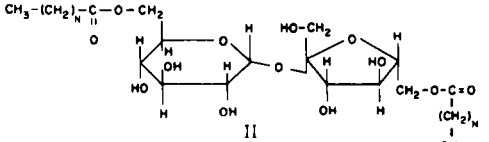
Attempts to synthesize fatty acid esters of sucrose date back to the work of Berthelot in 1860 (8). Other methods have been attempted (9–16), but none have received the wide acceptance accorded the method developed by Snell, Osipow, and Marra (1). Their method involves a transesterification, using as the reactants the methyl ester of a fatty acid, and potassium carbonate dissolved in N,N-dimethyl formamide. The raw materials involved are relatively inexpensive; as soon as the manufacturing method can be accomplished efficiently and cheaply it is apparent that there will be a very large market for these surfactants.

Initially it was thought (5) that these agents were completely nontoxic; however, a recent work (17) has provided evidence that while these surfactants are nontoxic orally, they are toxic upon injection. This prevents their proposed use in the formulation of intravenous emulsions.

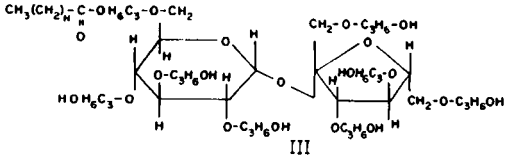
Another group of related surfactants was introduced a few years after the sucrose esters (18, 19). These are the fatty acid esters (III)



I



II



III

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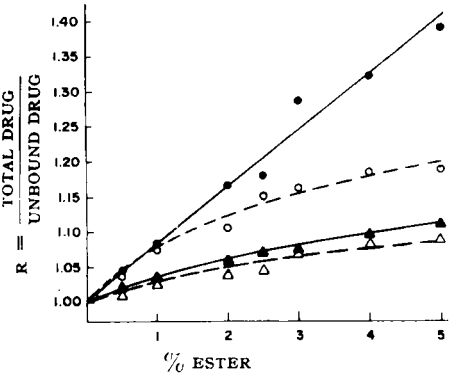


Fig. 1.—Binding of benzoic acid and three hydroxybenzoic acids by sucrose monolaurate in water at 30°. Key: ●——●, benzoic acid; O— — —O, *o*-hydroxybenzoic acid; ▲——▲, *m*-hydroxybenzoic acid; Δ— — —Δ, *p*-hydroxybenzoic acid.

of propoxylated sucrose which are chemically described as octakis (2-hydroxypropyl) sucrose. This agent has also been proposed as an emulsifying agent for cosmetics and pharmaceuticals. A recent work (20) describes a series of emulsions prepared with the aid of the propoxylated sucrose esters.

Because of their lack of long polyether chains, the sucrose esters have been recommended (4) for use in formulations to replace other nonionic surfactants such as the Tweens[1] Myrj 52,[2] and polyethylene glycols which have been shown (21–23) to interact with materials—such as preservatives incorporated in the formulation. It was the purpose of this investigation to observe whether and to what extent the esters of sucrose and/or propoxylated sucrose, which do not have as part of their molecule long polyether chains, would associate with a series of hydroxy and amino substituted benzoic acids.

EXPERIMENTAL

The interaction of benzoic acid and the substituted benzoic acids was studied using a dialysis method similar to those previously described in the literature (24–26). The method consisted of bringing two solutions into equilibrium across a semipermeable membrane. The membrane, Fisher cellulose casing,[3] permitted the low molecular weight compound in the solution outside of the dialysis sack to pass freely through the membrane; at the same time it did not permit the passage of the sucrose ester contained in the sack. Therefore, if the compound studied did not interact with the sucrose ester and was not bound by the membrane itself, the concentration of compound inside and outside of the dialysis sack was equal. If interaction did occur, the concentration of the compound on the ester side of the semipermeable membrane increased.

[1] Marketed by the Atlas Powder Co., Wilmington, Del.
[2] Polyoxyl 40 stearate. Marketed by the Atlas Powder Co., Wilmington, Del.
[3] Product of Fisher Scientific Co., Catalog No. 8-667.

The data obtained during the study were plotted to show the value *R*, the ratio of total drug in solution to the unbound drug, as a function of the concentration of the sucrose or hyprose ester. With the method used it was not possible to determine the stoichiometric ratio in which the reactants unite and thus accurately determine the formation constants. However, by use of the phase diagrams (Figs. 1–4) the degree of association between the various reactants can be readily seen.

Reagents.—Recrystallized benzoic acid, m.p. 122°, *o*-hydroxybenzoic acid, m.p. 157–159°, *m*-hydroxybenzoic acid, m.p. 200°, *p*-hydroxybenzoic acid, m.p. 212–213°, *o*-aminobenzoic acid, m.p. 145°, *m*-aminobenzoic acid, m.p. 174°, *p*-aminobenzoic acid, m.p. 186–187°, sucrose monostearate, sucrose monolaurate, sucrose dioleate, hyprose monostearate, hyprose monolaureate, hyprose monooleate, 0.001 *N* sulfuric acid were employed.

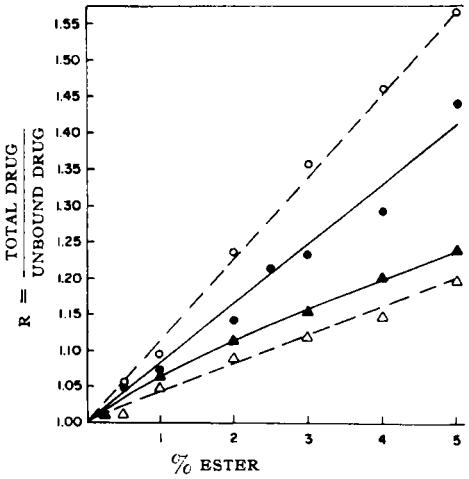


Fig. 2.—Binding of benzoic acid and three hydroxybenzoic acids by hyprose monolaurate. Key: ●——●, benzoic acid; O— — —O, *o*-hydroxybenzoic acid; ▲——▲, *m*-hydroxybenzoic acid; Δ— — —Δ, *p*-hydroxybenzoic acid.

Procedure.—Solutions of the aromatic acids in 0.001 *N* sulfuric acid were prepared. Amounts of acids used corresponded to half saturated solutions, *i.e.*, each solution contained in 50 ml. the following amount of acid: benzoic acid, 140 mg.; *o*-hydroxybenzoic acid, 460 mg.; *p*-hydroxybenzoic acid, 400 mg.; *o*-aminobenzoic acid, 180 mg.; *m*-aminobenzoic acid, 300 mg.; *p*-aminobenzoic acid, 170 mg. Fifty milliters of the solutions were pipetted into 125-ml. glass-stoppered bottles. Sacks were prepared from the Fisher cellulose casings and filled with 20 ml. of solutions containing varying concentrations of sucrose or propoxylated sucrose ester (0 to 5.0%). The sacks were tightly closed and immersed in a bottle containing a solution of the acid under study. The bottles were placed in a constant temperature water bath shaker at 30 ± 0.2° for 48 hours, after which time aliquots were pipetted from the external solution, diluted, and assayed for acid content using a Beckman model DU spectrophotometer and 1-cm. cells. For each series of determinations, a control bottle was prepared which differed from the other bottles since it contained no sucrose ester inside the sack. The internal and external solutions of each

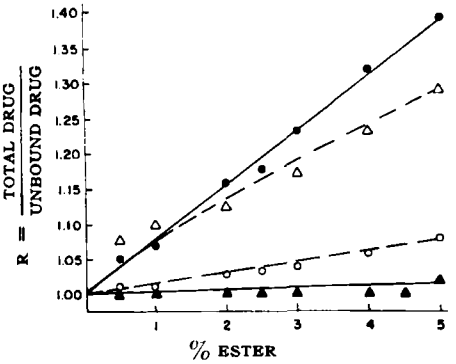


Fig. 3.—Binding of benzoic acid and three aminobenzoic acids by sucrose monolaurate in water at 30°. Key: ●——●, benzoic acid; O— — —O, *o*-aminobenzoic acid; ▲——▲, *m*-aminobenzoic acid; △——△, *p*-aminobenzoic acid.

blank were assayed for acid content to determine if equilibrium had been reached. Solutions were assayed for acid content at the following wavelengths: benzoic acid, 240 mμ; *o*-hydroxybenzoic acid, 240 mμ; *m*-hydroxybenzoic acid, 240 mμ; *p*-hydroxybenzoic acid, 240 mμ; *o*-aminobenzoic acid, 275 mμ; *m*-aminobenzoic acid, 267 mμ, and *p*-aminobenzoic acid, 275 mμ.

DISCUSSION

It can be noted that while the degree of association was not very great between the various reactants certain definite complexing tendencies can be discerned (Tables I–III). In the hydroxybenzoic acid series, *o*-hydroxybenzoic was the most reactive, the *m*-substituted compound less reactive, and the *p*-hydroxy less reactive. This order of reactivity varied from that of work previously reported with certain xanthines (27–29) and polymers (30–33). However, it is similar to some work reported (21) with the Tweens, which, in addition to the ethylene oxide polymer in the molecule, contains a hydrophobic moiety. Blaug and Ahsan (21) attributed the greater complexing ability of the Tweens, compared to polyethylene glycol, to the presence of a hydrophobic moiety in the molecule. The degree of reactivity of benzoic acid appears to depend on the nature of the ester. In the series of interactions with the esters of sucrose (Fig. 1), benzoic acid was more reactive than *o*-hydroxybenzoic acid. However, in the series of interactions with the propoxylated sucrose esters (Fig. 2), the benzoic acid was less reactive than the *o*-hydroxy compound but more reactive than the *m*-hydroxy acid. It is possible, in the case of the sucrose ester, that due to intramolecular bonding the *o*-hydroxy acid has less available sites for bonding than the benzoic acid molecule, making it less reactive. However, in the propoxylated sucrose ester molecule the availability of a greater number of bonding sites in close proximity to the acid molecule, partially fixed in place by a hydrophobic bond, allows the macromolecule to compete advantageously with the intramolecular bonding forces of the acid. Figures 1–4 are illustrative of the results obtained in these interaction studies.

The exact reason for the formation of molecular complexes is not completely known at the present time. A number of theories have been proposed (34–37) to account for the experimental data generally obtained. It was believed that hydrogen bonding was the major driving force of these interactions in the early work conducted with water soluble polymers (34). In a recent work (35) Higuchi and Drubulis stated that the degree of interaction shown by the hydroxy-aromatic acids and their salts was too great to be caused by hydrogen bonding or by hydrophobic bonding. They attributed the interaction to another donor-acceptor type of mechanism.

In the field of soap chemistry the solubilization of organic acids and phenols has been attributed (36) to micellar solubilization. Klevans (37), in

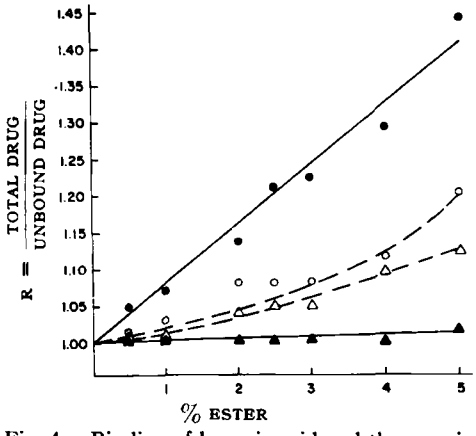


Fig. 4.—Binding of benzoic acid and three aminobenzoic acids by hyprose monolaurate in water at 30°. Key: ●——●, benzoic acid; O— — —O, *o*-aminobenzoic acid; ▲——▲, *m*-aminobenzoic acid; Δ— — —Δ, *p*-aminobenzoic acid.

his review of solubilization, stated that molecules can be solubilized by three possible mechanisms. The first was the adsorption of the added molecule on the surface of the micelle. The second was the dissolution of the added hydrocarbon in the lipophilic portion of the micelle. The third, thought to be the best explanation of the three, stated that the molecule becomes oriented in the palisade layer of the micelle. A group of English workers (36) studying soap interactions stated that the aromatic compounds studied appeared to orient themselves in the micelle in the same manner as the soap molecules. If this were the mechanism by which the compounds in the present study reacted, then a correlation based on the dipole moment should be valid. This was attempted by the authors but found unsuitable to explain the results obtained.

If we study the macromolecules in question, the sucrose, or the propoxylated sucrose esters, it can be seen that a fatty acid moiety is present which corresponds to the hydrophobic portion of a soap molecule. In contrast to the soap molecule, the hydrophilic portion of the sucrose or hyprose ester is nonionized. The hydrophilic portion does not possess the large number of binding sites found in the polyethers. Based on water solubility data,

TABLE I.—*R* VALUES FOR THE INTERACTION OF BENZOIC ACID AND SOME HYDROXY AND AMINO-SUBSTITUTED BENZOIC ACIDS WITH SOME SUCROSE AND PROPOXYLATED SUCROSE ESTERS (1% MACROMOLECULE CONCENTRATION)

Acid	Sucrose			Hyprose		
	Monostearate	Monolaurate	Dioleate	Monostearate	Monooleate	Monolaurate
Benzoic	1.03	1.08	1.08	1.03	1.05	1.09
o-Hydroxybenzoic	1.01	1.08	1.01	1.11	1.05	1.11
m-Hydroxybenzoic	1.01	1.03	1.01	1.03	1.03	1.07
p-Hydroxybenzoic	1.01	1.03	1.02	1.05	1.04	1.05
o-Aminobenzoic	1.01	1.01	1.01	1.06	1.04	1.02
m-Aminobenzoic	1.00	1.00	1.00	1.00	1.00	1.00
p-Aminobenzoic	1.00	1.08	1.01	1.03	1.07	1.01

TABLE II.—*R* VALUES FOR THE INTERACTION OF BENZOIC ACID AND SOME HYDROXY AND AMINO-SUBSTITUTED BENZOIC ACIDS WITH SOME SUCROSE AND PROPOXYLATED SUCROSE ESTERS (2.5% MACROMOLECULE CONCENTRATION)

Acid	Sucrose			Hyprose		
	Monostearate	Monolaurate	Dioleate	Monostearate	Monooleate	Monolaurate
Benzoic	1.07	1.20	1.21	1.07	1.15	1.20
o-Hydroxybenzoic	1.03	1.10	1.02	1.32	1.22	1.32
m-Hydroxybenzoic	1.01	1.07	1.02	1.07	1.06	1.13
p-Hydroxybenzoic	1.02	1.05	1.02	1.08	1.05	1.10
o-Aminobenzoic	1.02	1.03	1.02	1.09	1.07	1.06
m-Aminobenzoic	1.00	1.00	1.00	1.00	1.01	1.01
p-Aminobenzoic	1.00	1.05	1.00	1.05	1.10	1.05

TABLE III.—*R* VALUES FOR THE INTERACTION OF BENZOIC ACID AND SOME HYDROXY AND AMINO-SUBSTITUTED BENZOIC ACIDS WITH SOME SUCROSE AND PROPOXYLATED SUCROSE ESTERS (5% MACROMOLECULE CONCENTRATION)

Acid	Sucrose			Hyprose		
	Monostearate	Monolaurate	Dioleate	Monostearate	Monooleate	Monolaurate
Benzoic	1.10	1.40	1.34	1.30	1.17	1.44
o-Hydroxybenzoic	1.04	1.19	1.05	1.47	1.41	1.56
m-Hydroxybenzoic	1.01	1.11	1.05	1.15	1.13	1.25
p-Hydroxybenzoic	1.04	1.09	1.03	1.12	1.10	1.20
o-Aminobenzoic	1.03	1.28	1.05	1.13	1.14	1.20
m-Aminobenzoic	1.00	1.01	1.00	1.01	1.01	1.01
p-Aminobenzoic	1.01	1.07	1.01	1.07	1.12	1.12

it has been stated (5) that the oxygen atoms present in the sucrose portion or the molecule are equivalent to a minimum of ten ethylene oxide groups. This would make these esters more reactive than the Tweens. The results obtained indicated that the macromolecules in question were appreciably less reactive than those of the Tween series because of the lack of ethylene oxide groups in the sucrose or hyprose molecule.

The authors noted differences in the degree of reactivity between esters of the various fatty acids and between the esters of sucrose and those of hyprose. In most instances the esters of hyprose interacted to a greater degree than those of sucrose. The observed differences were probably due to the greater number of binding sites in the propoxylated sucrose molecule. The fatty acid portion of the ester molecule appeared to exert some effect on the degree of interaction. The laurate esters generally were the most reactive, while the stearate esters generally were least reactive. This latter effect was probably due, in part, to greater solubility of the laurate ester—hence greater availability of the molecule for interaction.

In studying the data obtained the possibility becomes apparent that more than one mechanism is responsible for the degree and order of reactivity of the acids studied. The theory proposed by Hyde, *et al.* (36), appears to explain best the results obtained. The aromatic acid is incorporated into the palisade layer of the sucrose or hyprose micelle in the same manner as the sugar ester molecules in the micelle. The stability of the complex can be attributed to two different types of binding. As represented in Fig. 5, the carboxyl and hydroxyl

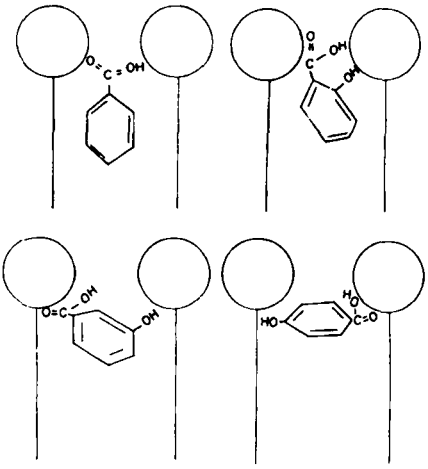


Fig. 5.—A schematic representation of the proposed mechanism of interaction between sucrose and hyprose ester, and benzoic acid and three hydroxybenzoic acids.

groups of the aromatic acids are associated with the hydroxyl groups and/or ether linkages present in the sucrose or hyprose molecule. The less polar portion of the interacting acid molecule associates with or becomes dissolved in the hydrocarbon tail of the ester in its micellar orientation. The greater degree of reactivity demonstrated by one acid molecule over another can be attributed to the ability of that molecule to make a better "fit," *i.e.*, the combination of hydrophilic bonding (hydrogen bonding or donor-acceptor type bonding) and hydrophobic bonding forming a more stable complex.

In the second series, the amino-substituted benzoic acids, Figs. 3 and 4, the orders of reactivity differed from those observed with the hydroxybenzoic acids. In most cases the *o*-substituted amino compound appeared to be most reactive, behaving similarly to the hydroxy counterpart. On the other hand, the *p*-aminobenzoic acid was next most reactive, instead of the *meta* compound showing opposite behavior from their hydroxy counterparts.

Since the studies were conducted in relatively strong acidic media, the amino compounds were present as the protonated species. Benzoic acid interacted to a greater degree than any of the aminobenzoic acids. This was expected as the protonated ion would not become involved in hydrogen bonding and would serve to inhibit any form of hydrophobic association. The phase diagrams presented indicate that the amino compounds, in general, were not as reactive as the hydroxybenzoic acids. The lack of reactivity of the *m*-aminobenzoic acid can be attributed, perhaps, to the steric configuration of the molecule, *i.e.*, the combination of hydrophilic and hydrophobic bonds forming a very weak complex.

TABLE IV.—RELATIVE COMPLEXING TENDENCIES OF SUCROSE AND HYPROSE ESTERS[a]

Sucrose Esters	Hyprose Esters
o-Hydroxybenzoic acid	Benzoic acid
Benzoic acid	*o*-Hydroxybenzoic acid
m-Hydroxybenzoic acid	*o*-Aminobenzoic acid
o-Aminobenzoic acid	*m*-Hydroxybenzoic acid
p-Hydroxybenzoic acid	*p*-Hydroxybenzoic acid
p-Aminobenzoic acid	*p*-Aminobenzoic acid
m-Aminobenzoic acid	*m*-Aminobenzoic acid

[a] Decreasing order of reactivity.

SUMMARY

A study was conducted to determine whether association would occur between a series of aromatic acids and some fatty acid esters of sucrose and a propoxylated sucrose.

Evidence of reactivity between the molecules studied was presented in addition to the degree to which this association occurred.

A mechanism was proposed to explain, to some degree, why association had occurred.

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Heterocyclic Thioureas II

Antitubercular Activity of Diheterocyclic Thioureas

By ARTHUR C. GLASSER and RICHARD M. DOUGHTY

The preparation and *in vitro* examination for antitubercular activity of a series of heterocyclic substituted (1-phenyl-2,3-dimethyl-5-pyrazolon-4-yl) thioureas are described. The second heterocyclic rings substituted on the molecules are examples of *N*- and *O*-heteroparaffinics, *N*-π deficient and *N*-π excessive rings. The compounds studied ranged in minimum inhibitory concentrations from 0.63 mg. per cent to a lower level of activity of 10 mg. per cent.

IT IS WELL KNOWN that certain 1,3-di(4-substituted phenyl)-thioureas have the ability to inhibit the growth of *M. tuberculosis* (1). Extensive series of related molecules of this general type have been investigated for this activity (2, 3). A previous communication of this series has reported on the activity of a series of 1-*p*-alkoxyphenyl-3-(5-pyrazolon-4-yl)- and (2-pyridyl)-thioureas (4). It was determined in the first study that one of the compounds, 1-*p*-isopropoxyphenyl-3-[(1-phenyl-2,3-dimethyl)-5-pyrazolon-4-yl] -2-thiourea, showed *in vitro* activity at the 0.16 mg. % level. This observation led to the synthesis of a second series of related thioureas in which both substituents of the molecule were of a heterocyclic nature, one of which was the 5-pyrazolone moiety of the before-mentioned compound. This study reports the effects upon the tuberculostatic activity of the second substituent being (*a*) *N* and *O* heteroparaffinic molecules, such as alkyl substituted piperidine, piperazine, and morpholine, (*b*) π-deficient *N*-heteroaromatic rings such as alkyl and dialkyl substituted pyridine, and (*c*) π-excessive *N*-heterocyclic ring such as substituted 5-pyrazolone.

As a group the *N*- and *O*-heteroparaffinics and the π deficient *N*-heterocyclic compounds showed about the same general level of activity as the *p*-alkoxyphenyl compounds reported previously. The most active compound of the present series at 0.63 mg. % was compound *17*. The rings involved here are of the π excessive type, and this type of electron abundance coupled with a thion–thiol enolization type reaction could possibly be used for a comparison to the presence of excessive electrons in the 4-position of such molecules as *p*-isopropoxyphenyl substituted pyrazolone thioureas reported previously. An attempt to place a center of electron density in the 4-position of some of the π-deficient molecules is now underway in this laboratory as a means of further investigating this observation.

The preparative methods used for the thioureas were essentially the same as reported previously in which the isothiocyanate, prepared by the action of thiophosgene on the appropriate amine in methylene chloride, was allowed to react with an amine in absolute alcohol or thiophene free benzene (5). The aminoalkylpyridines, morpholine, piperidine, and methylpiperazine used were commercial products purified by recrystallization or distillation just prior to use. The cyclic secondary amines, 4-ethyl, 4-*n*-propyl, and 4-isopropylpiperidine, were prepared by the reduction of the corresponding 4-alkylpyridine derivative using sodium and *n*-butanol reduction followed by catalytic hydrogenation in the presence of palladium-on-carbon in procedures similar to the method of Wawzonek (6).

The *in vitro* determination of the tuberculostatic activity was carried out generally as previously reported using the H37Rv strain of *M. tuberculosis* var. *hominis*[1] grown on Dubos media with added polysorbate 80[2] and beef serum (4). The insoluble nature of compounds *12* and *13* required the incorporation of methylcellosolve into the solvent used for the preparation of the original dilution for testing. This combined solvent showed inhibiting properties of significant nature, and thus detracted from the validity of the readings compared to those of the regular solvent which had been previously shown to be noninhibiting. However, it was felt that as members of the series it was desirable to have some sort of evaluation of these compounds, so they are included as qualified. A summary of structures, properties, and testing results are shown in Table I.

EXPERIMENTAL

1-Phenyl-2,3-dimethyl-5-pyrazolon-4-isothiocyanate.—A solution of 5.8 Gm. (0.05 mole) of thio-

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[1] American Type Culture Collection, 2112 M Street, N.W. Washington, D. C., 20037.

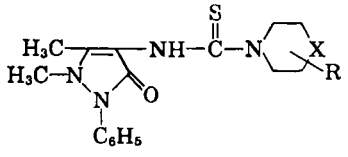
[2] Polyoxyethylene 20 sorbitan mono-oleate. Marketed as Tween 80 by the Atlas Powder Co., Wilmington, Del.

phosgene in 20 ml. of methylene chloride was added dropwise to a stirred solution of 10.2 Gm. (0.05 mole) of 4-aminoantipyrine in 30 ml. of methylene chloride contained in a 250-ml. flask surrounded by an ice bath. The reaction mixture was allowed to warm to room temperature and stand overnight. The resulting solid was filtered off and recrystallized from benzene-ether to give 6.4 Gm. (54%) of needles, m.p. 146 to 147°.

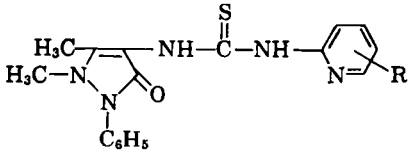
Anal.—Calcd. for $C_{12}H_{11}N_3OS$: C, 58.75; H, 4.52; N, 17.13. Found: C, 58.92; H, 4.50; N, 16.89.

4-Isopropylpiperidine.—A solution of 40.5 Gm. (0.3 mole) of 4-isopropylpyridine in 500 ml. of *n*-butyl alcohol was placed in a three-necked 1000-ml. flask equipped with an efficient condenser and stirrer. The solution was preheated to 50–60° and 70 Gm. of sodium added at such a rate that reflux was maintained. An additional 150 ml. of alcohol was added in portions to maintain the contents of the flask in a fluid condition. After the addition of the sodium was completed, the mixture was heated at reflux for 25 minutes to effect solution of all the sodium. The solution was cooled to about 75° and 200 ml. of ice water slowly added. After cooling to room temperature, the aqueous layer was separated and the alcoholic solution made acid to litmus with 6 *N* hydrochloric acid. The acidic solution was steam distilled to remove all the butyl alcohol, made alkaline to litmus with 10 *N* sodium hydroxide solution, and steam distilled until the distillate was no longer basic to litmus. The basic distillate was saturated with potassium carbonate and the amine extracted with ether. The ethereal solution of the amine was dried over anhydrous sodium sulfate, the ether removed by evaporation at reduced pressure, and the residue fractionated through a glass helices column to yield 8.5 Gm. of 4-isopropyl-1,2,5,6-

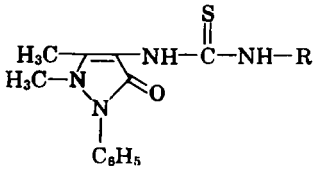
TABLE I.—DIHETEROCYCLIC THIOUREAS



No.	R	X	Yield, %	RS[c]	M.p., °C.[a]	Formula	*N*-Analyses[b] Calcd.	Found	Min. Inhib. mg. %
1	H	CH_2	62	Ac	188–189[e]	$C_{17}H_{22}N_4OS$	16.95	17.15	2.5
2	2-Methyl	CH_2	91	A	170–171	$C_{18}H_{24}N_4OS$	16.16	14.90	2.5
3	3-Methyl	CH_2	90	A	175–176	$C_{18}H_{24}N_4OS$	16.26	15.91	5.0
4	4-Methyl	CH_2	93	A	184–185	$C_{18}H_{24}N_4OS$	16.26	16.01	2.5
5	2,6-Dimethyl	CH_2	95	A	174–175	$C_{19}H_{26}N_4OS$	15.63	15.49	1.25
6	2-Ethyl	CH_2	85	A	149–150	$C_{19}H_{26}N_4OS$	15.63	15.74	5.0
7	4-Ethyl	CH_2	91	A	168 to 168.5	$C_{19}H_{26}N_4OS$	15.63	15.41	5.0
8	4-*n*-Propyl	CH_2	85	A	147 to 147.5	$C_{20}H_{28}N_4OS$	15.02	15.16	5.0
9	4-*i*-Propyl	CH_2	90	A	188–189	$C_{20}H_{28}N_4OS$	15.02	14.80	10.0
10	H	O	79	B	211–212[d]	$C_{16}H_{20}N_4OS$	16.81	16.46	5.0
11	H	$=N-CH_3$	70	C	168–169	$C_{17}H_{23}N_5OS$	20.27	20.59	2.5



12	H		65	A	248–249	$C_{17}H_{17}N_5OS$	20.63	20.41	0.63
13	4-Methyl		87	A	222–223	$C_{18}H_{19}N_5OS$	19.81	19.75	2.5
14	5-Methyl		73	A	240 to 241.5	$C_{18}H_{19}N_5OS$	19.81	19.47	2.5
15	6-Methyl		76	A	213 to 214.5	$C_{18}H_{19}N_5OS$	19.81	19.63	5.0
16	4,6-Dimethyl		86	A	223–224	$C_{19}H_{21}N_5OS$	19.06	19.17	2.5



17	4-Antipyryl		81	D	245–247 dec.	$C_{23}H_{24}N_6O_2S$	18.72	18.51	0.63

[a] Melting points determined on Fisher-Johns block and uncorrected. [b] Microanalyses by Weiler and Straus, Oxford, England and Galbraith Laboratories, Knoxville, Tenn. [c] Reported, Takahaski, T., and Kanematsa, K., *Yakugaku Zasshi*, **79** 162(1959), 190°. [d] Reported, *ibid.*, 213°, through *Chem. Abstr.*, **53**, 13138e(1959). [e] Recrystallization solvents: Ac—acetone, A—ethyl alcohol, B—benzene, C—chloroform, and D—methyl alcohol.

tetrahydropyridine, b.p. 176 to 177.5° at 738 mm., taken as a heart cut for a yield of 20%. Picrate 170–171°, (Lit. picrate 170 to 170.5°) (7).

Anal.—Calcd. for $C_8H_{15}N$: C, 76.74; H, 12.07; N, 11.19. Found: C, 76.83; H, 12.01; N, 10.93.

A solution of 8 Gm. of 4-isopropyl-1,2,5,6-tetrahydropyridine in 30 ml. of glacial acetic acid was subject to hydrogenation at 45 lb./in.^{2} in the presence of 2.5 Gm. of 10% palladium-on-charcoal until hydrogen was no longer taken up. After filtering off the catalyst, the solution was made alkaline to litmus with 10 *N* sodium hydroxide solution, and the alkaline solution steam distilled until the distillate was neutral to litmus. The distillate was saturated with potassium carbonate, and the amine separated and dried over potassium hydroxide pellets. Careful fractionation through a glass helices column resulted in 5.6 Gm. (69%) of 4-isopropylpiperidine, b.p. 171–172° at 733 mm., n_D^{20} 1.4577, chloroplatinate 172–173°. (Lit. b.p. 168–171°, chloroplatinate 172°) (8).

Anal.—Calcd. for $C_8H_{17}N$: C, 75.52; H, 13.47; N, 11.01. Found: C, 75.38; H, 13.42; N, 11.22.

1,1-(3-Isopropylpentamethylene)-3-(1-phenyl-2,3-dimethyl-5-pyrazolon-4-yl)-2-thiourea.—A solution of 0.57 Gm. (0.05 mole) of 4-isopropylpiperidine and 1.0 Gm. (0.04 mole) of 1-phenyl-2,3-dimethyl-5-pyrazolon-4-isothiocyanate in 10 ml. of absolute alcohol was refluxed on a steam bath for 2 hours. After evaporation of the reaction mixture to one-third its original volume, crystals formed on cooling. Filtration and recrystalization from alcohol gave 1.35 Gm. (90%) of the thiourea, m.p. 188–189°.

Anal.—Calcd. for $C_{20}H_{28}N_4OS$: C, 64.48; H, 7.57; N, 15.02. Found: C, 64.67; H, 7.61; N, 14.80.

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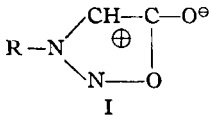
Prediction of Stability in Pharmaceutical Preparations XII

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By EDWARD R. GARRETT

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tetrahydropyridine, b.p. 176 to 177.5° at 738 mm., taken as a heart cut for a yield of 20%. Picrate 170–171°, (Lit. picrate 170 to 170.5°) (7).

Anal.—Calcd. for $C_8H_{15}N$: C, 76.74; H, 12.07; N, 11.19. Found: C, 76.83; H, 12.01; N, 10.93.

A solution of 8 Gm. of 4-isopropyl-1,2,5,6-tetrahydropyridine in 30 ml. of glacial acetic acid was subject to hydrogenation at 45 lb./in.^{2} in the presence of 2.5 Gm. of 10% palladium-on-charcoal until hydrogen was no longer taken up. After filtering off the catalyst, the solution was made alkaline to litmus with 10 *N* sodium hydroxide solution, and the alkaline solution steam distilled until the distillate was neutral to litmus. The distillate was saturated with potassium carbonate, and the amine separated and dried over potassium hydroxide pellets. Careful fractionation through a glass helices column resulted in 5.6 Gm. (69%) of 4-isopropylpiperidine, b.p. 171–172° at 733 mm., n_D^{20} 1.4577, chloroplatinate 172–173°. (Lit. b.p. 168–171°, chloroplatinate 172°) (8).

Anal.—Calcd. for $C_8H_{17}N$: C, 75.52; H, 13.47; N, 11.01. Found: C, 75.38; H, 13.42; N, 11.22.

1,1-(3-Isopropylpentamethylene)-3-(1-phenyl-2,3-dimethyl-5-pyrazolon-4-yl)-2-thiourea.—A solution of 0.57 Gm. (0.05 mole) of 4-isopropylpiperidine and 1.0 Gm. (0.04 mole) of 1-phenyl-2,3-dimethyl-5-pyrazolon-4-isothiocyanate in 10 ml. of absolute alcohol was refluxed on a steam bath for 2 hours. After evaporation of the reaction mixture to one-third its original volume, crystals formed on cooling. Filtration and recrystalization from alcohol gave 1.35 Gm. (90%) of the thiourea, m.p. 188–189°.

Anal.—Calcd. for $C_{20}H_{28}N_4OS$: C, 64.48; H, 7.57; N, 15.02. Found: C, 64.67; H, 7.61; N, 14.80.

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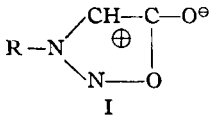
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among the alkyl sydnones alone, variations in R had a highly significant effect. The availability of the alkyl sydnones, R = methyl, propyl, phenethyl, isopropyl, *s*-butyl, furfuryl, *t*-butyl, and *t*-octyl (*i.e.*, 1,1,3,3-tetramethylbutyl), synthesized by Dr. Kier and associates (3, 4), gave an excellent opportunity to define the effects of various alkyl substituents on the stability of the sydnone ring.

EXPERIMENTAL

General Procedure.—The isolation and characterization of the sydnones studied has been reported (4, 5). In general, the molar absorptivity of the sydnones was ϵ = 6000–7000 in alkaline, acid, and neutral solutions as was expected (4), except for the phenethyl sydnone (ϵ = 3000). The wavelength of maximum absorbance was shifted 2–4 mμ to the violet in the aqueous solution compared to those reported in ethyl alcohol (4). The absorbance of the sydnones' maxima was 284 and 286 mμ. The absorbances and wavelengths of maximum absorbance did not significantly change with pH.

The general procedure for the kinetic studies was to dissolve a specific sydnone in nitrogen purged distilled water maintained at the temperature of the prospective kinetic study. The solution was subsequently diluted 1:1 with the appropriate nitrogen purged buffer solution, also thermally preequilibrated. In general, the final concentration was established at $10^{-4}M$ for optimum spectrophotometric absorbance reading, except for those specific cases where the effects of initial concentration on the apparent solvolytic rates were evaluated. In the phenethyl sydnone case the final concentration was adjusted to 2 × $10^{-4}M$ to heighten the absorbances.

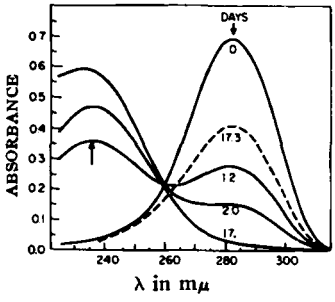


Fig. 1.—Typical spectrophotometric curves for the acid and alkaline degradation of alkyl sydnones. The absorbance of the 283 mμ band decreases with time in 0.5 *M* HCl (dashed line). A new 235 mμ band appears with time in 0.5 *M* NaOH. The sydnone is propyl sydnone (10^{-4} *M*) at 70°.

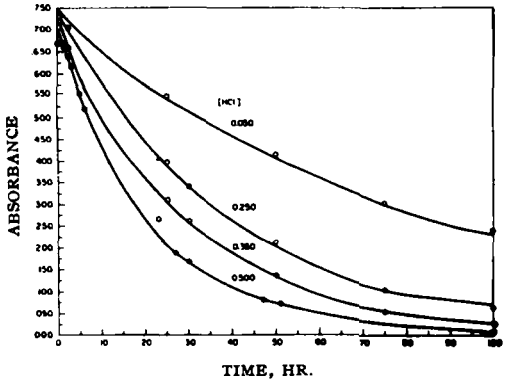


Fig. 2.—Typical curves for the loss of 284 mμ spectrophotometric absorbance of sydnones. The curves are obtained for the acid catalyzed solvolysis of *t*-butyl sydnone at 75°.

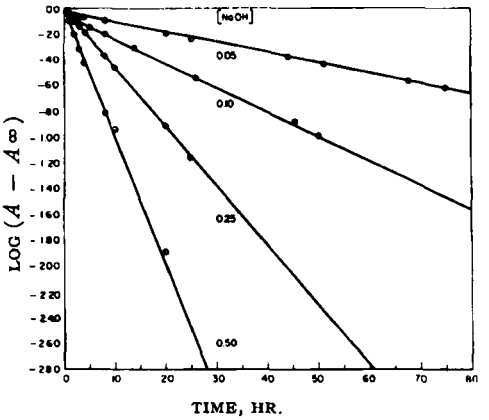


Fig. 3.—Typical first-order plots for the loss of 284 mμ absorbance (A) by alkaline catalyzed solvolysis for isopropyl sydnone at 75°. $A\infty$ is the asymptotic absorbance with time.

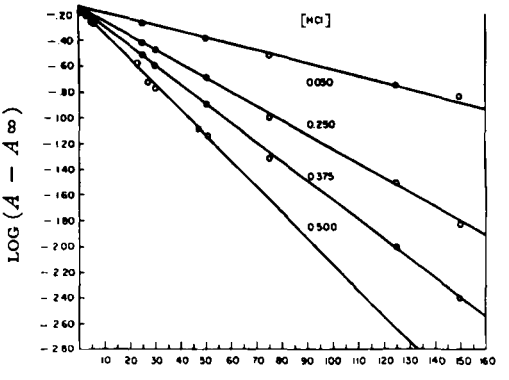


Fig. 4.—Typical first-order plots for the loss of 284 mμ absorbance. A, by acid catalyzed solvolysis, *t*-butyl sydnone at 75°. $A\infty$ is the asymptotic absorbance with time.

To abet solubilization, a few drops of ethanol were added to the sydnone prior to dissolution in the water. The alcohol percentage was negligible in a total final volume of 400 ml.

The aliquots to be assayed were rapidly cooled to room temperature before reading at the appropriate wavelengths on the Beckman model DU ultraviolet spectrophotometer.

Spectrophotometric Studies.—The absorbance, A, at the wavelength of the maximum for each sydnone (284 mμ, except 286 mμ for phenethyl and furfuryl sydnone) was read as a function of time until a small residual spectrophotometric reading, $A\infty$, remained constant or asymptotically approached zero.

In the cases of all sydnones but *t*-octyl a new band arose on alkaline catalyzed solvolysis but not on acid solvolysis. This new band had a maximum absorbance at wavelengths further toward the violet. Specifically, the maximum absorbance of the new absorption band was 232 mμ for propyl

TABLE I.—CONDITIONS AND RATE CONSTANTS (k IN SEC.$^{-1}$) FOR THE APPARENT FIRST-ORDER ACID CATALYZED DEGRADATION OF t-OCTYLSYDNONE[a] (10^{-4} M)

Temp., °C.	[HCl]	pH[b]	10^6k	$10^6(k - k_0)$[c]
50.0	0.050	1.39	0.390	0.13
50.0	0.125	1.02	0.518	0.25
50.0	0.250	0.73	0.781	0.42
50.0	0.375	0.56	1.07	0.71
50.0	0.500	0.44	1.43	1.08
54.7	0.050	1.39	0.710	0.07
54.7	0.125	1.02	1.03	0.39
54.7	0.250	0.73	1.50	0.86
54.7	0.375	0.56	1.95	1.31
54.7	0.500	0.44	2.47	1.83
61.0	0.050	1.39	1.82	0.18
61.0	0.125	1.02	2.61	0.97
61.0	0.250	0.74	3.37	1.73
61.0	0.375	0.57	4.36	2.72
61.0	0.500	0.44	6.59	4.95
64.9	0.125	1.02	4.21	1.86
65.2	0.250	0.74	5.58	3.23
64.9	0.375	0.57	7.71	5.36
64.9	0.500	0.44	9.96	7.61
69.0	0.0038	(2.31)	4.64	...
69.0	0.0063	(2.10)	4.54	...
69.0	0.0125	1.97	4.64	...
69.0	0.0188	1.78	5.60	1.00
69.0	0.025	1.60	5.63	1.03
69.0	0.050	1.39	6.23	1.63
68.3	0.100	1.11	7.93	3.33
68.3	0.200	0.83	9.49	4.9
69.6	0.250	0.74	11.4	6.8
68.3	0.300	0.66	12.8	8.2
68.3	0.500	0.45	18.2	13.6
75.0	0.050	1.39	11.6	1.1
74.9	0.125	1.02	14.7	4.2
75.0	0.250	0.74	24.0	13.5
75.0	0.375	0.58	29.6	19.1
75.0	0.500	0.45	38.3	27.8

[a] 1,1,3,3-Tetramethylbutylsydnone. [b] The pH values listed were calculated from pH = $-\log f_{HCl}[HCl]$, where the mean activity coefficients, f_{HCl}, were obtained or extrapolated from data in the literature for the appropriate temperatures. The parenthetical values were observed pH values. [c] The k_0 values were obtained from the k values for runs in 0.25 M NaCl.

sydnone, isobestic at 259 mμ; 256 mμ for furfuryl sydnone, isobestic at 268 mμ, 235 mμ for isopropyl sydnone; and 235 mμ for methyl sydnone. The t-butyl and s-butyl sydnones also showed a new band with maximum absorbance at 232 mμ when subjected to 0.2–0.5 M NaOH. The t-octyl sydnone showed no such new band with time in NaOH, HCl, or acetate and phosphate buffer solutions. Solvolysis of furfuryl sydnone in the neutral pH region also did not demonstrate the appearance of a new absorption band.

The rate of appearance of the new band on alkaline catalyzed solvolysis was the same as the rate of disappearance of the spectrophotometric absorption band of the sydnones. In general, the sum of the absorbances at the maxima of the disappearing and appearing bands was approximately constant.

Typical spectrophotometric curves as a function of time are given in Fig. 1 for propyl sydnone which demonstrate the appearance of a 235 mμ band on the disappearance of a 283 mμ band in alkaline solution. Typical curves for the disappearance of absorbance with time for the sydnones are given in Fig. 2 where the example given is for acid solvolysis.

CALCULATIONS AND RESULTS

Rate Constants.—The first-order rate constants, k, were calculated from the slopes of plots of the logarithm of the difference in absorbance, A, at time t and the final absorbance, $A\infty$, against time in accordance with:

$$\log (A - A\infty) = -kt/2.303 + \log A_0 \quad \text{(Eq. 1)}$$

Excellent first-order fits were obtained. Typical curves for alkaline and acid catalyzed solvolysis are given in Figs. 3 and 4.

The conditions and obtained rate constants for the acid catalyzed solvolysis of t-octyl sydnone which were studied in great detail are given in Table I. In addition, nine kinetic runs were studied in acetic acid–acetate buffers (0.05 M)

TABLE II.—CONDITIONS AND RATE CONSTANTS (k-SEC.$^{-1}$) FOR THE APPARENT FIRST-ORDER ACID CATALYZED DEGRADATION OF SYDNONES ($10^{-4}M$)[a]

Sydnone	Temp., °C.	[HCl][b]	10^6k	Sydnone	Temp., °C.	[HCl][b]	10^6k
Furfuryl	75.0	0.50	363	Phenethyl	75.1	0.375	0.196
	75.1	0.375	326		75.1	0.25	0.118
	75.1	0.25	312		75.1	0.05	0.026
	75.1	0.05	266		70.1	0.50	0.205
	70.0	0.50	199		61.6	0.50	0.075
	61.5	0.50	71.8				
t-Butyl	75.1	0.50	12.00	Isopropyl	75.2	0.50	0.408
	75.2	0.375	9.61		75.1	0.375	0.244
	75.2	0.25	7.07		75.1	0.25	0.195
	75.2	0.05	3.18		75.1	0.05	0.046
	70.0	0.50	6.40		70.1	0.50	0.219
	61.6	0.50	1.88		61.4	0.50	0.075
Propyl	74.9	0.50	0.306	s-Butyl	75.0	0.50	0.227
	75.1	0.375	0.259		75.0	0.375	0.171
	75.1	0.25	0.170		75.0	0.25	0.136
	75.1	0.05	0.038		75.0	0.05	0.043
	70.1	0.50	0.197		70.1	0.50	0.166
	61.6	0.50	0.061		61.6	0.50	0.077
Methyl	74.9	0.50	0.376		...	...	...
	75.1	0.375	0.286		...	...	...
	75.1	0.25	0.184		...	...	...
	75.1	0.05	0.048		...	...	...
	70.1	0.50	0.240		...	...	...
	61.6	0.50	0.072		...	...	...

[a] The phenethylsydnone kinetics were studied at 2.02×10^{-4} M. [b] The calculated pH at 75° for various [HCl] are 0.50 M 0.45; 0.375 M, 0.58; 0.25 M, 0.74; and 0.05 M, 1.39.

TABLE III.—CONDITIONS AND OBSERVED FIRST-ORDER CONSTANTS (k IN SEC.$^{-1}$) FOR THE APPARENT ALKALINE DEGRADATION OF SYDNONES ($10^{-4}M$)[a]

Sydnone	Temp., °C.	[NaOH][b]	10^5k	Sydnone	Temp., °C.	[NaOH][b]	10^5k
Furfuryl	75.1	0.50	39.1	Phenethyl	75.0	0.50	10.37
	75.1	0.25	29.1		75.0	0.25	5.41
	75.0	0.10	22.1		75.0	0.10	2.02
	75.1	0.05	23.0		75.0	0.05	1.04
	69.8	0.50	24.9		69.8	0.50	5.50
	65.3	0.50	17.7		65.3	0.50	3.70
	40.1	0.50	8.57		40.0	0.50	4.19
t-Butyl	75.1	0.50	4.34	Isopropyl	75.1	0.50	5.92
	75.1	0.25	2.12		75.1	0.25	2.91
	75.1	0.10	0.908		75.2	0.10	1.12
	75.1	0.05	0.469		75.1	0.05	0.516
	70.0	0.50	2.67		69.7	0.50	3.87
	65.3	0.50	1.74		65.3	0.50	2.50
	40.0	0.50	1.48		40.1	0.50	2.48
Propyl	75.0	0.50	8.57	*s*-Butyl	75.2	0.50	6.89
	75.0	0.25	4.41		75.2	0.25	3.38
	75.3	0.10	1.45		75.1	0.10	1.25
	75.0	0.05	0.863		75.2	0.05	0.446
	70.0	0.50	5.45		69.7	0.50	4.41
	65.3	0.50	3.45		65.3	0.50	2.66
	40.1	0.50	2.81		40.1	0.50	2.48
Methyl	75.0	0.50	10.45	*t*-Octyl	79.6	0.05	15.9
	75.0	0.25	5.29		75.1	0.50	13.0
	75.0	0.10	1.78		75.1	0.25	11.6
	75.0	0.05	0.933		75.1	0.10	10.5
	70.0	0.50	6.26		75.1	0.05	9.63
	65.3	0.50	3.97		69.9	0.50	6.74
	40.0	0.50	3.77		69.9	0.25	5.80
	...	...	...		69.9	0.10	5.33
	...	...	...		69.9	0.05	4.85
	...	...	...		69.9	0.005	5.18
	...	...	...		65.2	0.05	2.38
	...	...	...		61.1	0.50	2.07
	...	...	...		61.1	0.25	1.42
	...	...	...		61.0	0.05	1.41
	...	...	...		54.8	0.05	0.569
	...	...	...		50.0	0.05	0.313

[a] The phenethylsydnone kinetics were studied at 2.02×10^{-4} *M*. [b] Corresponding pH values can be calculated from $pOH = -\log f_{NaOH}[NaOH]$, $pH = pK_w - pOH$ where the activity coefficients, pOH, and the pK_w were given in or extrapolated from the data in the literature at 75°. Such calculated pH values are 12.24 for 0.50 *M* NaOH, 11.95 for 0.25 *M* NaOH, 11.61 for 0.10 *M* NaOH, and 11.34 for 0.05 *M* NaOH.

at 70.0° at pH values 2.98, 3.85, 4.29, 4.35, 4.51, 4.74, 4.92, 5.18, and 5.60; two runs were studied in phosphate buffers (0.10 *M*) at pH values 5.68 and 7.68; and four runs were studied in sodium chloride solutions at 0.01, 0.05, 0.10, and 0.30 *M*. The overall average 10^5k (sec.$^{-1}$) of these 15 runs was 4.82, standard deviation = 0.17. There was no significant effect of buffer, salt concentration, or pH on these rate values. Other studies on *t*-octyl sydnone hydrolysis in 0.25 *M* NaCl for various temperatures are: °C., 10^5k; 50.0°, 0.307; 54.8°, 0.637; 61.0°, 1.64; 65.2°, 2.35; 75.1°, 10.5; 79.6°, 15.7.

Conditions and rate constants for the apparent first-order acid catalyzed degradation of the other sydnones studied are given in Table II. The only compounds in this table that demonstrated the pH-independent solvolysis, k_o, characteristic of *t*-octyl sydnone were *t*-butyl sydnone (75°, pH 4.40, acetate buffer, $k = 2.5 \times 10^{-6}$ sec.$^{-1}$; pH 6.50, phosphate buffer, $k = 2.8 \times 10^{-6}$ sec.$^{-1}$) and furfuryl sydnone (75°, pH 4.40, acetate buffer, $k = 2.14 \times 10^{-4}$ sec.$^{-1}$; pH 6.50, phosphate buffer, $k = 2.27 \times 10^{-4}$ sec.$^{-1}$; 0.05 *M* NaCl, $k = 2.35 \times 10^{-4}$ sec.$^{-1}$; 0.25 *M* NaCl, 2.44×10^{-4} sec.$^{-1}$; 0.375 *M* NaCl, $k = 2.35 \times 10^{-4}$ sec.$^{-1}$; 0.50 *M* NaCl, $k = 2.66 \times 10^{-4}$ sec.$^{-1}$). The more general evaluations of k_o values are given below. All the other sydnones exhibited no significant change in spectrophotometric absorbance in the neutral pH regions, *viz.*, for 2 weeks at 75° in 0.5 *M* NaCl and also in acetate and phosphate buffers.

TABLE IV.—CONDITIONS AND APPARENT FIRST-ORDER RATE CONSTANTS (10^5k IN SEC.$^{-1}$) FOR THE SOLVOLYSIS OF *t*-OCTYL SYDNONE IN VARIOUS ETHANOL-WATER MIXTURES

Vol. % Ethanol	Temp., °C. 61.3	65.0	70.0	75.0
0	1.65	2.36	4.70	10.1
10	1.13	1.98	2.66	6.65
20	0.589	1.22	2.05	4.12
30	0.409	0.531	1.06	1.96
40	0.162	0.267	0.528	1.04
50	0.164	0.168	0.287	0.56
60	...	0.089	0.205	...
70	...	...	0.124	...

Conditions and rate constants for the apparent first-order alkaline degradation of the studied sydnones are given in Table III.

Effect of Sydnone Concentration on Rates.—There was no significant effect of sydnone concentration on the apparent first-order rate constant. In the following instances kinetic runs at 70° were

effected in 0.5 M HCl at 1.008 × $10^{-4}M$ and 2.016 × $10^{-4}M$ in sydnone (except for phenethylsydnone which was at twice these values). Aliquots of the higher concentration were diluted 1:1 with 0.5 M HCl so that it could be read at relatively the same absorbance as the lower concentration. The sydnones and the rate constants as 10^6k obtained at these two kinetic concentrations were: furfuryl, 199, 198; isopropyl, 0.219, 0.203; s-butyl, 0.166, 0.189; phenethyl, 0.205, 0.192; t-butyl, 6.40, 6.75; propyl, 0.197, 0.200; methyl, 0.240, 0.246.

The rate constants for the solvolysis of t-octyl sydnone in various ethanol-water mixtures and at several temperatures are given in Table IV.

Rate Dependency on Catalytic Species.—The log k-pH profiles for the solvolysis of sydnones that possess pH-independent regions of solvolysis (t-octyl, t-butyl, and furfuryl) are given in Fig. 5. The pH for the acidic region is calculated from

$$\text{pH} = -\log f_{\text{HCl}}[\text{HCl}] \qquad \text{(Eq. 2)}$$

where the mean activity coefficient f_{HCl} was obtained or extrapolated from data in the literature (8). The pertinent values are given in Tables I and II.

The pH for the alkaline region is calculated from

$$\text{pH} = \text{pK}_w - p\text{OH} = \text{pK}_w + \log f_{\text{NaOH}}\,[\text{NaOH}] \qquad \text{(Eq. 3)}$$

where the mean activity coefficient f_{NaOH} and the pK_w was obtained or extrapolated from data in the literature (8).

There are several ways (9) of quantifying kinetic expressions to define explicitly dependence of apparent first-order rate constants on the catalytic variables. Specific acid catalysis can be considered in the expression

$$k = k_{\text{HCl}}[\text{HCl}] + k_o \qquad \text{(Eq. 4)}$$

where k is the apparent first-order rate constant for solvolysis in the acid region and k_o is the pH-independent apparent first-order rate constant. It follows that k plotted against [HCl] should permit estimation of the slope k_{HCl}, the bimolecular rate constant for the HCl catalyzed solvolysis, and the intercept k_o. Such plots are given in Figs. 6 and 7 for the various sydnones at several temperatures and the derived k_{HCl} and k_o values are given in Table V.

Figure 6 is such a plot with nonzero intercepts for the sydnones that exhibited pH-independent solvolysis, whereas Fig. 7 is for those where pH-independent solvolysis was not significant.

An alternate method of rate constant determination is valid for the postulated rate dependence

$$k - k_o = k_{\text{H}^+}a_{\text{H}^+} \qquad \text{(Eq. 5)}$$

where k_{H^+} is the specific hydrogen ion catalytic rate constant and a_{H^+} is the activity of the hydrogen ion so that

$$\log (k - k_o) = \log k_{\text{H}^+} - \text{pH} \qquad \text{(Eq. 6)}$$

where pH can be calculated as in Eq. 2.

Such plots are given in Fig. 8 for t-octyl sydnone and t-butyl sydnone where the intercepts of the plots of the expected theoretical slope (*i.e.*, −1) are the log k_{H^+} values given on the semilog plot as k_{H^+}. An example of such a plot for a sydnone,

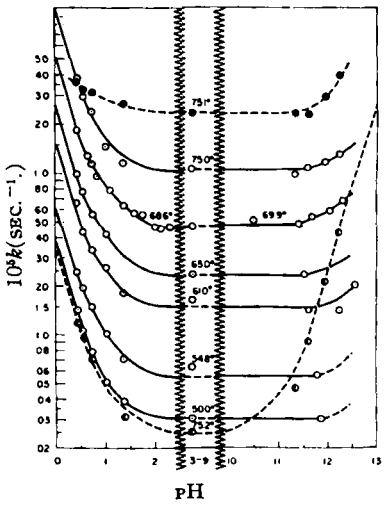


Fig. 5.—Profiles of the dependence of rate constants on pH for the solvolysis of sydnones that show a pH-independent region of solvolysis. Key: ⊙ t-octyl sydnone, ● furfuryl sydnone, ◐ t-butyl sydnone.

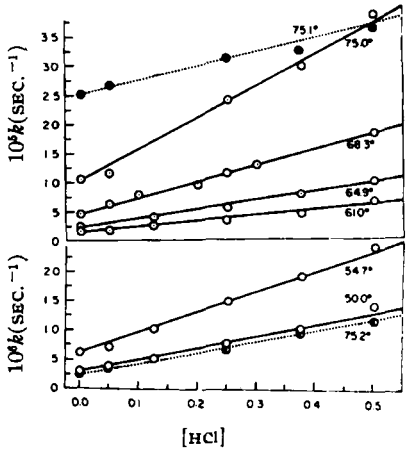


Fig. 6.—Apparent first-order rate constant, k, dependence on [HCl] at various temperatures for sydnones that exhibit pH-independent solvolysis. Key: ⊙ t-octyl sydnone, ● furfuryl sydnone, ◐ t-butyl sydnone.

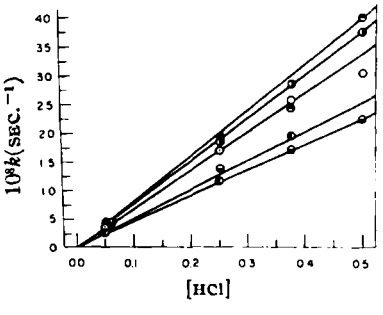


Fig. 7.—Apparent first-order rate constant, k, dependence on [HCl] at 75° for sydnones that do not exhibit pH-independent solvolysis. Key: ⊙ propyl, ◑, methyl, ◐, phenethyl, ◓ isopropyl, ◒ s-butyl.

TABLE V.—CATALYTIC CONSTANTS FOR THE ACID AND NEUTRAL SOLVOLYSIS OF THE ALKYL SYDNONES (k_i IN SEC.$^{-1}$)

Sydnone	Temp., °C.	10^5k_{HCl}[a]	10^5k_o[a]	$10^5k_{H^+}$[b]
t-Octyl	50.0	20.2	3.1	27.2
	54.7	35.2	6.3	49.3
	61.0	90.0	16.0	120
	64.9	148	25	203
	68.3	222	46	420
	75.0	530	105	725
t-Butyl	75.2	19.0	2.5	27
Furfuryl	75.1	232	250	350
Methyl	75.1	0.676	0.00	1.08
Propyl	75.1	0.760	0.00	0.96
Phenethyl	75.1	0.508	0.00	0.68
Isopropyl	75.1	0.800	0.00	1.08
s-Butyl	75.0	0.450	0.00	0.70

[a] Determined from the slopes and intercepts of plots of the apparent first-order rate constants k(sec.$^{-1}$) against [HCl] according to the expression $k = k_{HCl}[HCl] + k_o$. [b] Determined from the intercept of plots of log ($k - k_o$) against pH according to the expression log ($k - k_o$) = log k_{H^+} − pH where pH = −log f_{HCl}[HCl], where f_{HCl} is the mean activity coefficient obtained from the literature (8).

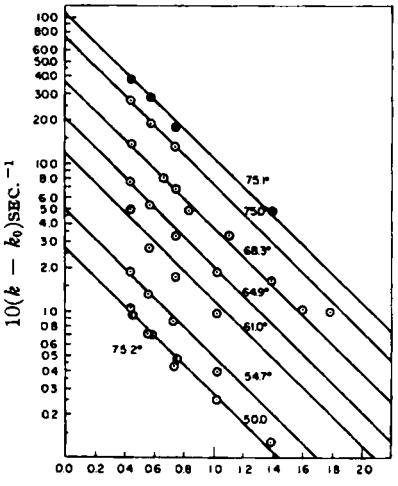


Fig. 8.—Semilogarithmic plots of the apparent first-order rate constants due to specific acid catalyzed solvolysis of sydnones, $k - k_o$, as a function of calculated pH. Key: ⊙, *t*-octyl, ◐, *t*-butyl. ● methyl.

methyl sydnone, whose degradation is nonsolvent catalyzed (*i.e.*, $k_o = 0$) is also given in Fig. 8. The k_{H^+} values so determined are also given in Table V. Either catalytic rate constant, k_{H^+} or k_{HCl}, is valid for the prediction of the apparent first-order rate constant, k, provided that it is used in the appropriate equation for the acidity function appropriately defined.

Similarly, the specific hydroxyl ion catalytic rate constants can be determined from the slopes of plots of the apparent first-order rate constants, k, against [NaOH] according to the expression

$$k = k_o + k_{NaOH}[NaOH] \qquad \text{(Eq. 7)}$$

Typical examples are given in Fig. 9, the pertinent data are given in Table VI.

Rate Dependency on Temperature.—The logarithmic version of the Arrhenius expression is

$$\log k = -\Delta H_a/RT + \log P \qquad \text{(Eq. 8)}$$

Such Arrhenius plots of the logarithms of various catalytic rate constants for the solvolysis of *t*-octyl sydnone are given in Fig. 10 for k_{H^+}, the specific acid catalytic rate constant where acidity is defined in terms of activity (Eqs. 5 and 6); k_{HCl}, where the acid is defined in terms of molar concentrations of hydrochloric acid (Eq. 4); k_o, the first-order rate constant for pH-independent solvolysis; and $k_{(0.5\ M\ NaOH)}$, the apparent first-order rate constant for the solvolysis; in 0.5 *M* NaOH. The slopes of such plots for the variously derived k_i values are not signficantly different, indicative of the fact that the heats of activation, ΔH_a = 30 Kcal./mole, for acid, neutral or alkaline solvolysis of *t*-octyl sydnone are the same. Also in Fig. 10 are Arrhenius plots for $k_{(0.5\ M\ HCl)}$ (apparent first-order rate constants for solvolysis in 0.5 *M* HCl) for *t*-butylsydnone, furfuryl sydnone, and isopropyl sydnone. The latter was typical of the other sydnones.

Arrhenius plots for solvolysis of other alkyl sydnones in 0.5 *M* NaOH are given in Fig. 11. The pertinent ΔH_a and log *P* values are listed in Table VII.

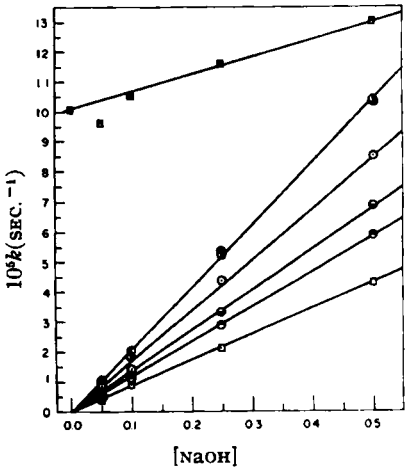


Fig. 9.—Apparent first-order rate constant, k, dependence on [NaOH] at 75° for *t*-octyl sydnone, ⊟, methyl sydnone, ◑; phenethyl sydnone, ◐; propyl sydnone, ⊙; *s*-butyl sydnone, ◒; isopropyl sydnone, ◓; and *t*-butyl sydnone, ⊡.

TABLE VI. —CATALYTIC CONSTANTS FOR THE ALKALINE AND NEUTRAL SOLVOLYSIS OF THE ALKYLSYDNONES (k_i IN SEC.$^{-1}$)

Sydnone	Temp., °C.	10^5k_{NaOH}	10^5k_o
t-Octyl	75.1	5.90	10.05
	69.9	3.46	4.95
Furfuryl	75.1	37.2	20.4
t-Butyl	75.1	8.70	0.25
Propyl	75.0	17.0	0.00
Methyl	75.0	20.8	0.00
Phenethyl	75.0	20.8	0.00
Isopropyl	75.1	11.8	0.00
s-Butyl	75.2	13.6	0.00

Rate Dependency on Ethanol Content.—The limiting case for a dipolar molecule–dipolar molecule reaction is usually independent of ionic strength but dependent on dielectric constants, D (10, 11).

$$\log k = \log k_{(D=\infty)} - K(1/D) \qquad \text{(Eq. 9)}$$

Plots of logarithms of the first-order rate con-

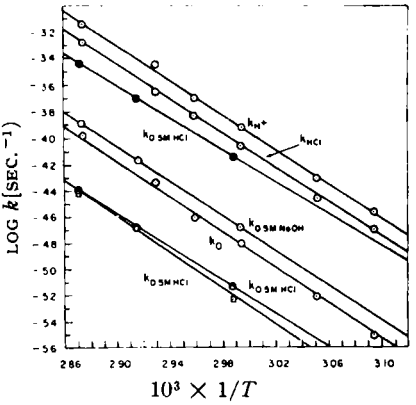


Fig. 10.—Arrhenius plots for the solvolysis of *t*-octyl sydnone, ⊙; *t*-butyl sydnone, ⊡; isopropyl sydnone, ◓; and furfuryl sydnone, ●; The k_i are: the specific hydrogen ion catalyzed solvolysis derived from hydrogen ion activity, k_{H^+}, and derived from hydrochloric acid molarity, k_{HCl}; and the apparent first-order rate constants in 0.5 *M* NaOH, $k_{0.5\ M\ NaOH}$ and in 0.5 *M* HCl, $k_{0.5\ M\ HCl}$.

stants (Table IV) for *t*-octyl sydnone solvolysis in ethanol-water mixtures against the reciprocal of the dielectric constants for such mixtures are given in Fig. 12. The values of K, log $k_{(D\ -\ \infty)}$ and $k_{(D\ -\ \infty)}$ for several temperatures are listed in Table VIII. The dielectric constant, D, was obtained from the values of such ethanol-water mixtures at 25° (8) after conversion of the per cent by volume data to per cent by weight values (12).

DISCUSSION

Prediction of Stability.—The stability of the sydnones in aqueous solutions can be calculated from the apparent first-order rate constants, k in sec.$^{-1}$, where the half-life in days (9),

$$t^1/_2 = 8.03 \times 10^{-6}/k \qquad \text{(Eq. 10)}$$

The apparent first-order rate constants, k_{HCl}[HCl] and k_{NaOH}[NaOH], for the solvolysis of alkyl sydnones that do not exhibit pH-independent solvolysis can be estimated from the $k_{0.5\ M}$ values derived from the Arrhenius parameters listed in Table VII.

$$k_{0.5\ M} = Pe^{-\Delta H_a/RT} \qquad \text{(Eq. 11)}$$

where $k_{HCl} = 2\ k_{0.5\ M\ HCl}$ and $k_{NaOH} = 2k_{0.5\ M\ NaOH}$ since a bimolecular rate constant can be estimated from the quotient of the apparent first-order rate constant and the molar concentration of the catalytic species. Estimates of a k_o' at an absolute temperature T' for those sydnones that do exhibit pH-independent solvolysis can be based on the same heat of activation as obtained in 0.5 *M* HCl (Table VII) and one k_o value for a particular temperature, T (Table V)

$$k_o' = k_o e^{-\Delta H_a/R(1/T\ -\ 1/T')} \qquad \text{(Eq. 12)}$$

Thus the first-order rate constants for the solvolysis of these sydnones can be calculated from

$$k = k_{HCl}[HCl] + k_{NaOH}[NaOH] + k_o \qquad \text{(Eq. 13)}$$

or

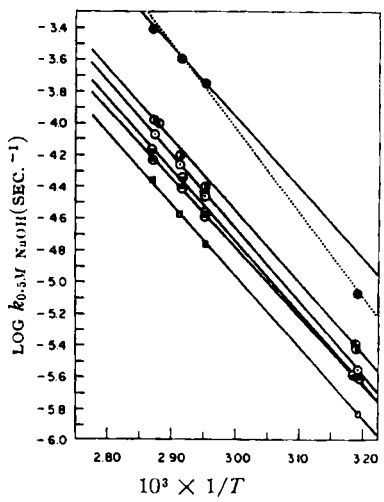


Fig. 11.—Arrhenius plots for the apparent first-order rate constants of solvolysis of alkyl sydnones in 0.5 *M* NaOH. Key: ⊡, *t*-butyl sydnone; ◓, isopropyl sydnone; ◒, *s*-butyl sydnone; ⊙, propyl sydnone; and ●, furfuryl sydnone. Two possible alternative relations are given for furfuryl sydnone.

$$k = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_o \qquad \text{(Eq. 14)}$$

dependent on the definition of the catalytic rate constants with respect to the catalytic species (9).

Application of these relations shows that the majority of the alkyl sydnones are quite stable at 30°. For example, the half-lives of the methyl, propyl, etc., sydnones are *ca.* 10 years in 0.1 *M* HCl and *ca.* 2 years in 0.5 *M* HCl; but the furfuryl sydnone has a half-life of 6 days in 0.5 *M* HCl.

The half-lives of the *t*-octyl and furfuryl sydnone in water alone at 30° can be predicted as measurable in days—13 days for furfuryl and 100 days for *t*-octyl (7 years for *t*-butyl). Such solutions would be unstable for pharmaceutical use.

Fortunately, the pH-independent solvolysis is highly restricted by dielectric constant. The plots

TABLE VII.—APPARENT THERMODYNAMIC QUANTITIES[a] FOR THE SOLVOLYTIC DEGRADATION OF THE ALKYL SYDNONES

Sydnone	—0.5 *M* [HCl]— ΔH_a	log *P*	—0.5 *M* [NaOH]— ΔH_a	log *P*
t-Octyl[b]	30.1	15.5	30.2	15.1
t-Butyl	31.0	14.6	20.5	8.52
s-Butyl	29.0	11.8	20.5	8.73
Propyl	29.0	11.8	21.2	9.23
Isopropyl	29.0	12.0	19.9	8.30
Methyl	29.0	12.0	21.7[c]	9.70[c]
Phenethyl	29.0	12.0	21.7[c]	9.70[c]
Furfuryl	27.6	13.9	20.4[d]	9.42[d]

[a] The quantities are derived from the logarithmic form of the Arrhenius relation: log $k = -(\Delta H_a/2.303\ R)\ (1/T) +$ log P, where the k is in sec.$^{-1}$ for solvolysis in 0.5 *M* HCl and 0.5 *M* NaOH, the ΔH_a is the heat of activation in Kcal./mole, R is the gas constant in cal./degree/mole, T is the absolute temperature, and log P is a constant associated with entropy. [b] For k_o, pH-independent solvolysis of *t*-octylsydnone, $\Delta H_a = 30.6$ and log $P = 14.9$. For k_{HCl} defined by $k_{HCl} = (k - k_o)/[HCl]$, where k is the apparent first-order rate constant in a given molarity of [HCl], $\Delta H_a = 29.7$ and log $P = 15.7$. For k_{H^+} defined by $k_{H^+} = (k - k_o)/[H^+]$, where $[H^+] = 10^{-pH}$ where pH = log f_{HCl}[HCl] where f_{HCl} is the mean activity coefficient as given by or extrapolated from literature values, $\Delta H_a = 29.8$ and log $P = 15.8$. [c] Alternative values omitting the 40° study are $\Delta H_a = 23.7$ and log $P = 10.9$. [d] Alternative values weighting the apparent deviate study at 40° are $\Delta H_a = 24.4$ and log $P = 11.96$.

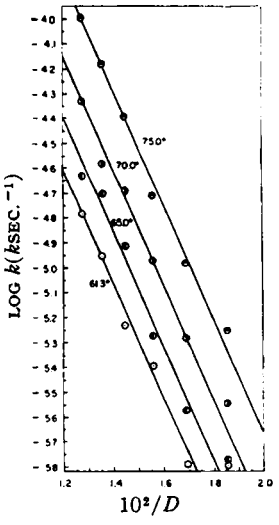


Fig. 12.—Plots of logarithms of first-order rate constants for the pH - independent solvolysis of *t*-octyl sydnone in ethanol - water mixtures against the reciprocal of the dielectric constant for such solvents.

or Fig. 12 and the values of Tables IV and VII show more than a tenfold decrease in rate from water to 50% ethanol-water. The magnitude of the slope, K, is equivalent to that observed for the great effect of dielectric constant on the rate of aspirin anhydride solvolysis in various dioxane-water mixtures (13).

The fact that Eq. 9 is applicable to the solvolysis of the *t*-octylsydnone that exhibits pH-independent solvolysis in the intermediate pH region is consistent with the model of a dipolar molecule attack on the dipolar molecule. It excludes the cases of like charged ions reacting and the attack of a positive ion on a dipolar molecule (9).

Order of Reactivities of Alkyl Sydnones.—The sequence of reactivities (Table VI) for alkaline catalyzed solvolysis of the sydnones at 75° is: furfuryl > methyl ~ phenethyl > propyl > *s*-butyl ≥ isopropyl > *t*-butyl > *t*-octyl. This is a reasonable sequence for expected inductive effects; *i.e.*, the more electron donating the substituent, the less reactivity to hydroxyl ion attack. However, this sequence is not capable of being assigned to significant variations in the heats of activation determined for hydrolysis in 0.5 *M* NaOH (Table VII) where inductive effects might conceivably be demonstrated (14). The heats of activation as determined in 0.5 *M* NaOH were 21 Kcal./mole for all within the error of estimate, except for *t*-octyl sydnone (30 Kcal./mole). In this case it must be realized that in 0.5 *M* NaOH the pH-independent solvolysis rather than the hydroxyl ion catalyzed solvolysis provides the major route of degradation. The lack of appearance of a spectrophotometric band capable of being assigned to the *N*-nitroso *t*-octylamino acid anion made this apparent, whereas a comparable band appeared in the cases of the 0.5 *M* NaOH solvolysis of the other sydnones.

TABLE VIII.—DEPENDENCE OF RATE CONSTANT (k IN SEC.$^{-1}$) FOR *t*-OCTYL SYDNONE SOLVOLYSIS ON THE RECIPROCAL OF THE DIELECTRIC CONSTANT, D, OF ETHANOL-WATER SOLUTIONS AS PER THE EXPRESSION LOG $k = -K(1/D) + \text{LOG } k_{(D=\infty)}$

Temp., °C.	K	log $k_{(D=\infty)}$	$10^2 k_{(D=\infty)}$
61.3	230	−1.85	1.41
65.0	230	−1.65	2.24
70.0	231	−1.38	4.18
75.0	230	−1.07	8.51

The sequence of alkaline catalyzed reactivities at 75° (Table VI) is almost the same as that for decreasing log P values (or decreasing entropies of activation) as listed in the second column of Table VII for solvolysis in 0.5 *M* NaOH. The values for the *t*-octyl and furfuryl sydnones must be excluded because the degradation by pH-independent solvolysis contributes greatly under the stated experimental conditions (0.5 *M* NaOH) for log P. In this instance methyl ~ phenethyl > propyl > *s*-butyl > *t*-butyl > isopropyl. The only radical slightly displaced is isopropyl. The reactivity and log P sequence is also consistent with expectation on the basis of branching of the substituent where the greater the branching the greater the decrease in entropy (*i.e.*, the lower the log P) on passing from reactants to the transition state (15, 16*a*). Also, the greater the positivity of the reacting sydnone ring, the less the decrease in entropy (or log P) on reaction with a negative ion (16*b*). In this manner the correlation of rate magnitude and log P on electron withdrawing substituents (primary > secondary > tertiary alkyls) could be rationalized.

The reactivity sequence, except for the fascinating case of the furfuryl sydnone, virtually reverses itself for the acid catalyzed solvolysis (Table V) of the sydnones at 75°—*t*-octyl > furfuryl ≫ *t*-butyl ≫ isopropyl ≥ propyl ≥ methyl > phenethyl ≥ *s*-butyl. This sequence is reasonably in accordance with the expected relation for electron donating groups (tertiary > secondary > primary alkyls), except for the furfuryl sydnone and the slight displacement of *s*-butyl in the series. There are no great differences among the heats of activation (29–30 Kcal./mole), except for the furfuryl sydnone which shows a decreased value. The tertiary alkyls have slightly higher values than the secondary and primary (Table VII).

The reactivity sequence (Table V) is almost the same as that for decreasing log P values (Table VII) obtained from solvolysis of the sydnones in 0.5 *M* HCl—*t*-octyl > *t*-butyl > furfuryl > methyl ~ isopropyl ~ phenethyl > *s*-butyl ~ propyl. However, the concept of branching decreasing the entropy of activation and log P values is not applicable here (15, 16*a*). When ions attack like-charged species, relatively large decreases in entropy are expected in the formation of the activated state (16*b*). The sequence of reactivities (tertiary > secondary or primary) can be rationalized as entropy effects since enhancement of the positive charge on the sydnone ring could be affected by electron withdrawing groups.

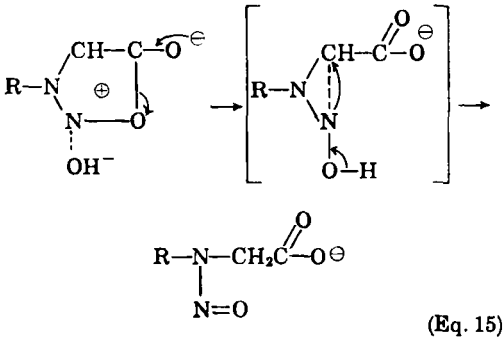
In the three cases of pH-independent solvolysis of the alkyl sydnones, the reactivity at 75° is in the sequence furfuryl > *t*-octyl ≫ *t*-butyl. The accentuated solvolytic attack by acid and the presence of highly significant pH-independent solvolysis with the highly branched *t*-octyl sydnone possibly implicates a degree of desolvation to the activated state. Such an explanation implies that solvation is greatest in the simple alkyl sydnones where the activated state for pH-independent and acid catalyzed solvolysis is relatively less solvated. It is reasonable to expect hydrogen ion attack to involve induction of the polarized *meso*-ionic sydnone to one

less so. Thus a relatively lower log P (or a greater decrease in entropy) would occur with the more solvated simpler alkyl sydnones. It is indicative that *t*-butyl, *t*-octyl, and furfuryl sydnones have higher melting points (4) and thus tend to become ordered more readily than the other sydnones.

Since the proton attacks the positively charged sydnone ring on acid catalyzed solvolysis, it is expected that the heat of activation would be greater than for hydroxyl ion attack (Table VII).

The higher log P values for acid catalyzed solvolysis of the sydnones over alkaline catalyzed solvolysis may be accounted for by a difference in mechanism. If the products of one reaction are greater in number than those of another, the entropy of the reaction usually increases (16*a*). However, a more explicit explanation can be related to the reaction mechanism

Reaction Mechanisms.—The alkaline catalyzed solvolysis of sydnones produces the respective *N*-nitroso acids (2) as was substantiated by the appearance of a spectral band capable of being assigned to these compounds. A plausible mechanism for this degradation[1] would be

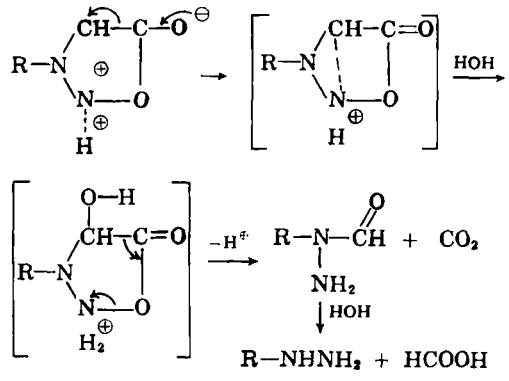


(Eq. 15)

The incipient 2,4 bond or quasidiazomethane is reasonable in light of literature observations of such additions (17, 18).

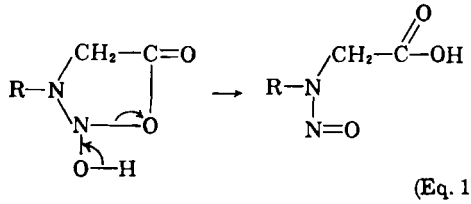
The point of attack of the hydroxyl ion could be at the nitrogen in the 2-position of the sydnone, the atom to which is assigned the greatest formal negative charge of the sydnone ring (2). It is apparent that an electronic redistribution must be induced on approach of the hydroxyl ion through the bonded p-orbitals of the atoms of the aromatic sydnone ring for this attack to be effective. However, proton attack on the 2-position should cause less "ordering" to form the activated state; thus a more positive entropy of activation is (Table VII) and should be present on proton catalyzed solvolysis than with hydroxyl ion catalyzed solvolysis. The reactions of oppositely charged species have more positive entropies of activation than the reactions of similarly charged species (16).

Since it has been reported that acid hydrolysis of sydnones gives an alkyl hydrazine, a carboxylic acid, and carbon dioxide (2, 6, 7), a possible detailed mechanism based on that given in the literature (2) would be



(Eq. 16)

Of course, water attack could occur similarly without prior protonation. An alternative mechanism could be visualized where water is added inversely across the incipient diazomethane

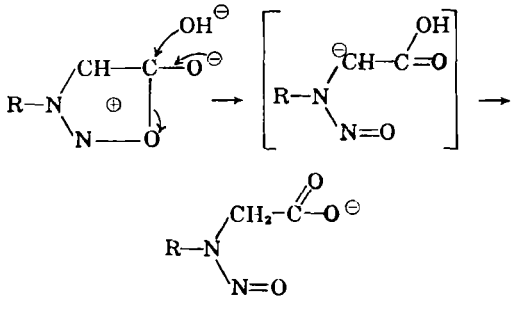


(Eq. 17)

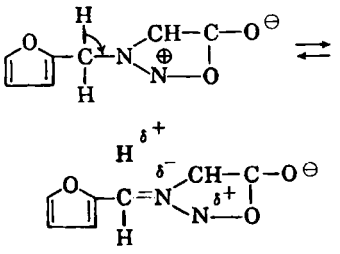
However, the lack of appearance of a spectral band for the *N*-nitroso alkylamino acetic acid on acidic and neutral solvolysis diminishes this possibility and implies similar mechanisms in acid and nonhighly alkaline solutions. The *N*-nitroso compound does show significant acid degradation but no significant change in ultraviolet absorbance in alkaline solution. Studies on the kinetics of hydrolysis of the corresponding *N*-nitroso alkylamino acetic acids are in process to clarify this point.

The most anomalous of the sydnones was the furfuryl which showed high reactivity in acid, alkaline, and intermediate solutions. If the furan ring is considered as an electron sink it may provide electron attracting properties to the furfuryl radical on hydroxyl ion attack. This implies that the intervening methylene group does not insulate the furan ring from polarizing the sydnone and suggests that the benzyl radical may act similarly. Also, the relatively unfilled p-orbital of the 3-position of the sydnone ring (2) may accept electrons readily from the furfuryl carbon and promote a *mesomeric or quasi conjugated system*—thus readily polarize the sydnone ring for hydrogen ion attack.

[1] An alternative mechanism could be postulated by an hydroxyl ion attack on the 5 position, the oxygen bonded carbon.



This position has an excess of positive formal charge (2). If this is a preferred mechanism, 5-alkylated sydnones should demonstrate significantly less susceptibility to hydroxyl ion attack than the sydnones studied herein.



(Eq. 18)

A molecular model of furfuryl sydnone indicates the possibility of stabilization of the activated complex by the oxygen of the furfuryl ring which is sterically positioned adjacent to the 2-nitrogen.

Furthermore, this conformation allows the maximum hyperconjugation between the methylene and 4-nitrogen position. The probability of hyperconjugation is further enhanced by the resulting extended conjugation since the two rings are coplanar in this conformation. If this explanation is valid, benzyl sydnone would not be so readily solvolyzed as furfuryl sydnone. However *o*-aminobenzyl or *o*-hydroxybenzyl or a pyrrole analog of furfuryl sydnone would have greatly accelerated solvolysis.

It is also possible to define the reaction sequence in alkali by hyperconjugative stabilization of the transition state by α-hydrogens since methyl sydnone has three—propyl, furfuryl, and phenethyl have two—isopropyl and *s*-butyl have one—and *t*-butyl and *t*-octyl have no α-hydrogens.

SUMMARY

1. The kinetics of solvolysis of a series of *meso*-ionic compounds, the 3-alkyl sydnones, have been studied spectrophotometrically in aqueous solutions by the measurement of absorbance at *ca.* 284 mμ.

2. There was no significant effect of buffer kind or buffer and sydnone concentration on the apparent first-order rate constants.

3. The reactivity on alkaline catalyzed solvolysis generally decreased in the order furfuryl, methyl, propyl, phenethyl, isopropyl, *s*-butyl, *t*-butyl, and 1,1,3,3-tetramethylbutyl sydnone. Except for the furfuryl sydnone, the order of reactivity generally reversed on acid catalyzed solvolysis. These sequences are in accordance with what would be expected from electron inductive effects.

4. The heats and entropies of activation were determined from rate studies and various temperatures. The variation in reactivities on alkaline catalyzed solvolysis is largely assigned to the entropy factor and can be explained by the branching of the alkyls, the variation in positivity of the sydnone ring due to the substituents which affects the attack of a negative ion, and/or the hyperconjugative stabilization of the transition state by α-hydrogens on acid catalyzed solvolysis. The variation in positivity of the sydnone ring due to the substituents which affects the attack of a positive ion could affect the entropy factor.

5. The highly branched alkylsydnones, *t*-butyl and 1,1,3,3, tetramethylbutyl, demonstrate a pH-independent solvolysis in the order implying a similar mechanism of attack as by acid catalyzed solvolysis. No spectrophotometric band assignable to a *N*-nitroso alkylaminoacetic acid was observed in acid and pH-independent solvolysis. Alternate points of possible attack of hydroxyl ion are at the 2 or 5 positions with the end product being the spectrophotometrically observed *N*-nitroso alkylaminoacetic acid.

6. The only truly anomalous compound in the sequence of reactivities is the furfuryl sydnone which is highly reactive on both acid and base catalyzed solvolysis and also exhibits a pH-independent solvolysis. Possible explanations are by intramolecular assistance of solvolysis by the furfuryl oxygen or by extension of conjugation because of a labile methylene hydrogen.

7. The solvolysis of 1,1,3,3-tetramethylbutyl sydnone in aqueous solvents of increasing ethanolic content is dramatically inhibited and can be related to the decrease in dielectric constant which implies a dipolar molecule attack on a dipolar molecule rather than a reaction of like charged ions or of a positive ion on a dipolar molecule in the rate-determining step.

8. On the basis of the heats of activation and entropy factors derived from the rate studies, stability of the various sydnones are predicted for various pH, acidic, and basic conditions.

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Effects of Selected U.S.P. Talcs on Acetylsalicylic Acid Stability in Tablets

By GERALD GOLD and JAMES A. CAMPBELL*

Talc U.S.P. varies in chemical composition according to its source and method of preparation. The effects of four different U.S.P. talcs on the stability of aspirin tablets at elevated temperature and humidity were studied. Data obtained indicated that different talcs varied significantly in their effects on the stability of aspirin. Aspirin stability was improved by acid washing the talcs. Selected talcs were analyzed and experiments conducted in an attempt to identify the factors adversely influencing aspirin stability.

As DEFINED IN the U.S.P. XVI (1) "Talc is a native, hydrous magnesium silicate, sometimes containing a small proportion of aluminum silicate." Chemically, talc is a hydrated magnesium silicate having the general formula, $3MgO \cdot 4SiO_2 \cdot H_2O$, in which layers of brucite are between silicon-oxygen layers with each layer being somewhat modified by isomorphous replacement. Talc is formed by the breaking down of tremolite, instatite, and other magnesium minerals (2). Purified talc is made by boiling very finely powdered talc with water containing about 2% hydrochloric acid, allowing the insoluble material to subside, decanting the supernatant liquid, and repeating the process with a weaker hydrochloric acid solution. The talc that has been freed of iron and other soluble impurities is thoroughly washed with water and dried at 110° (3).

Impurities which are reported to be present in talc are calcium carbonate, calcium silicate, and iron oxide (4). These impurities result in modification of the physical characteristics of the talc. When the calcium content is high and in the form of calcium silicate or crystalline calcium carbonate, the powdered material is abrasive. The presence of iron (either as ferric oxide or a complex magnesium ferric silicate) tends to make the talc grayish in appearance.

Talc is widely used in the pharmaceutical industry as a tablet lubricant and, as such, may influence tablet stability. The rate of aspirin decomposition in tablet formulations containing aspirin, phenacetin, and caffeine has been reported to be minimized when talc is used as the lubricant (5, 6). Since talc is a natural product, it does not have constant composition, but varies depending upon its origin and method of preparation. Analyses of talcs usually show variations in composition (7).

This investigation was undertaken to determine whether different U.S.P. talcs would vary in their effects on the stability of aspirin. The data obtained indicated that the stability of aspirin did vary with different talcs and an attempt was made to determine whether additional acid washing of talc would result in better aspirin stability. Selected talcs were analyzed and experiments conducted to identify the factors in talc adversely influencing aspirin stability.

EXPERIMENTAL

Materials Used.—Commercially available 20-mesh aspirin crystals of U.S.P. grade and commercially available U.S.P. talcs were used throughout this experiment. The talcs were designated talc *A*, *B*, *C*, and *D*. Other chemicals used were U.S.P. grade of calcium carbonate, C.P. grade of aluminum silicate, pharmaceutical grade of red iron oxide, and purified calcium silicate.

Acid Washing of Talcs.—Talcs *A*, *B*, *C*, and *D* were acid washed by the following procedure: 50 Gm. of talc was placed in 200 ml. of 10% hydrochloric acid and boiled for 10 minutes. The talc was filtered with suction through a sintered-glass filter and washed thoroughly with distilled water until the filtrate had the same pH as the washing solution. The talc was then dried in a vacuum desiccator over concentrated sulfuric acid.

pH Measurements.—The pH of a 2% suspension of talc in distilled water was measured with a Beckman model H2 pH meter.

Chemical Analyses of Talc.—Using standard methods of chemical analysis, talcs *A*, *C*, and acid washed talc *C* were assayed for the following in the order listed: loss on ignition, SiO_2, Fe_2O_3, Al_2O_3, CaO, and MgO. The anhydrous sodium carbonate fusion method was utilized to decompose the sample. Silica was volatilized by the hydrofluoric acid method and calculated by weight differences.

Gravimetric methods were employed for the determination of iron, aluminum, calcium, and magnesium. After preliminary separation of iron and aluminum as hydroxides, iron was precipitated from an acidic solution with cupferron, then ignited, and weighed as ferric oxide. Aluminum was precipitated with ammonium hydroxide, ignited, and weighed as aluminum oxide. Calcium was separated from mag-

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TABLE I.—STABILITY OF ASPIRIN IN TABLETS CONTAINING SELECTED TALCS

pH of talc →	Talc *A* 7.6	Talc *B* 7.7	Talc *C* 9.6	Talc *D* 9.2
Free Salicylic Acid in mg./Tablet at 40° C. and 90% Relative Humidity				
Time, wk.				
Original	<0.10	<0.10	<0.10	<0.10
1	0.13	0.30	0.93	0.28
2	0.23	0.69	2.61	0.57
4	0.41	1.07	5.85	1.12
8	0.32	2.05	13.00	1.46
12	0.80	3.35	25.80	2.96

nesium by a double precipitation with ammonium oxalate, then ignited, and weighed as calcium oxide. Finally, the magnesium was precipitated by the addition of ammonium phosphate, ignited to the pyrophosphate, weighed, and calculated as the oxide.

Preparation of Tablets.—Three series of aspirin tablets were prepared. Formulations of the first series contained talcs *A*, *B*, *C*, and *D* as commercially received. Formulations of the second series contained acid washed talcs *A*, *B*, *C*, and *D*, and formulations of the third series contained talc *A* plus known amounts of various talc impurities. Formulations of all three series contained in each tablet 325 mg. of aspirin and 13.5 mg. of talc. Formulations of the third series contained in addition 0.71 mg. per tablet of one of the following: aluminum silicate ($3\,Al_2O_3 \cdot 4SiO_2 \cdot H_2O$), red iron oxide ($Fe_2O_3$), calcium silicate ($CaSiO_3$), and calcium carbonate. Each ingredient was vacuum dried over concentrated sulfuric acid for not less than 16 hours prior to use and thereafter handled in a room in which the relative humidity did not exceed 19%. Tablets were compressed on a Colton single-punch machine using $^3/_8$-in. standard cup punches and die. Compressed tablets had a hardness of 3.0 to 4.0 Kg. measured with a Pfizer hardness tester and contained less than 0.2% moisture measured with a Cenco infrared moisture balance with a 125-watt infrared bulb at 90 volts for 10 minutes.

Accelerated Stability Conditions.—Tablets were placed in a desiccator and stored at 40° and approximately 90% relative humidity. The humidity in the desiccator was controlled by a saturated solution of monobasic ammonium phosphate in which an excess of salt was present to insure saturation. The relative humidity above a saturated solution of monobasic ammonium phosphate at 37.7° is 91.1% (8),

Free Salicylic Acid Assay.—Aspirin stability in tablets was evaluated in terms of free salicylic acid content. Twenty tablets were weighed, the average tablet weight determined, and the tablets ground to a fine powder. Approximately one tablet weight was accurately weighed and transferred to a glass-stoppered centrifuge tube and 25 ml. of benzene added. The tube was shaken on a mechanical shaker for 2 minutes and centrifuged for approximately 3 minutes. Five milliliters of clear benzene solution was transferred to a second glass-stoppered centrifuge tube and 10 ml. of ferric ammonium sulfate solution (0.1% ferric ammonium sulfate adjusted to pH 2.45 with sulfuric acid) was added. The tube was then shaken on a mechanical shaker for 30 seconds and centrifuged for 2 minutes. The benzene layer was aspirated off and the aqueous portion was read on an Evelyn photoelectric colorimeter using a 515-mμ filter. The instrument was adjusted to 100% transmittance with the reagent blank.

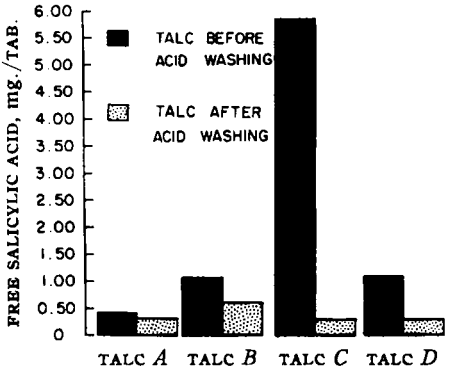


Fig. 1.—Effect of acid washed talcs on aspirin stability after 4 weeks at 40°C. and 90% relative humidity.

RESULTS AND DISCUSSION

The data from Table I indicate that different talcs vary significantly in their effects on the stability of aspirin. After 12 weeks at 40° and 90% relative humidity, tablets containing talcs *A*, *B*, *C*, and *D* had 0.80, 3.35, 25.80, and 2.96 mg. of free salicylic acid per tablet, respectively. Arranging the talcs in decreasing order of aspirin stability we have talc *A*, *D*, *B*, and *C*. It will also be noted that the pH of the talcs varied from 7.6 to 9.6. By arranging the pH of talcs in the same order of decreasing aspirin stability, we have 7.6, 9.2, 7.7, and 9.6. The pH of talc, therefore, did not appear to be directly related to aspirin stability.

Figure 1 illustrates the effects of acid washed talcs on aspirin stability. After 4 weeks at 40° and 90% relative humidity, aspirin tablets contained the following concentrations of free salicylic acid in milligrams per tablet: talc *A* 0.41; acid washed talc *A*

TABLE II.—CHEMICAL ANALYSES OF VARIOUS TALCS, %

	SiO_2 %	MgO %	CaO %	Al_2O_3 %	Fe_2O_3 %	Free Moisture %	Loss on Ignition %
Talc *A*	61.89	30.90	0.36	0.54	0.31	<0.2	0.53
Talc *C*	48.20	32.10	4.36	3.71	1.07	<0.2	6.44
Acid washed talc *C*	52.63	30.50	0.36	4.00	1.18	<0.2	1.38

TABLE III.—STABILITY OF ASPIRIN IN TABLETS CONTAINING TALC *A* AND VARIOUS IMPURITIES

Free Salicylic Acid in mg./Tablet at 40°C. and 90% Relative Humidity					
Time, wk.	Talc *A*	Talc *A* + Red Iron Oxide	Talc *A* + Calcium Silicate	Talc *A* + Aluminum Silicate	Talc *A* + Calcium Carbonate
Original	<0.10	<0.10	0.12	<0.10	<0.10
1	<0.10	0.13	1.11	0.11	2.61
2	0.19	0.25	1.85	0.19	4.66
4	0.22	0.33	3.20	0.23	8.01

0.30; talc *B* 1.07, acid washed talc *B* 0.60; talc *C* 5.85, acid washed talc *C* 0.30; talc *D* 1.12, acid washed talc *D* 0.30. Therefore, aspirin stability was improved by acid washing the talcs.

The data in Table II present the chemical analyses of talcs *A*, *C*, and acid washed talc *C* which were assayed for SiO_2, Al_2O_3, CaO, and MgO to determine whether talcs which varied in their effects upon the stability of aspirin would also vary in their chemical composition. Talcs *A* and *C* were selected for analyses because they represented the talcs which were affected the least and the most, respectively, by acid washing. Acid washed talc *C*, which had previously been shown to have good aspirin stability, had relatively high concentrations of Fe_2O_3 and Al_2O_3. This suggests that aluminum or iron in talc does not adversely influence aspirin stability. Talc *C*, which was associated with poor aspirin stability, had a loss on ignition of 6.44% which exceeds the U.S.P. limit of 5%. The loss on ignition did not represent free moisture. Talc *C* also had a relatively high concentration of CaO which would reflect a calcium carbonate or calcium silicate impurity. A calcium carbonate impurity would also contribute to a high loss on ignition. By acid washing talc *C*, the loss on ignition and CaO content were reduced and aspirin stability significantly improved. A high loss on ignition and CaO content, therefore, may be indicative of impurities in talc which adversely influence aspirin stability.

The effect that impurities in talc have on aspirin stability is shown in Table III. A different aspirin lot was used in this phase of the experiment which may explain the low free salicylic acid levels obtained. It will be noted that after 4 weeks at 40° and 90% relative humidity, aspirin tablets containing talc *A* had 0.22 mg. of free salicylic acid per tablet. The series of tablets also prepared with talc *A*, but with added aluminum silicate, red iron oxide, calcium silicate, or calcium carbonate, had free salicylic acid values of 0.23, 0.33, 3.20, and 8.01 mg. per tablet, respectively. Aspirin stability, therefore, is adversely influenced by the presence of calcium carbonate or calcium silicate and not significantly influenced by the presence of aluminum silicate or red iron oxide. This is in agreement with the conclusions from the analyses of selected talcs in which aspirin decomposition was associated with the calcium content and not associated with aluminum or iron.

SUMMARY AND CONCLUSIONS

The effects of different U.S.P. talcs on the stability of aspirin tablets have been investigated. From the results obtained the following conclusions can be drawn.

Different U.S.P. talcs varied significantly in their effect on the rate of aspirin decomposition.

The stability of aspirin was improved by acid washing the talcs.

The pH of talc did not appear to be directly related to aspirin stability.

When using commercially available talcs, a high-calcium content and high loss on ignition were associated with increased aspirin decomposition. The aluminum and iron content were not associated with aspirin decomposition.

Aspirin stability in tablets was adversely affected by the presence in talc of added calcium silicate and calcium carbonate and not affected by aluminum silicate or red iron oxide.

As a result of this investigation, the authors are of the opinion that variations in talc could alter the stability of other pharmaceuticals containing or utilizing talc in their preparation.

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Metal Chelates as Potential Reactivators of Organic Phosphate Poisoned Acetylcholinesterase

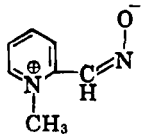
By SANFORD BOLTON and ARNOLD BECKETT†

As part of a study to investigate metal chelates as possible reactivators of phosphate poisoned acetylcholinesterase, it is shown that chelates act as inhibitors of the enzyme. In addition, copper and nickel chelates of 2-pyridinealdoxime catalyze the decomposition of DFP and sarin. Compared to 2-PAM, however, these chelates are very poor reactivators, probably because of an improper alignment of the oxime oxygen in the chelate-enzyme complex.

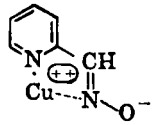
CERTAIN ESSENTIAL FEATURES are evident in reactivators of acetylcholinesterase (ACHase) poisoned by fluorophosphate esters. The following properties are important: (*a*) The reactivator should act as a reversible competitive inhibitor of the enzyme. In particular, attachment to the free anionic site of the poisoned enzyme is important and is, therefore, enhanced by the presence of a positively charged group in the reactivator molecule. Wilson and co-workers (1) and Green and Smith (2) demonstrated that complex formation takes place between the reactivator and the inhibited enzyme during reactivation. (*b*) The reactivator should have a nucleophilic atom capable of reacting with and decomposing the phosphate poison; in the case of 2-pyridinealdoxime methiodide (2-PAM), Wilson, *et al.* (3), demonstrated that the oximate ion is the active species. However, there appears to be an optimum pKa value; some compromise between too high or low nucleophilic character should be reached. Substances which have been shown to increase the rate of decomposition of the organic phosphate poisons include hydroxamic acids (4), oximes (5, 6), and certain chelates (7, 8). The presence of aquo groups, weak chelate stability, and positive charge are contributing factors toward the catalytic properties of the chelates (8). (*c*) The nucleophilic atom in the bound reactivator should be properly oriented in relation to the poisoned site. A certain degree of rigidity is also important.

Wilson (9) has reviewed and discussed the roles of these features relating to the discovery of 2-PAM.

The above work has involved compounds in which the positively charged center is a quarternary ammonium group, *e.g.*, I. It is, therefore, of interest to see whether similar effects can be obtained by the use of tertiary nitrogen compounds into which the positively charged site is introduced *via* chelation, *e.g.*, II. Such an approach appeared to be relevant because of the catalytic



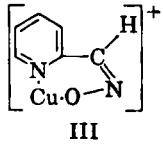
I



II

effect of copper chelates on the decomposition of the organic phosphates. Also, the introduction of a doubly positively charged center might be a contributing factor toward increased binding to ACHase.

Since the present investigation is concerned principally with chelates of 2-pyridinealdoxime (2-PA), it is of importance to consider whether chelate formation takes place *via* the oxime nitrogen (II) or oxygen (III). Reactivating activity would be expected to be better if II is the more stable structure since the oxygen is free to react with the bound phosphate. The generally greater stability of five membered chelate rings compared with six membered rings, the fact that nickel complexes of 2-PA have been isolated in which the oxime hydrogen is retained (10), and the high water solubility of the nickel and copper chelates of 2-PA (11) suggest that II is the preferred structure.



III

In the present work, inhibition of ACHase activity by metal chelates suggests that binding takes place between the chelate and the anionic site of the enzyme. In addition, copper and nickel chelates of 2-PA catalyze the decomposition of diisopropyl phosphonfluoridate (DFP)

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and methyl isopropyl phosphonfluoridate (sarin). The reactivation of poisoned acetylcholinesterase by these chelates is, however, much weaker than 2-PAM probably due, in part, to a lack of stereospecificity.

The affinity of diacetyl monoxime (DAM), pyruvaldehyde aldoxime (MINA), and 2,3-pentanedione 3-oxime (POX) (all of which have been investigated as antidotes for organic phosphate poisoning (12)) for cupric, nickelous, and cobaltous ions were examined in this report, but the stabilities of these chelates were too weak to warrant further investigation.

EXPERIMENTAL

Reagent.—2-PA, recrystallized from water, m.p. 113°; MINA, prepared by the method of Freon (13), recrystallized from petroleum ether and benzene, m.p. 65°; DAM, recrystallized from water and charcoal, m p. 75°; POX, recrystallized from petroleum ether, m.p. 59°; acetylcholine (ACH), m.p. 165–166°; sodium chloride, magnesium chloride, and potassium nitrate, all analytical reagent grade; cupric nitrate, cobaltous nitrate, and nickelous nitrate were all analytical grade and their purities were checked by titration with the disodium salt of EDTA according to methods suggested by Chaberek and Martell (14*a*), using Eriochromeschwarz T and murexide as indicators. The ACHase was prepared from bovine serum and was the same as that used by Clitherow (15). The enzyme decomposed 2.8 μm. of ACH in 30 minutes, per milligram of protein, at 25°, near the optimum substrate concentration.

Chelation Studies.—The extent of chelation of DAM, MINA, and POX with the appropriate metal ions was followed potentiometrically by observing equilibrium pH values after addition of increments of base to the metal-oxime mixtures. Equilibrium occurred very slowly as indicated by the gradual drifting of pH in these systems. Therefore, the data were determined by preparing a series of metal-oxime solutions containing different amounts of base and allowing the solutions to stand for at least 24 hours before pH readings were taken. All measurements were made with a Cambridge bench type pH meter; the temperature was kept constant at 25 ± 0.2°. The ionic strength of all solutions was maintained at 0.10 *M* by the addition of potassium nitrate. To insure the exclusion of carbon dioxide, nitrogen was passed through the solutions.

Acetylcholine Hydrolysis Studies.—The rate of hydrolysis of ACH by ACHase, alone and in the presence of inhibitors, was followed by neutralizing the acid produced by titrating, manually, with 0.01 *N* sodium hydroxide to keep the pH constant at 7.4 ± 0.05. (The results for the hydrolysis of ACH by uninhibited ACHase obtained by this method compared favorably with those obtained manometrically). In general, the chelate mixture was first brought to a pH of 7.4; then 5 ml. of the enzyme suspension, containing 75 mg. of enzyme per 100 ml., was added. The final volume of solution was adjusted to 15 ml. If the enzyme and metal mixture came into contact before the pH adjustment, inhibition was greatly increased, probably due to an irreversible inhibition produced by the excess of free metal ion at low pH values. At higher substrate concentrations (>3 × 10^{-3}*M* ACH), decomposition was linear as a function of time for about 30 minutes. At lower substrate concentrations, the reaction velocity was calculated from the initial decomposition. The temperature was kept constant at 25 ± 0.2° and a stream of nitrogen was passed over the solutions to exclude carbon dioxide in all studies. All reagents were dissolved in an unbuffered solution containing 0.9% NaCl and 0.266% $MgCl_2$.

Hydrolysis of DFP and Sarin.—The nonenzymic hydrolysis of the organic phosphate compounds was followed by neutralizing the acid produced in an unbuffered solution containing 1.25 × 10^{-3} *M* mixture of the appropriate chelate. The pH was kept constant, as above, at 7.4 and the temperature maintained at 25 ± 0.2°.

Reactivation Studies.—A double strength solution of esterase (150 mg./100 ml.) was used in these stud-

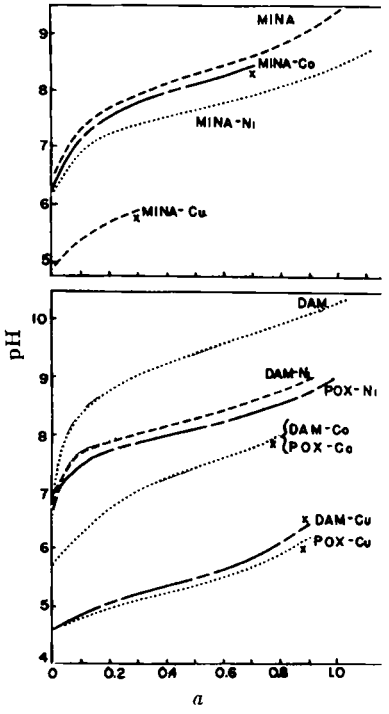


Fig. 1.—Potentiometric titration curves of oximes and oxime-metal mixtures. Ratio of oxime to metal is 2:1. Concentration of oxime is 4 × 10^{-3} *M*. "*a*" is equivalent of base/oxime; x = precipitation.

TABLE I.—APPROXIMATE VALUES OF LOG K_1K_2[a] FOR OXIME-METAL CHELATES

Chelating Species	Log K_1K_2
DAM-Copper	13.3
DAM-Cobalt	7.2
DAM-Nickel	7.6
MINA-Copper	5.0 (Log K_1)
MINA-Cobalt	2.8 (Log K_1)
MINA-Nickel	6.6
POX-Copper	13.5
POX-Cobalt	9.2
POX-Nickel	8.0

[a] K_1 = [Oxime-Metal]/[Oxime][Metal]; K_2 = [Oxime 2-Metal]/[Oxime][Oxime-Metal].

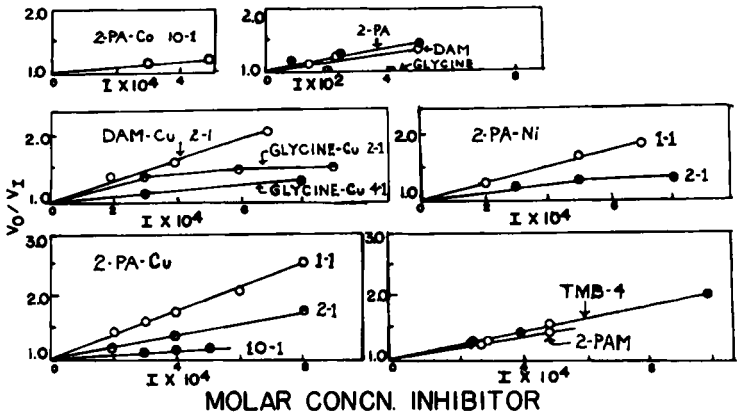


Fig. 2.—The effect of inhibitor concentration (I) on the velocity of ACH hydrolysis in the presence of AChase. V_0/V_I = (velocity in absence of I)/(velocity in presence of I); 1:1, etc., indicates ratio of oxime to metal ion.

ies so that reactivation effects would be more noticeable. A 0.1-mcg. quantity of DFP or 0.02 mcg. of sarin was added to 5 ml. of the esterase suspension and allowed to remain in contact for 1 hour at room temperature before the reactivators were added. Again, as above, the pH of the chelate solutions was adjusted to 7.4 before addition to the poisoned enzyme. Enzyme activity was determined in the presence of 3×10^{-3} *M* ACH and controls were run in all cases. Wilson and Ginsburg (16) have observed that dilution of the poisoned enzyme prior to addition of the reactivator greatly increased the rate of reactivation. This result may be due to excess poison in the system being diluted to an innocuous concentration. Unfortunately, the enzyme used in this study had an initial activity too low to allow the adaptation of this technique. The choice of concentration of poison, therefore, was based on its ability to poison the enzyme and on the ability of 2-PAM to reactivate the poisoned enzyme in a reasonably short period of time. The concentration of sarin used (0.02 mcg./5 ml.) almost completely inactivated the enzyme in 1 hour while DFP (0.10 mcg./5 ml.) reduced the activity by approximately 50% in 1 hour. After 24 hours, DFP inhibited enzyme had virtually no activity.

RESULTS

Chelation Studies.—The results of the potentiometric chelation studies of MINA, DAM, and POX are shown in Fig. 1. These chelates were unsuitable as reactivators because of their instability. All the systems containing copper precipitated $Cu(OH)_2$ at pH 7.4, whereas the nickel and cobalt chelates were incompletely formed.

Attempts at evaluating stability constants on the basis of stepwise chelate formation for the 1:1 and 2:1 chelates from this data yielded inconsistent values. It appeared that there was strong overlapping of the formation constants or that true equilibrium had not been reached. Approximate values of log K_1K_2 were calculated from a plot of $\bar{n}$ *versus* pA according to Bjerrum (17) and are recorded in Table I.[1]

As in the 2-PA system (11), cobalt chelates of DAM and POX appear to be more stable than the corresponding nickel chelates. With MINA, the more usual stronger nickel chelate occurs.

Inhibition Studies.—Figure 2 illustrates the AChase inhibition by the pertinent species in this study. Data on 2-PAM and TMB-4 are included for purposes of comparison.

The free oximes and glycine inhibit AChase activity to a small extent compared to the inhibition exhibited by their metal chelates. The inhibition demonstrated in the chelate systems could not be accounted for by the free metal ion. In fact, separate studies indicated that a concentration of 10^{-6} *M* copper ion, which is, with the exception of the DAM-copper chelate (see below), greater than that which would be present in these systems, exerts a small *potentiating* effect upon AChase activity. This is in accord with a similar observation made by Godfraind and Godfraind (18).

The fact that the glycine-copper and the DAM-copper complexes inhibit the activity of the enzyme suggests that inhibition by copper chelates is probably a general phenomenon. A saturated solution of $Cu(OH)_2$ at pH 7.4 reduced the hydrolytic activity of AChase by approximately 25%. This result was taken into consideration when plotting the DAM-copper data as these solutions contained a mixture of chelating species and precipitated $Cu(OH)_2$.

Several generalizations may be made on the basis of the results shown in Fig. 2. The 1:1 chelates are the strongest inhibitors. Increasing the ratio of oxime to metal ion decreases inhibition. This is to be expected if inhibition occurs by binding *via* the positively charged metal; increased ratio of ligand to metal would not only decrease the positive nature of the metal but would also exert a shielding effect. Cobalt chelates have little inhibitory effect while copper chelates exert the stronger effect, probably because of the stronger electropositive character of the copper ion.

Since the 2-PA chelates at pH 7.4 in these systems are mixtures of different species (11), it is difficult to ascertain the effects of individual components.

TABLE II.—CONCENTRATIONS OF 1:1 AND 2:1 GLYCINE-COPPER CHELATE SPECIES IN 2:1 GLYCINE-COPPER MIXTURES

Molar Concn. 2:1 Glycine-Copper × 10^4	Molar Concn. 1:1 Chelate × 10^4	Molar Concn. 2:1 Chelate × 10^4
3	0.66	2.34
6	1.00	5.00
9	1.25	7.75
12	1.46	10.54
15	1.64	13.36

[1] $\bar{n}$ = [Bound ligand]/[total metal]; pA = negative log of concentration of free oximate ion.

In 1:1 2-PA-copper and nickel mixtures, this analysis is further complicated by the presence of hydrolytic and/or polymeric species of unknown composition (11).

At pH 7.4, solutions containing chelating agent and metal in the ratio 2:1, contain both 1:1 and 2:1 chelate species; if the 1:1 chelate is preferentially bound, a shift in equilibrium might be expected as

$$\text{2-PA–Metal–2-PA} \underset{+\text{2-PA}}{\overset{-\text{2-PA}}{\rightleftharpoons}}$$

$$\text{Metal-2-Pa} \underset{-\text{Enzyme}}{\overset{+\text{Enzyme}}{\rightleftharpoons}} \text{2-PA-Metal Enzyme}$$

As more 1:1 than 2:1 chelate is removed from solution by binding with the enzyme, the equilibrium between the two chelate species will be shifted to the right, forming more of the 1:1 chelate. If the shift occurs to an appreciable extent, the inhibition caused by the solutions containing metal-ligand ratios of 2:1 (or higher) would approach that of the 1:1 mixtures of corresponding concentration. Since this is not the case, it can be assumed that this effect occurs only to a very limited extent.

If the equilibrium between 1:1 and 2:1 chelate species is not appreciably disturbed by the presence of enzyme, the composition of glycine-copper mixtures can be calculated with a knowledge of chelate stability constants.[2] Unfortunately, 1:1 mixtures of glycine and copper precipitated at pH 7.4, but the composition of 2:1 mixtures is shown in Table II. At a glycine-copper ratio of 4:1, the solutions consist of practically all 2:1 species.

It is apparent from Table II that the ratio of 1:1 to 2:1 chelate decreases with increasing concentration of the 2:1 mixture and this could, qualitatively, explain the leveling of the inhibition curve in Fig. 2. This phenomenon also occurs in the 2:1 2-PA–nickel mixture. That this leveling is not apparent in the 2-PA–copper system may be due to the great difference between the 1:1 and 2:1 chelate stability constants (11) which causes a much less pronounced effect of concentration on the ratio of 1:1 and 2:1 chelate species.

ACHase activity as a function of substrate concentration is shown in Fig. 3. In the presence of both 2-PAM and 1:1 2-PA–copper mixtures, the optimum substrate concentration is shifted to higher substrate concentrations. This shift, as noted by Augustinsson and Nachmansohn (19), is indicative of competitive inhibition. A more quantitative demonstration of the nature of the inhibition of the 1:1 2-PA–copper mixture may be seen in Figs. 4 and 5. Data of enzymic activity as

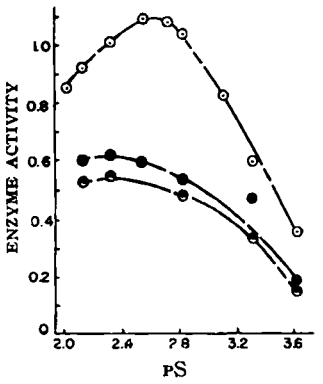


Fig. 3.—Enzymic activity as a function of substrate concentration. Activity = ml. of 0.0095 N base consumed in 30 minutes. Key: O—absence of inhibitor; ●—presence of 5×10^{-4} M 2-PAM; ◐—presence of 4×10^{-4} 2-PA-Cu, 1:1.

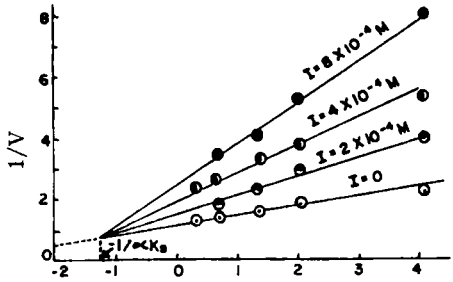


Fig. 4.—Plot of 1/V *vs.* 1/(substrate concentration) in the presence of 1:1 2-PA–Cu mixtures (I). V = velocity of ACH hydrolysis in ml. of 0.0095 N base consumed in 20 minutes (extrapolated if necessary); K_s = Michaelis-Menten constant.

functions of both substrate and inhibitor concentrations have been analyzed according to methods suggested by Friedenwald and Maengwyn-Davies (20). In the present example, the inhibition, according to this analysis, is a mixture of both competitive and noncompetitive types with an α[3] value of approximately 3. The leveling of the curve in Fig. 5 also indicates that the 1:1 chelate mixture exhibits both types of inhibition (see *Reference* 20). The Michaelis Menten constant for the ACH-ACHase interaction calculated from the data in Fig. 4 is 2.85×10^{-4} which is in close agreement with the value of 2.6×10^{-4} previously reported (21). The inhibitor constant for the 1:1 2-PA–copper inhibition is 2×10^{-4} is calculated from the data plotted in Fig. 6; this is a composite value of all the species present in the 1:1 mixture.

Hydrolysis of DFP and Sarin.—The catalytic effect of 1:1 and 2:1 mixtures of 2-PA and copper and nickel is shown in Table III.

As expected, the 1:1 chelate of copper proved to be the strongest catalyst (8). However, the overall effect is not particularly dramatic when the results are compared with those obtained with copper chelates used by Courtney and co-workers (8). For example, one of the most potent catalysts

[2] $[A_t]$ = Concn. of total ligand; $[A]$ = concn. of ligand anion; K_a = acid dissociation constant of ligand; $[MA]$ = concn. of 1:1 chelate; $[MA_2]$ = concn. of 2:1 chelate; $[M_t]$ = concn. of total metal; $[M]$ = concn. of free metal

$$[A_t] = [A](1 + [H^+]/K_a) + [MA] + 2[MA_2] \quad \text{(Eq. 1)}$$

$$[M_t] = [M] + [MA] + [MA_2] \quad \text{(Eq. 2)}$$

$$K_1 = [MA]/[M][A] \quad \text{(Eq. 3)}$$

$$K_2 = [MA_2]/[A][MA] \quad \text{(Eq. 4)}$$

Combining Eqs. 1, 2, 3, and 4

$$[A_t] = ([A](1 + [H^+]/K_a)) + (K_1 + 2K_1K_2[A]) \times [M_t]/(1 + K_1[A] + K_1K_2[A^2]) \quad \text{(Eq. 5)}$$

(A) can be determined by a method of successive approximations. With a knowledge of $[A]$ and $[M_t]$, $[MA]$ and $[MA_2]$ can be calculated from Eqs. 2, 3, and 4. Values of K_a, K_1, and K_2 for glycine were taken from "Organic Sequestering Agents" (14b).

[3] "α," as defined by Friedenwald and Maengwyn-Davies (20), is a constant related to the dissociation of the enzyme-inhibitor-substrate complex. In cases of complete competitive and noncompetitive inhibition, "α" assumes the special values of ∞ and 1, respectively.

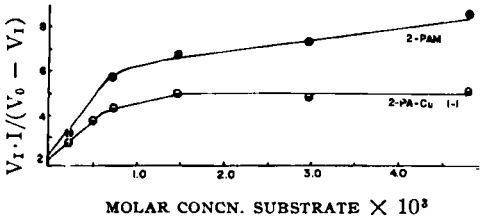


Fig. 5.—Plot of $V_I - I/(V_0 - V_I)$ *vs.* substrate concentration. V_0 = ACH hydrolysis velocity in absence of inhibitor, V_I = ACH hydrolysis velocity in presence of inhibitor, I = molar concn. of inhibitor.

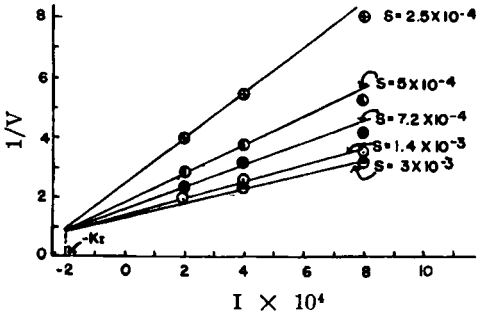


Fig. 6.—1/(Rate of ACH hydrolysis) as a function of inhibitor concentrations at various substrate concentrations. I = molar concentration of 1:1 2-PA–Cu mixtures; V = ml. of 0.0095 *N* base consumed in 20 minutes (extrapolated if necessary); Ki = inhibitor constant.

reported by them was the dipyridyl-copper chelate which half decomposed sarin and DFP in 3 and 23 minutes, respectively. It is of interest to note the marked effect of the nickel chelates on sarin decomposition.

Reactivation Studies.—The results of reactivation studies are shown in Table IV. The percentage of reactivation was calculated by two methods: one on the basis of the activity of the partially poisoned enzyme when the reactivator was added, and the other on the activity at the time that reactivation was measured (1, 22). This approach makes little difference in the results of sarin poisoned enzyme but does affect significantly DFP results. The results for 2-PAM are included for purposes of comparison. None of the chelates showed evidence of significant reactivation in the period of time in which 2-PAM was effective. Therefore, the reactivators were allowed to remain in contact with the poisoned enzyme for approximately 1 to 2 days before activity was measured. During these periods of time, the nickel chelates showed marked activity in regenerating sarin poisoned enzyme while the copper chelates were quite ineffective. The reverse effect was noted in the DFP poisoned enzyme where the nickel chelates had negligible activity. The data also suggest that the 2:1 chelate mixture is a more effective reactivator than the 1:1 mixture in the 2-PA–nickel reactivation of sarin poisoned enzyme.

DISCUSSION

Chelates of 2-PA possess two of the three properties of reactivators, as mentioned in the introductory section of this paper, which enhance the rate of reactivation of organic phosphorus poisoned ACHase. (*a*) The fact that electropositive nature of the chelate enhances its inhibitory effect and that the 1:1 copper chelate exhibits some competitive character in its inhibition of the enzyme indicate an attachment at the anionic site of the ACHase surface. The 1:1 copper chelate is, in fact, a stronger inhibitor than 2-PAM. Wilson and Ginsburg (23) have suggested that binding of the reactivator to the enzyme is an important factor in improving reactivating capacity. (*b*) The chelates are capable of catalytically decomposing the organic phosphates, although their activity in this respect was less than might have been anticipated. Perhaps the strong affinity of the oximes for the metals decreases the catalytic activity (8). Also, the fact that the oxime acts as a very strong acid in its chelated form indicates that the oxime oxygen loses much of the nucleophilic character which it possesses in its nonchelated state. It should be mentioned, however, that high reactivity with the phosphates—although, no doubt, advantageous—is by itself not the criterion of a good reactivator. For example, hydroxamic acids, although potent in decomposing the organic phosphates, are very poor reactivators *in vivo* (24). Apparently, detoxification does not occur by direct decomposition of the phosphate (1, 2, 25, 26). An attachment to the poisoned enzyme by the reactivator, followed by decomposition of the bound phosphate by the properly oriented nucleophilic group has been termed a "Promoting Effect" by Wilson. Wilson has demonstrated that it is this crucial feature which accounts for the excellent reactivating capacity of 2-PAM (9).

It seems that the rather poor reactivating capacity of the chelates in this study is mainly due to an improper orientation of the oxime oxygen in the bound chelate in relation to the esteratic site to which the phosphate is bound. If the oxime oxygen in 2-PAM is directed toward the bound phosphate (see structure I), it is apparent that chelation results in a shift of the oxygen away from the phosphorylated site (see structure II). The introduction of a second molecule of oxime in the 1:1 copper chelate results in a planar configuration (IV). It is clear that the second oxime molecule can be in no better position than the first for reactivation. Nickel chelates can assume an octahedral (V) as well as a planar configuration. Although the planar structure of the nickel chelate seems to be more acceptable on the basis of steric considerations, structure V is not unreasonable. In fact, measurements of the magnetic moments of 2:1 2-PA–nickel chelates by Krause and Busch

TABLE III.—CATALYTIC EFFECT OF 2-PYRIDINE-ALDOXIME CHELATES ON THE DECOMPOSITION OF SARIN AND DFP AT pH 7.4 AND 25°

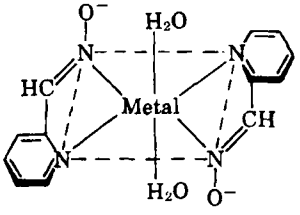
Chelate Composition Concn. = 1.25 × $10^{-3}M$	$t_{1/2}$ Life (hr.) of Sarin of Initial Concn. 3.5 × $10^{-4}M$	$t_{1/2}$ Life (hr.) of DFP of Initial Concn. 2.65 × $10^{-4}M$
2-PA–copper 1:1	2.6	8.2
2-PA–copper 2:1	6.3	10.2
2-PA–nickel 1:1	3.7	28
2-PA–nickel 2:1	10.3	39
No chelate	App. 60	3 days

TABLE IV.—REACTIVATION OF POISONED ACETYLCHOLINESTERASE BY NICKEL AND COPPER CHELATES OF 2-PYRIDINEALDOXIME

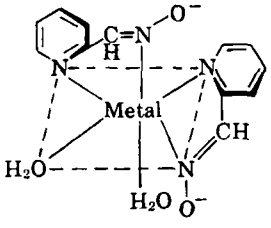
Reactivator[a]	Concn.	Time, hr.	Poison	% Reactivation I[b]	% Reactivtion II[b]
2-PAM	5×10^{-4}	1	DFP	20	13
		2¾		52	49
	1×10^{-4}	0.83	Sarin	67	
	2.5×10^{-4}	2		100	
Cu-2-PA 1:1	1×10^{-3}	51.0	DFP	66	43
		48		61	34.5
	2.5×10^{-4}	21.25		77	49
	1×10^{-3}	45	Sarin	...	
	2.5×10^{-3}	24		13	
Cu-2-PA 1:2	1.10^{-3}	51.5	DFP	56.0	27
		48.25		50	15
	2.5×10^{-3}	22.0		77	60
	1×10^{-3}	45	Sarin	...	
	2.9×10^{-3}	24.5		36	
Ni-2-PA 1:1	1×10^{-3}	46	DFP	...	...
	2.5×10^{-3}	22.75		9	...
	1×10^{-3}	48	Sarin	26	
	2.5×10^{-3}	23.5		66.5	
Ni-2-PA 1:2	1×10^{-3}	46	DFP	...	...
	2.5×10^{-3}	23.25		8.5	...
	1×10^{-3}	48	Sarin	50	
	2.5×10^{-3}	23		92	

[a] The ratios indicate [total concentration of metal]/[total concentration of oxime] in the solutions tested for reactivation. [b] % Reactivation (I) = [(poisoned enzyme activity with reactivator)-(activity at time of reactivation)]/[(activity with no poison)-(activity at time of reactivation)] × 100. % Reactivation (II) = [(poisoned enzyme activity with reactivator). (activity at time of addition of reactivator)]/[(activity with no poison)-(activity at time of addition of reactivator)] × 100.

(10) indicate a nonplanar structure. If the 2:1 2-PA–nickel chelate assumes an octahedral configuration, one of the oximes may be, by chance, better oriented to detoxify the poisoned enzyme. This could account for the increased effectiveness of the 2:1 nickel chelate as a reactivator of sarin poisoned AChase.



IV



V

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Effect of Various Concentrations and Frequency of Application of Gibberellic Acid on Growth and Formation of Metabolic Products of *Datura stramonium*

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In a 2 × 4 × 5 factorial experiment employing 320 plants in randomized blocks with eight replications the following salient points were noted from a statistical analysis of the data: the greatest increase in height was induced with the 192-mcg. dosage of giberellic acid (GA). All treated plants at the 12, 48, and 192-mcg. dosages of GA grew more rapidly than controls. Height increases were significantly different at each dosage level. The most favorable response on height was between the 12-mcg. and 48-mcg. levels. The total dry weights of treated plants were significantly affected by dosage and age. The growth rate varied with the dose of GA used. The frequency of application (whether one or two installments were given weekly) did not significantly affect the aforementioned variables. Analyses of pooled samples of treated plants indicated the following general trends: reduced concentration of alkaloids in the plant organs, greatest reductions usually noted at the higher dosage levels, decreased per cent of chlorophyll, marked increases in the total sugars and starch content of the leaf-tops, considerable decreases in the petroleum ether extract of the leaf-tops, and qualitative differences in the ether extract of the leaf-tops.

THE METABOLIC EFFECTS of gibberellic acid (GA) on many plants (1–4) and with some medicinal plants (5) have been described. Among the members of the Solanaceae which have been investigated are *Datura stramonium* (6–8), *D. innoxia* (9), *D. meteloides* (10), *Atropa belladonna* (6, 7, 11, 12), *Hyoscyamus niger* (13), and tobacco (14–16). Gibberellin treatment generally induced a reduction in the concentration of alkaloids in the aerial parts of the plants and increases in plant height and stem weight. Variable results were noted on root growth and root alkaloid production (5–16)

The objectives of this research were: (*a*) to determine the concentration of GA which would produce the most favorable response on growth; (*b*) to determine whether frequency of application of the chemical, *i.e.*, whether a given dosage given once weekly *versus* one-half dose given twice weekly, would elicit different responses on growth; (*c*) to submit all of the data obtained during the growth phase to statistical analysis in order to determine which variables were statistically significant; (*d*) to ascertain whether GA treatment would alter the quantity of various metabolic products produced by the plant

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EXPERIMENTAL

Procedure.—*Datura stramonium* plants employed in this study were grown under greenhouse conditions. Seeds obtained from a single plant grown in the Oregon State University drug garden in 1958 were germinated in flats containing a soil mixture of two parts of loam and one part of sand. About 100 Gm. of complete organic fertilizer[1] was incorporated into the soil of each flat. On June 15, 1959, 350 thirty-five-day-old seedlings were transplanted into individual 1-gallon metal containers that had been filled with the sandy loam mixture. Approximately 4 Gm. of complete fertilizer had been thoroughly mixed into the soil of each container prior to transplantation. At zero time on June 23, 320 of the most uniform plants were selected for this study.

Explained statistically this was a 2 × 4 × 5 factorial experiment in randomized blocks with eight replications. The entire experiment employed 320 plants. The first factor was the number of installments used in applying the GA. The same dosage may be applied once weekly or in two installments twice weekly, half a dose each time. Therefore, this factor had two levels. The second factor had four levels, namely, 0, 12, 48, and 192-mcg. dosages of GA. The third factor was the age of the plants at the time of harvesting. This factor had five levels, namely, 0, 1, 2, 3, and 4 weeks. Each replication included plants which were about the same height.

At zero time (June 23) height and leaf measurements were made, treatment with GA was given to the designated randomized plants, and the eight replications from each of the eight series were harvested. The harvested plants were divided into three portions—leaf-tops, stems, and roots. The plant parts were cut into small segments; fresh weights were taken immediately. The plants were

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis—5% total nitrogen, 3% available phosphate, 2% available potash.)

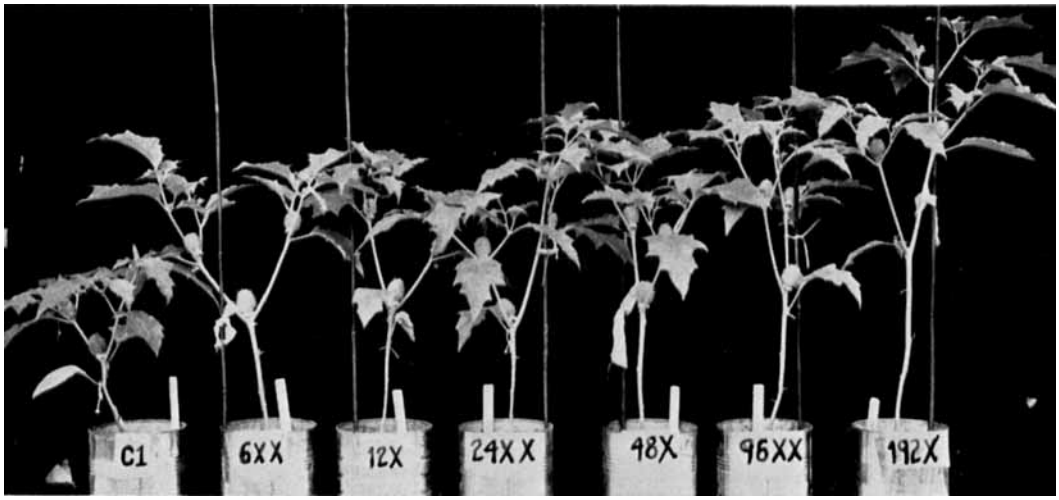


Fig. 1.—Effect of GA-treatments on the growth of *Datura stramonium*. Key = C_1, control; 6XX, 6 mcg. twice weekly; 12X, 12 mcg. weekly; 24XX, 24 mcg. twice weekly; 48X, 48 mcg. weekly; 96XX, 96 mcg. twice weekly; 192X, 192 mcg. weekly. Plants at the final harvest were about 10 weeks old.

then dried for 48 hours at 48.5° in a circulating hot-air drier and were stored in a desiccator until dry weight was determined. At this time the parts were pooled according to treatment and pulverized to a No. 40 powder in the Wiley mill. However, an exception to this procedure was made at the first harvest (zero time) when all samples were pooled and designated as controls, since treatment had not begun with the zero harvest series. This was done to assure a sufficient quantity of plant material for subsequent analytical work. The powder was stored in colored glass containers and a desiccator for subsequent analyses. This same procedure was followed at each week thereafter until all plants were harvested by the end of the 4-week treatment period. The day temperature in the greenhouse during the 4-week observation period was often high, frequently ranging from 100–110° F. for several hours in the afternoon.

Height and leaf measurements were taken three times weekly during the treatment period. All plants were dusted lightly thrice weekly with a 4% malathion dust. Aqueous solutions of GA[2] were prepared fresh each treatment day from a stock alcoholic solution of the chemical. The calculated dosage, in 0.02-ml. portions, was delivered from a micropipet onto the surface of the youngest unfounding leaf. The groups of plants which were designated to be treated twice weekly received the second weekly treatment 3 days after the first dosage was applied.

Growth Effects.—Characteristic gibberellin effects were noted in the treated plants (Fig. 1). There was marked elongation at the internodes which was more evident with plants receiving the higher doses of GA. The leaves of treated plants appeared chlorotic, narrower, and thinner. Abscission of the lower leaves of treated plants was more rapid than in the controls. The stems of the controls at the final harvest appeared more succulent and less woody than those of treated plants. Furthermore, a higher per cent of fruit-set was noted in the control group. All treated plants were taller than controls (Fig. 2). The greatest increase in height was noted in the plants receiving the largest dose (192 mcg.) of GA. This group was 73% taller than controls at the final harvest.

Fresh and Dry Weights.—Mean dry weight data indicated that stem growth of treated plants was generally significantly increased throughout the 4-week observation period (Table I). About a two-fold increase or more was noted in the stems of all treated plants at the end of the second week. At the final harvest the 192-mcg. series demonstrated the highest stem weight, 217% that of controls. It appeared that maximal stimulation of stem growth was attained at the end of 2 weeks. Variable results were noted in leaf-tops and root weights during

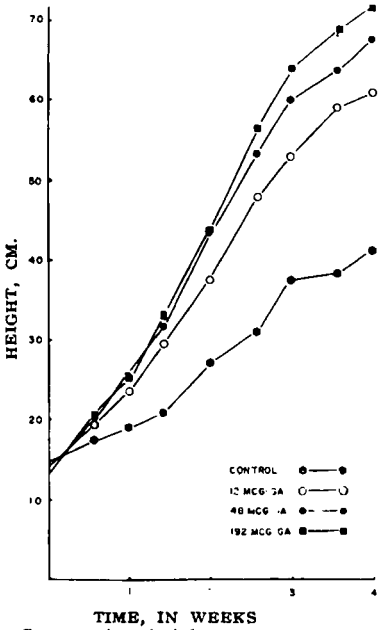


Fig. 2.—Stramonium height measurements. Plants were about 6 weeks old at zero time.

[2] The gibberellic acid employed in this study was furnished through the courtesy of Dr. Edwin F. Alder, Head of Plant Science Research, Eli Lilly and Co., Greenfield, Ind.

TABLE I.—MEAN WEIGHTS OF STRAMONIUM PLANT PARTS

Treatment mcg.	Harvest Time, wk.	Total Weight			Leaf-Tops			Stems			Roots		
		Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
Control[a]	0	5.29	0.539	...	2.18	0.306	...	1.27	0.123	...	1.84	0.134	...
12	0	5.00	0.498	92.3	2.15	0.280	91.5	1.06	0.100	81.3	1.80	0.117	87.3
6 (2×)	0	6.04	0.577	107.0	2.67	0.317	103.5	1.43	0.131	106.5	1.94	0.130	97.0
48	0	4.44	0.451	83.6	1.88	0.248	81.0	1.09	0.095	77.2	1.48	0.108	80.5
24 (2×)	0	5.09	0.555	102.9	2.05	0.282	92.1	1.17	0.116	94.3	1.87	0.157	117.1
192	0	4.76	0.491	91.0	2.21	0.286	93.4	1.32	0.125	101.6	1.23	0.080	59.7
96 (2×)	0	5.00	0.529	98.1	2.28	0.297	97.0	1.36	0.132	107.3	1.36	0.100	74.6
Control[a]	1	14.02	1.133	...	6.92	0.680	...	3.25	0.242	...	3.86	0.211	...
12	1	15.15	1.235	109.0	6.75	0.713	104.8	3.95	0.260	107.4	4.46	0.262	124.1
6 (2×)	1	12.33	0.999	88.1	5.04	0.584	85.8	3.33	0.220	90.9	3.96	0.195	92.4
48	1	14.11	1.269	112.0	5.95	0.699	102.7	4.55	0.301	124.3	3.61	0.269	127.4
24 (2×)	1	12.14	1.029	90.8	5.21	0.554	81.4	3.74	0.264	109.0	3.19	0.211	100.0
192	1	15.43	1.260	111.2	6.95	0.721	106.0	4.88	0.312	128.9	3.58	0.227	107.5
96 (2×)	1	14.74	1.158	102.2	6.93	0.710	104.4	4.66	0.301	124.3	3.15	0.192	90.9
Control[a]	2	30.97	2.644	...	17.92	1.708	...	6.77	0.480	...	6.28	0.445	...
12	2	38.55	3.139	118.7	18.09	1.739	101.8	12.07	0.868	180.8	8.40	0.532	119.5
6 (2×)	2	32.33	2.720	102.8	15.62	1.600	93.6	10.41	0.770	160.4	6.19	0.350	78.6
48	2	34.30	3.540	133.8	18.50	1.970	115.3	14.10	1.180	245.8	6.71	0.390	87.6
24 (2×)	2	36.96	3.131	118.4	15.74	1.656	96.9	12.66	0.927	193.1	8.58	0.548	123.1
192	2	41.29	3.601	136.1	18.73	1.992	116.6	14.69	1.058	220.4	7.87	0.551	123.8
96 (2×)	2	37.57	3.644	137.8	15.77	1.799	105.3	14.10	1.348	280.8	7.70	0.497	111.6
Control[a]	3	56.11	7.345	...	30.74	4.338	...	14.15	2.008	...	11.22	0.996	...
12	3	62.67	8.294	112.9	30.78	4.433	102.1	21.48	2.845	141.6	10.41	1.015	101.9
6 (2×)	3	63.44	7.526	102.4	30.88	4.029	92.8	20.21	2.524	125.6	12.36	0.973	97.6
48	3	64.54	8.175	111.3	29.36	4.071	93.8	24.27	3.246	161.6	10.91	0.870	87.3
24 (2×)	3	55.18	7.358	100.1	23.77	3.363	77.5	22.32	3.167	157.7	9.09	0.828	83.1
192	3	60.37	8.172	111.2	28.32	4.017	92.6	23.55	3.430	170.8	8.50	0.725	72.7
96 (2×)	3	59.84	7.946	108.1	25.46	3.573	82.3	25.92	3.668	182.6	8.28	0.705	70.7
Control[a]	4	70.84	11.24	...	40.56	6.82	...	16.87	3.35	...	13.41	1.05	...
12	4	71.63	12.92	114.9	35.22	6.16	90.3	24.84	5.39	160.8	11.57	1.37	130.4
6 (2×)	4	76.87	14.21	126.4	41.82	7.42	108.7	24.45	5.39	160.8	10.59	1.40	133.3
48	4	69.68	13.52	120.2	32.62	5.84	85.6	28.95	6.53	194.9	8.11	1.41	134.2
24 (2×)	4	81.48	14.21	126.4	39.95	6.92	101.4	26.45	5.80	173.1	15.07	1.49	141.9
192	4	85.27	15.38	136.8	38.81	6.53	95.7	32.69	7.28	217.3	13.77	1.57	149.5
96 (2×)	4	69.73	12.44	110.6	31.34	5.50	80.6	26.34	5.65	168.6	12.05	1.29	122.8

[a] Average of two control groups of eight plants each.

TABLE II.—TOTAL ALKALOID CONTENT[a] OF STRAMONIUM PER PLANT AND ORGAN

Treatment, mcg.	Harvest Time, wk.	Per Plant		Leaf-Tops		Stems		Roots	
		mg.	Control, %	mg.	Control, %	mg.	Control, %	mg.	Control, %
Control[b]	0	1.07	...	0.58	...	0.33	...	0.16	...
Control[b]	1	3.08	...	2.10	...	0.87	...	0.11	...
12	1	2.29	74.3	1.35	64.2	0.65	74.7	0.29	263.6
6 (2×)	1	1.79	58.1	0.99	47.1	0.57	65.5	0.23	209.0
48	1	1.82	59.1	1.05	50.0	0.61	70.1	0.16	145.4
24 (2×)	1	1.48	48.0	0.88	41.9	0.47	54.0	0.13	118.1
192	1	1.95	63.3	1.22	58.0	0.59	67.8	0.14	127.2
96 (2×)	1	1.65	53.5	0.99	47.1	0.51	58.6	0.15	136.3
Control[b]	2	5.69	...	4.28	...	1.01	...	0.40	...
12	2	5.63	98.9	3.50	81.7	1.74	172.2	0.39	97.5
6 (2×)	2	4.64	81.5	3.04	71.0	1.39	137.6	0.21	52.5
48	2	6.18	108.6	4.14	96.7	1.65	163.3	0.39	97.5
24 (2×)	2	5.08	89.2	3.15	73.5	1.49	147.5	0.44	110.0
192	2	4.45	78.2	2.99	69.8	0.85	84.1	0.61	152.5
96 (2×)	2	4.56	80.1	3.06	71.4	0.95	94.0	0.55	137.5
Control[b]	3	10.26	...	7.95	...	1.61	...	0.70	...
12	3	12.02	117.1	8.42	105.9	2.99	185.7	0.61	87.1
6 (2×)	3	9.61	93.6	6.85	86.1	2.27	140.9	0.49	70.0
48	3	9.07	88.4	5.70	71.6	2.93	181.9	0.44	62.9
24 (2×)	3	8.34	81.2	5.38	67.6	2.54	157.7	0.42	60.0
192	3	9.35	91.1	7.74	97.3	1.37	85.0	0.24	34.2
96 (2×)	3	8.22	80.1	6.07	76.3	1.84	114.2	0.31	44.2
Control[b]	4	18.93	...	14.19	...	3.69	...	1.05	...
12	4	24.69	130.4	17.25	121.5	5.93	160.7	1.51	143.8
6 (2×)	4	26.35	139.1	20.03	141.1	4.85	131.4	1.47	140.0
48	4	20.38	107.6	12.85	90.5	6.40	173.4	1.13	107.6
24 (2×)	4	21.57	113.9	13.15	92.6	7.08	191.8	1.34	127.6
192	4	21.78	115.0	14.69	103.5	5.72	155.0	1.37	130.4
96 (2×)	4	17.88	94.4	12.10	85.2	4.52	122.4	1.26	120.0

[a] Alkaloid content for plant parts calculated from dry weight and alkaloid analyses data; total plant alkaloids = leaves and tops + stems and roots; based on av./plant/groups. [b] Average of two control groups of eight plants each.

the first 3 weeks. However, at the fourth week considerable increases in the weights of the roots of the treated plants were noted (Table I). Variable results were noted in the total dry weight of treated plants the first week, but increased growth (dry wt.) was observed thereafter.

Analysis for Alkaloids.—One-gram samples of the dried plant parts, employing pooled samples, were assayed for total alkaloids according to the Witt-Youngken method (17), substituting chloroform for benzene as the immiscible solvent. A minimum of three duplicate determinations was carried out on each sample. All of the treatments induced significant reduction in the concentration of alkaloids in the leaf-tops during the first 2 weeks. Generally, the leaf-tops of the treated plants had decreased concentrations at the third week, whereas increased concentrations were noted at the fourth week. The stems of the treated plants generally indicated markedly decreased concentration of alkaloids. The stronger treatment (192-mcg. dose) generally induced the greatest reduction. Variable results were noted in the roots of the treated plants. The general trend was a higher concentration than controls during the first 2 weeks and a lower concentration the last 2 weeks.

Total Plant Alkaloids.—The total alkaloids per plant and per plant organ were obtained by multi-

TABLE III.—CHLOROPHYLL *a* AND *b* CONTENT[a] OF STRAMONIUM AERIAL PARTS

Treatment, mcg.	Harvest Time, wk.	Leaf-Tops			Stems		
		Chl. *a*	Control, %	Chl. *b*	Chl. *a*	Control, %	Chl. *b*
Control	0	0.167	...	0.087	0.0075	...	0.0055
Control	1	0.274	...	0.193	[b]	[b]	[b]
12	1	0.268	98.0	0.165	[b]	[b]	[b]
48	1	0.228	83.2	0.169	[b]	[b]	[b]
192	1	0.273	99.6	0.194	[b]	[b]	[b]
Control	2	0.286	...	0.191	0.021	...	0.018
12	2	0.259	90.5	0.157	0.014	67	0.011
48	2	0.225	78.6	0.198	0.010	48	0.009
192	2	0.230	80.4	0.122	0.018	86	0.016
Control	3	0.276	...	0.200	0.010	...	0.0084
12	3	0.187	67.7	0.109	0.010	100	0.0070
48	3	0.196	71.0	0.102	0.013	130	0.0065
192	3	0.251	93.5	0.153	0.014	140	0.011
Control	4	0.165	...	0.058	0.0056	...	0.0044
12	4	0.043	26.1	0.028	0.0052	93	0.0035
48	4	0.056	34.5	0.039	0.0046	82	0.0038
192	4	0.063	38.2	0.043	0.0043	77	0.0028

[a] Results expressed as per cent of dry wt.; 1-Gm. samples for leaf-top determinations; 2-Gm. samples for stems. [b] Insufficient material remained for chlorophyll determinations.

TABLE IV.—SELECTIVE SOLVENT EXTRACTIONS[a] OF STRAMONIUM LEAF-TOPS

Harvest Time, wk.	Treatment: Control	48 mcg.	192 mcg.
	Petroleum Ether		
0	1.44[b]	1.44[b]	1.44[b]
1	1.13	0.58	0.98
2	2.21	1.59	1.74
3	4.81	4.10	2.95
4	5.70	3.38	2.46
	Ether		
0	0.81[b]	0.81[b]	0.81[b]
1	0.75	0.55	0.53
2	1.13	0.95	1.09
3	0.84	1.09	0.83
4	0.85	1.05	1.12

[a] Expressed as per cent of dry wt.; based on 1-Gm. samples. [b] Pooled sample of 64 plants from all eight series.

TABLE V.—TOTAL SUGAR[a] AND STARCH[b] CONTENT OF STRAMONIUM LEAF-TOPS

Treatment, mcg.	Harvest Time, wk.	Total Sugars: mg./Gm.	Control, %	Starch: mg./Gm.	Control, %
Control	0	33.7[c]	...	7.33[c]	...
Control	1	14.9	...	3.41	...
48	1	17.9	120.	3.92	115.
192	1	37.9	254.	2.51	74.
Control	2	32.5	...	4.60	...
48	2	15.0	46	6.99	141.
192	2	32.1	99.	7.11	155
Control	3	21.1	...	9.76	...
48	3	28.8	184.	10.24	105.
192	3	42.5	201.	11.37	116.
Control	4	22.8	...	8.58	...
48	4	59.0	259.	10.67	124.
192	4	56.0	246.	13.36	156.

[a] Method of McCready (20). [b] Method of Viles and Silverman (22). [c] Pooled sample of 64 plants from all eight series.

plying the dry weight of the plant part by the per cent of alkaloids obtained from the alkaloid analysis and expressing the results in milligrams (Table II).

The total alkaloid content per treated plant was generally markedly reduced during the first 3 weeks but increased at the final harvest. The favorable effect the fourth week was due primarily to increased growth in the treated plants. The leaf-tops of the treated plants indicated a similar trend, except that both decreased and increased content were noted at the terminal harvest. On the other hand, the content of alkaloids in the stems was significantly decreased the first week but usually increased the remaining 3 weeks. Variable results were noted in total root alkaloids.

Chlorophyll Content.—The chlorophyll analysis was conduted by a method described by Gjerstad (18), but modified by employing dried material instead of fresh samples and using a Soxhlet extraction apparatus. A minimum of two duplicate determinations was carried out on each sample. The chlorophyll was completely extracted from the powdered material with a 20% water-in-acetone solvent. The results of the chlorophyll analysis are shown in Table III. The chlorophyll content of the treated plants was markedly reduced in the treated plants. The decreases of chlorophyll *a* content in the leaves of the treated plants at the final harvest were considered significant. Surprisingly, at this harvest, the group treated with the lowest concentration of GA (12 mcg.) contained the least amount of chlorophyll *a*. The amount of stem chlorophyll, except at the third harvest, was considerably less in the treated plants. The ratio of chlorophyll *a* to *b* was somewhat higher in the stems than in the leaves. The decreased content of chlorophyll in the leaves confirms our observation that the leaves of treated plants appeared chlorotic.

Selective Solvent Extraction.—To determine the effect of gibberellin treatment on the formation of other types of metabolic products, 1-Gm. samples of the untreated, 48-mcg., and 192-mcg. groups were completely extracted with petroleum ether and then with anhydrous ether. The method of Dragendorff (19) was employed with use of the Soxhlet extractor. At least two duplicate determinations were carried out on each sample. The amount of petroleum ether extractive was considerably less in the treated plants at each harvest (Table IV). The decreases of 41% in the 48-mcg. group and of 57% in the 192-mcg. group at the final harvest were considered significant. The lower dosage of GA caused a greater reduction during the first 2 weeks. On the other hand, the plants treated with 192 mcg. of GA displayed greater decreases at the latter two harvests (Table V). The content of ether extractive in the treated plants was considerably less than controls at the first two harvests and somewhat greater than controls at the final harvest. Of greater importance was the apparent qualitative difference in the ether soluble extractives of the treated plants. The waxy residue from the controls was dark green in color, whereas that from the treated plants was a yellowish brown color.

Carbohydrate Content.—The procedure for the extraction of total sugars was adapted from that of McCready (20). A 250-mg. sample of leaf material

TABLE VI.—ANALYSIS OF VARIANCE FOR GROWTH DATA

Source of Variation	Degrees of Freedom	Mean Square: Height	Total Dry Wt.	Leaf-Tops Dry Wt.	Stem Dry Wt.	Root Dry Wt.
Replication	7	192.36	26.26[a]	13.90[a]	2.75[a]	0.09
Installment	1	84.46	4.69	0.63	1.37	0.07
Dosage	3	4,049.34[a]	12.23[a]	1.09	18.05[a]	0.08
Age	4	24,826.19[a]	1,796.09[a]	432.04[a]	310.89[a]	16.17[a]
Ins. × Dosage	3	0.23	1.50	0.79	0.12	0.05
Ins. × Age	4	26.25	0.81	1.32	1.37[a]	0.03
Dosage × Age	12	568.97[a]	4.62[a]	0.74	5.28[a]	0.20[a]
Ins. × Dos. × Age	12	13.57	2.55	1.02	0.58	*0.04*
Error	273	36.53	2.48	0.95	0.49	0.05
Total	319					

[a] Significant at $p = 0.05$.

was shaken with 15 ml. of hot, neutral (to litmus), 80% ethanol for 30 minutes on a wrist-action shaker. The mixture was centrifuged and the supernatant liquid decanted into a 100-ml. volumetric flask containing 25 ml. of 15% neutral lead acetate solution. The residue was washed three times with ethanol solution and centrifuged. Each time the supernatant liquid was decanted into the flask containing the lead acetate solution. Powdered sodium oxalate was added to remove the lead ions and the mixture was brought to the 100-ml. mark with distilled water. The mixture was centrifuged and aliquots taken from the supernatant liquid. Total sugar was determined by the anthrone–sulfuric acid color reaction (21) employing 0.5-ml. aliquots of the clarified extract. The residue from the sugar extraction procedure was extracted and analyzed for starch (amylose) according to the method of Viles and Silverman (22). The results of these analyses are presented in Table VI.

The gibberellin treatments induced considerable increases in the total sugar content of the plants, except at the second week (Table VI). The increased content in the treated groups at the third and fourth week was considered significant. Marked increases in the starch content of treated plants were noted in the treated plants from the second through the fourth week.

STATISTICAL ANALYSIS OF GROWTH DATA[3]

Analysis for Height.—The analysis of variance for the height data is given in Table VI which shows that only dosage, age, and dosage times age interaction are significant. This indicates that both dosage and age affect the heights of plants. The significant interaction indicates that the rate of growth varies with dosage.

The number of installments does not affect the height of plants. Whether a certain dose is applied all at once or in two installments with half a dose each time will not make a difference on the average heights of plants.

Since dosage times age interaction was significant, a more detailed analysis of the growth rates of plants at various doses was made. The mean heights are shown in Fig. 2. The analysis of variance is shown in Table VII.

The Table shows that for control and 12-mcg. groups the deviation from linearity is not significant, while that for 48 and 192-mcg. groups is significant. This implies that the growth curves for control and 12 mcg. are approximately linear, while those for 48 and 192 are not. The average growth rates, b_1 and b_2, for control and 12 mcg. are 7.16 and 12.27 cm. per week, respectively. This difference is significant (Table VII). In other words, the plants receiving 12 mcg. of GA grow faster than control plants. For the two higher doses, the average rates of growth cannot be compared, because the growth curves cannot be approximated by straight lines. To determine whether these two curves are identical, the dosage times age interaction for 48 and 192 mcg. was tested. This interaction is not significant. The implication is that the two growth curves are parallel.

[3] The author acknowledges the assistance of Dr. Jerome C. R. Li, Chairman of Department of Statistics, Oregon State University, for assisting in the experimental design and performing the statistical analysis of the growth data.

TABLE VII.—ANALYSIS OF VARIANCE FOR GROWTH RATE FOR HEIGHT

Source of Variation	Degrees of Freedom	Mean Square	F
Control			
Linear regression	1	8,203.93	
Deviation from linearity	3	65.87	1.80
12 mcg.			
Linear regression	1	24,090.92	
Deviation from linearity	3	79.99	2.19
24 mcg.			
Linear regression	1	33,857.85	
Deviation from linearity	3	239.77	6.56[a]
192 mcg.			
Linear regression	1	38,146.06	
Deviation from linearity	3	225.57	6.17[a]
Control and 12 mcg.			
b_1 *vs.* b_2	1	2,088.99	57.19[a]
48 and 192 mcg.			
Dosage × age	4	21.01	0.58
Error	273	36.53	

[a] Significant at $p = 0.05$.

From the analyses of variance (Tables VI and VII), one can draw the following conclusions. (*a*) For the same amount of GA applied, whether one or two installments is used, the height of the plant is not affected. (*b*) A larger dose of GA will make the plants grow faster in height, but the effect of dosage is limited. The growth curves for 48 mcg. and 192 mcg. are not significantly different.

Analysis of Dry Weights of Leaf-Tops.—Only the age of the plants affect the dry weight of leaves and tops. Dosage and the number of installments are not significant (Table VI).

Analysis of Stem Dry Weights.—Table VI shows that both dosage and age effect the dry weight of stems. Significant dosage times age interaction indicates that the growth rate varies with the dosage of GA. The growth rate also varies slightly with the number of installments used in applying the acid. The conclusions can also be observed from Table I. The analysis of growth rate given in Table VIII indicates that the weight of stems grows faster with a higher dosage of GA, but the two higher doses, 48 and 192 mcg., have the same growth rate. This conclusion is also true for the height of plants.

Analysis of Root Dry Weights.—The dry weight of roots is not significantly affected by the dosage of GA or the number of installments. The roots do develop with age. The rate of growth of the roots, however, varies slightly with the dosage as indicated by the significant dosage times age interaction shown in Table VI. The analysis of the growth rate is

TABLE VIII.—ANALYSIS OF GROWTH RATE, DRY WEIGHT OF STEMS

Source of Variation	Degrees of Freedom	Mean Square	F
Dosage × Age	12	5.28	10.87[a]
0 and 12 mcg.	4	5.74	11.81[a]
48 and 192 mcg.	4	0.27	0.56
Low *vs.* High	4	9.84	20.24[a]
Error	273	0.48	

[a] Significant at $p = 0.05$.

TABLE IX.—ANALYSIS OF VARIANCE FOR GROWTH RATE, DRY WEIGHT OF ROOTS

Source of Variation	Degrees of Freedom	Mean Square	F
Dosage × Age	12	0.20	4.46[a]
Among Treated	8	0.08	1.89
Treated *vs.* Untreated	4	0.43	9.61[a]
Error	273	0.04	

[a] Significant at $p = 0.05$.

shown in Table IX. The growth rates of the roots are not significantly different among the treated plants, 12, 48, and 192 mcg. The control plants have a slightly different growth rate.

Analysis of Total Dry Weight.—Table VI indicates that the total dry weight is not affected by the number of installments, but is significantly affected by dosage and age. Significant interaction between dosage and age indicates that the growth rate varies with dosage. These conclusions can also be observed from Table I. The analysis for growth rate is given in Table X which shows that the treated plants (*i.e.*, those of the 12, 48, and 192-mcg. groups) have the same growth rate in total dry weight, but the control plants grow at a slower rate.

Statistical analysis of leaf growth data (such as leaf-blade width, leaf-blade length, and petiole length) did not indicate significant results from the dosage applied. It was noted, however, that the leaves of treated plants grew at a different rate than controls.

DISCUSSION AND CONCLUSIONS

The treated plants displayed characteristic gibberellin effects such as taller and spindlier plants, greater internodal elongation, the appearance of chlorotic leaves, and significantly increased stem growth. These effects have been reported in other solanaceous alkaloid-producing plants (5–16). Other effects generally noted in the treated plants included less succulent shoots, woodier stems, less fruit-set than controls, and a more rapid abscission of the lower leaves. There is a correlation between the statistical analysis of height data and stem dry weight data, in that the significant factors were dosage, age, and dosage times age interactions. All treated plants grew more rapidly than controls; the height and stem weights were significantly different at each dosage level; and the rate of growth varied with the dosage. The stem dry weight was the only variable significantly affected by the number of installments. The statistical analyses of both variables indicated that the most favorable response on growth was induced by the GA between the 12 and 48-mcg. dosage levels. Previously unpublished work performed in our laboratory on stramonium which was treated with weekly doses of 10, 25, 50, and 100-mcg. doses of GA indicated that the most favorable response on height was induced with the 50-mcg. dose. Thus, it was concluded that the most beneficial response on growth was near the 48-mcg. weekly dosage level.

TABLE X.—ANALYSIS OF VARIANCE FOR GROWTH RATE, TOTAL DRY WEIGHT

Source of Variation	Degrees of Freedom	Mean Square	F
Dosage × Age	12	4.62	1.86[a]
Among Treated	8	0.39	0.16
Treated *vs.* Untreated	4	13.08	5.27[a]
Error	273	2.48	

[a] Significant at $p = 0.05$.

A study of dry weight data (Table I) indicates that treatments at all dosage and frequency levels resulted in increased total plant weight at the terminal harvest. At this time reduced leaf-tops weight was generally noted; significantly increased stem weight (with over a twofold increase in the 192-mcg. group) and significantly increased root weight were found. The largest increase in stem weights were induced with the higher concentrations of GA and correlates with the height increases. A similar trend was found in the root weights. The reduced leaf-tops weight was most likely due to two factors: more rapid abscission of the lower leaves than in the controls, and less fruit-set which resulted in fewer capsules being formed in the treated plants. Generally, stem growth was significantly increased in the treated groups throughout the 4-week observation period. Variable results were noted in the weights of the other organs during the first 3 weeks. Conclusions regarding plant growth were that generally a favorable response on growth was induced by the treatments, the increased stem growth was considered significant, treated plants grew at a more rapid rate than controls, and the growth rate generally varied with the dose of GA applied.

The general trend was a reduced concentration of alkaloids in the organs of the treated plants. The largest decreases were usually noted with the higher dosage levels of GA. However, this was not true in all cases. When using the single weekly doses of 12, 48, and 192-mcg. as a basis, it was found that reduced concentrations of alkaloids were induced in the leaf-tops of these groups at the first two harvests, while increased concentrations were noted at the final harvest. A similar trend was observed in the concentration of stem alkaloids, except that significant reductions were noted at all four harvests in the 192-mcg. series. The reductions in the stems were attributed to dilution of the alkaloids which resulted from markedly increased stem growth. The roots generally demonstrated increased concentrations of alkaloids at the latter two harvests. The general trend of reduced concentration of alkaloids in the aerial parts of gibberellin-treated plants is in agreement with other reports in the literature (5–16). However, increased concentration of alkaloids such as that found in the aerial parts at the terminal harvest has not been previously reported for GA-treated plants. Brummett (23) found that, when stramonium was administered a single 100-mcg. dose of GA, significantly reduced concentrations of alkaloids occurred in all the plant organs at the first and second-week harvests. On the other hand, slight increases were found in the leaf-tops and roots at the fourth-week harvest. Brummett's plants were approximately the same age during the treatment and harvest periods as those employed in this experiment. This suggests that mature plants do not respond to

GA in the same manner as do younger plants. Further, the dosage per unit of plant weight was about twentyfold greater at the time of initial treatment than at the terminal harvest. This dilution of about $^1/_{20}$ in dosage, which was due to the increased weight of the mature plants, could also account for the increased concentration of alkaloids in the aerial parts of the treated plants at the terminal harvest.

The total alkaloid content per plant was generally considerably less in the treated groups at the first three harvests. This was due mainly to lower concentrations in most of the plant organs and a general reduction in leaf-tops and root growth. This trend was reversed at the final harvest when marked increases were usually found in the treated groups. The increases were due to a favorable effect on growth by the GA treatments and a higher concentration of alkaloids in the leaf-tops of the treated plants.

The per cent of chlorophyll in the leaf-tops of the 12, 48, and 192-mcg. groups was considerably less than controls throughout. The decreases (compared with controls) at the final harvest of 74, 65, and 62%, in the corresponding groups, were considered significant. The reduced per cent of chlorophyll in the aerial parts of treated stramonium confirms our observations in this experiment and our previous publications (5–7) that the leaves of treated plants appeared chlorotic. This is also in agreement with that reported for *Datura meteloides* (10) and spearmint (24), but does not agree with the results reported for foxglove (25) and peppermint (26).

Calculations were made from the results of the chlorophyll analysis and from the dry weight data to indicate the milligrams of chlorophyll *a* present in the leaf-tops of the plants. The average per control plant was 1.84, 4.96, 12.15, and 11.59 mg. at the first, second, third, and fourth harvests, respectively. This indicates that the maximal chlorophyll content occurred at the third harvest. Only slight differences compared with controls were found in the treated groups at the first and second harvests. However, the chlorophyll *a* content was markedly reduced by the GA-treatment at the third and fourth harvests. The figures for the 12, 48, and 192-mcg. groups at the third harvest were 8.42, 8.14, and 10.05 mg., respectively. The corresponding figures at the fourth harvest were 2.46, 3.50, and 3.92 mg., respectively. The treated groups attained their maximal content of chlorophyll at the third harvest, as did the controls. The latter, however, contained up to 50% more chlorophyll *a* at that time than did the treated groups. At the final harvest the controls had about a three to fivefold greater amount of chlorophyll *a* than did the treated groups. It was apparent that chlorophyll synthesis progressed at about the same rate in treated and untreated groups during the first 2 weeks of the observation period. A study of the aforementioned data, however, indicates that catabolism of the pigment between the third and fourth-week harvests was significantly different in the treated groups. It was concluded that the significant reductions in the chlorophyll *a* content of the treated groups were probably due to a greatly increased rate of degradation of the pigment.

Considerable increases in the total sugar content of the 48 and 192-mcg. groups were found in the leaf-tops throughout the 4-week observation period, except at the second harvest. The increases at the third and fourth harvests were considered significant. It was noted that the sugar content in the leaf-tops of the controls decreased with age. A complete reversal of this trend was noted in the treated plants. Increased sugar content from GA treatment has been reported in *Digitalis purpurea* (25), *Digitalis lanata* (27), and buckwheat (28). Likewise, marked increases in the starch content of the leaf-tops of treated plants were noted from the second through the fourth weeks. Apparently GA stimulated carbohydrate production in the leaf-tops of stramonium. In a previous publication (6) it was suggested that GA induced changes in the carbohydrate and/or protein metabolism of stramonium. The results of this experiment indicate that GA increased the synthesis of carbohydrates in the leaf-tops and confirms our previous report (6) that the leaves of treated plants had a higher dry matter content than controls. This increased leaf density was attributed primarily to the increased carbohydrate content. It was doubtful that protein metabolism was directly affected. Brummett (23) has found generally no significant differences in the total, soluble, and protein nitrogen of the leaves of treated and untreated stramonium. However, significant decreases were noted in the nitrate nitrogen of the leaves of treated plants (23). These facts strongly suggest that the reduced concentration of alkaloids usually found in the aerial parts of stramonium treated with GA is due to an alteration in the normal metabolic pathways whereby alkaloids are synthesized by the plant. This might be explained in several ways. First, the increased carbohydrate was utilized for more extensive stem growth and synthesis of structural components of the plant. Second, the stimulation of carbohydrate production and subsequent storage diverted the utilization of carbohydrates, which might have been used for alkaloid synthesis, into other metabolic pathways. Either would result in the alteration of the usual pathways of alkaloid biogenesis normally existing in the plant.

The decreases of up to 57% in the petroleum-ether extract of the treated leaf-tops of stramonium suggest further that lipid biosynthesis was depressed. This solvent extracts volatile oils, fixed oils, and waxes. Stramonium seeds are rich in fixed oil. However, since the capsules and enclosed seeds were not weighed separately when leaf-tops weights were made, this experiment did not ascertain if the reduced lipid content was because of inhibition of lipid synthesis or a lower seed content in the treated plants. Further work is proceeding on this problem. Considerable decreases were noted in the content of the ether-soluble extractive of the leaf-tops at the first two harvests, while appreciable increases were found at the final harvest. Of greater interest was the apparent qualitative difference between the extracts found in the treated and untreated plants. The former were a yellowish-brown color, whereas the latter were dark green in color. A study is being continued to determine the difference in the composition of the ether-soluble extractive of the treated and untreated plants.

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Interaction of Pharmaceuticals with Schardinger Dextrins III

Interactions with Mono-Halogenated Benzoic Acids and Aminobenzoic Acids

By JOHN L. LACH and TING-FONG CHIN

A series of mono-halogenated benzoic acids and aminobenzoic acids were shown to undergo definite interactions with the cyclodextrins. The complexes formed are considered to be due, in part, to inclusion formation and to other attractive forces existing between the guest and host molecules. Data are presented to illustrate the effect of steric hindrance, polarity, resonance structure and inductive effect with respect to this interaction. Some stoichiometric data are also presented and several formation constants calculated.

THE CYCLIC STRUCTURE of the dextrins confers upon these compounds the ability to form mono-molecular inclusion compounds, where the guest is enclosed within the cyclodextrin void. In the case of these cyclodextrins there is just one host molecule formed by several constituents (glucose units) united through ordinary chemical bonds, which enclose one single molecule of the guest. This makes the cage of a permanent nature as opposed to clathrates (these may also have a cage-like hollow space in the center as in the dextrins) which are derived from crystalline lattices.

Generally speaking, formation of solid crystalline inclusion compounds can be explained on the basis that the molecules are held together by virtue of their spatial configuration, and these compounds are defined as chemically inert toward each other. True inclusion formation therefore implies that no attractive force or forces are of paramount importance between guest and host. However, in aqueous solution inclusion compounds of the cyclodextrins and various organic substances do exist (as reported from these laboratories) (1, 2), indicating that some attractive force or forces are operative which stabilize the guest molecule in the cage-like hollow space or the guest molecules are bonded to the cyclodextrin in some manner other than inclusion formation. The purpose of this investigation was to study this interaction further in terms of polarity, inductive effect, resonance structure, and steric hindrance with respect to the degree of interaction of these dextrins with various agents. A series of mono-halogenated and aminobenzoic acid derivatives were selected for this study.

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EXPERIMENTAL

Reagents:

α-Cyclodextrin $[\alpha]_D^{25}$ in water = +150.5 ± 0.5; β-cyclodextrin $[\alpha]_D^{25}$ in water = +162.5 ± 0.5;

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EXPERIMENTAL

Reagents:

α-Cyclodextrin $[\alpha]_D^{25}$ in water = +150.5 ± 0.5; β-cyclodextrin $[\alpha]_D^{25}$ in water = +162.5 ± 0.5;

p-chlorobenzoic acid, recrystallized, m.p. 235–236°; *m*-chlorobenzoic acid, recrystallized, m.p. 158–159°; *o*-chlorobenzoic acid, recrystallized, m.p. 140–141°; *p*-bromobenzoic acid, recrystallized, m.p. 250–251°; *m*-bromobenzoic acid, recrystallized, m.p. 154–155°; *o*-bromobenzoic acid, recrystallized, m.p. 148–150; *p*-iodobenzoic acid, recrystallized, m.p. 263–265°; *m*-iodobenzoic acid, recrystallized, m.p. 185–187°; *o*-iodobenzoic acid, recrystallized, m.p. 161–162°; *p*-aminobenzoic acid, recrystallized, m.p. 186° dec.; *m*-aminobenzoic acid, recrystallized, m.p. 175° dec.; *o*-aminobenzoic acid, recrystallized, m.p. 145–146°; cinnamic acid, recrystallized, m.p. 133°, were utilized.

Apparatus:

A constant temperature water bath, set at 30 ± 0.5° with rotating spindle; 20-ml. capacity vials with gum rubber stoppers and aluminum caps; Beckman DU spectrophotometer with 1-cm. silica cells.

Procedures:

Preparation and Separation of Cyclodextrins.—The method used for the preparation and separation of cyclodextrins is the modified method of French (3).

Determination of Molecular Complex Formation.—The solubility method of Higuchi and Lach (4) was used to study complex formation. The total concentration of the drug in the solution was measured spectrophotometrically since the cyclodextrins show no absorption at the wavelengths and concentration employed. Absorptivities had previously been determined for each of the drugs studied at the following wavelengths: *p*-chlorobenzoic acid, 238 mμ; *m*-chlorobenzoic acid, 280 mμ; *o*-chlorobenzoic acid, 238 mμ; *p*-bromobenzoic acid, 240 mμ; *m*-bromobenzoic acid, 280 mμ; *o*-bromobenzoic acid, 240 mμ; *p*-iodobenzoic acid, 244 mμ; *m*-iodobenzoic acid, 282 mμ; *o*-iodobenzoic acid, 244 mμ; *p*-aminobenzoic acid, 278 mμ; *m*-aminobenzoic acid, 300 mμ; *o*-aminobenzoic acid, 318 mμ· and cinnamic acid, 270 mμ.

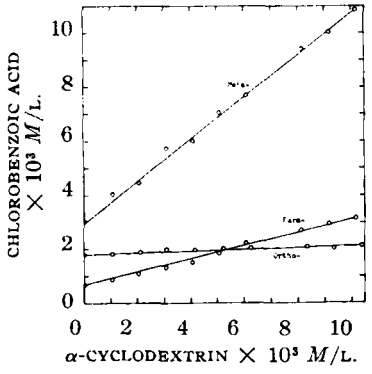


Fig. 1.—Interaction of chlorobenzoic acids with α-cyclodextrin at 30°.

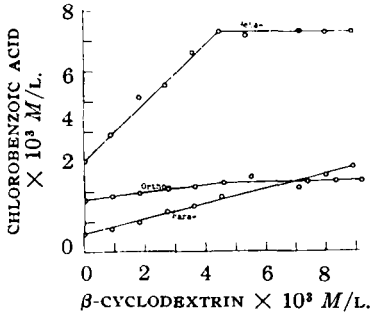


Fig. 2.—Interaction of chlorobenzoic acids with β-cyclodextrin at 30°.

RESULTS AND DISCUSSION

A number of methods were tested for this molecular complexation study. These methods included partition distribution, equilibrium dialysis, and solubility method in nonaqueous solutions.

The partition distribution method proved unsuccessful. The addition of various concentrations of cyclodextrin to a chloroform-water system containing benzoic acid resulted in no change in the distribution of this organic acid between the two phases, indicating that no interaction of the dextrin

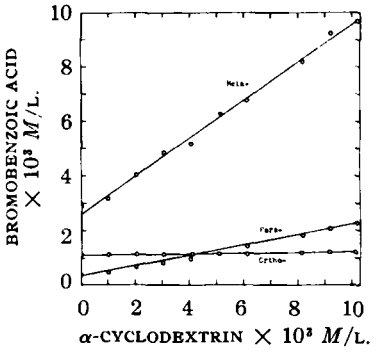


Fig. 3.—Interaction of bromobenzoic acids with α-cyclodextrin at 30°.

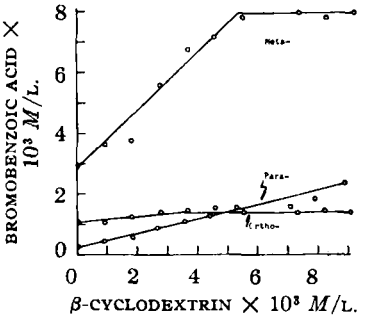


Fig. 4.—Interaction of bromobenzoic acids with β-cyclodextrin at 30°.

and benzoic acid had taken place. The use of ether, carbon tetrachloride, skellysolves, and benzene in this distribution study was also unsuccessful. This was somewhat surprising since benzoic acid and cyclodextrin readily interact in aqueous solution. This lack of interaction in the two-phase system is probably because the affinitive force of cyclodextrin with water and the affinitive force of benzoic acid with the organic solvent are greater than the attractive force of the cyclodextrin and benzoic acid.

A solubility method employing organic solvents to minimize any interaction due to hydrogen bonding or polar effects was also investigated. The solubility effect of benzoic acid as a function of cyclo-

dextrin in solvents such as chloroform, carbon tetrachloride, ether, 1,4 dioxane, skellysolves, and benzene was studied. However, as with the partition study, no interaction was observed. This lack of interaction of benzoic acid with the dextrins in such solvents does indicate that attractive forces must play a major role in interactions of this type in solution. However, it should be pointed out that this lack of reactivity may also be partly because benzoic acid forms dimers in many organic solvents (5). This lack of reactivity was also noticed by Schlenk (6), who pointed out that water seems to be a necessary component for complex formation of the cyclodextrins. Schlenk reported no interaction when the anhydrous cyclodextrins were heated and shaken with myristic acid in methanol. Also, these cyclodextrins failed to react with dry vapor of tri-

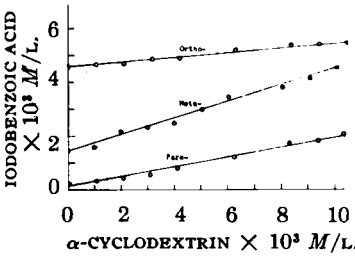


Fig. 5.—Interaction of iodobenzoic acids with α-cyclodextrin at 30°.

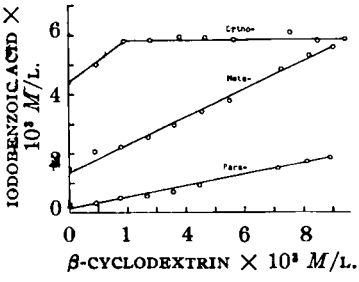


Fig. 6.—Interaction of iodobenzoic acids with β-cyclodextrin at 30°.

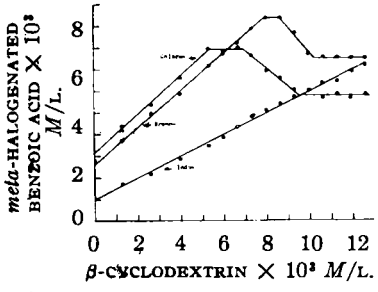


Fig. 7.—Interaction of *meta*-halogenated benzoic acids with β-cyclodextrin at 30°.

chloroethylene or bromobenzene, both known to be typical adduct formers.

The solubility method employing an aqueous solution was, therefore, used to study these interactions. Two series of agents were employed—mono-halogenated benzoic acid derivatives to study inductive and polar effects, and aminobenzoic acid derivatives to study resonance structure effects with respect to this cyclodextrin interaction. The results of these interactions are shown in

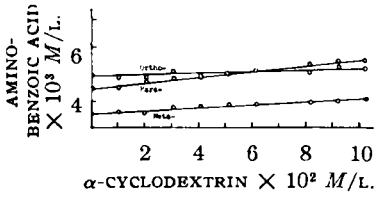


Fig. 8.—Interaction of aminobenzoic acids with α-cyclodextrin at 30°.

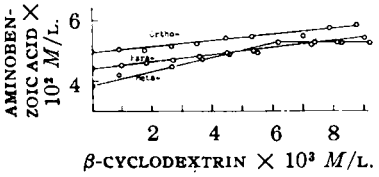


Fig. 9.—Interaction of aminobenzoic acids with β-cyclodextrin at 30°.

Figs. 1–9. A definite increase in the equilibrium solubility of the mono-halogenated benzoic acids as a function of cyclodextrin added is observed in Figs. 1–6. Since the concentration of the free benzoic acids in solution is an invariant, exact stoichiometric relationships cannot be calculated from these phase diagrams; however, the linear dependency of these interactions is indicative of a first-order relationship with respect to the cyclodextrin concentration. Since the solubility limit of β-cyclodextrin was not exceeded in this system it was felt that some information regarding the stoichiometric relationship would be obtained using this higher dextrin concentration. Figure 7 shows a phase diagram obtained for the interaction of *m*-chlorobenzoic acid, *m*-bromobenzoic acid, and *m*-iodobenzoic acid with β-cyclodextrin. From the plateau region of these diagrams the stoichiometry of *m*-chlorobenzoic acid and *m*-bromobenzoic acid can be calculated according to the method of Higuchi and Lach (4). A 1 to 1 relationship is obtained for these two acids. The formation constant for both acids was calculated to be 2.24×10^3. A formation constant of this magnitude is considerably greater than those previously reported for interactions of this type (7–9). This is certainly indicative of the formation of a strong cyclodextrin complex.

A comparison of the interactions of the halogenated derivatives and the cyclodextrins can be approximated from the slopes of these isotherms and are presented in Tables I and II.

An examination of data presented in these tables reveals the following observations:

1. The degree of interaction for chlorobenzoic acid is greater than bromobenzoic acid which in turn is greater than iodobenzoic acid.

2. The interaction of *meta* halogenated benzoic acids is greater than for the *para* halogenated benzoic acids which is in turn greater than the *ortho* halogenated benzoic acids.

3. The interaction of β-cyclodextrin is greater than that of the α-cyclodextrin.

It is difficult at this time to interpret these data mathematically. However, certain physical and chemical properties of these halogenated acids are evident. As already pointed out, the degrees of interaction of chlorobenzoic acids are greater than

TABLE I.—SLOPES OF ISOTHERMS OF INTERACTION OF THE MONO-HALOGENATED BENZOIC ACIDS WITH α-CYCLODEXTRIN

Position of Halogen Substituted	Benzoic Acid Derivatives		
	Chloro	Bromo	Iodo
Meta	0.76	0.68	0.31
Para	0.24	0.19	0.18
Ortho	0.03	0.01	0.08

those of bromobenzoic acids which in turn are greater than those of iodobenzoic acids. This order of reactivity may be due, in part, to inclusion formation. Since the void space in the cyclodextrin molecule is fixed, it is certainly reasonable that the smaller molecule would interact to a greater degree by virtue of its ease of entrance into the void. Attempts to correlate this activity of the mono-halogenated *para* and *meta* benzoic acids with the

TABLE II.—SLOPES OF ISOTHERMS OF INTERACTION OF THE MONO-HALOGENATED BENZOIC ACIDS WITH β-CYCLODEXTRIN

Position of Halogen Substituted	Benzoic Acid Derivatives		
	Chloro	Bromo	Iodo
Meta	0.96	0.93	0.47
Para	0.25	0.24	0.19
Ortho	0.13	0.09	0.74

Hammett sigma constants was only partially successful suggesting that, in addition to hydrogen bonding, steric factors and other attractive forces are also operative in this interaction. Interestingly, the order of reactivity of these halogenated aromatic acids parallels the electronegativity of the halogen substituent on the aromatic ring and not solely on the polarity of the carboxyl group. This may be rationalized on the basis of an inductive effect brought about by the halogen substituent. Since the inductive effect of the *meta* isomer is greater than that of the *para* isomer, the result is a lower electron density at the carboxyl group, facilitating a greater degree of intermolecular hydrogen bonding. This difference in degree is evident in the greater interaction of the *meta* isomer. Although the electron density of the carboxyl group is lowest in the *ortho* acid, the low degree of interaction can be rationalized on the basis of a steric effect. The bulky halogen atom in this *ortho* position decreases the hydrogen bonding tendency of the carboxylic acid and increases the overall size of the terminal position of the molecule to hinder the ease of entrance into the void space of the cyclodextrin. However, the reason for the *o*-iodobenzoic acid interaction with β-cyclodextrin being greater than the *para* and *meta* isomers cannot be explained at this time.

Data obtained in a study of a series of aminobenzoic acids are presented in Figs. 8 and 9. Table III lists the slopes of these isotherms.

The results of this aminobenzoic acid series are, at this time, somewhat difficult to understand, particularly in the interactons of the α-cyclodextrin. The order of reactivity of the *para* and *meta* isomers does not parallel the order observed in the halogenated and hydroxylated series (1). It does, as expected, parallel this order in the β-cyclodextrin interaction. This may be due, in part, to the greater freedom of entrance of the interacting molecule afforded by the larger void in the β-cyclodextrin; consequently, steric considerations would be less than in the restricted α-cyclodextrin molecule. The greater observed activity of the *para* isomer in the α-cyclodextrin over the *meta* isomer is also not readily obvious. A consideration of the resonance effect with respect to the polarity of the carboxyl group in the *para* isomer should lead to a lesser degree of interaction than the *meta* isomer. Since this is not

TABLE III.—SLOPE OF THE ISOTHERMS OF INTERACTION OF THE AMINOBENZOIC ACIDS WITH CYCLODEXTRINS

Aminobenzoic Acids	α-Cyclodextrin	β-Cyclodextrin
m-Aminobenzoic acid	0.58	2.25
p-Aminobenzoic acid	0.98	1.09
o-Aminobenzoic acid	0.29	0.91

the case the data suggest that the steric effect is more dominant in the α-cyclodextrin containing the smaller void.

It is interesting to compare the cyclodextrin interaction with cinnamic acid (Fig. 10) and that of benzoic acid which has been reported by Lach and Cohen (1). The difference in these two molecules lies in a carbon double bond. Lach and Cohen reported a slope of approximately 1.0 for the ben-

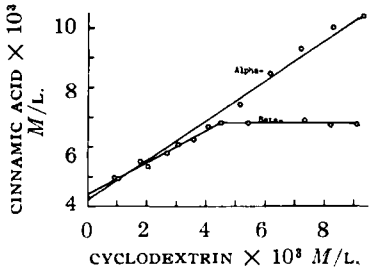


Fig. 10 — Interaction of cinnamic acid with cyclodextrins at 30°.

zoic acid interaction. A slope of approximately 0.6 is obtained here for the cinnamic acid study. The greater degree of interaction of benzoic acid may be attributed to the higher polarity of the carboxylic group due to resonance consideration. Comparison of this interaction based on size only should not produce such a great change in slope which is a measure of the degree of reactivity.

Investigations of this type do indicate the complexity of such interactions. Studies with other molecules and the cyclodextrins are being conducted in these laboratories to elucidate further the nature of these interactions.

CONCLUSION

Data presented show that the cyclodextrins form molecular complexes with the mono-halogenated benzoic acids and the aminobenzoic acids in aqueous solution. It is difficult, at this time, to explain fully the mechanism involved in this interaction since no crystallographic data of the complexes formed are available. However, it can be pointed out that, in addition to inclusion formation, other attractive forces must also be operative in the net interaction. Several formation constants and stoi-

chiometric ratios have been calculated. The higher order of magnitude of these formation constants indicates a high degree of stability of the complex.

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Potential Hypoglycemic Sulfonylureas

By KARL A. NIEFORTH†, GLENN L. JENKINS, and ADELBERT M. KNEVEL

A series of *N'*-alkyl-*N*-*p*-phenylbenzenesulfonylureas and *N,N'*-bis(*N*-alkylcarbamyl) 4,4'-biphenyldisulfonamides has been prepared for pharmacological studies of their hypoglycemic activities.

ALTHOUGH SULFONYLUREAS have been reported in the literature for several years, it was not until 1955 that the hypoglycemic activity of these compounds was recognized and reported. Since that time, the interest in synthesizing compounds of this type has increased considerably and has resulted in a few marketable compounds. The purpose of this paper is to extend research in this area to include the biphenyl nucleus.

Early work in the area of hypoglycemics was centered around derivatives of guanidine (1, 2). Several years later, various derivatives of isopropylthiadiazole were investigated and found to be active hypoglycemics (3). This work (3) may also be considered to be the start of the use of sulfonamides in controlling the symptoms of diabetes. In 1955, a sulfonylurea compound was found to be very active in lowering the blood sugar concentration in diabetics (4). After this work was reported, new active compounds began appearing at a rapid rate (5–8). It is now generally accepted that the sulfonylurea function is a source of hypoglycemic compounds. The work in this paper is based on that assumption and is conducted in two parts. The first part is the synthesis of *N'*-alkyl-*N*-*p*-phenylbenzenesulfonylureas (VII); the second part is the synthesis of *N,N'*-bis(*N*-alkylcarbamyl)4,4'-biphenyldisulfonamides (XIV).

Biphenyl (I) was sulfonated according to a published procedure (9) and gave good yields of 4-biphenylsulfonic acid (II). This in turn was reacted with phosphorus pentachloride using carefully controlled conditions to form 4-biphenylsulfonyl chloride (III). If the conditions were not controlled, varying amounts of 4,4'-biphenyldisulfonyl chloride (X) were found in the reaction mixture. The sulfonyl chloride was heated in ammonia water forming 4-biphenylsulfonamide (IV). The sulfonylurea (V) was prepared (10) upon reaction of the sulfonamide with *n*-butylisocyanate in the presence of triethylamine. Because of the difficulty in preparing the various isocyanates needed in this project, an alternate route was used to prepare the remainder of the compounds in Part I (11).

In Part II, biphenyl(VIII) was sulfonated with an excess of sulfuric acid to form 4,4'-biphenyldisulfonic acid (IX) (12). The reaction between 4,4'-biphenyldisulfonic acid and phosphorus pentachloride was controlled to prevent the formation of 4,4'-dichlorobiphenyl. 4,4'-Biphenyldisulfonyl chloride (X) was heated with ammonia water to form 4,4'-biphenyldisulfonamide (XI). From this point, the reactions in Part II are the same as those utilized in Part I. One compound (XII) was synthesized by the reaction of 4,4'-biphenyldisulfonamide and *n*-butylisocyanate. The remainder of the compounds was prepared by the alternate route (XI-XIV).

EXPERIMENTAL[1]

4-Biphenylsulfonic Acid and 4,4'-Biphenyldisulfonic Acid.—These compounds were prepared by reacting biphenyl with sulfuric acid. The quantities of sulfuric acid depended on the desired acid (9, 12). The salts of the sulfonic acids were formed by dissolving the acids in alcohol and adding concentrated solutions of potassium or sodium hydroxide to the alcoholic solutions.

4-Biphenylsulfonyl Chloride.—Twenty grams

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[1] Sulfur determinations were carried out by Dr. G. Weiler and Dr. F. B. Strauss, Microanalytical Laboratory, 164 Banbury Road, Oxford, England, and Alfred Bernhardt, Mikroanalytisches Laboratorium im Max-Planck-Institut fur Kohlenforschung, Hohenweg 17, Mulheim (Ruhr), Germany.

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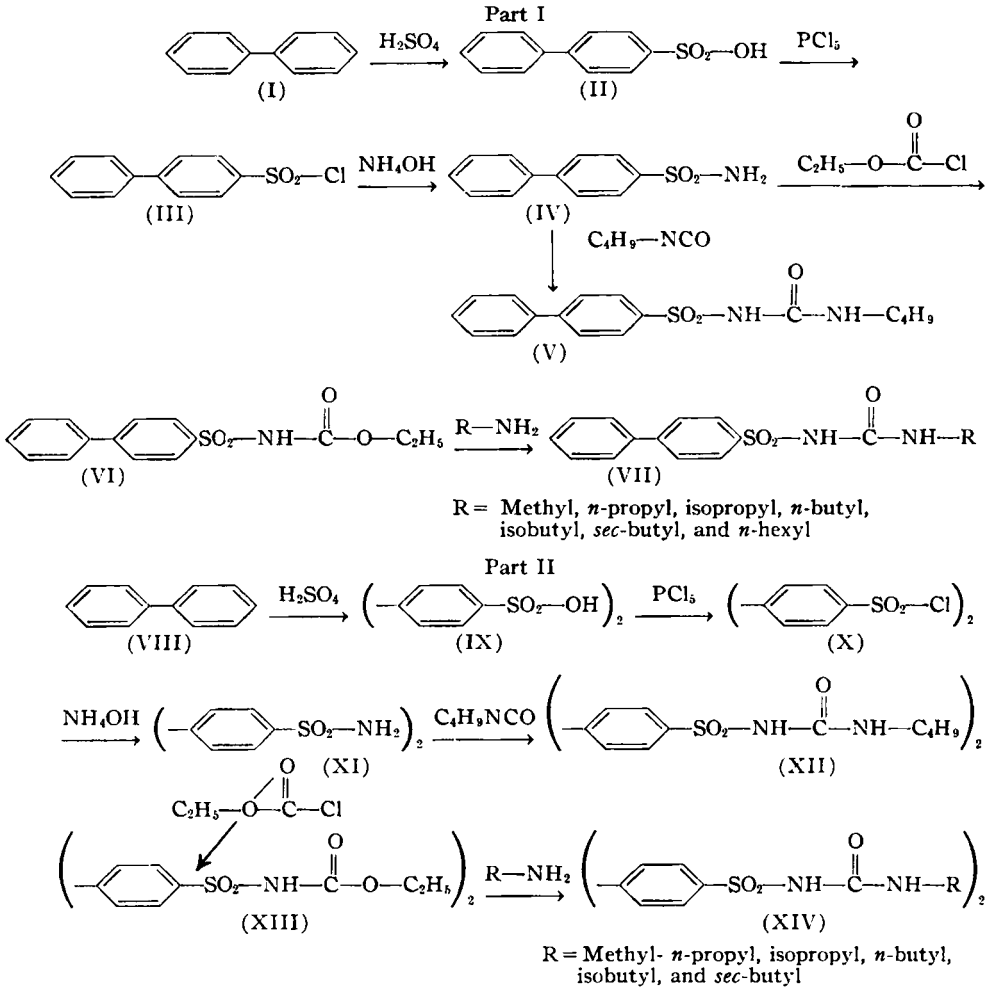
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(0.075 mole) of sodium 4-biphenylsulfonate was heated at 100° for 1 hour in an oil bath with 16 Gm. (0.075 mole) of phosphorus pentachloride. At the end of the heating period, the semisolid mass was poured into a beaker and allowed to solidify (13). The melting point of a purified sample agreed with the reported melting point of 115°. The crude sulfonyl chloride was washed with several small portions of cold water to remove any of the starting materials and by-products before using it to prepare the sulfonamide.

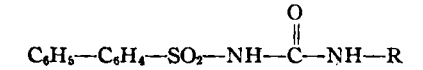
4,4′-Biphenyldisulfonyl Chloride.—In a 1-L. flask fitted with a condenser were placed 122 Gm. (0.32 mole) of potassium 4,4′-biphenyldisulfonate and 175 Gm. (0.85 mole) of phosphorus pentachloride. The flask was shaken thoroughly to mix the powders and heated on an oil bath at 100° for 20 hours. The reaction mixture was poured into ice water and filtered. The residue was filtered and washed with three portions of cold water to remove any starting material and by-products. For identification purposes, one batch was recrystallized from glacial acetic acid and was melted at the reported melting point of 205–206°. All other batches were reacted in the crude state.

Biphenylsulfonamides.—Both sulfonamides used in this work were prepared simply by boiling the crude sulfonyl chlorides with an excess of concentrated ammonia water for 1 hour. The only precaution taken was to add the ammonia water to the sulfonyl chloride slowly enough to prevent a rapid reaction and resulting effervescence.

Sulfonylureas.—*Method A* (*10*).—In a 100-ml. flask fitted with a condenser were placed the sulfonamide and at least a twofold excess of *n*-butylisocyanate dissolved in 10 ml. of triethylamine. The mixture was heated on an oil bath at 100° for 20 hours. The resin which formed was dissolved in 4% sodium hydroxide and treated with charcoal. The clear colorless solution was acidified by pouring it into a cooled solution of acetic acid. The product was filtered and recrystallized from 70% aqueous alcohol. Data for these compounds are listed in Tables I and II.

Method B (*11*).—The first step in this method involved the synthesis of a sulfonylurethan. Fourteen grams (0.06 mole) of 4-biphenylsulfonamide, 200 ml. of anhydrous acetone, and 15 Gm. of anhydrous potassium carbonate were placed in a three-necked flask. The suspension was heated to boiling and 10 Gm. (0.1 mole) of ethyl chlorocarbonate was added dropwise over a period of 1 hour.

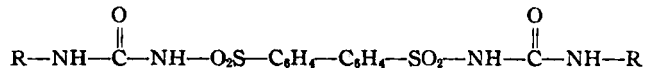
TABLE I.—ALKYL-*N*-*p*-PHENYLBENZENESULFONYLUREAS



R	Molecular Formula	Method of Synthesis[a]	Yield, %	M. p.[b]	Sulfur Analysis—Calcd.	Sulfur Analysis—Found
Methyl	$C_{14}H_{14}N_2O_3S$	B	48	240–241	11.05	10.96
n-Propyl	$C_{16}H_{18}N_2O_3S$	B	60	186.5 to 188	9.82	9.78
Isopropyl	$C_{16}H_{18}N_2O_3S$	B	52	183–184	9.82	9.99
n-Butyl	$C_{17}H_{20}N_2O_3S$	A	28	177–178	9.42	9.35
Isobutyl	$C_{17}H_{20}N_2O_3S$	B	63	211–212	9.42	9.14
sec-Butyl	$C_{17}H_{20}N_2O_3S$	B	45	180–181	9.42	9.55
n-Hexyl	$C_{19}H_{24}N_2O_3S$	B	53	151 to 152.5	7.77	7.92

[a] The methods refer to *Methods A* and *B* discussed in the *Experimental* section; the yield is based on the final reaction. [b] The melting points were determined by the open capillary method and are uncorrected.

TABLE II.—*N*,*N*′-BIS(*N*-ALKYLCARBAMYL)4,4′-BIPHENYLDISULFONAMIDES



R	Molecular Formula	Method of Synthesis	Yield, %	M. p.[b]	Sulfur Analysis—Calcd.	Sulfur Analysis—Found
Methyl	$C_{16}H_{18}N_4O_6S_2$	B	67	220	15.03	15.15
n-Propyl	$C_{20}H_{26}N_4O_6S_2$	B	70	215	13.29	13.31
Isopropyl	$C_{20}H_{26}N_4O_6S_2$	B	83	227	13.29	13.15
n-Butyl	$C_{22}H_{30}N_4O_6S_2$	A[a]	80	237	12.36	12.69
Isobutyl	$C_{22}H_{30}N_4O\,S_2$	B	63	228	12.36	12.06
sec-Butyl	$C_{22}H_{30}N_4O_6S_2$	B	81	218	12.36	12.65

[a] *Method B* resulted in 32% yield. [b] The melting points of the compounds in Table II were determined on a Kofler melting point apparatus. All compounds decomposed upon melting. The decompqsition was evidenced by the evolution of a gas and the formation of 4,4′-diphenyldisulfonamide which melted at 305°.

The mixture was stirred at reflux for 12 hours. When the reaction was complete, any insoluble material was filtered off and the solution carefully acidified with diluted hydrochloric acid. The precipitate was dissolved in sodium carbonate solution and any insoluble material removed. The solution was acidified and extracted with several small portions of ether. The ethereal extract was dried over exsiccated sodium sulfate; then the ether was removed. The product, *N*-*p*-phenylbenzenesulfonylurethan (VI) was recrystallized from 80% aqueous ethanol yielding 11.5 Gm. (47%) of an analytical sample melting at 81 to 82.5°.

Anal.—Calcd. for $C_{15}H_{15}NO_4S$: S, 10.50. Found: S, 10.59.

N,*N*′-Bicarbethoxy-4,4′-biphenyldisulfonamide (XIII) was prepared in the same manner with the following modification. Since the product was not very ether soluble, it was recrystallized after acidification of the sodium bicarbonate solution. Starting with 5 Gm. (0.016 mole) of 4,4′-biphenyldisulfonamide, the yield of final product was 6 Gm. (82%) melting at 180–182°.

Anal.—Calcd. for $C_{18}H_{20}N_2O_8S_2$: S, 14.03. Found: S, 13.63.

The sulfonylureas were prepared from the corresponding sulfonylurethan in the following manner. The sulfonylurethan obtained above was dissolved in a primary amine warming to 80° if necessary. The excess amine was removed using a water aspirator leaving a gummy amine salt of the sulfonylurethan. This was pyrolyzed at 130° and 5 mm. Hg for 3 to 6 hours depending on the time necessary for the salt to lose its gummy appearance or for the bubbling to stop. After pyrolysis, the product was dissolved in sodium bicarbonate solution and treated with charcoal if deemed necessary. The alkaline solution was acidified with diluted hydrochloric acid and the resulting solid recrystallized from 70% aqueous alcohol. Data for these compounds are listed in Tables I and II.

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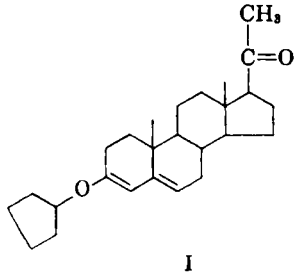
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Quingestrone—Determination of Minute Quantities of Decomposition Products by Paper Chromatography

By JOSEPH M. TALMAGE, MELVIN H. PENNER, and MILTON GELLER

A comprehensive paper chromatographic assay procedure has been developed for quingestrone and its decomposition products. The complete assay procedure consists essentially of three steps: (*a*) separation and identification of the decomposition products, (*b*) quantitative determination of the decomposition products, and (*c*) quantitative assay of the parent compound. As little as 1 per cent of decomposition products can be determined with an accuracy of ±5 per cent. Stability data is presented for quingestrone in crystalline form, in solutions, and in the pharmaceutical dosage form.

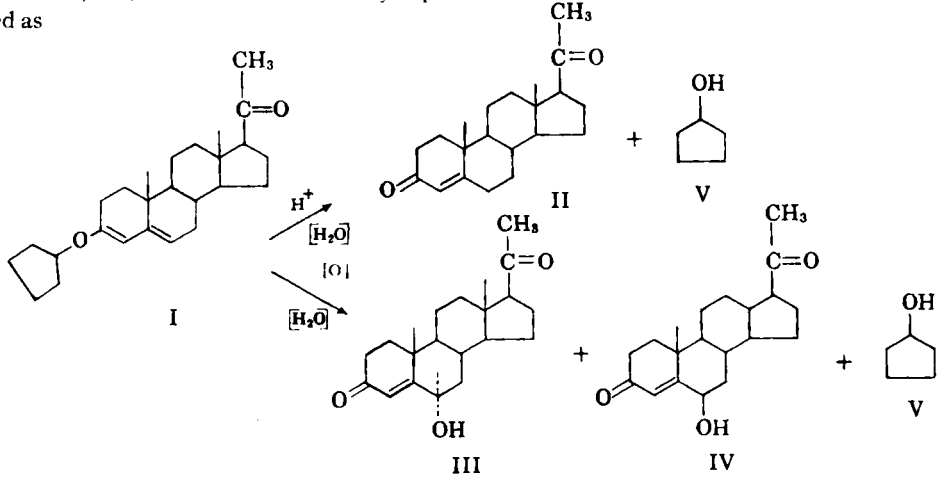
QUINGESTRONE[1] was supplied by Ercoli (1, 2), *et al.* It has the structural formula



I

To investigate the stability of quingestrone (I), a rapid, accurate analytical method was required which would differentiate quingestrone (I) from its decomposition products. Ercoli (3) reported the identification of progesterone (II), 6-α-hydroxyprogesterone (III), and 6-β-hydroxyprogesterone (IV) in addition to cyclopentanol (V) as the most likely decomposition products.

The apparent mechanism for the hydrolytic conversion of I in the presence of acids and/or oxygen to II, III, and IV is schematically represented as



These decomposition products could be identified using the E4 paper chromatographic system of Eberlein and Bongiovanni (4). However, this system was too time consuming and cumbersome for routine analytical work.

Several functional group procedures were investigated in an attempt to differentiate the 3-carbonyl group characteristic of each of the decomposition products from the 3-cyclopentyl enol ether of the parent compound. Reaction with 2,4-dinitrophenylhydrazine (5), or isonicotinic acid hydrazide (6) proved to be unsuitable when the reaction conditions gave quantitative conversion to progesterone. Reaction with 2,6-di-*tert*-butyl-*p*-cresol (7) was found to be unsuitable because of decomposition of quingestrone under the reaction conditions. Attention was then focussed upon developing procedures involving mild conditions to avoid *in situ* hydrolysis of quingestrone.

Ultraviolet spectroscopy as an analytical tool was unsuitable because of the proximity of absorption maxima and absorptivity of the decomposition products to that of quingestrone. Infrared evidence for the presence of nonetherified progesterone can be confirmed by the presence of the characteristic Δ^4-3-ketone band at 1680

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[1] The cyclopentyl enol ether of progesterone. Marketed as Enol Luteovis by Vister Laboratories, Milan, Italy.

TABLE I.—DETERMINATION OF THE ABSORPTIVITY OF THE PRINCIPLE DECOMPOSITION PRODUCTS

Progesterone Standard			6-β-Hydroxyprogesterone		
mg./10 ml.	A at 415 mμ	a	mg./10 ml.	A at 415 mμ	a
0.025	0.050	20.0	0.0235	0.026	11.1
0.050	0.106	21.2	0.0470	0.051	10.8
0.100	0.209	20.9	0.0940	0.109	11.5
0.200	0.428	21.4	0.1880	0.211	11.3
	Mean	20.9 ± 0.4		Mean	11.2 ± 0.2

cm.$^{-1}$ Quantitation based uponthis band was unsuccessful when applied to the dosage form containing quingestrone in sesame oil because of interference from the latter and poor resolution of the 1680 cm.$^{-1}$ band.

Several investigators (8–12) have reported the use of Zaffaroni type paper chromatographic solvent systems for the quantitative analysis of steroids in pharmaceutical dosage forms. Two previously reported (13) Zaffaroni systems herein described provided the nucleus for the development of a rapid, reproducible, precise, and sensitive tool for the stability investigation of quingestrone. The method is capable of separating and quantitatively measuring as little as 1% of the known decomposition products in the parent compound.

EXPERIMENTAL

Preparation of Standards

Solutions of progesterone, 6-α-hydroxyprogesterone, and 6-β-hydroxyprogesterone are prepared in absolute methanol at a concentration of 2 mg./ml. of solution. A standard solution of quingestrone is prepared in reagent grade heptane at a concentration of 2 mg./ml.

Preparation of Samples

Samples under investigation contained quingestrone in sesame oil, packaged in sealed ampuls and soft gelatin, one-piece capsules at concentrations of 50, 40, and 25 mg./ml. Aliquots of these oil solutions are diluted to a quingestrone concentration of 20 mg./ml. with reagent grade heptane just prior to application to the paper. For stability studies in various solvents, solutions of quingestrone are prepared at a concentration of 20 mg./ml.

Isoniazid Eluting Solvent

Dissolve 1 Gm. of U.S.P. isonicotinic acid hydrazide in 1 L. of absolute methanol containing 1.25 ml. of concentrated hydrochloric acid.

Method A

This is used for the separation and identification of decomposition products.

Filter Paper.—Whatman No. 1 is cut into 6 × 18-in. strips. A horizontal pencil line is drawn 3 in. from one end and five equidistant points are marked off. The paper is impregnated by dipping into a solution of 35–65 propylene glycol-methanol and blotted between paper towels.

Developing Solvent.—Toluene saturated with propylene glycol.

Chromatographic Chamber.—Any conventional chamber used for descending paper chromatography.

Paper Chromatographic Procedure.—To the five designated points, 0.010 ml. of each standard solution and 0.025 ml. of the sample solution is spotted using micro volumetric transfer pipets. The paper is then placed in the chromatographic chamber, the spots resting on the antisiphon rod, and the tab end placed into the trough which contains the developing solvent. When the solvent front is within 1 in. of the bottom of the paper (about 3 hours), the strips are removed from the chamber and air dried for 30 minutes.

The positions of the steroids on the chromatogram are located by examination through a fluorescent screen over a short wavelength ultraviolet light source (14). The presence or absence of decomposition products is confirmed by comparing the sample spots with the standard spots. 6-α-Hydroxyprogesterone, 6-β-hydroxyprogesterone, and progesterone can be seen as separate, dark spots having R_f values of 0.27, 0.38, and 0.80, respectively, while quingestrone is at the solvent front.

Method B

This is used for the quantitative determination of the decomposition products.

Filter Paper.—Whatman No. 3 MM is cut into 3 × 18-in. strips and a horizontal pencil line is drawn 3 in. from one end. The paper is impregnated by dipping into ethylene glycol monomethyl ether and blotted between paper towels.

Developing Solvent.—Heptane saturated with ethylene glycol monomethyl ether.

Paper Chromatographic Procedure.—To each of two paper strips, 0.250 ml. of sample solution is applied by streaking across the penciled line from a micro volumetric transfer pipet, leaving a 1/4-in. margin on either side. A third strip serves as a blank. The papers are then placed in the chromatographic chamber, the streaked sample located just below the antisiphon rod, and the tab end placed

TABLE II.—DETERMINATION OF PER CENT RECOVERY

mg. Applied	mg. I in Sesame Oil Applied	mg. Found	Theory, %
0.025 (II)	0	0.023 (II)	92
0.050 (II)	0	0.047 (II)	94
0.100 (II)	0	0.100 (II)	100
0.025 (II)	5.0	0.024 (II)	96
0.050 (II)	5.0	0.048 (II)	96
0.100 (II)	5.0	0.095 (II)	95
0.250 (II)	5.0	0.247 (II)	98.8
0.0235 (IV)	0	0.0268 (IV)	114
0.047 (IV)	0	0.0474 (IV)	101
0.094 (IV)	0	0.097 (IV)	103
0.186 (IV)	0	0.180 (IV)	97
0.0235 (IV)	5.0	0.025 (IV)	106
0.047 (IV)	5.0	0.050 (IV)	106
0.094 (IV)	5.0	0.100 (IV)	106
0.186 (IV)	5.0	0.178 (IV)	96

TABLE III.—STABILITY OF CRYSTALLINE QUINGESTERONE

Storage Conditions	—1 Gm. of Sample Contains— Quingesterone, mg.	Progesterone, mg.	6-β-Hydroxyprogesterone, mg.
4°C. under nitrogen	995	3	2
4°C. under air	930	24	50
25°C. under air	40	140	653

into the trough which contains the developing solvent. When the solvent front is within 1 in. of the bottom of the paper (about 3 hours), the strips are removed from the chamber and air dried for 30 minutes.

The positions of the decomposition products on the chromatograph are located by observing in a darkened room over an ultraviolet light source. The progesterone and 6-β-hydroxyprogesterone can be seen as darkened zones having R_f's of 0.30 and 0.10, respectively, while the quingestrone and sesame oil can be seen as a darkened zone from R_f 0.80 to the solvent front.

The decomposition product zones are cut out, cut into $^1/_2 \times {}^1/_2$-in. pieces and placed in a glass-stoppered 125-ml. Erlenmeyer flask. Appropriate zones of the blank chromatogram are treated as the corresponding sample zones. Ten milliliters of the isoniazid eluting solvent are pipeted into each flask, stoppered, and shaken mechanically for 1 hour.

The solution is then drawn into a pipet through cotton wrapped around the tip, to filter out paper fibers, and transferred to a 1-cm. corex cell. The absorption of the eluted steroid is determined at 415 mμ using the blank solution in the reference cell on a Beckman DU spectrophotometer.

In a similar manner, a series of standards, both with and without quingestrone in sesame oil, were analyzed to establish the accuracy and precision of the method.

Method C

This is used for the quantitative determination of quingestrone.

Filter Paper.—Same as *Method B*, except the paper is impregnated by dipping the paper into ethylene glycol monomethyl ether containing 0.1% α-tocopherol. The α-tocopherol is needed to prevent decomposition of the quingestrone during the drying of the chromatogram.

Developing Solvent.—This is the same as *Method B*.

Paper Chromatographic Procedure.—To each of two paper strips, 0.100 ml. of a $^1/_{10}$ heptane dilution of the sample solution is streaked. To a third strip, 0.100 ml. of a standard quingestrone solution is streaked, while a fourth strip serves as a blank. The chromatographs are developed, dried, the quingestrone detected, cut out, eluted, and measured as the decomposition products in *Method B*.

RESULTS AND DISCUSSION

In all the samples examined, only trace amounts of 6-α-hydroxyprogesterone can be detected, while in some samples substantial amounts of 6 β-hydroxyprogesterone and progesterone are found. The separation of progesterone and quingestrone in the sesame oil was not always satisfactory in the toluene–propylene glycol solvent system of *Method A* because the sesame oil distributed itself between the two zones. For this reason, quantitative analysis was done by *Methods B* and *C*.

TABLE IV.—STABILITY OF QUINGESTRONE IN SOLVENTS

Solvent	Conditions	Dec., % Progesterone	Dec., % 6-β-Hydroxyprogesterone
Chloroform	$^1/_2$ hr., RT	0.5	...
Chloroform	7 hr., RT	50	3
Chloroform	2 wks., RT	55	18
Methanol	7 hr., RT	trace	...
Methanol	2 wks., RT	24	49
Heptane	2 wks., RT	...	15
Sesame oil	4 mo., RT	2.2	5.7
Sesame oil	3 wks., 45°C.	15	36

Since the $\Delta^{5,6}$ analog of progesterone is a possible hydrolytic cleavage product which would not be detected by the indicated ultraviolet method, several chromatograms of decomposed samples were stained with 2,4-dinitrophenylhydrazine with the result that no non-ultraviolet absorbing 3-ketones were detected. This indicated that this compound is not among the decomposition products.

The precision and adherence to the Beer-Lambert law was confirmed by reacting varying amounts of progesterone and 6-β-hydroxyprogesterone with 10 ml. of the isoniazid solution. The absorbance of the resulting chromaphore was then determined at 415 mμ on a Beckman DU spectrophotometer. The results of this study are tabulated in Table I.

The described method is capable of detecting and quantitating 0.025 mg. of progesterone and 6-β-hydroxyprogesterone in the presence of 5 mg. of the parent compound. The precision and accuracy were established by the standard addition method. The standard quingestrone sample used in this study showed no evidence of decomposition.

The amount of progesterone or 6-β-hydroxyprogesterone present was calculated by the simple formula: mg. present $= (A/a) \times 10^{-3}$, where A = absorbance at λ 415 mμ found. The results of this investigation are summarized in Table II.

The accuracy of determining the indicated levels of decomposition products can be calculated to be ±5% from data in Table II.

Crystalline quingestrone is stable only if stored under an inert atmosphere such as nitrogen and preferably at low temperatures. If allowed to remain at room temperature under atmospheric conditions, decomposition can be detected after only 3 days, the major decomposition product being 6-β-hydroxyprogesterone together with some progesterone.

TABLE V.—STABILITY OF QUINGESTRONE IN THE PHARMACEUTICAL DOSAGE FORM

Condition	Quingestrone Analysis	Dec., % Progesterone	Dec., % 6-β-Hydroxyprogesterone
2 mo., RT	99.1	trace	0.9
6 mo., RT	100.0	...	0.1
2 yrs. RT and 4°C.	98.7	...	1.0
6 mo., 37°C.	101.0	...	1.5
1 mo., 45°C.	98.5	trace	1.0
4.5 mo., 45°C.	95.5	4.5	1.3
67 hr., 105°C.	98.5	0.3	0.9

If stored in the refrigerator under nitrogen and opened only occasionally to remove samples and subsequently blanketed with nitrogen, no decomposition is observed after several months.

Typical analyses of solid crystalline quingestrone after 5 months' storage are tabulated in Table III.

In solution, quingestrone is stable in the absence of acids, bases, and oxidizing agents, including atmospheric oxygen. It is less stable in chloroform and methanol than in heptane and exhibits best stability in a good grade vegetable oil such as sesame oil. In Table IV, stability data in solvents stored under the indicated conditions are presented. No precaution was taken to exclude air in these studies.

The pharmaceutical dosage form consists of quingestrone dissolved in sesame oil at concentrations of 50, 40, and 25 mg./ml. contained in sealed glass ampuls and/or in sealed, soft gelatin capsules. It is of the utmost importance that the peroxide content of the sesame oil be kept minimal, *i.e.*, less than 50 mcq per ml. as determined by the method of the American Oil Chemists Society (15). As a further precaution, an antioxidant, namely α-tocopherol is added at a concentration of 0.1% of the weight of sesame oil used.

In Table V, stability data are presented for this final dosage form.

SUMMARY

A quantitative paper chromatographic method has been developed which not only permits the determination of quingestrone, but also simultaneously provides a precise method for assaying as little as 1% of the known decomposition products, progesterone and 6-β-hydroxyprogesterone.

Data have been presented to indicate that a solution of the cyclopentyl enol ether of progesterone (quingestrone) in sesame oil of low peroxide content with addition of α-tocopherol and exclusion of atmospheric oxygen represents the best means for preserving the product in a pharmaceutical dosage form.

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Effect of Deuterium Oxide on the Growth of Peppermint (*Mentha piperita* L.) I

Morphological Study

By M. I. BLAKE, F. A. CRANE, R. A. UPHAUS, and J. J. KATZ

The effect of varying concentrations of D_2O on the growth of peppermint plants was studied over a period of at least 50 days. The extent of deuterium uptake is reported. This appears to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant.

THE EFFECTS of deuterium on living organisms have attracted the attention of many investigators since this heavy, stable isotope of hydrogen was first discovered in 1933. Early work has been reviewed by Morowitz and Brown (1). Current developments are described by Katz (2) and are the subject of a recent monograph (3). Interest in this general area has been stimulated by the discovery that a considerable variety of organisms can be grown in 99.8% D_2O (4, 5). Since it has proved possible to replace essentially all of the hydrogen in a variety of algae, bacteria, and molds, it becomes of interest to examine the behavior of higher plants in response to isotopic substitution. The effects of deuterium on mice (6), rats (7), and dogs (8) have been reported, but the present paper presents what we believe to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant.

Since deuterium oxide is now available in adequate quantities and at a moderate cost, it is possible to study the effect of high concentrations of this solvent on the growth of higher plants over an extended period of time. Peppermint plants are ideal for such an investigation since they can

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be conveniently grown from rooted cuttings in an aqueous nutrient solution and require no special attention (9).

The present report describes the effect of varying concentrations of deuterium oxide on the growth of mint plants for a period of at least 50 days, at which time the control plants initiated inflorescences. The extent of deuterium uptake by the plant, as determined by direct analysis, is also reported.

EXPERIMENTAL

Growth Studies.—Cuttings from fully grown mint plants (*Mentha piperita*) were rooted in moist vermiculite. A root system had developed in about 2 weeks. The stem length ranged from 3 to 6 cm. At this time the plants usually bore four to five pairs of small leaves. No sign of lateral shoots was evident. The plants weighed between 1 and 2 Gm. Erlenmeyer (250 ml.) flasks fitted with cork stoppers served as convenient culture flasks. The plant cutting was inserted into a slit cut from the center to the edge through the length of the stopper and narrow enough to hold the cutting erect, the stem extended above the cork and the root system was directed into the flask. The surface of the cork was covered with masking tape to ensure an air tight system and to minimize isotopic exchange with the water vapor in the air.

A concentrated nutrient stock solution was prepared which required a fourfold dilution. This facilitated the preparation of solutions containing

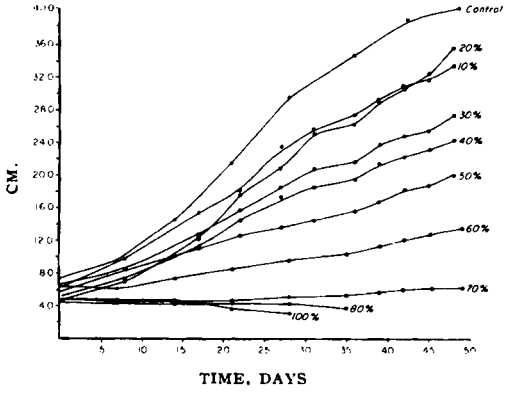


Fig. 1.—Effect of D_2O concentration on growth of stem in peppermint plants.

different concentrations of deuterium oxide. The final nutrient solution had the following composition (Gm./L.): $Ca(NO_3)_2 \cdot 4H_2O$, 1.18; KNO_3, 0.30; KH_2PO_4, 0.14; $MgSO_4 \cdot 7H_2O$, 0.49; $NaNO_3$, 0.60; and FeEDTA, 0.005. The following trace elements were present (p.p.m.): boron; 0.50; manganese, 0.50; zinc, 0.05; copper, 0.01; and molybdenum, 0.01. The deuterium oxide used in this study had an isotopic purity of 99.6% and was distilled from alkaline permanganate in a Barnstead conductivity water apparatus.

Nutrient solution for these studies was prepared by transferring 62.5 ml. of concentrate (1:4) from a buret to a 250-ml. volumetric flask. The volume of deuterium oxide calculated to give the desired D_2O concentration was added from a second buret to the volumetric flask. The flask was then filled to the mark with distilled water. For those experiments in which the deuterium oxide concentration was 80% or above, 4:1 nutrient concentrate was prepared in deuterium oxide. Sufficient water and deuterium oxide were added to give the desired final deuterium oxide concentration. The final nutrient solution was transferred to the culture flask and the cork stopper with plant attached was placed in position. The entire flask was covered tightly with black plastic sheeting to prevent growth of algae in the solution. A 1-ml. sample of nutrient solution was removed periodically, prior to the addition of fresh nutrient. This was done each 7 days in the early stages of plant growth and every 3 or 4 days as the plant elongated more rapidly. The sample was analyzed for deuterium oxide by the method described earlier (10). This served to detect whether the deuterium oxide content of the nutrient solution was being reduced by absorption of H_2O from the atmosphere, or whether isotopic exchange had occurred by other means.

A series of culture flasks was prepared which contained 10, 20, 30, 40, 50, 60, 70, 80, and 100% deuterium oxide in the nutrient solution. Controls were run concurrently. When a sample of solution was removed for deuterium analysis and fresh nutrient was added, the following measurements were made on all plants: stem length from top of cork stopper, lateral shoot length from center of stem, root length, width of root system, and volume of fresh nutrient added. The experiment was terminated after 50 days because the controls went into the flowering stage and normal elongation terminated. The effect of varying degrees of deuteriation on the stem length of the mint plants is shown in Fig. 1. Figures 2 and 3 show photographs of the mint plants prior to termination of the experiment.

Harvesting of Plants.—The plants grown in nutrient solutions ranging in D_2O concentration from 0 to 100% were harvested after 50 days. Those plants in 70% D_2O nutrient were grown as long as 130 days before being harvested. Each plant was divided into leaves, stems, and roots; each portion was weighed. Adequate samples of each plant part were placed in glass vials which were sealed tightly and placed in the deep freeze until analyzed for deuterium content. Samples of the plant parts were killed in Randolph's modified Navashin fluid (11). These were reserved for a histological study to be reported later. The remaining portions of



Fig. 2.—Peppermint plants grown in liquid nutrient solutions containing 0 to 50% D_2O. Plant C is the control.

each plant were combined and steam distilled in a Clevenger distillation apparatus. The volatile oil fraction of each plant was collected and set aside for future study.

Deuterium Analysis.—The deuterium content of the nutrient solution, as previously mentioned, was determined throughout the course of the experiment by analyzing a sample of the medium prior to the addition of fresh nutrient. The harvested plants were analyzed for labile and fixed deuterium. In this context, labile deuterium refers to deuterium present as interstitial and cellular water. Fixed deuterium includes deuterium present in both exchangeable and nonexchangeable portions in the organic components of the plant. A vial, containing leaves, stems, and roots of an experimental plant and which was stored in the deep freeze, was transferred immediately to a distilling tube after removing the cap. Care was taken to avoid exchange of labile and exchangeable deuterium atoms with atmospheric H_2O. The distillation tube was attached to a vacuum line and the labile water was removed from the plant under reduced pressure, condensed in a $-78°$ trap, and transferred to an ampul which was then sealed off. The sample was then analyzed for D_2O content. The dried plant residue was removed from the distillation tube and divided into leaves, stems, and roots. Equal weights of each plant part were placed in a combustion boat. The boat was placed in a combustion apparatus and the sample was burned in oxygen at 700°. The water of combustion was collected in a $-78°$ trap and transferred to an ampul which was then sealed off. The sample was analyzed for deuterium oxide

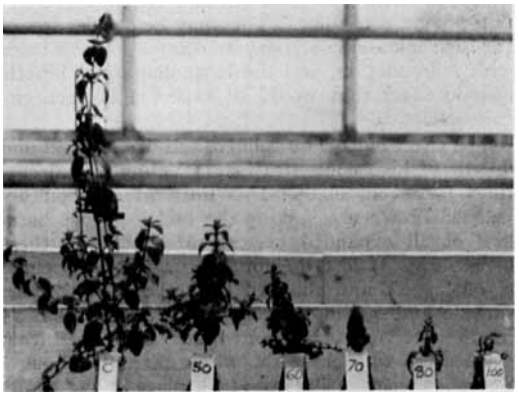


Fig. 3.—Peppermint plants grown in liquid nutrient solutions containing 50 to 100% D_2O.

content. This represents the deuterium in the dry tissues. The data for these analyses are shown in Fig. 4 where the concentration of deuterium in the water and the organic components of the plant are plotted against nutrient D_2O content.

DISCUSSION

The black Mitcham strain of peppermint grown in full nutrient solution by the water culture technique usually develops from a rooted cutting of about 8-cm. length with four pairs of expanded leaves and a weight of about 1.5 Gm., to a mature plant in about 50 days. The mature primary shoot grown on long day photoperiod (14 hours of daylight or longer) generally reaches a height of 35 to 50 cm.; it bears six to eight pairs of long lateral shoots, the lowest of which normally reaches a length approaching that of the primary axis. The leaves, well expanded on the erect stems, elongate to approximately 5 by 8 cm. and are normally bright green.

Elongation of both primary and lateral stems ceases when their apical meristems are stimulated to floral initiation and an inflorescence develops. After flowering occurs, development of secondary lateral shoots takes place at the base of the primary stem (normal ground level of plants grown in soil). These secondary lateral shoots from the same nodes where an erect flowering shoot has arisen are stolons that grow horizontally or pendulous; their leaves remain small and scale-like.

Plants grown on short days (10 hours or less of light photoperiod) do not initiate flowers, are lighter green than long-day plants, and produce many more stolons. When plants are grown for 50 or more days on short photoperiod all of the erect stem apices become stolon-like and even the main apex hangs down and appears as a stolon.

All of the plants described in this work were grown on long-day photoperiod. The natural summer day was extended to 16 hours by a bank of fluorescent and incandescent lamps suspended on an open frame above the plants so that they did not shade during the day, and which provided about 600 f.-c. on the upper parts of the plants during the time the lamps were turned on.

Control plants followed the characteristic growth pattern for peppermint. The major deviation in this experiment from earlier work in this laboratory was that a single plant was grown in a 250-ml. flask, whereas previously several plants (four to eight) were grown in a larger volume of nutrient solution (4 to 12 L.). When plants have 30 or more pairs of leaves they transpire large volumes of water. The nutrient solution had to be replenished or replaced in the 250-ml. Erlenmeyer flasks every 48 to 72 hours in the later phase of the growth cycle. Aside from this problem the peppermint plants grew well in this type of culture flask.

The effect of varying concentrations of deuterium oxide on the elongation of plant is evident in Fig. 1. With the exception of the 20% plant, increasing concentrations of D_2O in the nutrient solution caused a nearly proportionate reduction of elongation growth of the axial shoot. This pattern of elongation was reflected in the absorption of nutrient solution by the roots. The volumes necessary to replenish or replace that taken up for growth or transpiration by the plants were progressively less for each D_2O level. The regular progession of growth shown in Fig. 1 implies limitation of a major factor in the

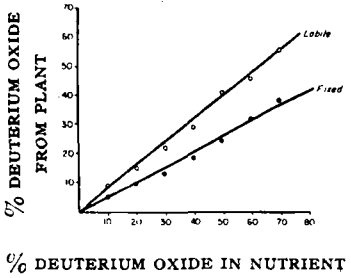


Fig. 4.—Deuterium content of labile water and water of combustion of peppermint plants. Labile, water obtained by distillation; fixed, water obtained by combustion of dried plants.

ability of the plant's primary shoot to elongate under conditions in which rapid elongation did occur in control plants furnished aqueous nutrient solution.

Elongation of the lateral shoots was affected by deuterium in two ways. At lower D_2O concentrations (10 to 40%), the lateral stems grew more nearly like short-day lateral stems. The lateral stems hung recumbent and were elongated more than the corresponding erect lateral branches of the controls. Such stolon-like branches occurred on the controls only as secondary laterals in small numbers after the erect shoots developed inflorescences at their apices. At higher concentrations of D_2O, the pattern of elongation of the lateral shoots was essentially the same as that of the primary shoots. There was a greater reduction in shoot length and greater retardation of leaf development with increase in D_2O concentration. Lateral shoots of the 70% plants were so short that after 50 days of growth they were still difficult to measure. They were overlapped by primary leaves of the main shoot. Both of these growth responses are probably due to the inability of the plants to synthesize adequately their structural, storage, and hormonal materials required for normal growth. The inability of the shoot apices to orient themselves in the erect manner and to stimulate their leaves to elongate to the size of vegetative photosynthesizing leaves reflects a decrease in normal stem orienting hormone. Such lateral shoots, hanging unoriented, elongated to the degree of which they were capable under the constraint of the D_2O concentration on their synthesizing system.

Reduction in elongation of leaf blades and petioles followed the same general pattern as that of the stems. At low D_2O concentrations leaves of the primary shoot elongated nearly as much as the corresponding leaves of the controls. They were held in the normal erect position, were the normal green color, and apparently functioned in a near normal way photosynthetically. At concentrations of 50% D_2O or higher (and in the later phases at lower concentrations), leaves appeared epinastic and recurved downward, clasping the stem or lower leaves, thus making a compact mass that was quite brittle or fragile (Fig. 3, 60 and 70%). The leaves of the plant in 80% D_2O nutrient did not elongate more than a few millimeters, remained epinastic throughout their existence, and eventually became necrotic, brown, and dry.



Fig. 5.—Rooted peppermint cutting after 24 hours in 99.6% D_2O nutrient solution. Brown necrotic areas are outlined in black.

Peppermint commonly develops red or purple coloration under deficiencies of nitrogen, phosphorus, or sulfur. This coloration is due to anthocyanins, water-soluble pigments, which accumulate under conditions in which the normal products of photosynthesis do not continue to build structural or metabolic carbohydrates and proteins. It appears that deuterium oxide interferes with the biochemistry of the plant and exerts its effect on the normal anabolic reactions of the leaf mesophyll by inhibiting the rate of the photosynthetic reactions and ensuing growth. The results are morphological symptoms resembling those of a plant growing under a deficiency of the elements needed for normal growth. Thus, by decreasing the rates in a chain of reactions, the D_2O causes the leaf cells to appear incapable of making normal use of the nitrogen, phosphate, sulfate, and other nutrients that are available to them, even in abundance.

The root system reflected to a lesser degree the same pattern of response to D_2O. At low concentrations the root system was broad, well branched, and nearly as large as that of the controls. Individual roots were turgid, pearl-white, and not noticeably reduced in length. At higher D_2O concentrations the entire amber to brown root system was reduced in extent and appeared shriveled. The ability to absorb water, even from an abundant supply as a liquid nutrient solution, was reduced by D_2O. The total volumes of liquid taken up at higher D_2O concentrations were progressively smaller.

The ability of plants growing on any concentration of D_2O to initiate inflorescence appears to be impaired. Plants were maintained in nutrient solution until the control plants had either initiated inflorescences or had produced full-blown flowers, but in no case were there floral primordia at the apex of any plant grown in D_2O. This seems to parallel the results in animals reported by Hughes and Calvin (12) and Czajka and Finkel (13), whereby substitution of D_2O for a part of the drinking water produced sterility or otherwise affected the reproduction potential in mice. This may be a reflection of the high metabolic activity associated in both cases with reproduction, and the large deuterium kinetic isotope effect that would be expected in such circumstances.

The effect of D_2O on cellular activity is rapid and drastic at high concentrations. Young rooted cuttings placed in 99.6% D_2O nutrient solution developed brown areas along the midrib of the basal half of all expanded leaves. Mesophyll in these areas (Fig. 5) lost its normal turgidity, appeared water-logged, and changed from the normal green to pale amber and then to dark brown within 24 hours. Necrosis of adjacent tissues progressed from these areas to include the whole plant in about 1 week. No elongation ever took place in the plants furnished 99.6% D_2O. Effects on plants furnished 80% D_2O nutrient were similar, but the cycle was accomplished in two to three times the interval of time.

Several plants were maintained at a 70% D_2O level up to 130 days. In these plants the progression of epinastry, necrosis, coloration, and death occurred, as previously described, only after axillary shoots elongated and appeared to grow in a near-normal fashion, but which eventually recapitulated the progression. Then other shoots would continue the sequence and eventually die. This occurred until the apex of the plant became a dense mass of dead primary and lateral shoots. The shoot apex, it would appear, made a valiant effort to achieve the best possible growth it could attain under the constraints of the experimental treatment.

Several cuttings were removed from a plant grown in 50% D_2O nutrient for 40 days. One cutting was transplanted into a culture flask containing 50% D_2O nutrient solution, and the other was placed in a flask containing aqueous nutrient and no D_2O. The former cutting showed no sign of developing a root system and succumbed within 3 to 4 weeks. The latter grew a root system within 2 weeks and continued growing in a normal manner similar to a typical control plant. It was hoped that if a root system would develop in the 50% D_2O medium, it would be possible to propagate a deuteriated plant; and possibly with successive cuttings in constantly increasing concentrations of D_2O a plant could be grown in a highly deuteriated medium. Thus far, this has not been successful, but further work is in progress with this aspect of the investigation.

In Fig. 4 comparison is made between the deuterium content of the water distilled from the deep frozen fresh plants and the water of combustion of the dried plants. The distilled or labile water contains deuterium from water loosely bound by the plant tissues and represents essentially completely exchangeable deuterium. The deuterium content of the labile water from the plant is approximately 75% that of the nutrient solution in which the plant was grown. The water obtained from combustion of the dried plants, representing organically bound exchangeable and nonexchangeable deuterium, contains about 50% of the deuterium content of the nutrient solution. The ratio of organically bound to labile deuterium is about 0.66, somewhat higher than that reported by Katz, *et al.* (6), for certain tissues in mice. This may reflect the nonequilibrium aspects of the situation resulting from the continuous transpiration of water. The active water metabolism and the ensuing fractionation may contribute significantly to the observed isotope effects.

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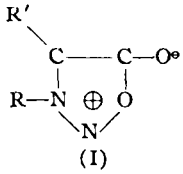
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Synthesis of Several Disubstituted Sydnones

By DEVINDRA DHAWAN and LEMONT B. KIER*

A number of new 3,4-dialkylsydnones have been prepared to compare their pharmacological properties with those of a series of 3-alkylsydnones. Both series were similar in their central convulsive activity. The CD_{50} and partition coefficient values of both series were compared. The CD_{50} values for the 3,4-dialkylsydnones were significantly lower than for the 3-alkylsydnones. In addition, the CD_{50} values for the 3,4-dialkylsydnones were close to one another and were independent of the partition coefficient.

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was a potent central nervous system stimulant with a particularly stimulating effect on respiration. It did not potentiate acetylcholine, nor did it produce acetylcholinesterase inhibition in these studies. It produced a more favorable respiratory response and blood pressure rise than the same dose of pentylenetetrazole when administered intravenously to a pentobarbitalized dog.

With these preliminary findings in hand, a systematic study of a number of sydnones was initiated with the purpose of further elaborating the pharmacological properties and any structure-activity relationships. In the previous communication from this laboratory (2), a number of 3-alkylsydnones were synthesized. The pharmacological response of these compounds was qualitatively similar to that of 3-*sec*-butylsydnone, but one response in particular—the onset of a charac-

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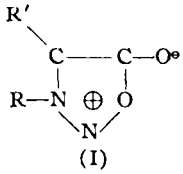
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teristic convulsive pattern—varied appreciably concerning the minimum dose necessary to initiate it. One physical parameter of these compounds was closely correlated with the convulsive dose, that of the oil-water partition coefficient. If the pharmacological action was chiefly central, as preliminary data indicated, then the sydnones with a greater affinity for lipid solvents would be expected to penetrate the lipid barrier into the CNS with greater facility. This was precisely the case for the 3-alkylsydnones. Those with a higher partition coefficient required a lower dose (mmoles/Kg.) to bring on convulsions in 50% of the animals (CD_{50}).

This current study was initiated with several objectives in mind. First, since the previous study dealt only with the 3-alkylsydnones, the effect on activity by substitution at both 3 and 4 positions should be assessed. Second, the study would afford a comparison between the two series with respect to pharmacological activity and physical properties. Finally, a more detailed pharmacological evaluation of representative members of both groups would be conducted and some generalizations made regarding the activity of the ring itself and structural features enhancing this activity.

A group of 3,4-dialkylsydnones was selected in order to maintain a relatively short chain length at both the 3 and 4 position to achieve partition coefficients approximating those of the 3-alkyl series.

Essentially the same method of synthesis was employed in this series as that previously reported (3). Trifluoroacetic anhydride was tried as a ring closing reagent with III in the final step of the synthesis, but this did not improve the yield and, in fact, frequently resulted in a lower yield of sydnone. (See Tables I and II). Generally, ring closure to form dialkylsydnones proceeded with greater difficulty than was encountered with the 3-alkylsydnones. This is quite probably due to the inductive effect of the 4-alkyl group which lowers the acidity of the 4-hydrogen atom in IV. This in turn would result in a higher activation necessary for its removal from the transition state (II) based upon Baker's proposed mechanism (4).

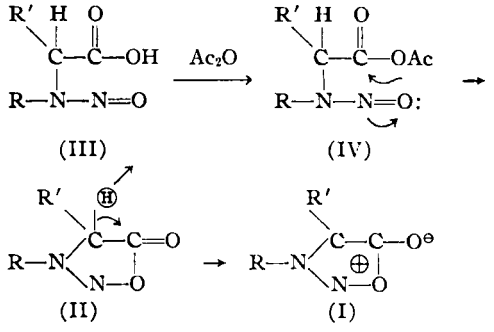
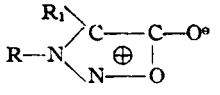


TABLE I.—ALKYLAMINOESTERS

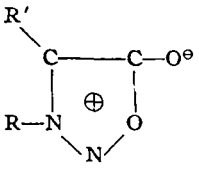
Name	Formula	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N	B.p.	Yield, %
Methyl *N*-cyclohexyl-aminopropionate	$C_{10}H_{19}O_2N$	64.83	10.34	7.56	64.65	10.16	7.56	75°/1mm.	51
Methyl *N*-ethylamino-propionate	$C_6H_{13}O_2N$	54.94	9.99	10.68	55.23	9.79	10.53	61°/5mm.	51
Ethyl *N*-*tert*-butylamino-propionate	$C_9H_{19}O_2N$	62.39	11.05	8.09	61.91	11.24	8.16	100°/20mm.	41
Ethyl *N*-butylamino-propionate	$C_9H_{19}O_2N$	62.39	11.05	8.09	62.41	10.82	7.91	59°/10mm.	47
Ethyl *N*-propylamino-butyrate	$C_9H_{19}O_2N$	62.39	11.05	8.09	62.31	10.86	8.28	60°/5mm.	53
Methyl *N*-butylamino-butyrate	$C_9H_{19}O_2N$	62.39	11.05	8.09	62.41	10.82	7.91	81°/10mm.	47
Ethyl *N*-butylamino-hexanoate	$C_{12}H_{25}O_2N$	66.93	11.70	6.51	67.08	11.52	6.91	84°/3mm.	27

TABLE II.—3,4-DIALKYLSYDNONE



R	R_1	Formula	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N	λC–O I.R.	U.V. max.	U.V. E.	B.p. or m.p.	Yield %
Cyclohexyl	CH_3	$C_9H_{14}O_2N_2$	59.32	7.69	15.38	59.41	7.82	15.16	5.83	294	6652	151°	53
Ethyl	CH_3	$C_6H_8O_2N_2$	46.87	6.29	21.87	47.07	6.82	21.96	5.80	295	5866	133°/0.5mm.	40
tert-Butyl	CH_3	$C_7H_{12}O_2N_2$	53.83	7.74	17.94	53.67	7.91	17.91	5.78	293	5850	153°	50
Butyl	CH_3	$C_7H_{12}O_2N_2$	53.83	7.74	17.94	53.93	7.89	18.03	5.80	295	6441	149°	31
Propyl	C_2H_5	$C_7H_{12}O_2N_2$	53.83	7.74	17.94	53.92	7.71	17.62	5.79	293	6028	128°/5mm.	35
Butyl	C_2H_5	$C_8H_{14}O_2N_2$	56.45	8.29	15.46	56.74	8.40	15.51	5.77	296	6658	130°/4mm.	41
Propyl	CH_3	$C_5H_{10}O_2N_2$	50.69	7.04	19.71	51.19	7.68	19.88	5.80	295	5905	135°/13mm.	48

TABLE III.—SYDNONE PHYSICAL AND PHARMACOLOGICAL DATA



R	R′	PC (*n*-Amyl Alcohol) H_2O	CD_{50} mmole/Kg.	R	R′	PC (*n*-Amyl Alcohol) H_2O	CD_{50} mmole/Kg.
Isopropyl	H	2.3	2.96	Cyclohexyl	CH_3	1.85	0.24
Propyl	H	2.5	1.60	Ethyl	CH_3	2.05	...
tert-Butyl	H	4.9	0.87	Propyl	CH_3	2.8	0.34
sec-Butyl	H	6.4	1.13	Butyl	CH_3	2.9	0.13
tert-Octyl	H	8.0	0.25	Butyl	C_2H_5	4.1	0.19
				tert-Butyl	CH_3	6.7	0.71

The partition coefficients of the 3,4-dialkylsydnones could not be satisfactorily run in chloroform in order to compare these values with those obtained for the 3-alkylsydnones; hence, both series were run in amyl alcohol. The CD_{50} values were obtained for the 3,4-dialkylsydnones as previously described (5). (See Table III).

A comparison of the CD_{50} values for both sydnone series and their partition coefficients revealed some significant differences. Although both series of compounds were similar in their central convulsive activity, the CD_{50} values for the 3,4-dialkylsydnones were significantly lower than the 3-alkylsydnone values. (See Fig. 1). In addition, the CD_{50} values for the 3,4-dialkylsydnones appeared to be very close to one another and were independent of the partition coefficient.

One possible explanation for this difference is that in the case of the 3-alkylsydnones, the absolute lipid solubility is sufficiently low so that the partition coefficient becomes a limiting factor concerning the amount of drug which reaches the CNS. In the case of the 3,4-dialkylsydnones, the absolute lipid solubility is appreciably higher so that in spite of a less favorable partition coefficient in some compounds, there is sufficient solubility in the biolipid phase to permit an active level of sydnone to reach the site of action to elicit a response. In other words, because of a high-absolute solubility in the biolipid phase, the partition coefficient of the 3,4-dialkylsydnones is not a limiting factor to activity.

On the other hand, if the 3,4-dialkylsydnones are not more soluble in the lipid phase, then an alternative explanation is that the intrinsic activity of the 3,4-dialkylsydnones is greater than that for the 3-alkylsydnones. Thus throughout the range of partition coefficient values studied for the 3,4-dialkylsydnones, there is sufficient drug being absorbed to elicit a response. This minimal amount necessary to elicit a response must then be less than the amount which is possible to be absorbed with a given partition coefficient value.

A more detailed pharmacological study of these compounds will be published elsewhere (6). This study revealed that representative members of both groups possessed diuretic activity as well as a chloruretic, naturetic, and glucosuretic effect. In a comparative study, 3-*sec*-butylsydnone had a moderate effect in lowering the blood pressure of a dog and was

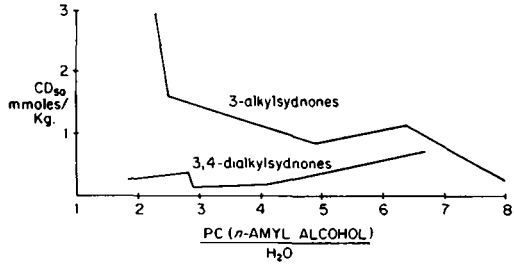


Fig. 1.—Partition coefficients *vs.* minimum convulsive dose of 3-alkyl- and 3,4-dialkylsydnones.

superior to 3-butyl-4-ethylsydnone in this regard. In higher doses, both compounds caused respiratory difficulty, labored breathing, and lung hemorrhages.

EXPERIMENTAL[1]

Alkylaminoester Synthesis.—Since all of these syntheses were essentially the same, the synthesis of methyl *N*-ethylaminopropionate is described as typical. A solution of methyl α-bromopropionate (83.5 Gm., 0.5 mole) was added dropwise to a solution of ethylamine (45.09 Gm., 1.0 mole) dissolved in dry benzene. The mixture was stirred for 1 hour, then refluxed for 2 hours. The ethylamine hydrobromide was filtered out and the benzene removed from the filtrate under vacuum. The residue was distilled, b.p. 60°/5 mm., yield 51%. See Table I for physical constants, yields, and analyses.

Sydnone Synthesis.—The synthesis of 3-ethyl-4-methylsydnone is described as a typical example. Twenty grams of methyl *N*-ethylaminopropionate was hydrolyzed by refluxing with 14 Gm. of sodium hydroxide in 200 ml. of water. The solution was then cooled to −5° and made acid to pH 2. Sixteen grams of sodium nitrite dissolved in 50 ml. of water was added to this reaction mixture. The addition was made slowly to keep the temperature below 0°. This was stirred for 3 hours, then extracted with ether. The residue from the ether gave a positive Liebermann nitroso test. The nitrosoaminoacid was not isolated but was treated directly with 150 ml.

[1] All melting points were done on the Kofler apparatus and are corrected. Analyses were performed by Weiler and Strauss Laboratories, Oxford, England, and by Galbraith Laboratories, Knoxville, Tenn.

of freshly distilled acetic anhydride and allowed to stand in the dark for 2 days. The volatile products were then removed under vacuum, and the last traces of acetic acid were removed by azeotropic distillation with xylene. The remaining oil was distilled under vacuum, b.p. 133–135°/0.5 mm., yield 40%. (See Table II for physical constants, yields, and analyses.)

Partition Coefficients.—The partition coefficients of both the 3-alkylsydnones and the 3,4-dialkylsydnones were obtained between water saturated *n*-amyl alcohol and *n*-amyl alcohol saturated water. Several concentrations of each compound in water saturated *n*-amyl alcohol were equilibrated at 23° with varying quantities of *n*-amyl alcohol saturated water. The concentration in the *n*-amyl alcohol before and after equilibration with the aqueous phase was determined from the ultraviolet absorption at the maximum. An average of at least four determinations for each compound was used. (See Table III).

Determination of CD_{50} Values.—The same procedure previously referred to (5) was used to determine the CD_{50} values. The CD_{50} values for the 3-alkylsydnones were reported in a previous communication (2). (See Table III). The partition coefficient *versus* CD_{50} values are graphically portrayed in Fig. 1.

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(1) Kier, L. B., Fox, L. E., Dhawan, D., and Waters, I. W., *Nature*, **195**, 817(1962).
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Color Reactions of Veratrum Alkaloids with Sulfuric Acid and Sulfuric Acid Reagents

By HORACE D. GRAHAM†

Veratrum alkaloids, when treated with concentrated sulfuric acid, or "sulfuric acid reagents" give red, purple, yellow, or violet with characteristic absorption maxima. This reaction can be used as the basis of a simple, rapid colorimetric method for the determination of isolated samples of this class of alkaloids. The final concentration of sulfuric acid is very critical for maximum color development and, since color intensity is decreased if water is present to the extent of greater than 1 per cent, an anhydrous medium is recommended for maximum development. The typical color is best obtained when ethyl alcohol or methyl alcohol is used as the solvent for the alkaloids. Except for hydrochloric acid, no color is obtained with acids other than sulfuric. After heating for several minutes in hydrochloric acid, some color developed, but the intensity was always much less than that produced after treatment of the same amount of alkaloid with sulfuric acid. Inorganic salts, sodium benzoate, and glucose, above certain limiting concentrations, will interfere with color development.

CONSIDERABLE INTEREST has developed in the veratrum alkaloids because of their potent hypotensive action. This was forcibly emphasized by Kupchan (1), who recently reviewed the hypotensive veratrum ester alkaloids, paying particular attention to the relationship between structure and hypotensive activity. However, despite their growing importance in medicine and pharmacology, simple, rapid methods for the quantitative assay of veratrum alkaloids are still lacking. As far as can be ascertained, present methods of detection and assay involve mainly biological means such as the pigeon emetic response or intricate physical, chemical, or other methods (2–18). Dadlez, *et al.* (19), reported on the color reactions of alkaloids, including veratrine, with sulfuric acid and sulfuric acid in combination with several other reagents such as furfural, *p*-dimethylaminobenzaldehyde, and vanillin. Moraes and Palma (20) employed both concentrated nitric acid and concentrated sulfuric acid in studying the color reactions of several alkaloids including veratrine, following separation of the alkaloids by paper chromatography. Mandelin reagent (concentrated sulfuric acid plus formalin) and Fröehde reagent (concentrated sulfuric acid plus ammonium molybdate) were also used.

Reference has been made (21) to the red, purple, or violet developed by certain veratrum alkaloids on treatment with concentrated sulfuric acid. Since alkaloids of many other classes do not give these colors, a study of the reaction was undertaken to delineate those factors which

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† Present address: College of Agriculture and Mechanic Arts, University of Puerto Rico Mayaquez.

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TABLE I.—COLORS PRODUCED BY VERATRUM ALKALOIDS AND OTHER ALKALOIDS WITH SULFURIC ACID AND SULFURIC ACID REAGENTS

Alkaloid Tested	Reagent No.[a,b] 1	2	3	4	5
Veratrine sulfate	R	P	P	R	R
Veriloid powder	Pk-Y	P	P	R-Br	Pk-O
Total alkaloids of *Veratrum viride*	Pk-Y	Br-Y	Br-Y	R-Br	Pk-O
Jervine	Y	Y	Y-Br	G-Y	Y
Protoveratrines A and B	P-Pk	P	P	R	Pk
Protoveratrine A	Pk-P	P	P	P	Pk
Protoveratrine B	P	P	Pk-P	P	R
Cevadine	R	P	P	R	R
Veratridine	R	P	P	R	R
Veratrine alkaloid	R	P	P	R	R
Alpha veratrine	R	P	P	R	R
Veratramine	Y	Y-Br	G-Y	R-Br	Y-G
Veratric acid	NC	NC	NC	Y	Y
Tomatine	Y	Br	P	Br	G-Br
Tomatidine	Y	G-Br	P	R	G-Y
Solanine	Y	Y-G	Br	O-Br	G-Y
Conessine	Y	Y	Y	Y	G-Y
Colchicine	Y	Y	Y	Y	G-Y
Sitosterol acetate	Y	P-Br	P-Br	O-Br	G-Y
Stigmasterol	O	P-Br	P-Br	O-Br	Br
Cholesterol	O-Y	Br	R-Br	O-Br	O-Br
Ergosterol	O-Br	Br	P-Br	Br	O-Br
Digitonin	Y	Br	Y	O-Br	O-Br
Reagent blank	NC	NC	NC	NC	Y

[a] *1*, Concentrated H_2SO_4; *2*, concentrated H_2SO_4 + $FeCl_3$; *3*, concentrated H_2SO_4 + $FeNH_4(SO_4)_2 \cdot 12H_2O$; *4*, concentrated H_2SO_4 + *p*-dimethylaminobenzaldehyde; *5*, hydroquinone + H_2SO_4. [b] R = Red; Pk = pink; Y = yellow; B = blue; Br = brown; O = orange; G = green; P = purple; NC = no color.

influence the reaction and, specifically, to exploit it for rapid quantitative determination of these alkaloids.

Sulfuric acid alone or in combination with other inorganic or organic reagents has been used frequently for the colorimetric determination of many important alkaloids and steroids (22–24). A preliminary survey showed that, in addition to the colors produced in concentrated sulfuric acid, the veratrum alkaloids gave pronounced colors with some of the reagents reported by Jakovljevic (25) in his method for vinblastine. This communication summarizes the results of experiments on the many factors which may influence the reaction between the veratrum alkaloids and concentrated sulfuric acid. Among those investigated were the final acid concentration necessary for maximum color development, the influence of the presence of water, added salts and other excipients in the reaction medium, color stability, the influence of various solvents for the alkaloids, and the substitution of other acids for sulfuric acid. Color development with various sulfuric acid reagents was also studied.

EXPERIMENTAL

Materials.—The alkaloids are listed in Table I. Sulfuric acid, reagent grade—95–98%, sp .gr. 1.8407 to 1.8437; absolute ethyl alcohol and methyl alcohol; vanillin U.S.P.; ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; ferric chloride, $FeCl_3 \cdot 6H_2O$; *p*-dimethylaminobenzaldehyde, m.p. 74–75°; ferric ammonium sulfate, $FeNH_4(SO_4)_2 \cdot 12\ H_2O$; and inorganic salts, organic salts, and other chemicals (see Table II) were employed. All of U.S.P. or C.P. grade obtained from commercial sources and used without further purification.

The sulfuric acid–ferric chloride reagent was prepared according to the directions given by Zak, *et al.* (23). The ferric ammonium sulfate

TABLE II.—INFLUENCE OF ADDED SALTS, GLUCOSE, AND OTHER COMPOUNDS ON THE COLOR REACTION BETWEEN VERATRINE SULFATE AND SULFURIC ACID

Salt Added	Limiting Concn. (*M*)
(Monovalent)	
Sodium chloride	5×10^{-4}
Sodium biphosphate	5×10^{-4}
Ammonium chloride	1×10^{-4}
Ammonium carbonate	1×10^{-4}
Sodium carbonate	1×10^{-4}
Potassium chloride	2×10^{-4}
Lithium chloride	2×10^{-4}
(Divalent)	
Barium chloride	2×10^{-4}
Magnesium chloride	2×10^{-4}
Magnesium sulfate	2×10^{-4}
Mercuric chloride	2×10^{-4}
(Trivalent)	
Ferric chloride	1×10^{-6}
Aluminum chloride	0.6×10^{-6}
(Organic Salts and Other Compounds)	
Sodium benzoate	3×10^{-4}
Sodium acetate	5×10^{-4}
Hexamethonium chloride	0.5 mg.
Promazine hydrochloride	0.01 mg.
Promethazine	0.01 mg.
Potassium acid phthalate	2×10^{-4}
Glucose	0.01 mg.

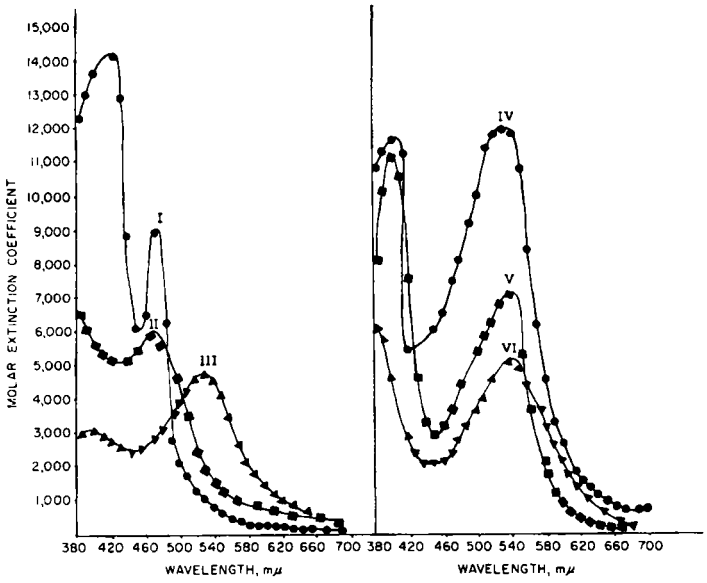


Fig. 1.—Absorption spectra of veratrum alkaloids in concentrated sulfuric acid. Key: I, veratramine; II, jervine; III, protoveratrine A; IV, cevadine; V, veratridine; VI, protoveratrine B.

solution was prepared in a manner similar to the ferric chloride reagent. The vanillin and *p*-dimethylaminobenzaldehyde reagents were prepared as 1% (w/v) solutions of the respective compounds in concentrated sulfuric acid.

Equipment.—Coleman model 14 universal spectrophotometer, 30-ml. Pyrex glass-stoppered test tubes, vacuum oven, and 50-ml. acid buret were utilized.

General Procedure for Screening the Color Reactions of the Various Alkaloids with Sulfuric Acid and Sulfuric Acid Reagents.—For routine screening, the alkaloids were dissolved in absolute methyl alcohol or glacial acetic acid. One milliliter of each stock solution containing 500 mcg. of the particular alkaloid was placed in a glass-stoppered Pyrex test tube. At the end of this period, 9 ml. of concentrated sulfuric acid or sulfuric acid reagent was added and the tubes allowed to stand at room temperature. The color developed was noted immediately after the addition of the sulfuric acid, after 30 and 60 minutes, and again after 24 hours. The results are summarized in Table I.

Determination of Wavelengths of Maximum Absorption of the Colors Developed with the Various Reagents and the Molar Extinction Coefficients or Some of the Veratrum Alkaloids.—One milliliter of a 10^{-3}–10^{-5} *M* solution of the particular alkaloid was placed in a ground-glass-stoppered test tube, and 9 ml. of the sulfuric acid or sulfuric acid reagent was added. The tubes were shaken to mix the ingredients thoroughly and allowed to stand at room temperature for 60 minutes. The wavelength of maximum absorption was then determined by measuring the optical density of the solutions over the wavelength range of 380–700 mμ and plotting the optical density as a function of the wavelength. Molar extinction coefficients were calculated for six of the alkaloids used; the plots

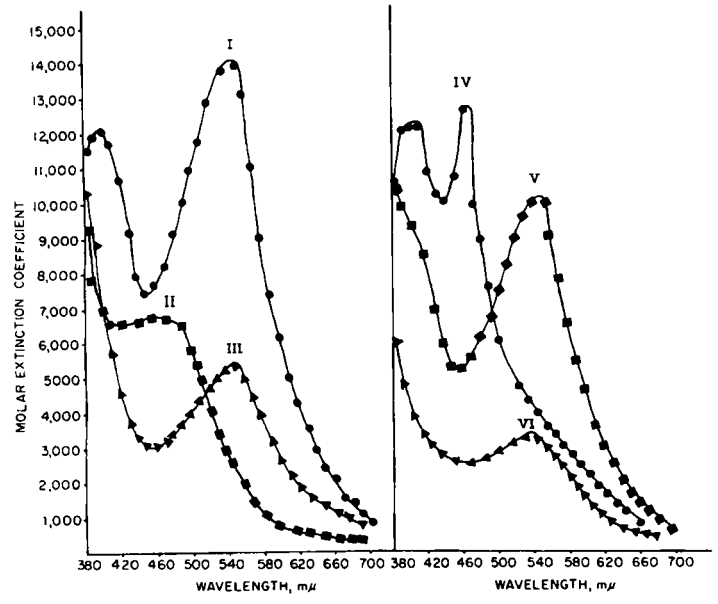


Fig. 2.—Absorption spectra of veratrum alkaloids in concentrated sulfuric acid plus ferric chloride. Key: I, cevadine; II, jervine; III, protoveratrine A; IV, veratramine; V, veratridine; VI, protoveratrine B.

TABLE III.—ABSORPTION CHARACTERISTICS OF VERATRUM ALKALOIDS IN CONCENTRATED SULFURIC ACID AND CONCENTRATED SULFURIC ACID PLUS FERRIC CHLORIDE

Alkaloid	In H_2SO_4		In H_2SO_4-$FeCl_3$	
	λ Max.	Mol. Ext. Coeff.	λ Max.	Mol. Ext. Coeff.
Veratrine sulfate	390	...	380	...
	540	...	550	...
Veriloid powder	410	...	400	...
Total alkaloids of *Veratrum viride*	390	...	380	...
Veratrine	400	...	400	...
	540	...	...	...
Jervine	470	5.95×10^3	420–480	6.7×10^3
Veratramine	410	16.76×10^3	410	13.01×10^3
	470	8.93×10^3	460	12.84×10^3
Protoveratrine A	390	3.20×10^3	530	3.37×10^3
	530–540	4.60×10^3	...	...
Protoveratrine B	540	5.10×10^3	550	5.30×10^3
Cevadine	400	11.70×10^3	400	12.20×10^3
	520–530	11.90×10^3	540	13.98×10^3
Veratridine	400	11.3×10^3	540–550	10.01×10^3
	540	7.00×10^3	...	...

of molar extinction coefficients *versus* wavelength are shown in Figs. 1 and 2.

Data for Figs. 1 and 2 were obtained from measurements against reagent blanks. Data for the other alkaloids listed in Table III were obtained from measurements against distilled water.

Influence of Variables on the Reaction of Veratrum Alkaloids with Sulfuric Acid.—For all experiments on the influence of variables on the reaction, veratrine sulfate only was used. A constant quantity (500 mcg.) of veratrine sulfate was treated with sulfuric acid according to the general procedure, except that the variables were tested over the following ranges: acid normality (3.6 to 32.4*N*), final concentration of water (0.0 to 5.0%), and time for color development (0.0 minutes to 72 hours).

Acids substituted for sulfuric acid were: concentrated hydrochloric, glacial acetic, phosphoric (85%), formic, and lactic acids. The intensity of the color developed was measured after 60 minutes at 540 mμ against a reagent blank.

Influence of Added Salts, Glucose, and Other Compounds on the Color Reaction of Veratrine Sulfate with Sulfuric Acid.—The influence of added salts was determined by adding varying amounts of the particular salt to a tube containing a constant amount (500 mcg.) of veratrine sulfate and developing and measuring the optical density of the resulting color after 60 minutes. This influence is considered of importance since as noted in the "Merck Index" (26) the alkaloids of *Veratrum viride* are usually administered after dilution with saline. Since dilution with 5% glucose may also be done prior to administration (26), the influence of this sugar on color development was also assessed. The severity of interference from other compounds such as promazine hydrochloride, sodium benzoate, etc., was also determined.

The term "limiting concentration" is used here to mean the maximum concentration of added salt or other compound which will cause a deviation in optical density of the color developed of not more than 1% of the color density given by the control system, *i.e.*, by 500 mcg. of veratrine sulfate in 1 ml. of absolute methanol and 9 ml. of concentrated sulfuric acid. The results are summarized in Table II.

Quantitative Response of Alkaloids in Sulfuric Acid.—After the influence of the several variables

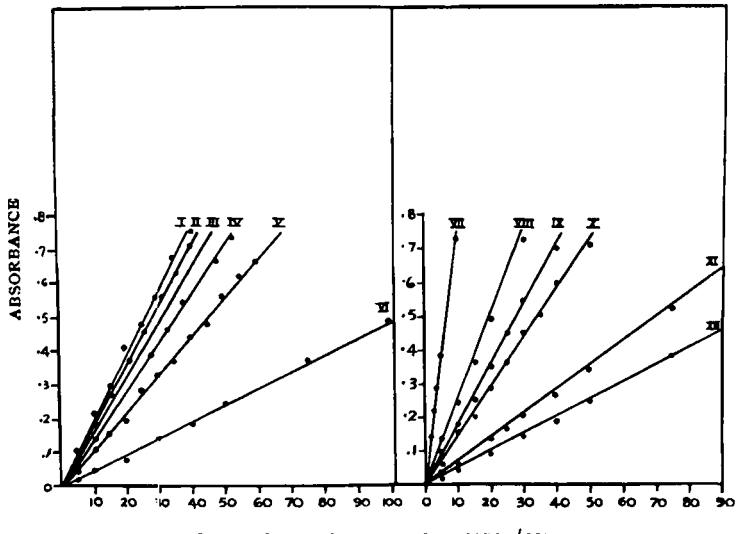


Fig. 3.—Quantitative color response of veratrum alkaloids when treated with concentrated sulfuric acid. Key: I, veratridine[a]; II, veratrine alkaloid[b]; III, jervine[d]; IV, veriloid[c]; V, veratrine sulfate[a]; VI, protoveratrines A and B[a]; VII, veratramine[c]; VIII, α-veratrine[a]; IX, cevadine[b]; X, total alkaloids of *Veratrum viride*[e]; XI, protoveratrine A[b]; XII, protoveratrine B[a]. Letters indicate wavelengths at which measurements of color intensity were made. Key: a, 540 mμ; b, 530 mμ; c, 410 mμ; d, 470 mμ; e, 390 mμ.

was delineated, it was decided to test the quantitative response of the alkaloids in sulfuric acid. For this, 0.0 to 1.0 ml. of a stock solution containing varying amounts of the alkaloids was added to consecutive ground glass-stoppered test tubes. Where necessary, the volume was made up to 1 ml. with absolute methanol. Nine milliliters of concentrated sulfuric acid was added to each test tube and, after standing at room temperature (30°) for 60 minutes, the colors developed were measured at the appropriate wavelengths of maximum absorption. The results are summarized in Fig. 3.

DISCUSSION

In the proposed method, the final concentration of sulfuric acid is very critical for maximum color development. As the acid concentration increased, the density of the color also increased, but over the range tested (3.6 to 32.4 *N*) , no constancy in the density of the color developed was observed. Therefore, it is recommended that the highest final normality of acid (32.4 *N*) be used. Several other acids were substituted for sulfuric acid but, except for concentrated hydrochloric acid, no color development occurred. On heating with concentrated hydrochloric acid, veratrine sulfate gave some color. Water, if present to the extent of greater than 1% of the total volume of the reaction vessel, will diminish color intensity. It can be removed simply by heating with a recovery of 96 to 100.1% of the alkaloid. Maximum color development occurred after 30–60 minutes; the colors are stable for as long as 12 hours.

Salts and glucose interfere with the development of the color. Such interference is minimal at the limiting concentrations or amounts shown in Table II. Substances such as promazine hydrochloride and promethazine interfere because they, too, produce colors in concentrated sulfuric acid. Only slight differences in the sensitivity of the various reagents were noted and, because of its gross simplicity, concentrated sulfuric acid alone is highly suitable. Other related alkaloids—for example, tomatine, tomatidine, solanine, and conessine also give colors with the reagents (Table I), but the colors are different from those produced by the veratrum alkaloids. With most of the veratrum alkaloids, two distinct absorption maxima occurred usually at 390–410 mμ and 530–550 mμ (Table III); with the tomatine, tomatidine, etc., only a single absorption maximum was observed, generally at 400–420 mμ.

Figures 1 and 2 show plots of molar extinction coefficients *versus* wavelength for six defined alkaloids in sulfuric acid and sulfuric acid–ferric chloride. Although slight shifts in wavelengths of maximum absorption of the colors developed with these reagents occurred (Table III), the molar extinction coefficients of any particular alkaloid do not differ greatly from one reagent to the other. However, with either reagent and especially at the higher wavelength of maximum absorption, the molar extinction coefficient of the alkaloids differ greatly, thus allowing for some differentiation. Figure 3 shows that the method as outlined is quantitative and that within the concentrations employed, Beer's law was obeyed.

The sulfuric acid–ferric chloride reagent (reagent *2*) provides some distinction between jervine and veratramine. The former exhibited a broad absorption maximum at 420–480 mμ while the latter absorbed maximally at 410 and 460 mμ. The presence of a benzene ring in veratramine probably could have contributed to the difference in absorption maxima. If these two alkaloids must be separated, veratramine can be precipitated by digitonin, whereas jervine will not be precipitated. In many cases the optical density of the color produced by the veratrum alkaloids was greater at the lower wavelength of maximum absorption, usually 390–410 mμ. However, if the iron reagents are used, these do exhibit some absorption in this region. For this reason, measurements at the higher wavelength of maximum absorption (530–550 mμ) are preferable. Although veratrum alkaloids produced colors with two of the classical steroid reagents—namely, the Lieberman-Burchard and Salkowski reagents, color development was so slow that these reagents are of no advantage. Likewise, sulfuric acid plus vanillin or ammonium molybdate or dichromate are too nonspecific. When veratrine sulfate was used as the representative alkaloid, the typical color was obtained if ethyl alcohol, methyl alcohol, ether, chloroform, or benzene were used as solvents. Best results were obtained when ethyl or methyl alcohol were used. With isopropyl alcohol and amyl alcohols, yellowish pink colors and a muddy appearance of the mixture resulted.

The mechanism of the reaction of the veratrum alkaloids with sulfuric acid and sulfuric acid reagents is not definitely known, but dehydration is certainly involved since water inhibits the reaction. The production of color may also be due partially or wholly to degradation products of the alkaloids (27–30).

The "heat of the reaction" resulting from the addition of the sulfuric acid or sulfuric acid reagents to the alkaloid solution provides enough heat for the immediate development of the red color formed from the reaction of veratrine sulfate. If cold (deep freeze) H_2SO_4 is added to frozen samples of veratrine sulfate and the mixture kept in an ice water bath, a yellow color develops. After 3 hours at room temperature (30°), the typical red color appeared; after 8 hours it reached the intensity of a sample treated by the general procedure. If the tubes to which the cold acid was added were heated, the typical red developed after 10–15 minutes at 40°. At 70°, the intensity of the color developed after 8 minutes of heating was equivalent to that produced from the same amount of alkaloid treated according to the general procedure. This indicates that, although the heat of reaction leads to the immediate production of the typical color, in H_2SO_4 of 32.4 *N*, this typical color will still result even at relatively low temperatures, provided a long enough reaction time is allowed.

SUMMARY

The color produced by veratrum alkaloids in concentrated sulfuric acid or concentrated sulfuric acid containing ferric chloride or ferric ammonium sulfate can serve as a rapid, simple test, for the quantitative determination of these alkaloids

The presence of water in the reaction vessel reduced color intensity which reaches a maximum after 60 minutes. The color developed is stable for at least 6 hours. With most of the alkaloids used, two absorption maxima, usually at 390–410 mμ and 530–550 mμ, were observed. With the sulfuric acid–ferric chloride reagent, jervine and veratramine absorbed at 420–480 mμ and 410 and 460 mμ, respectively. Several of the steroids tested produced colors. However, they exhibited a single absorption maximum which was generally always around 400–420 mμ.

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Stability of the Cobalamin Moiety in Buffered Aqueous Solutions of Hydroxocobalamin

By ARNOLD D. MARCUS and JOSEPHINE L. STANLEY

The degradation of hydroxocobalamin has been studied kinetically in various buffer systems. Degradation was first order with respect to the substrate in all cases. The most stable solution studied was composed of a pH 4.3, 0.05 *M* acetate buffer made isotonic with sodium chloride. The apparent heat of activation has been found in the above system to be 26.95 Kcal./mole. Extrapolations to 25 and 30° show hydroxocobalamin to be sufficiently stable to permit formulation of injectable solutions under practical conditions. Even when the data are constrained to give a poor stability picture at 25 and 30°, the predicted stability remains excellent. At worst, a solution of hydroxocobalamin in pH 4.3 acetate buffer will retain at least 90 per cent of claimed cobalamin at 30° for 170 weeks if a modest 20 per cent overage is included in the solution. The poorer stability in other buffer systems indicates the degradative reaction(s) to be subject to general base catalysis. The influence of some amines on the reaction rate tend to support this inference.

HYDROXOCOBALAMIN is an analog of vitamin B_{12} in which a hydroxyl function has replaced the cyano group in the cobalt coordination complex. Recent clinical studies (1) have provided dramatic evidence that parenterally administered hydroxocobalamin yields considerably more prolonged high blood levels of biologically active cobalamin than does cyanocobalamin. Unfortunately, hydroxocobalamin has acquired over the years the reputation of being unstable in solution (2, 3). We have found that, far from being unstable, hydroxocobalamin in suitably buffered solutions is actually quite stable. Even when viewed pessimistically, hydroxocobalamin is sufficiently stable to allow the preparation of injectable solutions under practical conditions which will retain the claimed cobalamin content for long periods of time.

Preliminary stability tests (4) indicated that an apparently stable solution of hydroxocobalamin could be obtained in pH 4.3, 0.05 *M* acetate buffer made isotonic with sodium chloride. The

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presence of chloride ion seemed important in achieving a stable solution. When the buffer concentration was increased to 0.1 *M* acetate, the cobalamin was less stable. In a buffer composed of 0.1 *M* dibasic sodium phosphate and 0.05 *M* citric acid at pH 4.3, the hydroxocobalamin appeared to be just as stable as in the isotonic 0.05 *M* acetate buffer.

These observations indicated the need for a more careful evaluation of the entire stability picture. It was necessary to determine, if possible, the precise stability of hydroxocobalamin in each system in order to attempt to predict the stability under "ordinary" conditions. It is the purpose of this communication to report such evaluations and the prediction of stability as determined by kinetic studies under accelerated conditions. This report confirms the preliminary observations that hydroxocobalamin is a stable species in the proper solution environment. The response of hydroxocobalamin to various buffers, however, indicates that the degradative reaction(s) may involve general base catalysis.

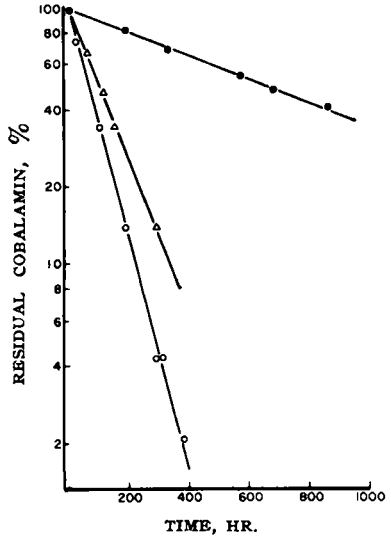


Fig. 1.—Plots showing degradation of hydroxocobalamin to be first order with respect to the substrate under a variety of experimental conditions. Key: ●, pH 4.3, 0.05 *M*, acetate at 70°; ○, pH 4.3, 0.05 *M*, acetate at 90°; △, pH 4.3, citrate-phosphate at 80°.

TABLE I.—RATE CONSTANTS FOR THE DEGRADATION OF HYDROXOCOBALAMIN IN VARIOUS BUFFERS[a]

Buffer Compn.	Temp., °C.	k, hr.$^{-1}$ × 10^{-2}
pH 4.3, 0.05 *M* acetate[b]	90	10.5
pH 4.3, 0.05 *M* acetate[b]	80	3.35
pH 4.3, 0.05 *M* acetate[b]	70	1.07
pH 4.3, 0.05 *M* acetate	90	10.5
pH 4.3, 0.05 *M* acetate	80	3.36
pH 5.5, 0.05 *M* acetate	90	20.0
pH 5.5, 0.05 *M* acetate	80	7.2
pH 4.3, citrate-phosphate	90	25.0
pH 4.3, citrate-phosphate	80	6.90
pH 5.5, citrate-phosphate	90	22.2
pH 5.5, citrate-phosphate, no NaCl	90	22.1
pH 5.5, citrate-phosphate	80	7.85

[a] All buffers contained 8.2 mg./ml. sodium chloride unless otherwise noted. [b] Solution contained 200 mcg./ml. hydroxocobalamin initially. These data were used for the Arrhenius-type plot of Fig. 2. All other solutions contained 1000 mcg./ml. initially.

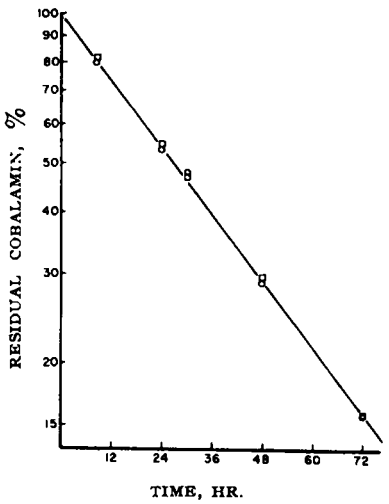


Fig. 2.—A plot showing the degradation of hydroxocobalamin in a pH 5.5 citrate-phosphate buffer (90°) to be unaffected by inclusion of sodium chloride. Key: ○, no chloride; □, 0.14 *M* in NaCl.

In the buffered aqueous solutions of hydroxocobalamin used in this study, the designation *hydroxo*cobalamin is actually inaccurate. Numerous cobalamins or cobalaminium species could actually be present. However, the original term will be retained to simplify the text and discussion. Furthermore, the inherent biological activity resides in the cobalamin moiety; the associated anions, coordinated functions, or presence of a formal charge can influence only stability and compatibility, not the qualitative activity. That the buffer constituents could affect the ability of hydroxocobalamin to provide prolonged high blood levels was recognized, but this would be revealed by clinical studies.

EXPERIMENTAL

Reagents.—All chemicals were Merck, reagent grade, except for the hydroxocobalamin. This was crystalline material assaying at least 95% hydroxocobalamin on an anhydrous basis. All buffers were prepared to give the desired pH at the actual temperature of the run.

Procedure.—The solutions were flushed with nitrogen and carefully filled into 5-ml. ampuls to avoid streaking the ampul stems. This prevented charring during the flame-sealing and avoided introduction of extraneous materials which might influence the rate of degradation. The ampuls were placed in constant temperature baths regulated to within ±0.05°. After thermal equilibrium was

attained, three samples were removed, chilled, and stored at 4°. These were designated our "zero" hour samples. Other samples were removed at suitable intervals and quenched in the same manner. All samples were assayed for residual hydroxocobalamin by the microbiological procedure of Skeggs, *et al.* (5, 6).

RESULTS AND DISCUSSION

As shown in Fig. 1, the degradation of hydroxocobalamin is first order with respect to the substrate under a variety of experimental conditions. The various rate constants are given in Table I. The apparent activation energy for the degradation of hydroxocobalamin in pH 4.3, 0.05 *M* acetate buffer was calculated from the slope of the line in Fig. 2. This value was a rather high 26.95 Kcal./mole. Although the apparent heat of activation could have been used to calculate the rate constants for lower temperatures, the values given in the following discussion were actually obtained by graphical extra-

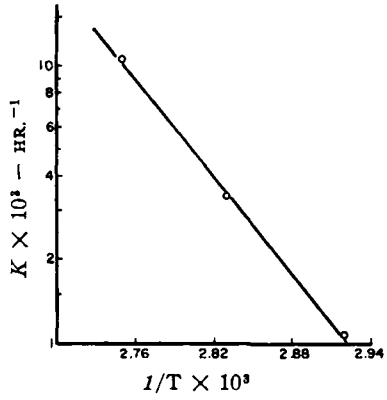


Fig. 3.—An Arrhenius-type plot illustrating the effect of temperature on the degradation of hydroxocobalamin in pH 4.3, 0.05 *M*, acetate buffer. The apparent activation energy calculated from the slope of the line is 26.95 Kcal./mole.

polation. When the calculated and extrapolated values were compared, the differences were insignificant.

Examination of the extrapolated rate constants shows that at 30° the rate is 6.2×10^{-6} hours^{-1}. As a result, even at this relatively elevated temperature, solutions of hydroxocobalamin in pH 4.3, 0.05 *M* acetate buffers are sufficiently stable to retain at least 90% of the claimed cobalamin content for 125 weeks. The same solution at 25° could be expected to retain no less than 90% of the claimed hydroxocobalamin content for at least 220 weeks. These data make it clear that under suitable conditions hydroxocobalamin is quite stable in aqueous solutions.

Despite the optimistic nature of the preceding treatment, the possibilities for error inherent in microbiological assays and long-range extrapolations were recognized. It seemed judicious, therefore, to try to make predictions from extrapolations tending to give the least favorable 30 and 25° stability patterns. When this was done, the rate constants were $k_{30} = 1.0 \times 10^{-5}$ hours^{-1} and $k_{25} = 4.6 \times 10^{-6}$ hours^{-1}. Calculations based on these "new" values indicated a loss of 10% of the initial cobalamin content after 125 weeks at 25° and 60 weeks at 30°. Despite the still good stability at 25°, a temperature of 30° is not unusually high for the storage of pharmaceuticals. It seemed important, therefore, to attempt to insure good stability under such storage conditions.

Calculations based on $k_{30} = 1.0 \times 10^{-5}$ hours^{-1} showed that the use of a 20% average of hydroxocobalamin would yield a solution retaining at least 90% of "label claim" for no less than 170 weeks at 30°. Under such conditions, therefore, and employing extrapolations prejudiced to show greatest degradation, it was apparent that hydroxocobalamin is not "unstable" in solution.

When the degradation of hydroxocobalamin in other buffers was studied, some unexpected results were obtained. In an isotonic pH 5.5, 0.05 *M* acetate buffer, for example, the rate of degradation was approximately twice the rate at pH 4.3. Since in this pH range the hydroxocobalamin-aquocobalamin equilibrium favors the "aquo" form overwhelmingly, pH alone did not appear to be the major degradative influence. This was shown, in part, by rate studies in a buffer prepared to be 0.1 *M* in dibasic sodium phosphate and 0.05 *M* in citric acid. The rate in this buffer was also twice that in the isohydric acetate buffer. Since all three systems contained the same concentration of chloride ion, the differences in rate were inexplicable on the basis of the relative coordinating ability of chloride and other anions. Indeed, as shown in Fig. 3, the actual need for chloride ion is problematical. However, this single run at 90° may not accurately reflect the conditions and reactions which hold at conventional temperatures.

Since it appears somewhat doubtful that chloride ion functions to exert any "protective" effect and acetate is also an unlikely candidate for this type of action, an explanation of the "buffer effect" was sought elsewhere. Although no precise evidence was available, it seemed likely that this response stemmed from nucleophilic attack by the buffer anions. In this sense, acetate is not a "protective" agent but actually a less objectionable species. This would certainly tie in well with the rates at pH 5.5 in acetate systems. The effect of the citrate-phosphate system could be explained in like manner. Another facet of the present study which lends support to this possibility is the observed degradative influences of aniline and pyridine. In a rather qualitative two-point study we have found that both of these nucleophiles, present at 0.1 *M*, in pH 4.3, 0.05 *M* acetate buffer, caused an approximately twofold increase in the rate of degradation of hydroxocobalamin. The precise effects of these and similar agents is being studied further.

The apparent general base catalyzed degradation of hydroxocobalamin is being studied currently in greater detail. The effect of pH, *per se*, also will be evaluated. However, it should not be at all surprising that this type of degradation could occur in the cobalamin moiety. There are seven amide functions and two ester linkages in the molecule. Within the past 10 years, the number of instances of general acid-base catalyzed degradation of amides and esters has been well documented (7–9).

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Interaction of Some Pharmaceuticals with Macromolecules I

Effect of Temperature on the Binding of Parabens and Phenols by Polysorbate 80 and Polyethylene Glycol 4000

By N. K. PATEL and N. E. FOSS

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IN RECENT YEARS considerable attention has been centered on the interaction of preservatives with nonionic macromolecules (1–5) and the importance of considering such interactions when determining proper preservative concentrations (2). These interactions are particularly noticeable with phenolic preservatives in the presence of surfactants which are polyoxyethylene derivatives of fatty acid esters. Several review articles have been published describing the possible mechanism of the interaction of phenolic compounds with surfactants (6–9).

Higuchi and Lach (10) and Guttman and Higuchi (11) hypothesized that the complex formation between phenols and polyethylene glycols could be due to hydrogen bond formation between the hydrogen of the phenolic hydroxyl group and basic oxygen atom of polyethylene glycol. It would thus be expected that proton donors like phenol and *p*-chlorophenol might form molecular complexes with surfactants containing polyoxyethylene groups.

Guttman and Higuchi (11) have indicated that addition of thermal energy to the phenol-PEG interaction might result in a decrease in association between phenol and PEG. Thus, if the principal binding force between a phenolic preservative and a nonionic surfactant is due to hydrogen bond formation, the increase in temperature might decrease the intermolecular association in such a system. Furthermore, nonionic surfactants usually contain a relatively large number of hydrophobic groups in the molecule and contribution of hydrophobic interactions (10) to the magnitude of binding of a phenolic preservative by the macromolecule might be of considerable importance.

In the present investigation an equilibrium dialysis technique was utilized to obtain information regarding the extent of binding of methyl, propyl, and butylparaben, phenol and *p*-chlorophenol with polysorbate 80[1] at various temperatures, for the purpose of relating these interactions to the reported possible mechanism of binding. The solubility method was employed to study the interaction of methyl and propylparaben with PEG 4000 at 20°. The selection of the phenolic compound was based on the relative proton donating power of the compound and on the length of the hydrocarbon chain of the *p*-hydroxybenzoic acid esters.

EXPERIMENTAL

Reagents.—Recrystallized methyl *p*-hydroxybenzoate,[2] m.p. 127–128°; recrystallized propyl

Received May 8, 1963, from the School of Pharmacy, Duquesne University, Pittsburgh, Pa.
Accepted for publication June 13, 1963.
This work was carried out at the School of Pharmacy, University of Maryland, Baltimore.
The authors express sincere thanks to Dr. H. B. Kostenbauder, School of Pharmacy, Temple University, Philadelphia, Pa., for his valuable suggestions.
Presented to the Pharmaceutical Sciences Section, American Association for the Advancement of Science, Philadelphia meeting, December 1962.

[1] Polyoxyethylene (20) sorbitan monooleate. Marketed as Tween 80 by the Atlas Powder, Co., Wilmington, Del.
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p-hydroxybenzoate,[3] m.p. 96–98°; recrystallized butyl *p*-hydroxybenzoate,[4] m.p. 70–72°; phenol, reagent grade (Mallinckrodt Chemical Works); *p*-chlorophenol, reagent grade (Eastman Organic Chemicals); polysorbate 80, a commercial sample; polyethylene glycol 4000.[5]

Equilibrium Dialysis Method.—The experimental technique was identical in principal to that used by Patel and Kostenbauder (1) in studying the interaction of parabens with polysorbate 80. Because the nylon membrane[6] employed during the present investigation was in the form of a thin film, a dialysis cell similar to that suggested by Kostenbauder (12) was utilized.

A typical dialysis cell is shown in Fig. 1. Each dialysis cell consists of two plexiglas[7] blocks $2^1/_2 \times 2^1/_2 \times 1^1/_{16}$ in., each having a cavity of 20-ml. capacity and four $^3/_{16}$ in. holes at the corners. The dialysis membrane was placed between the two blocks with the cavities facing each other. The blocks were held firmly together by the use of four stainless steel head bolts $3^1/_2 \times {}^3/_{16}$ in., inserted in the corner holes and secured with stainless steel wingnuts. Each block has a threaded hole entering the cavity used for the introduction of the solution or the removal of sample for analysis. After the two blocks were securely tightened, 10 ml. paraben solution was placed in the cavity on one side of the membrane and 10 ml. polysorbate 80 solution was placed on the other side of the membrane. The entire assembly was then stoppered tightly by means of plexiglas screw-plugs with thin polyethylene gaskets and agitated at a constant temperature until equilibrium was attained (1).

Nylon membranes were unsatisfactory for the dialysis studies involving phenol and *p*-chlorophenol, since aqueous solutions of these substances converted the structure of nylon from a thin membrane to a gummy, resinous mass. A thin rubber membrane[8] was found to be highly satisfactory, since it proved to be permeable to phenol and impermeable to polysorbate 80. The experimental procedure was identical to that reported previously (1).

After equilibration, aliquots were removed from both sides of the membrane and concentrations of the compounds under study were determined spectrophotometrically at a wavelength of 255 mμ for methyl, propyl, and butylparaben, 270 mμ for phenol, and 278 mμ for *p*-chlorophenol, using a Beckman DU spectrophotometer.

Solubility Method.—The solubility of methyl and propylparaben was determined in the presence of varying concentrations of polyethylene glycol 4000. The experimental procedure (3), method of assay, and treatment of the data have been described previously (1).

RESULTS

Effect of Temperature on the Binding of Parabens by Polysorbate 80.—Binding of methyl and propylparaben by polysorbate 80 at 30° has been reported previously (1). Blaug and Ahsan (5) determined the solubility of propyl and butylparaben in aqueous solutions of polysorbate 80 at 0°. In the present study the magnitude of binding of methyl, propyl, and butylparaben by polysorbate 80 at varying temperatures was determined by the dialysis method.

Figures 2–4 show that the paraben-polysorbate 80 interaction exhibits a marked temperature dependency. The slope values given in these figures are indicative of the relative magnitude of binding of parabens by polysorbate 80. It is evident from these slope values that an increase in temperature decreases the extent of binding of methyl, propyl, and butylparaben by polysorbate 80.

Figures 2–4 illustrate further that the degree of binding increases in general order of 1:6:18 from methyl to propyl to butylparaben; the corresponding slope values increase from 0.7 to 4.4 to 12.9 at 30° and 0.63 to 3.9 to 11.3 at 40°.

Effect of Temperature on the Binding of Phenols by Polysorbate 80.—The phenol-polysorbate 80 interaction shows only a minor temperature de-

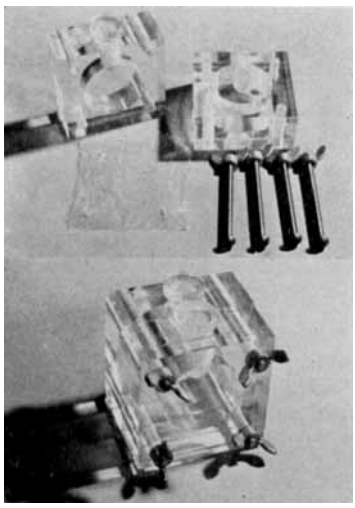


Fig. 1.—Dialysis cell.

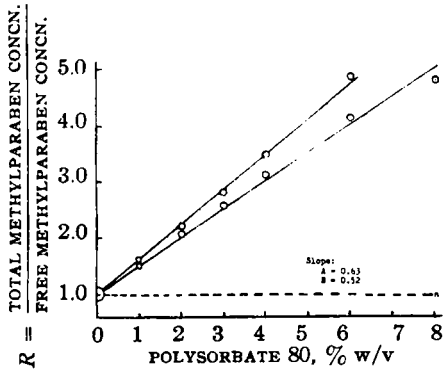


Fig. 2.—Binding of methylparaben by polysorbate 80 in aqueous solution at 40 and 50°. A, 40°; B, 50°. The broken line represents a theoretical line which would be obtained if no binding were to have taken place. Such a line will be labelled "n" in the figures of this paper.

[3]Propyl Parasept, purified; supplied through the courtesy of Heyden Newport Chemical Corp., New York, N. Y.
[4]Butyl Parasept, purified; supplied through the courtesy of Heyden Newport Chemical Corp., New York, N. Y.
[5]"Carbowax" 4000; supplied through the courtesy of Union Carbide Chemicals Co., New York, N. Y.
[6]Plaskon Nylon, 0.5 mil thickness; supplied through the courtesy of Central Research Laboratories, Allied Chemical Corp., Morristown, N. J.
[7]Methyl methacrylate. Marketed as Plexiglass by the Rohm and Haas Co., Philadelphia, Pa.
[8]Supplied through the courtesy of Julius Schmid, Inc., New York, N. Y.

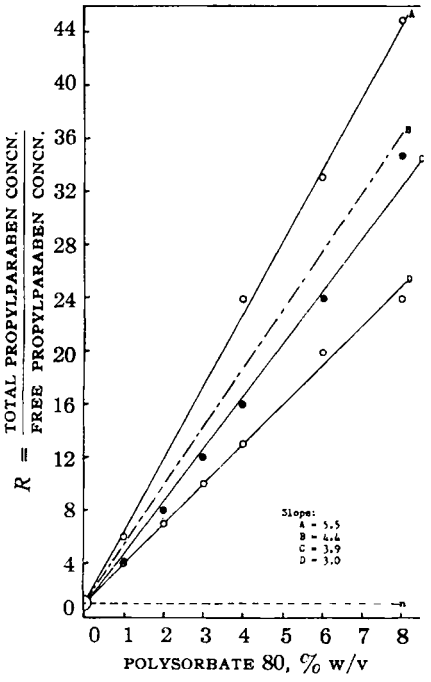


Fig. 3.—Binding of propylparaben by polysorbate 80 in aqueous solution at various temperatures. A, 20°; B, 30°. Data from Patel and Kostenbauder (1): C, 40°; D, 50°.

pendency as shown in Fig. 5. There was no indication of any change in the degree of binding of *p*-chlorophenol by polysorbate 80 at 40 and 50° (Fig. 6). Figures 5 and 6 illustrate that the binding tendency of polysorbate 80 toward phenols increases from phenol to *p*-chlorophenol. The slope value is 1.4 for *p*-chlorophenol compared to 0.25 for phenol, indicating that the magnitude of binding of *p*-chlorophenol with polysorbate 80 is in the order of approximately six times that of phenol.

Effect of Temperature on the Binding of Parabens by PEG 4000.—Solubilities of methyl and propylparaben were determined in aqueous solutions of PEG 4000 at 20° to observe the effect of this temperature on the extent of interactions compared to results previously reported at 30° (3). Curves *A* and *B* of Figs. 7 and 8 indicate that the extent of interaction of both the parabens with PEG increases with the increase in temperature.

DISCUSSION

Polysorbate 80.—Micellar solubilization of the phenolic preservative (7, 13) or the formation of molecular complexes of the type described by Higuchi and co-workers (10, 11) has been suggested as the possible mechanism of the interaction between a phenolic compound and polysorbate 80. A micelle of a nonionic surfactant (7) might be expected to provide an ideal model for the association with a phenol providing for both the possibility of hydrogen bond formation and hydrophobic interactions.

The hydrogen bonding tendency can easily be shown by correlating the magnitude of binding with proton-donating power of the phenolic compounds. The affinity of polysorbate 80 for *p*-chlorophenol is approximately six times greater than that for phenol. The substitution of a chlorine atom in the benzene ring increases the proton-donating power of phenol, making it more susceptible to hydrogen bond formation (14).

Higuchi and Lach (10) demonstrated that compounds such as phenol with acidic hydrogen would associate with electrophilic atoms such as oxygen; and they further suggested that such an interaction could be rendered favorable by "squeezing together" of the hydrophobic portions of the interacting molecules. This is a possible explanation of the experimental findings that as the hydrophobic character of the phenolic compound was increased from methyl to propyl to butylparaben, the binding affinities of these compounds for polysorbate 80 increased in the order of approximately 1:6:18.

Thermal agitation would be expected to exert two antagonistic tendencies on the binding of phenol by polysorbate 80. It would result in a decrease in association of phenol with polysorbate 80 by weakening attractive forces between them (11). On the other hand, it might be favored by the thermal desolvation of the polyoxyethylene chain. These two tendencies apparently nullify each other in the binding of phenol and *p*-chlorophenol by polysorbate 80. Furthermore, the molecular structure

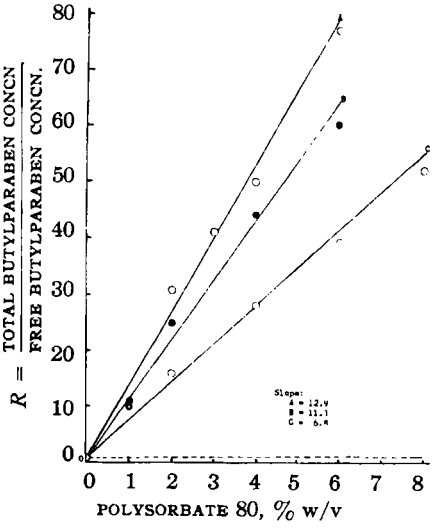


Fig. 4.—Binding of butylparaben by polysorbate 80 in aqueous solution at various temperatures. A, 30°; B, 40°; C, 50°.

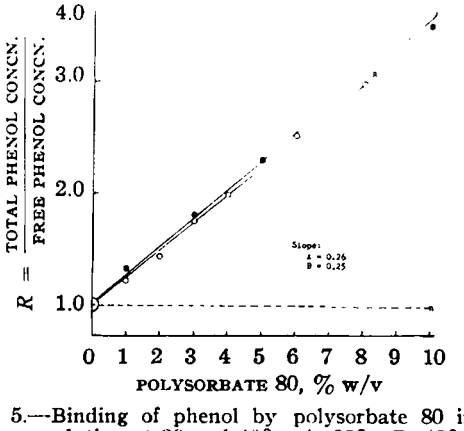


Fig. 5.—Binding of phenol by polysorbate 80 in aqueous solution at 30 and 40°. A, 30°; B, 40°.

of the interacting compounds and the steric hindrance may account for the decreased magnitude of binding of parabens by polysorbate 80 at higher temperatures. Addition of heat to such a system tends to increase rotation and vibration of the hydrocarbon moieties (7) of the ester linkages in both polysorbate 80 and paraben molecules. It seems probable that hydrogen-oxygen bonding would be sterically and statistically less favored at an elevated temperature. Phenol and *p*-chlorophenol with the absence of any ester linkage showed no temperature dependency, while butylparaben, having the largest ester linkage, showed a maximum temperature dependency.

Polyethylene Glycol 4000.—The effect of temperature on PEG-paraben interaction indicates that the binding increases with temperature. These interactions would be favored by thermal desolvation of the polymer chain, thus providing more sites for attachment for the competing interaction with paraben (11). Addition of heat might result also in a decrease in association of paraben with the polymer by weakening attraction forces between them. In addition, there is no steric hindrance due to ester linkage which is present in polysorbate 80. Thus, it is likely that desolvation of the polymer chain, allowing the competing paraben molecules to take part in the hydrogen bonding and "squeezing together" of the hydrophobic moieties (10) without any steric hindrance contribute to the stability of the complex at a higher temperature.

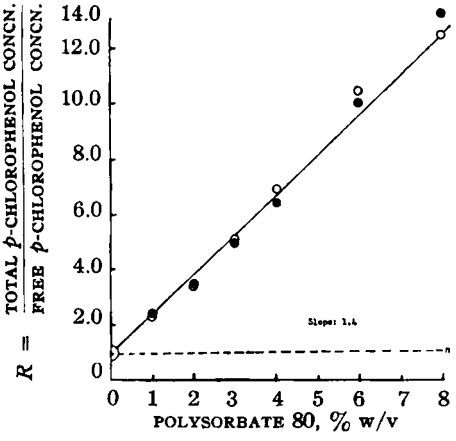


Fig. 6.—Binding of *p*-chlorophenol by polysorbate 80 in aqueous solution at 40 and 50°. O, 40°; ●, 50°.

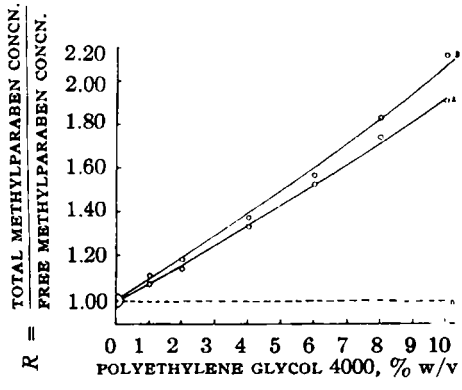


Fig. 7.—Ratio, *R*, of total methylparaben concentration to free methylparaben concentration as a function of the concentration of PEG 4000 at 20 and 30°. A, 20°; B, 30°. Data from Miyawaki, Patel, and Kostenbauder (3).

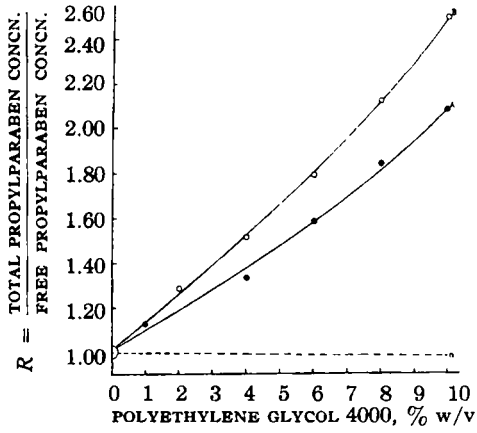


Fig. 8.—Ratio, *R*, of total propylparaben concentration to free propylparaben concentration as a function of the concentration of PEG 4000 at 20 and 30°. A, 20°; B, 30°. Data from Miyawaki, Patel, and Kostenbauder (3).

SUMMARY

The interaction of methyl, propyl, and butylparaben, phenol and *p*-chlorophenol with polysorbate 80 has been studied quantitatively by means of a dialysis technique utilizing a plexiglas dialysis cell and a semipermeable membrane.

The degree of binding of polysorbate 80 by methyl, propyl, and butylparaben increased approximately in the order of 1:6:18.

The extent of this intermolecular association was dependent upon the relative proton-donating properties of the phenolic compound.

The binding affinity of polysorbate 80 for the parabens employed in this study decreased with increase in temperature, whereas that of polyethylene glycol 4000 showed an increase. The phenol-polysorbate 80 interaction was found to be temperature independent.

Interpretation of these data regarding the possible mechanism of interaction of a phenolic compound with macromolecules of the polyoxyethylene type has been discussed.

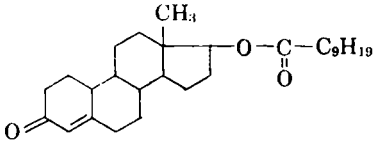
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Qualitative and Quantitative Tests for Nandrolone Decanoate

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drugs concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation. ***Drug Standards Laboratory***

19-NOR-Δ^4-ANDROSTENE-17β-OL-3-ONE decanoate; $C_{28}H_{44}O_3$; M.W. 428.66. The structural formula of nandrolone decanoate may be represented as follows:



Physical Properties.—Nandrolone decanoate occurs as a white to creamy white crystalline powder which is odorless or may have a slight odor, m.p. 33–37° (U.S.P. XVI, Class Ia). It is very soluble in acetone, in alcohol, in dioxane, and in methanol, and is practically insoluble in water.

Identity Tests.—A 1:100,000 solution of nandrolone decanoate in alcohol exhibits an ultraviolet absorbance maximum at about 239 mμ [absorptivity (1%, 1 cm.) about 400]. The ultraviolet absorption spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of nandrolone decanoate in potassium bromide in a disk of about 0.82-mm. thickness is shown in Fig. 2.

Mark a 4 × 19-in. strip of filter paper (Whatman No. 1 or the equivalent) with a fold line 3 in. from one end. Mark the starting line 2¼ in. below the fold line. Impregnate the strip with immobile solvent consisting of a 2:1:3 mixture of propylene glycol, 2-phenoxyethanol, and methanol. Blot the strip with dry filter paper and hang it in a hood for a few minutes to dry. Prepare 1% solutions of nandrolone decanoate (*Test preparation*) and nandrolone decanoate reference standard (*Standard preparation*) in methanol and spot the paper at the starting line as follows: Apply 5.0 μl. of the *Test preparation* (50 mcg.) to one spot, 5.0 μl. of the *Standard preparation* (50 mcg.) to a second spot, and 2.5 μl. each of the *Test preparation* and of the *Standard preparation* to a third spot. Place the strip in a tank arranged for descending chromatography, add an excess of *n*-heptane to the bottom of the tank, and equilibrate the system for 1 hour. Add the mobile solvent, consisting of a 2:1:200 mixture of propylene glycol, 2-phenoxyethanol, and *n*-heptane, seal the tank, and allow the solvent front to pass to the bottom of the strip. Remove the strip from the tank, and hang it in a hood for a few minutes to air-dry. Heat the strip in an oven at 100° for 30 minutes. allow it to cool to room temperature, and pass it through a 0.25% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. Alternatively, spray the strip with the reagent by means of a suitable atomizer Red spots appear slowly at room temperature. The spot obtained with the *Test preparation* agrees in color and R_f value with that obtained with the *Standard preparation*. The chromatogram obtained with the mixed spot is monodisperse.

Purity Tests.—Dry about 250 mg. of nandrolone decanoate, accurately weighed, in a vacuum desiccator over phosphorus pentoxide for 4 hours. The loss in weight does not exceed 0.5%.

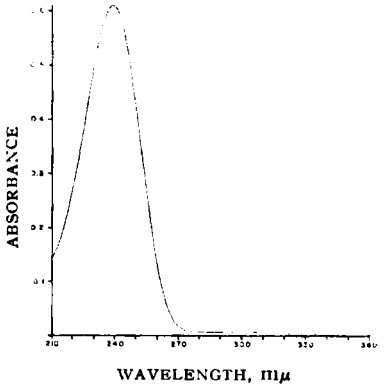


Fig. 1.—Ultraviolet absorption spectrum of nandrolone decanoate in alcohol (15 mcg. per ml.); Beckman model DK-2A spectrophotometer.

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Accepted for publication October 14, 1963.

Organon, Inc., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

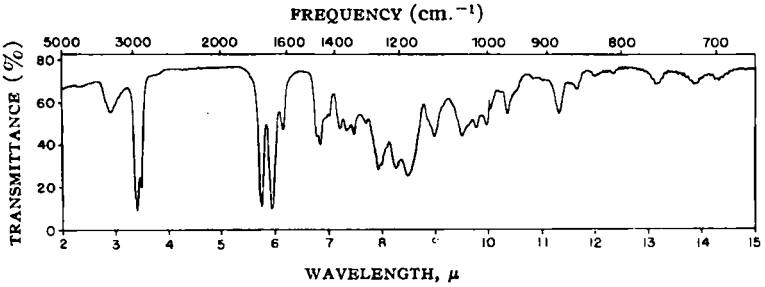


Fig 2.—Infrared spectrum of nandrolone decanoate in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

Determine the optical rotation of a 1% solution of nandrolone decanoate in dioxane by the method of U.S.P. XVI. The specific rotation is between +32 and +36°.

Prepare a 2% solution of nandrolone decanoate in dioxane: the solution is clear and is free of mechanical impurities.

Assay

Assay Preparation.—Transfer about 25 mg. of nandrolone decanoate, accurately weighed, to a 100-ml. volumetric flask, dilute to volume with methanol, and mix. Transfer 10.0 ml. of this solution to a 100-ml. volumetric flask, dilute to volume with methanol, and mix.

Isoniazid Reagent.—Transfer 500 mg. of isoniazid to a 500-ml. volumetric flask, dissolve it in methanol, add 0.625 ml. of hydrochloric acid, dilute to volume with methanol, and mix.

Procedure.—Pipet 5 ml. of the *Assay Preparation* into a 10-ml. volumetric flask, add isoniazid reagent to volume, and mix. Allow to stand for 1 hour with occasional shaking. Determine the absorbance in a 1-cm. cell at 380 mμ with a suitable spectrophotometer *versus* a blank prepared by diluting isoniazid reagent with an equal volume of methanol. Calculate the quantity, in mg., of $C_{28}H_{44}O_3$ in the portion of nandrolone decanoate taken by the formula $W \times A_u/A_s$, where A_u is the absorbance of the solution, A_s is the absorbance of a similarly prepared solution of nandrolone decanoate reference standard, and W is the weight of nandrolone decanoate reference standard taken. The amount of nandrolone decanoate found, on the anhydrous basis, is not less than 96.0% and not more than 104.0% of $C_{28}H_{44}O_3$.

DOSAGE FORMS OF NANDROLONE DECANOATE

Nandrolone Decanoate Injection

A sterile solution of nandrolone decanoate in vegetable oil.

Identity Test.—Prepare a 2 × 19-in. strip of filter paper as in the assay for nandrolone decanoate injection. Apply 10 μl. each of the *Assay preparation* and of the *Standard preparation* to the starting line, 3/4 in. apart, and develop the chromatogram in the same manner as in the assay. After drying at 100° for 30 minutes, allow the strip to cool to room temperature, and pass it through a 0.25% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. Alternatively, spray the strip with the reagent by means of a suitable atomizer. Red spots appear slowly at room temperature. The spot obtained with the *Assay preparation* agrees in color and R_f value with that obtained with the *Standard preparation*. No secondary spots appear.

Assay.—*Standard preparation.*—Dissolve about 50 mg. of nandrolone decanoate reference standard, accurately weighed, in sufficient acetone to make 10.0 ml., and mix.

Assay preparation.—By means of a pipet calibrated "to contain," transfer a volume of nandrolone decanoate injection, equivalent to about 50 mg. of nandrolone decanoate, to a 10-ml. volumetric flask, rinse the pipet with small portions of acetone, dilute to volume with acetone, and mix.

Isoniazid reagent.—Transfer 500 mg. of isoniazid to a 1000-ml. volumetric flask, dissolve it in methanol, add 0.625 ml. of hydrochloric acid, dilute to volume with methanol, and mix.

Procedure.—Prepare six 1 1/2 × 19-in. strips of filter paper (Whatman No. 1 or the equivalent) as follows. Mark the fold line 3 in. from one end, and the starting line 2 1/4 in. below the fold line. Wash the strips twice with methanol and hang them in a hood to air-dry. Impregnate the strips with immobile solvent, consisting of a 1:1:2 mixture of propylene glycol, 2-phenoxyethanol, and methanol. Blot the strips with dry filter paper, and hang them in a hood for a few minutes to dry.

By means of micropipets, spot three of the strips at the starting line with 10.0 μl. of the *Standard preparation* and the other three with 10.0 μl. of the *Assay preparation*. Place the strips in a tank arranged for descending chromatography, add an excess of *n*-heptane to the bottom of the tank, and equilibrate the system for 1 hour. Add the mobile solvent, consisting of a 1:1:200 mixture of propylene glycol, 2-phenoxyethanol, and *n*-heptane, seal the tank, and allow the chromatogram to develop until the solvent front reaches the end of the paper (2–3 hours). Remove the strips from the tank, hang them in a hood for 15 minutes to air-dry, and heat them in an oven at 100° for 30 minutes. By means of a short wavelength ultraviolet lamp (maximum emission at about 254 mμ), locate the positions of the spots, which appear dark purple against a light purple background. Outline the spots lightly with pencil, and cut the strips transversely at a distance of 3 cm. above and below the center of the spot. For the blank determinations, cut a second 6-cm. segment from the portion of each strip which lies immediately below the excised segment. Roll the twelve 6-cm. segments and place each in an 18 × 150-mm. test tube. To each tube add 4.0 ml. of isoniazid reagent, thoroughly wetting the paper. Close the tubes and allow them to stand for 1 hour at room temperature with occasional agitation. Decant the contents of each test tube into separate 15-ml. centrifuge tubes, and centrifuge at 5000

r.p.m. to remove any filter paper fibers. Decant the clear solutions into 1-cm. cells and determine their absorbances in a suitable spectrophotometer at 380 mμ *versus* the isoniazid reagent. Correct the absorbances for their respective blanks, and average the three absorbances obtained with the *Standard preparation* and the three obtained with the *Assay preparation*. Record the average absorbance obtained with the *Standard preparation* as A_s and that obtained with the *Assay preparation* as A_u and calculate the quantity, in milligrams, of $C_{28}H_{44}O_3$ in the volume of the sample taken by the formula $W \times A_u/A_s$, in which W represents the quantity, in milligrams, of nandrolone decanoate reference standard taken. The amount of nandrolone decanoate is not less than 90.0% and not more than 115.0% of the labeled amount of $C_{28}H_{44}O_3$.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Nandrolone decanoate[1] is a long-acting anabolic agent which is chemically related to the androgenic steroids. Nandrolone differs from testosterone in the absence of a methyl group in the 19 position of the molecule.

Identity Tests.—Due to its possession of physical and chemical properties which are closely related to those of similar steroidal compounds, a specific paper chromatographic identity test is included in the monograph for the bulk material. A direct comparison of the R_f value (about 0.4 in the system used) with that of authentic material serves to aid in the identification of the compound. The 2,4-dinitrophenylhydrazine reagent which is used to locate the spots on the developed chromatogram further aids in identification by establishing the compound as a member of the ketosteroid class.

Quantitative Methods.—The quantitative methods included in the monograph were chosen from many which have been applied to this class of compounds. Due to the presence of an α,β-unsaturated carbonyl group in ring A of the steroid molecule, nandrolone decanoate may be assayed spectrophotometrically (1, 2), gravimetrically as the semicarbazone (3) or as the 2,4-dinitrophenylhydrazone (4), and colorimetrically following its reaction with isoniazid (5) or other reagents (6).

[1] Marketed as Deca Durabolin by Organon, Inc., West Orange, N.J.

The colorimetric determination of nandrolone decanoate with isoniazid (isonicotinic acid hydrazide) is suitably accurate and precise as an assay for the bulk material. Beer's law is obeyed for final solutions covering an approximate concentration range of 5–40 mcg. per ml. Hence, the reaction also possesses the sensitivity required of an assay method to be used in conjunction with paper chromatography.

The quantitative paper chromatographic assay for nandrolone decanoate in the dosage form is highly specific for the intact molecule. Washing of the paper with methanol prior to impregnation with the immobile phase removes traces of material which could later contribute to the absorbance measurements. Variations in blank absorbances were noted when separate paper strips were used for the blank determinations. These variations were reduced considerably by applying a blank correction obtained with a segment of each paper used in the assay. While this may not be an acceptable practice in many instances, no interfering absorbance due to artifacts was found in the segments used, and a general increase in precision was obtained. Where interference, due to decomposition products or other extraneous material in the dosage form exists, this method of blank determination should not be used. A discussion of the factors involved in this type of assay may be found in a recent publication by Roberts and Florey (7).

Analysis of a commercial preparation of nandrolone decanoate injection gave an average value of 97.4 ± 2.4%[2] of the labeled amount. A synthetic sample (similar in composition to the commercial product) containing 50 mg. per ml. of nandrolone decanoate plus 10% benzyl alcohol in sesame oil was prepared and assayed. An average recovery of 98.9 ± 3.2%[2] of the theoretical amount of nandrolone decanoate was obtained. Recoveries and precisions agree with published figures for a similar assay method (7).

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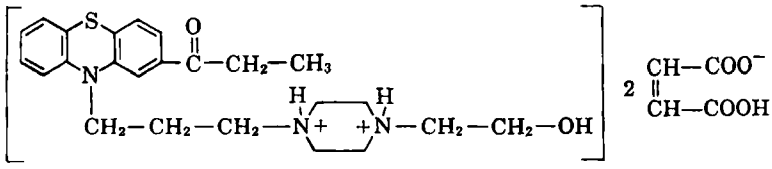
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[2] Maximum deviation from the mean value.

Qualitative and Quantitative Tests for Carphenazine Maleate

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drugs concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation. ***Drug Standards Laboratory***

1 - {10 - (3 - [4 - (2 - HYDROXYETHYL) - 1 - PIPERAZINYL] - PROPYL)PHENOTHIAZIN - 2 - YL} - 1 - PROPANONE BIS(HYDROGEN MALEATE); $C_{24}H_{31}N_3O_2S \cdot 2C_4H_4O_4$; M.W. 657.75. The structural formula of carphenazine maleate may be represented as



Physical Properties.—Carphenazine maleate occurs as a finely divided, odorless, yellow powder, m.p. 162–167°, with decomposition, U.S.P. XVI Class Ia. It is slightly soluble in alcohol and in water, and practically insoluble in ether. The pH of a 1% suspension in carbon dioxide–free water is between 2.5 and 3.5.

Identity Tests.—Dissolve 15 mg. of carphenazine maleate in 10 ml. of water. Place 5 drops of the solution in a depression of a white spot plate and add 1 drop of a solution of chloramine-T (1 in 10): a violet precipitate forms, slowly changing to gray. To another 5 drops of the solution add 1 drop of the buffered palladous chloride solution used in the *Assay:* a permanent red develops.

Dissolve 15 mg. of carphenazine maleate in 10 ml. of alcohol and place 1 drop of the solution on a piece of filter paper. Allow the solvent to evaporate and examine the residue under a short wavelength ultraviolet lamp (maximum emission at about 254 mμ): a yellow fluorescence is observed.

A 1 in 50,000 solution of carphenazine maleate in 0.1 *N* hydrochloric acid exhibits ultraviolet absorbance maxima at about 243 mμ [absorptivity (1%, 1 cm.) about 385] and 277 mμ, and minima at about 228 and 260 mμ. The ratio of the absorbances at 243 and 277 mμ is between 1.27 and 1.41. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of carphenazine maleate in potassium bromide, in a disk of about 0.82-mm. thickness, is shown in Fig. 2.

Purity Tests.—Determine the water content of carphenazine maleate by the titrimetric (Karl Fischer) method: carphenazine maleate contains not more than 1% water.

Char about 1 Gm. of carphenazine maleate, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until the evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.2%.

Assay

Palladous Chloride Stock Solution.—Transfer 500 mg. of palladous chloride to a 250-ml. beaker, add 5 ml. of hydrochloric acid, and warm the mixture on a steam bath. Add 200 ml. of hot water in small portions until solution is complete, cool, transfer the solution to a 500-ml. volumetric flask, dilute to volume with water, and mix.

Buffered Palladous Chloride Solution.—Transfer 75 ml. of the *Palladous chloride stock solution* to a 500-ml. volumetric flask, add 50 ml. of 1 *N* sodium

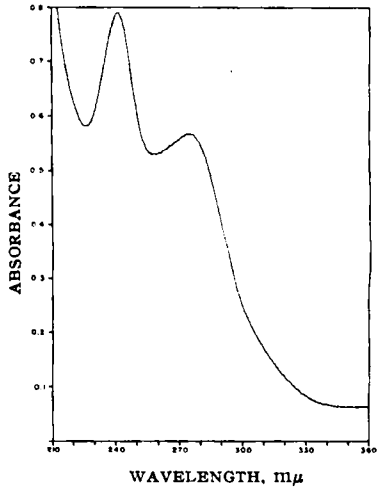


Fig. 1.—Ultraviolet absorption spectrum of carphenazine maleate in 0.1 *N* hydrochloric acid (2 mcg. per ml.); Beckman model DK-2A spectrophotometer.

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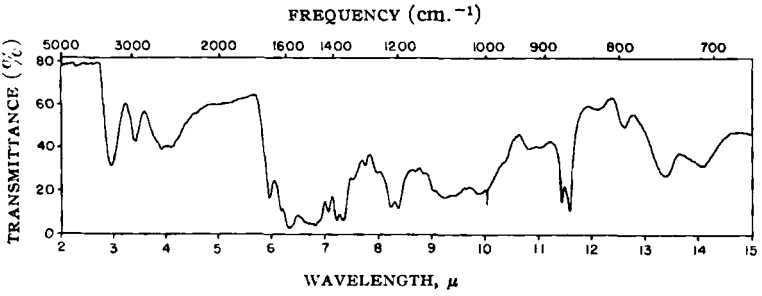


Fig. 2.—Infrared spectrum of carphenazine maleate in potassium bromide disk (0.5%): Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

acetate solution and 44 ml. of 1 *N* hydrochloric acid, dilute to volume with water, and mix.

Standard Preparation.—Transfer about 50 mg. of carphenazine maleate reference standard, previously dried in vacuum over phosphorus pentoxide for 18 hours and accurately weighed, to a 250-ml. volumetric flask. Dissolve it in about 100 ml. of diluted alcohol, add diluted alcohol to volume, and mix.

Procedure.—Transfer about 50 mg. of carphenazine maleate, accurately weighed, to a 250-ml. volumetric flask; dissolve it in about 100 ml. of diluted alcohol, add diluted alcohol to volume, and mix. Pipet 4 ml. of *Buffered palladous chloride solution* into each of three glass-stoppered test tubes. Pipet 3 ml. of the assay solution and of the *Standard preparation*, respectively, into separate tubes and prepare a blank by adding 3.0 ml. of diluted alcohol to the remaining tube. Mix the contents of each tube thoroughly; within 10 minutes spectrophotometrically determine the absorbances of the solutions containing carphenazine maleate, in 1-cm. cells, at the wavelength of maximum absorbance at about 490 mμ, using the blank to set the instrument to zero absorbance. Record the absorbance of the solution from the *Standard preparation* as A_s and that from the assay solution as A_u and calculate the weight, in milligrams, of carphenazine maleate in the portion of carphenazine maleate taken by the formula $W \times A_u/A_s$, where W is the weight, in milligrams, of carphenazine maleate reference standard taken. The amount of carphenazine maleate found, on the anhydrous basis, is not less than 98.0% and not more than 102.0% of the weight of the sample taken

DOSAGE FORMS OF CARPHENAZINE MALEATE

Carphenazine Maleate Tablets

Identity Tests.—Transfer to a glass-stoppered 250-ml. conical flask an amount of powdered tablets equivalent to about 25 mg. of carphenazine maleate. Add 100 ml. of alcohol, shake occasionally during 15 minutes, and filter through a medium porosity sintered-glass filter, discarding the first few milliliters of filtrate. Evaporate 6 ml. of the filtrate to dryness on a water bath with the aid of a current of air and dissolve the residue in 1 ml. of water: the solution responds to the two color identity tests under carphenazine maleate.

Evaporate 4 ml. of the filtrate obtained in the previous test to dryness on a water bath with the aid of a current of air and dissolve the residue in 50 ml. of 0.1 *N* hydrochloric acid: the solution exhibits ultraviolet absorbance maxima at about 243 and 277 mμ, and the ratio of the absorbances at these wavelengths is between 1.27 and 1.41.

Assay.—Weigh and finely powder not less than 20 carphenazine maleate tablets. Transfer an amount of powder equivalent to about 20 mg. of carphenazine maleate, accurately weighed, to a medium porosity sintered-glass funnel. Fit the funnel into the neck of a vacuum bell jar so that the stem discharges into a 100-ml. volumetric flask containing 50.0 ml. of water. Extract the powder with successive 5-ml. portions of alcohol until the flask is filled almost to the mark. Dilute to volume with alcohol, mix, and readjust the volume to the mark with alcohol. Proceed as directed in the *Assay* under carphenazine maleate, beginning with "Pipet 4 ml. of *Buffered palladous chloride solution*. . . ." The amount of carphenazine maleate found is not less than 95.0% and not more than 110.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Carphenazine maleate[1] is a phenothiazine derivative which is used in the management of chronic psychosis.

Identity Tests.—Carphenazine maleate was found to undergo the typical color reactions of phenothiazines with the eight test reagents described by Yung and Pernarowski (1). The reactions obtained with the chloramine-T and palladous chloride reagents are particularly distinctive and together serve to differentiate carphenazine maleate from the 13 phenothiazine tranquilizers reported in their paper. The fluorescence test is nonspecific and represents a typical class property. Identification is further aided by the characteristic ultraviolet and infrared spectra.

Attempts to prepare sharp-melting solid derivatives of carphenazine maleate were unsuccessful. The picrate, reineckate, and 2,4-dinitrophenylhydrazone derivatives were obtained but could not be recrystallized to yield sharp-melting products.

When the ultraviolet absorption identity test was performed on commercial 25-mg. tablets, the ratio of the absorbances at 243 and 277 mμ was 1.310. A similar test sample was purified by extraction with ether from alkaline medium, evaporation of the solvent, and dissolution of the residue in dilute hydrochloric acid. The ratio of absorbances obtained with this solution was 1.316. Purification, therefore, was not necessary for identification purposes.

[1] Marketed as Proketazine Maleate tablets by Wyeth Laboratories, Philadelphia, Pa.

Quantitative Methods.—The red palladium (II)–carphenazine color complex developed in the colorimetric assay forms immediately on addition of the test solution to the palladous chloride reagent and is stable for about 15 minutes. The color fades slowly thereafter, and the absorbance of the solution decreases about 2.5% in 3.5 hours. With reagent containing 0.015% palladous chloride, the colors produced by solutions of carphenazine maleate obeyed Beers law in the concentration range of 0.0 to 0.3 mg. per ml. Reagents containing 0.005 and 0.010% palladous chloride failed to produce linear concentration-absorbance plots with the same concentration range of carphenazine maleate. The acidity of the reagents was kept constant throughout these tests (pH 2.1 to 2.2). Analysis of commercial tablets labeled to contain 25 mg. of carphenazine maleate gave an average value of 96.7 ± 1.0%.[2]

[2] Maximum deviation from the mean value.

Repetitive analyses performed on the same tablet extracts were more precise so that the gross deviation may be attributed mainly to variations in the extraction procedure.

Samples of carphenazine maleate dissolved in glacial acetic acid and in a 4:1 mixture of chloroform and acetonitrile were titrated potentiometrically with 0.1 *N* perchloric acid in glacial acetic acid and in dioxane, respectively. The former system gave a sharp break (about 100 mv.) in the curve, equivalent to 98.1% of the amount of carphenazine maleate taken; the latter solvent system was unsatisfactory. In both systems a small amount of a dark gummy precipitate (soluble in acetone) formed as the titration progressed.

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(1) Yung, D. K., and Pernarowski, M., This Journal, 52, 365(1963).

Technical Articles

Improved Viable Counting Method for Petrolatum-Based Ointments

By W. T. SOKOLSKI and C. G. CHIDESTER

A filtration method for recovery of viable micro-organisms from petrolatum-based ointments is described. In this method the ointment is dissolved in isopropyl myristate and passed through a filter. Advantages of the method are that all the micro-organisms in the ointment sample are concentrated on the filter pad, and the filter pad can be rinsed to remove antibacterial agents and traces of petrolatum. Markedly higher recoveries were obtained by the proposed method than by methods in common use.

Current viable counting methods for petrolatum-based ointments involve either (*a*) smearing the ointment directly on the surface of an agar plate (1, 2) or (*b*) extracting the micro-organisms from ointment by shaking with water (3, 4) and plating the aqueous phase. There have been conflicting reports concerning the sterility of ophthalmic ointments. Using an extraction method, Vander Wyk and Granston (3) found that 85% of the ointments they tested were contaminated with micro-organisms; Bowman and Holdowsky (4) found only 10% contaminated in a similar method. Neither report included a test of the effectiveness of the extraction method—such as an attempt to recover a known inoculum from ointment.

One objection to the use of aqueous extraction methods for ointments that contain antimicrobial agents is that the resulting concentrations

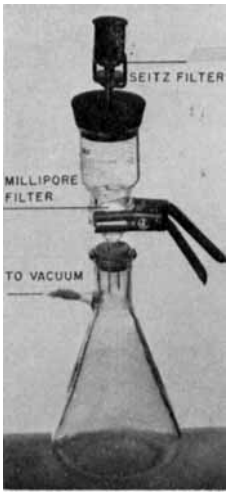


Fig. 1.—Filter unit for viable count or sterility testing of petrolatum-based ointments.

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The authors are grateful to Mrs. L. K. Schadewald, Miss A. Dilley, Mr. E. M. Stapert, and Mr. J. W. Slade, The Upjohn Co., for their technical assistance, and to Dr. Eino Nelson, University of Buffalo, Buffalo, N. Y., for suggesting isopropyl myristate as an ointment solvent.

Quantitative Methods.—The red palladium (II)–carphenazine color complex developed in the colorimetric assay forms immediately on addition of the test solution to the palladous chloride reagent and is stable for about 15 minutes. The color fades slowly thereafter, and the absorbance of the solution decreases about 2.5% in 3.5 hours. With reagent containing 0.015% palladous chloride, the colors produced by solutions of carphenazine maleate obeyed Beers law in the concentration range of 0.0 to 0.3 mg. per ml. Reagents containing 0.005 and 0.010% palladous chloride failed to produce linear concentration-absorbance plots with the same concentration range of carphenazine maleate. The acidity of the reagents was kept constant throughout these tests (pH 2.1 to 2.2). Analysis of commercial tablets labeled to contain 25 mg. of carphenazine maleate gave an average value of 96.7 ± 1.0%.[2]

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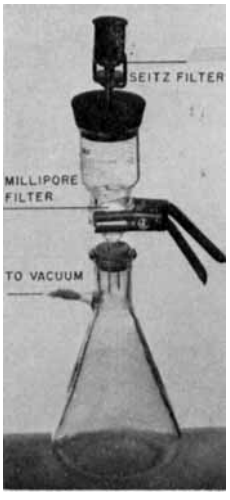


Fig. 1.—Filter unit for viable count or sterility testing of petrolatum-based ointments.

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TABLE I.—RECOVERY OF *Bacillus subtilis* FROM SEEDED CORTISONE ACETATE OPHTHALMIC OINTMENT

Experiment	Viable Count of Inoculum (Calcd. per Gm. Ointment)	Method of Viable Cells Recovered per Gm. Ointment: Proposed (Filtration)	I Agar Smear, 0.5 Gm./Plate (two plates)	II Water Extract	III Broth Extract
	32	22	0, 2	0	4
	29	11	0, 0	0	3
	36	9	1, 1	1	7
1	..	9	..	..	3
	39	26	1, 2	35[a]	..
	..	22	..	0	2
	..	..	..	1	4
Av. per Gm.	34	16	2	0–1	4
	54	39	3, 0	2	2
	48	4	1, 0	1	1
2	54	53	1, 0	4	4
	60	98	0, 0	2	1
	..	79	3, 1	0	0
	..	30	..	0	0
Av. per Gm.	54	50	2	1–2	1–2
	51	17	0, 0	4	2
	34	25	1, 0	1	0
3	30	15	0, 0	0	1
	34	24	1, 0	3	1
	..	44	0, 0	3	1
	..	..	..	0	1
Av. per Gm.	37	25	0–1	2	1
	43	50[a]	2, 0	0	0
	44	37	1, 0	1	3
4	..	35	0, 0	0	0
	..	35	1, 0	0	9
	..	..	1, 0	1	3
	..	..	..	..	2
Av. per Gm.	44	36	1	0–1	3
	98	42	1, 0	0	3
	..	52	0, 0	2	10
5	99	47	1, 0	3	2
	..	65	0, 0	1	22
	..	64	0, 0	3	2
	..	252[a]	0, 0	0	4
Av. per Gm.	99	54	0–1	1–2	7

[a] Aberrant observations according to U.S.P. XVI, Table I, p. 874. Responses not included in average.

of these agents in the aqueous phase and in the final growth medium could be high enough to inhibit growth. It is likely that many of the organisms become enveloped in petrolatum without access to nutrients with either an extraction method or an agar smear method. They may be viable but unable to multiply.

An effective viable counting method for ointments would be desirable—first, to determine whether the incidence of contamination in ophthalmic ointments is high enough to be a serious problem—and second, because the method could possibly be adapted to the sterility testing of ophthalmic ointments if this should become a requirement.

The purpose of this paper is to describe a filtration method similar to the one used by Holdowsky in a sterility test for antibiotics (5). In this method the entire ointment sample is dissolved and filtered, and the filter pad is rinsed for the removal of antimicrobial agents and traces of petrolatum. Comparisons of recoveries obtained by the proposed method and by current methods are made.

PROPOSED METHOD

Materials and Equipment.—Isopropyl myristate;[1] brain heart infusion broth (BHI) and nutrient broth;[2] polysorbate[3] 80; 47 mm., 0.45 μ, type HA filter pads, 47-mm. absorbent pads, and 250-ml. filter holders;[4] No. 13½ rubber stoppers; Seitz filter pads and filter holders; fine-tipped forceps; 50 mm.-Petri dishes with covers, were utilized.

Preparation of Materials and Equipment.—Prepare rinse and growth media. The basic rinse medium is nutrient broth with 1% polysorbate 80 added. The basic growth medium is BHI with

[1] Marketed as Deltyl Extra by Givaudan-Delawanna, Inc., New York, N. Y.
[2] Baltimore Biological Laboratories of Difco Laboratories, Baltimore, Md.
[3] Marketed as Tween 80 by Atlas Powder Co., Wilmington, Del.
[4] Millipore Filter Corp., Bedford, Mass.

TABLE II.—RECOVERY OF BACTERIA FROM OPHTHALMIC OINTMENTS AFTER SEEDING WITH APPROXIMATELY 70 CELLS/GM.

Ointment[a]	Active Ingredients per Gm. of Ointment	Viable Cells Recovered/ Gm.
Neo-Medrol	Neomycin sulfate, 5 mg. Methylprednisolone, 1 mg.	10
Neo-Delta-Cortef	Neomycin sulfate, 5 mg. Prednisolone acetate, 2.5 mg.	32
Neo-Cortef	Neomycin sulfate, 5 mg. Hydrocortisone acetate, 0.5 mg.	68
Baciguent	Bacitracin, 500 units Phenacaine hydrochloride, 20 mg.	21
Myciguent	Neomycin sulfate, 5 mg.	26
Mycitracin	Neomycin sulfate, 5 mg. Polymyxin B sulfate, 5000 units Bacitracin, 500 units	40
Penicillin G potassium	Potassium penicillin G, 100,000 units	19
Sulfathiazole	Sulfathiazole, 50 mg.	18
Neosone	Cortisone acetate, 15 mg. Neomycin sulfate, 5 mg.	35
Neo-Cortef with tetracaine	Neomycin sulfate, 5 mg. Tetracaine hydrochloride, 5 mg. Hydrocortisone acetate, 5 mg.	93
Mercury yellow oxide	Mercury yellow oxide, 10 mg.	0

[a] Trade names of The Upjohn Co., Kalamazoo, Mich.

0.1% polysorbate 80 added. For neomycin ointments, add 3% sodium chloride to the rinse and growth media; add 0.1% ascorbic acid to the growth medium. For penicillin ointments, add 200 kinetic units penicillinase per milliliter to the rinse and growth media after they have been autoclaved. For sulfonamide ointments, add 100 mcg. *p*-aminobenzoic acid per milliliter to the rinse and growth media.

Sterilize 100-ml. volumes of isopropyl myristate by dry heat at 200° for 2 hours. Autoclave the following for 20 minutes at 121°: growth medium; 500 ml. volumes of rinse medium; covered Petri dishes, each containing 2 absorbent pads; and individually wrapped forceps (for use in aseptic handling of bacterial filters). Sterilize filter pads by exposure to ethylene oxide. Assemble filter units as shown in Fig. 1 but do not include filter pads. Place absorbent pads between the sintered glass supports and the funnels for protection, and autoclave the complete unit at 121° for 30 minutes.

Procedure.—For each ointment to be tested, bring 500 ml. rinse medium and 100 ml. isopropyl myristate to temperature in a 47° water bath. Place a filter pad in a filter unit and moisten it with 0.3 ml. rinse medium; dissolve 1 Gm. of the ointment in the 47° isopropyl myristate. The sample should not be dissolved very long in advance of filtration because prolonged exposure to a temperature of 47° may be damaging to vegetative cells (6, 7).

Add the isopropyl myristate–ointment solution to the funnel over the filter pad. Apply a vacuum capable of pulling 550 mm. of mercury (ordinary house vacuum in our tests) until all the solution has passed through the filter pad. Rinse twice with 250-ml. aliquots of rinse medium. Allow all the liquid to pass through the filter pad, but do not let the filter pad become dry. With ointments containing antibacterial agents, the rinse medium should be added in five or more aliquots to ensure adequate rinsing. With neomycin ointments, the second or third rinse should be allowed to stand in the funnel for about 15 minutes.

Add 4 ml. of growth medium to a Petri dish containing absorbent pads. Aseptically remove the filter pad and place it on the absorbent pads. All the micro-organisms which were originally in the ointment sample should now be on the filter pad. Incubate the Petri dish at 32–35° and examine the filter pad for growth after 24 and 48 hours.

EXPERIMENTAL

Comparison of Methods

In five experiments, the proposed method was compared for efficiency in recovery of a known inoculum to three other procedures commonly used for the microbial examination of ointments. Since the time of exposure to the ointment environment necessarily varied between methods, conditions favoring survival of the inoculum in the ointment seemed desirable. For this reason we decided to inoculate with spores and to use an ointment which did not contain antibacterial agents.

Procedure for Each Experiment

Preparations for the proposed method (*see above*) were made. In addition, a Waring Blendor, a 30-ml. syringe, and 50-ml. Erlenmeyer flasks containing glass beads and either (*a*) 7 ml. distilled water or (*b*) 7 ml. BHI broth with 0.1% polysorbate 80 were sterilized by autoclaving for 20 minutes at 121°. Twenty by 100-mm. Petri dishes containing 28 ml. sterile BHI agar with 0.1% polysorbate 80 were prepared, and melted tubes of the same agar (for use in making poured plates) were placed in a 47° water bath. Twenty-eight grams of 1.5% cortisone acetate[5] ointment were thoroughly blended with 1 ml. of an aqueous *Bacillus subtilis* UC 564 spore suspension containing approximately 10^8 spores/ml. Poured-plate viable counts of 0.1-ml. aliquots of the spore suspension were made. The seeded ointment was heated to 47° and distributed from a syringe.

The proposed method, as described previously, was followed.

[5] The Upjohn Co., Kalamazoo, Mich.

TABLE III.—RECOVERY OF VEGETATIVE CELLS FROM ISOPROPYL MYRISTATE

Organism	Viable Count of Inoculum	Viable Cells Recovered
K. pneumoniae	72	22
P. vulgaris	Spreaders[a]	Spreaders[a]
Ps. aeruginosa	*ca.* 560	Spreaders[a]
S. aureus	*ca.* 1000	*ca.* 800
S. lutea	46	29
C. albicans	52	43
S. cerevisiae	61	52
Penicillium sp.	Dense growth[a]	Very dense growth[b]

[a] Unable to count because colonies grew together. [b] Colonies were too numerous to count.

Method I.—Each 1-Gm. sample of seeded ointment was divided between two Petri plates. The ointment was added directly to the surface of the agar and spread with a sterile glass rod.

Method II.—Each gram of seeded ointment was added to 7 ml. water. The flasks were heated for 10 minutes in a 47° water bath, then shaken vigorously for 1 hour on a wrist action shaker at room temperature. The entire aqueous phase was plated with 28 ml. of agar.

Method III.—This was the same as *Method II*, except that broth was used to extract the ointment instead of water and the flasks were shaken at 35°. This method was included in the comparison because some oil and petrolatum products have been tested for sterility by incubation in nutrient media (8–10).

All plates were incubated at 35° and examined for growth after 24 and 48 hours. Table I gives 48-hour counts.

Tests of Unseeded Cortisone Acetate

The cortisone acetate ointment used in the comparison of methods was not sterilized. To determine the number of organisms present in the ointment prior to seeding, seven 1-Gm. samples were tested by the proposed method.

Tests of Additional Ointments

Recovery of a known inoculum by the proposed method was also tested with 11 additional ophthalmic ointments, all containing antibacterial agents. Because it would have been difficult to blend an inoculum uniformly in 1-Gm. quantities of ointments, the ointment-isopropyl myristate solutions were inoculated and mixed by vigorous shaking. Each solution was inoculated with 0.1 ml. of an aqueous *B. subtilis* spore suspension. Poured-plate viable counts were made to determine the number of spores in the inoculum.

RESULTS AND DISCUSSION

The data in Table I show that markedly higher recoveries of bacteria from seeded ointment were obtained with the proposed filtration method than with the other methods. This was probably due to the removal of the organisms from the petrolatum environment. Poor recoveries with *Method I* were not surprising since one would expect most of the organisms to be coated with petrolatum. However, recoveries with extraction *Methods II* and *III* were not much higher. Possibly the majority of organisms were not extracted. If they were extracted they may have retained a petrolatum coating.

The test ointment used in the recovery study (Table I) did not contain antibacterial agents. If sulfonamides or water-soluble antibiotics had been present, they would have been transferred to the viable count media in *Methods I, II,* and *III*. Since most antimicrobial agents are more active against growing cells than against dormant cells, cells which could survive in ointment might be inhibited in the viable count medium. Although the proper additives can antagonize the activity of some drugs, it would be advantageous to remove the viable cells from the drug environment.

In viable counts of unseeded cortisone acetate, an average of seven bacteria per gram were recovered of which six resembled *Bacillus sp.* The recoveries given in Table I include all colonies resembling the inoculum.

Recoveries obtained by the proposed method from seeded ointments containing antibacterial agents are given in Table II. Viable cells were recovered from all ointments except mercury yellow oxide.[6] The addition of thioglycollic acid to the medium for antagonizing the mercury salt (11) was not attempted. In preliminary tests of neomycin ointments, recoveries were very low. This may have been due to adsorption of neomycin to the cellulose ester filter pads. Sodium chloride, which is known to desorb neomycin from cellulose (12), was added to the rinse medium and the second or third rinse was allowed to stand in the funnel for 15 minutes. As an additional precaution, two known inhibitors of neomycin activity, sodium chloride (12) and ascorbic acid (13), were added to the growth medium. In subsequent tests of neomycin ointments, recoveries were satisfactory.

The next consideration was the possibility that isopropyl myristate would be toxic to vegetative microbial cells. Isopropyl myristate is reported to be nontoxic to humans since "drugs and cosmetics containing this substance may be safely applied topically and parenterally" (14). This oil is the isopropyl ester of a saturated fatty acid and should be less dehydrating than unsaturated vegetable oils. Table III indicates that recoveries of viable vegetative cells from isopropyl myristate were comparable to recoveries of spores.

The proposed method could be used for sterility testing of ointments with simple modifications. As described by Holdowsky (5), a 25-mm. disk (approximately one-half the filtering area) could be cut from the filter pad for incubation in fluid thioglycollate medium while the remainder is incubated in Sabouraud's medium. An apparatus consisting of a series of filter units is now commercially available.[7] The units are fitted so that rinse medium may be supplied aseptically. However, it is necessary to remove the rubber stopper from the funnel to add the test solution. To reduce the risk of contamination from air, an additional opening could be made in the rubber stopper to accommodate a hypodermic needle. The test solution could then be added with a syringe.

SUMMARY

A filtration method for the recovery and enu-

[6] The Upjohn Co., Kalamazoo, Mich.
[7] Millipore Filter Corp., Bedford, Mass.

meration of viable organisms from petrolatum-based ointments was described and shown to be more efficient than methods now commonly used. An application of the method to the sterility testing of ointments was discussed.

REFERENCES

(1) *Federal Register*, 12, 2223(April 4, 1947), p. 141.8 (c).
(2) Lehrfeld, L., and Donnelly, E. G., *Arch. Opthalmol.*, 40, 39(1948).
(3) Vander Wyk, R. W., and Granston, A. E., THIS JOURNAL, 47, 193(1958).
(4) Bowman, F. W., and Holdowsky, S., *ibid.*, 48, 95(1959).
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(9) "United States Pharmacopeia," 14th rev., Mack Printing Co., Easton, Pa., p. 87.
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(11) Marshall, M. S., Gunnison, J. B., and Luxen, M. T., *Proc. Soc. Exptl. Biol. Med.*, 43, 672(1940).
(12) Sokolski, W. T., and Lummis, N. E., *Antibiot. Chemotherapy*, 11, 271(1961).
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Notes

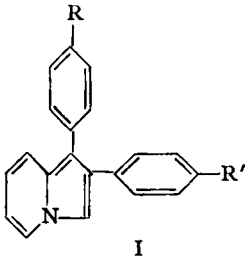
Preparation of *m*- and *p*-Nitrobenzoyl Derivatives of Some Arylindolizines

By VINCENT S. VENTURELLA†

Investigations into the benzoylations of substituted arylindolizines has shown that the reaction is reduced but not prevented by steric or electronic effects.

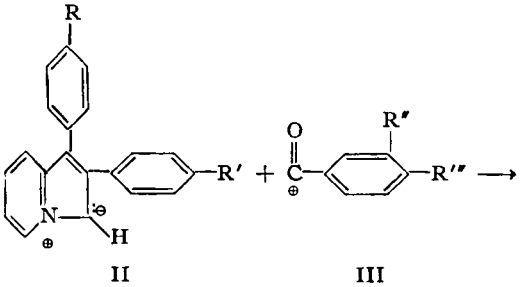
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Since hydrolysis of the end product was previously (1) found not appreciable if the reaction was performed in benzene solution below 60° for 1 hour, followed by allowing the mixture to stand 48 hours at room temperature, any change in the formation of the benzoyl derivatives would presumably be because of an increase or decrease in the formation of III.

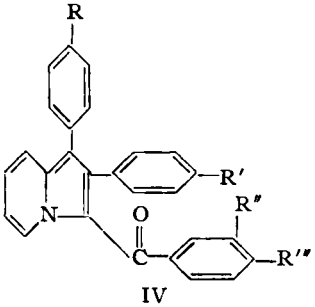


I

I *a*, R = H, R′ = Br
b, R = Cl, R′ = Br
c, R = H, R′ = NO_2
d, R = Cl, R′ = NO_2



II III



IV

IV *a*, R = R″ = H, R′ = Br, R‴ = NO_2
b, R = R‴ = H, R′ = Br, R″ = NO_2
c, R″ = H, R = Cl, R′ = Br, R‴ = NO_2
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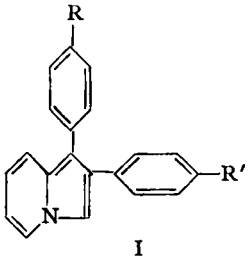
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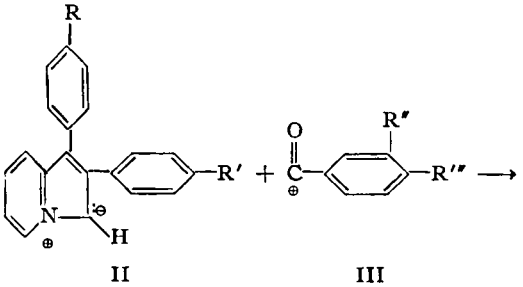
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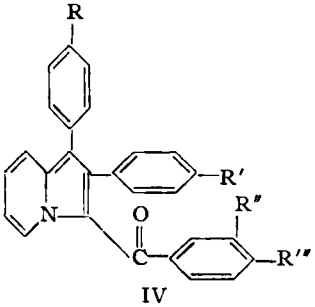


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TABLE I.—SUBSTITUTED BENZOYLINDOLIZINES

Compd.	M.p., °C.(Recrystn. Solv.)	Yield, %	Anal. Calcd. C	H	N	Found C	H	N
IV*a*	220.5 to 222.0, dec. (hot C_6H_6)	66	65.20	3.42	5.63	65.60	3.45	5.34
IV*b*	177 to 178.5 (boiling EtOH)	89	65.20	3.42	5.63	65.55	3.60	5.26
IV*c*	218 to 219.5, dec. (C_6H_6-Petroleum ether)	38	61.01	3.01	5.27	61.14	3.22	5.33
IV*d*	202 to 203.5 (C_6H_6)	64	61.01	3.01	5.27	60.61	3.18	5.48
IV*e*	245.5 to 247.0 (hot C_6H_6)	54	69.99	3.67	9.07	70.29	3.90	9.22
IV*f*	222 to 223.5	46	69.99	3.67	9.07	70.18	3.77	8.78

To test this theory, Compounds I *a*, *b*, and *c* were reacted with *p*- and *m*-nitrobenzoyl chlorides. The *p*-nitro would tend to decrease the formation of III by the conjugative mechanism (the inductive effect being negligible) and the *m*-nitro group would tend to favor the formation of III by a combination of conjugative and inductive effects (3).

These assumptions are followed with Compound I *a*, but fail in the reaction of I *b* with *m*-nitrobenzoyl chloride because the *p*-chlorophenyl substituent present in the 3 position of the indolizine does not favor the formation of II. This effect is also shown to provide a sharp decrease in the formation of IV *c*. A similar anomoly is shown between I *c* and *m*-nitrobenzoyl chloride because the steric repulsion of two proximal nitro groups is unfavorable to the formation of IV. However, the reaction of *p*-nitrobenzoyl chloride with I *c* follows the pattern set by Compound I *a*, *i.e.*, the *p*-nitro group has little effect upon the reactions in this series unless accompanied by a strong tendency to decrease the nucleophilic character of the 1 position of the indolizine nucleus.

Compounds IV *a* through *f* were prepared in the manner previously described (1); the analytical data appear in Table I.

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(1) Venturella, V. S., THIS JOURNAL, **52**, 868(1963).
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(3) Gould, E. S., "Mechanism and Structure in Organic Chemistry," Henry Holt and Co., New York, N. Y., 1959, pp. 218-219.

Quantitative Fluorometric Determination of Panthenol in Multivitamin Preparations

By RENÉ G. PANIER and JEAN A. CLOSE

A new procedure is described for the estimation of panthenol in a multivitamin preparation highly concentrated in sugar. The method involves the quantitative elimination of sugar by crystallization in the presence of ethanol, extraction of panthenol from the dry residue with chloroform, purification of the extract on ion-exchange resins, and final fluorometric determination of β-alanol after alkaline hydrolysis of the effluent. Fluorescence is developed by reaction of β-alanol with ninhydrin and *n*-butanal, for 45 minutes at 60° in the presence of carbonate buffer, pH 9.1. The fluorescent intensity measured at 465 mμ is proportional to the β-alanol concentration in the range between 0.1 and 1 mcg./ml.

DURING THE LAST 10 years, several papers have been published on the quantitative analysis of panthenol in multivitamin pharmaceutical preparations. Chemical methods have been proposed to replace the time consuming microbiological assay. It seems, however, that the problem of the determination of panthenol in highly concentrated sugar preparations has not been solved satisfactorily, and we were recently confronted with that question.

In 1949, Crockaert (1) showed that panthenol and calcium pantothenate could be determined after alkaline hydrolysis as β-alanol and β-alanine, respectively, with the use of 1,2-naphthoquinone-4-sulfonate, while Wollish and Schmall (2) proposed the dosage of pantoyl lactone as ferric hydroxamate, after acid cleaving of the panthenol.

More recently, Zappala and Simpson (3) described a colorimetric method for the determination of panthenol in multivitamin tablets containing 15% sugar. According to these authors, the method of Schmall and Wollish (4) is not applicable to the preparation highly concentrated in sucrose because of the darkening of the solution during the alkaline hydrolysis. They solved the difficulty by extraction of the powdered sample and further purification on ion-exchange resin before estimating β-alanol by chlorination and subsequent iodometry. Their method is unfortunately inapplicable to liquid multivitamin preparations, particularly to syrups with a very high sugar content (more than 50% sucrose).

Our purposes were to determine the panthenol in multivitamin preparations and to perfect a very sensitive routine method capable of answering our control and research problems.

EXPERIMENTAL

Reagents

All the reagents were analytical grade.

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Quantitative Fluorometric Determination of Panthenol in Multivitamin Preparations

By RENÉ G. PANIER and JEAN A. CLOSE

A new procedure is described for the estimation of panthenol in a multivitamin preparation highly concentrated in sugar. The method involves the quantitative elimination of sugar by crystallization in the presence of ethanol, extraction of panthenol from the dry residue with chloroform, purification of the extract on ion-exchange resins, and final fluorometric determination of β-alanol after alkaline hydrolysis of the effluent. Fluorescence is developed by reaction of β-alanol with ninhydrin and *n*-butanal, for 45 minutes at 60° in the presence of carbonate buffer, pH 9.1. The fluorescent intensity measured at 465 mμ is proportional to the β-alanol concentration in the range between 0.1 and 1 mcg./ml.

DURING THE LAST 10 years, several papers have been published on the quantitative analysis of panthenol in multivitamin pharmaceutical preparations. Chemical methods have been proposed to replace the time consuming microbiological assay. It seems, however, that the problem of the determination of panthenol in highly concentrated sugar preparations has not been solved satisfactorily, and we were recently confronted with that question.

In 1949, Crockaert (1) showed that panthenol and calcium pantothenate could be determined after alkaline hydrolysis as β-alanol and β-alanine, respectively, with the use of 1,2-naphthoquinone-4-sulfonate, while Wollish and Schmall (2) proposed the dosage of pantoyl lactone as ferric hydroxamate, after acid cleaving of the panthenol.

More recently, Zappala and Simpson (3) described a colorimetric method for the determination of panthenol in multivitamin tablets containing 15% sugar. According to these authors, the method of Schmall and Wollish (4) is not applicable to the preparation highly concentrated in sucrose because of the darkening of the solution during the alkaline hydrolysis. They solved the difficulty by extraction of the powdered sample and further purification on ion-exchange resin before estimating β-alanol by chlorination and subsequent iodometry. Their method is unfortunately inapplicable to liquid multivitamin preparations, particularly to syrups with a very high sugar content (more than 50% sucrose).

Our purposes were to determine the panthenol in multivitamin preparations and to perfect a very sensitive routine method capable of answering our control and research problems.

EXPERIMENTAL

Reagents

All the reagents were analytical grade.

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Sodium hydroxide (0.5 *N*), hydrochloric acid, absolute ethanol, sucrose, and chloroform were used.

Solution A.—Composed of acetate buffer, pH 4.7, 1 *M*.

Solution B.—Composed of acetate buffer, pH 4.7, 0.5 *M*.

Solution C.—Composed of a 14 m*M* quantity of ninhydrin solution in carbonate/bicarbonate buffer, pH 9.1, 1 *M;* must be prepared freshly.

Solution D.—A standard solution of β-alanol—5 mg. pure panthenol dissolved in a few milliliters of distilled water was hydrolyzed with 2 ml. NaOH, 0.5 *N*, in a boiling water bath for 1 hour, neutralized to pH 7, diluted with distilled water to 50 ml., and then to 100 ml. with *Solution A*.

Solution E.—Composed of a freshly prepared 0.2 *M* quantity of *n*-butanal aqueous solution.

Ion-Exchange Resins.—Dowex 50X4 (200–400 mesh), Amberlite C.G. 400 (100–200 mesh) were employed. The resins are prepared as usual: repeated washings with distilled water and elimination of the fines by pouring the supernatant; alternate washings with hydrochloric acid and sodium hydroxide until clear and colorless filtrates are obtained. Finally, prepare Dowex 50 under H^+ form and Amberlite C.G. 400 under OH^- form; wash the resins with water until neutral reaction of the effluent.

Procedure

A.—Transfer a known volume of the multivitamin syrup, equivalent to 2–3 mg. panthenol in a flat-bottomed porcelain basin. A few crystals of sucrose are added; 9 volumes of chilled absolute ethanol are poured over the syrup. Allow the solution to stand overnight in the refrigerator at 4°; a slow crystallization of sucrose is obtained. Partial or total evaporation of the alcoholic solvent occurs during the night; evaporation to total dryness is achieved in a desiccator under vacuum.

B.—Thoroughly and finely powder the crystalline residue in the basin with an agate pestle; transfer the powder quantitatively in a 150-ml. glass-stoppered Erlenmeyer flask. Wash the basin carefully with a few milliliters of absolute ethanol which are added to the flask, add 80 ml. $CHCl_3$. Mechanically shake the stoppered flask for 30 minutes; then filter the chloroformic suspension on fritted glass. The flask and the sugar crystals on filter are washed out three times with 25-ml. $CHCl_3$ fractions.

Collect the main filtrate and washings together in a round bottom flask and evaporate under vacuum the clear (sometimes lightly colored) solution to dryness at low temperature (30–35°) in a rotating evaporator. The dry residue is quantitatively dissolved in 20–25 ml. distilled water in several fractions.

C.—Allow the aqueous solution to pass through a Dowex 50X4 (H^+) column—25 mm. diam. × 25 mm. height—at a rate of 6 ml./minute; immediately wash the column with 60 ml. of water. The solution and washing are collected separately. Both effluents are then passed successively through an Amberlite C.G. 400 (OH^-) column—10 mm. diam. × 100 mm. height—at a flow rate of 3 ml./minute, then collected together in a 100-ml. volumetric flask. The solution is perfectly clear and colorless; it must not give any fluorescence by U.V. irradiation.

D.—Add 5 ml. NaOH, 0.5 *N*, and heat for 1 hour in a boiling water bath. Cool the hydrolyzate and neutralize with normal hydrochloric acid; dilute to the mark with distilled water.

E.—Fluorometric Determination.—*Unknown.*—Pipet 0.5 ml. of the sample hydrolyzate into a test tube and add successively 0.5 ml. buffer solution, pH 4.7 (*Solution A*); 0.5 ml. butanal solution (*Solution E*); and 0.5 ml. ninhydrin solution (*Solution C*).

Standard and Blank.—Prepare standard and blank solutions as follows: 1 ml. standard hydrolyzate (*Solution D*); 0.5 ml. *n*-butanal solution (*Solution E*); and 0.5 ml. ninhydrin solution (*Solution C*). For the blank, use 1 ml. buffer solution, pH 4.7, 0.5 *M* (*Solution B*), 0.5 ml. butanal solution, and 0.5 ml. ninhydrin solution.

Mix well by swirling and allow the loosely capped tubes to stand for 45 minutes in a water bath at 60°. Cool the tubes and dilute the solutions with 18 ml. distilled water. Read the fluorescent intensity of the blank, sample, and standard solutions at an excitation wavelength of 385 mμ and fluorescence of 465 mμ with a convenient spectrophotofluorometer.

The authors use the Zeiss spectrophotofluorometer

TABLE I.—COMPARISON OF RESULTS BY FLUOROMETRIC AND MICROBIOLOGICAL METHODS

Sample Syrup[a]	Theoretical Amount of Panthenol, mg./100 ml.	Panthenol Found—Fluorometric	Panthenol Found—Microbiological[c]
1	66	64.7	60.0
2	66	66.0	64.5
3	33	31.0	...
4	66	65.7	66.0
5	66	66.3	...
6	33	32.80	...
Tablets[b]	mg./Tablet		
1	20	20.0	20.8
2	20	19.5	23.0
3	20	...	21.5
4	20	...	19.7

[a] Composition: vitamins B_1, B_2, B_6, nicotinamide, nipagine, sucrose (more than 50%). [b] Composition: vitamins B_1, B_2, B_6, B_{12}, nicotinamide, lactose (30%). [c] We are indebted to Dr. Brouwers, Microbiology Department, Union Chimique Belge, for the microbiological data. The determinations were made with *Acetobacter suboxydans* according to E. De Ritter and S. H. Rubin (6).

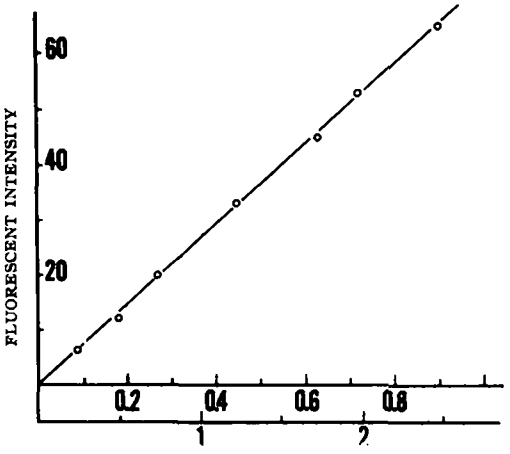


Fig. 1.—Relationship between fluorescent intensity and β-alanol concentration.

(Z.M.F.4.C.) under the following operating conditions. Calibration is made with a quinine sulfate solution (5); 1 mm. monochromator's slits are used. The sensitivity of the instrument is set so that a full scale deflection of the galvanometer spot is obtained for 3.5 mcg./ml. concentration of panthenol in reading the diluted solution, using quartz cells—1 cm. light path—for the measurements.

F.—Calculate the amount of panthenol in the unknown syrup by

$$\frac{Ps\,(Iu - Ib)}{Vu\,(Is - Ib)} \times 200 = \text{mg. panthenol in 100 ml. syrup}^1$$

where Iu = fluorescent intensity of the unknown, Is = fluorescent intensity of the standard. Ib = fluorescent intensity of the blank, Ps = standard panthenol content in mg./100 ml., and Vu = volume of the sample of the unknown syrup.

RESULTS AND DISCUSSION

In this laboratory the described method has given reproducible results, both with multivitamin syrups and tablets. With the latter, the first step of the procedure was neglected: the tablets were directly powdered and extracted as described in step *B*.

As shown in Table I, the mean deviation between the observed results was ±2% which indicates a good reproducibility in the range of concentration studied.

As mentioned by Zappala and Simpson (3), the recoveries of panthenol after treatment on ion-exchange resins are low because of a partial retention of the vitamin by the resins. Several assays with internal standards have shown that the mean retention in our experimental conditions was approximately 6% of the total panthenol content of the solution. A correction factor of 6% was thus introduced in the final calculation of panthenol in the syrups and tablets.

The quantitative determination of β-alanol in the final hydrolyzate could have been made by one of the methods described in the literature (1, 3, 4). It seemed interesting to use the new fluorometric method perfected by Close, *et al.* (7), which is routinely used in our laboratory for other purposes. This method is easy, rapid, precise, and very sensitive.

As illustrated in Fig. 1, the relationship between the fluorescent intensity and the β-alanol concentration is perfectly linear in a range of concentration between 0.1 and 1 mcg./ml. of β-alanol in the diluted solution (or 0.27 and 2.7 mcg./ml. of panthenol). This range is highly convenient for the needs of the quantitative estimation of panthenol in the pharmaceutical preparations.

We believe that the method proposed can be used as a routine procedure for the panthenol determination in the pharmaceutical preparations with a high sugar content, and that it can also be the basis for the assay of the other vitamins contained in the same preparations.

[1] Uncorrected value; see next paragraph for the correction factor.

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By M. L. KHORANA and M. M. SANGHAVI

Fractional precipitation and chromatographic studies have shown that *C. angustifolia* pods contain, besides sennosides A and B, glucosides of rhein and chrysophanic acid. The latter were best isolated by acidification of the aqueous extract to pH 3. Biologically, a mixture of these new anthraquinone glucosides and the bianthranol glycosides was more active than either. The possibility of the presence of traces of aloe-emodin or emodin glucoside has also been indicated.

In 1949, STOLL (1) isolated sennosides A and B from the leaves and pods of senna (*C. angustifolia* Vahl and *C. acutifolia* Delile). However, Fairbairn (2) showed that these two glycosides did not represent the full potency of the drug. Further studies of the drug with the purpose of isolating other principles, representing the residual potency of the drugs, were therefore warranted. During recent years *C. acutifolia* has been studied by Fairbairn (3) and Vickers (4), and isolation of a number of glycosides has been claimed.

This work deals with similar studies on *C. angustifolia* pods, and evidence for the presence of two other glycosides is presented.

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EXPERIMENTAL

Aqueous Extract

A coarse powder prepared from 600 Gm. of senna pods was divided into three portions. One portion, after maceration with 36 ml. of 1 *N* sodium bicarbonate and menstruum (water saturated with chloroform), was percolated with the menstruum to obtain 2 L. of the percolate. The second and third portions of the drug were similarly extracted, using first the extract from the previous batch as

(Z.M.F.4.C.) under the following operating conditions. Calibration is made with a quinine sulfate solution (5); 1 mm. monochromator's slits are used. The sensitivity of the instrument is set so that a full scale deflection of the galvanometer spot is obtained for 3.5 mcg./ml. concentration of panthenol in reading the diluted solution, using quartz cells—1 cm. light path—for the measurements.

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TABLE I.—CHROMATOGRAPHY OF *C. angustifolia* GLUCOSIDES

Substance	Spot No.	R_f	Color	
			Visible	Ultraviolet Light
Sennoside A	1	0.00	Yellow changing to brown	Bright yellow
Sennoside B	2	0.30	Yellow changing to brown	Bright yellow
Acid precipitate	3	0.35	Yellow changing to brown	Bright yellow
	4	0.44	Yellow changing to brown	Bright yellow
	5	0.99 Tailing to ...	Reddish brown	Reddish brown

menstruum supplemented by fresh menstruum as required to obtain 2 L. of the percolate from each portion. Two liters of percolate were thus obtained from 600 Gm. of the drug.

Fractionation of the Aqueous Extract

(a) **Acid Precipitate.**—The aqueous extract acidified to pH 3 with concentrated hydrochloric acid and preserved with chloroform was allowed to stand overnight. The acid precipitate (*a*), separated by vacuum filtration through filter paper supported on muslin, was washed with water and dried; yield, 3.7%.

(b) **Neutral Precipitate.**—Sufficient ammonium chloride and anhydrous calcium chloride were added to the combined filtrate and washings from (*a*) to obtain concentrations of 2 and 5%, respectively; pH was adjusted to 7 with liquor ammonia. The yield of the neutral precipitate after filtration, washing with water, and drying, was 2.1%.

(c) **Alcoholic Precipitate.**—Sufficient 95% alcohol was added to the combined filtrate and washings from (*b*) to cause complete precipitation. The yield of the precipitate after filtration, washing with alcohol, and drying, was 13.8%.

Chromatographic Studies of the Acid Precipitate

The acid precipitate (*a*) (0.2 Gm.) was extracted with 15 ml. of 70% methanol; the filtered extract was spotted on Whatman filter, chromatographic grade 1. Solutions of sennoside A in cellosolve and sennoside B in 70% methanol were spotted for comparison. The chromatogram was developed by the ascending technique with 85 ml. of absolute isopropanol and sufficient 1.94 *N* acetic acid to make 100 ml. The chromatograms, sprayed with 1 *N* alcoholic sodium hydroxide, were examined in visible as well as ultraviolet light. The results are given in Table I.

A comparison of colors in visible and ultraviolet light as given in Table I indicates that Spots 3 and 4 of the acid precipitate are most probably due to glycosides. Comparison of R_f values indicates that these spots are due to compounds other than sennosides A and B. Spot 5, because of its color, seemed to be due to free anthraquinones or some coloring matter.

Identification of the New Aglycones

Hydrolysis of the Acid Precipitate.—The acid precipitate (8 Gm.), extracted with ether to remove free anthraquinones, was hydrolyzed by heating with 40 ml. of 3 *N* hydrochloric acid in a boiling water bath for 15 minutes. The anthraquinones from the cooled hydrolyzate were extracted with ether and the aqueous layer set aside for *Identification of Sugars*. The ether extract, after being washed twice with distilled water and dried over sodium sulfate, was concentrated to about 5 ml.

TABLE II.—CHROMATOGRAPHY OF TWO NEW AGLYCONES FROM *C. angustifolia* PODS

No.	Substance Name	R_f	Color
A	Aloe-emodin	0.94	Pink
B	Emodin	0.88	Pink
C	Chrysophanol	0.98	Pink
D	Rhein	0.00	Pink
E	Sennidin A	0.00	Yellow[a]
F	Sennidin B	0.00	Yellow[a]
G	Benzene concentrate	0.98	Pink
H	Alcoholic concentrate	(*1*) 0.00 (*2*) 0.52 (*3*) 0.98	Pink
I	C + G	0.98 (*1*) 0.00	Pink
J	D + H	(*2*) 0.52 (*3*) 0.98	Pink

[a] Yellow slowly changing to violet.

Fractionation and Chromatography of the Ether Extract.—The concentrated ether extract was extracted serially with (*A*) 1 *N* sodium bicarbonate, (*B*) 5% sodium carbonate, and (*C*) 1 *N* sodium hydroxide. The sodium carbonate extract (*B*), had a color that was too weak and was not studied further. Each of the other two alkali extracts was made acidic with hydrochloric acid and re-extracted with ether. After washing the ether extracts with water, the solvent was removed; the residues were repeatedly extracted with hot, sodium-dried, benzene, using 5 ml. each time. The extracts were concentrated to 5 ml. and poured on columns of $CaSO_4 \cdot {}^1/_2\, H_2O$ (E. Merck). The columns were prepared and washed with benzene.

The sodium bicarbonate–soluble fraction (*A*) gave a yellow band on the column, and a faintly colored benzene eluate, but the reverse was the case with the sodium hydroxide-soluble fraction (*C*). The eluate was more colored than the band on the column. The two eluates were combined, concentrated to about 5 ml., and the concentrate used for spotting (*G*).

The bands on the two columns were extruded and extracted with alcohol containing 0.5% acetic acid. The two extracts were combined, concentrated to about 5 ml., and the concentrate used for spotting (*H*). Sennidin A in alcohol, sennidin B in chloroform, and some known anthraquinone (Table II) in benzene were spotted on the same paper. The chromatograms were developed with toluene and sprayed with alcoholic 1 *N* sodium hydroxide. The results are reported in Table II.

Though all the four spots, *D*, *E*, *F*, and *H*(*1*) showed a similar behavior in having R_f 0.00; they differed in the color produced with 1 *N* sodium hydroxide; *E* and *F* developed bright yellow which slowly changed to violet on standing, and *D* and *H*-(*1*) under the identical conditions gave a pink color.

This differentiated the spots *E* and *F* due to bimolecular anthranols from the spots *D* and *H(1)* due to monomolecular anthraquinones. Hence it was clear that while benzene concentrate *G* contained chrysophanol, the spot *H(1)* most probably was due to rhein; the spot *H(2)*, however, did not correspond to any of the aglycones chromatographed.

Chromatography with a second solvent system: lower phase of water:acetone:benzene (2:1:4) gave the following R_f values: chrysophanol, 0.00; rhein, 0.74; benzene solution, 0.00; alcohol solution, (*1*) 0.74, (*2*) 0.89; *C* + *G*, 0.00; *D* + *H*, (*1*) 0.74 and (*2*) 0.89. All the spots were colored pink with 1 *N* sodium hydroxide.

In the above paragraph *H(1)* is evidently due to rhein as indicated by R_f values of rhein and *D* + *H* (*1*). Similarly, comparison of chrysophanol, benzene solution, and *C* + *G* indicated that the second aglycone was chrysophanol.

Identification of Sugars

The aqueous acidic layer (mentioned in *Identification of the New Aglycones*) was cooled, neutralized with sodium hydroxide pellets, and concentrated to dryness using high vacuum. The 1:3 filtered aqueous pyridine extract of the residue was used for spotting on Whatman No. 1 paper. For comparison, a glucose solution and a mixture of the two were spotted simultaneously.

The chromatograms were developed with *n*-butanol:glacial acetic acid:water (4:1:5), by radial chromatographic techniques, and sprayed with Partridge's reagent (5). The average of four R_f values were found to be—glucose, 0.32; aldose from the acid precipitate, 0.30; and a mixture of glucose and the aldose, 0.33. This clearly indicated that the sugar of the glycosides present in the acid precipitate was glucose.

DISCUSSION

In this study the drug was extracted with water instead of alcohol. This not only made the process more economical, but also permitted better separation of the acid precipitate. Since the glucosides present in the drug were thermolabile, all concentrations by heat even under reduced pressure were avoided, and a concentrated extract was obtained by following the procedure of repercolation.

Precipitation of the aqueous extract at different pH, from 1.0 to 7.0, showed that the highest yield of the so-called acid precipitate was obtained at pH 3. This precipitate showed the presence of a laxative principle when tested on albino mice. Tested similarly, the second fraction obtained from the filtrate on addition of calcium chloride and ammonium chloride at pH 7 was inactive, but the third fraction obtained on addition of sufficient alcohol to the mother liquor from the second fraction was again active. A detailed report on the biological assay of the different fraction will be published separately. The fraction II (*C*) contained sennoside A and B, the presence of which is already reported by Stoll (1).

The glucosides present in the acid precipitates could not be crystallized, but they have been shown to be different from sennosides A and B by chromatographic techniques. In addition, the new glycosides have been shown to be those of rhein and chrysophanic acid by cochromatography from two different solvent systems. The sugar part of the glycosides is glucose in both the cases.

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Two Disintegrating Agents *versus* Cornstarch for Compressed Tablets

By G. HECHT† and C. L. HUYCK‡

Cornstarch, Dry-Flo, and Solka-Floc BW 40, were tested for suitability as disintegrating agents. The Dry-Flo and the cornstarch proved favorable for the sodium bicarbonate and the acetylsalicylic acid tablets. It was noted that Solka-Floc BW 40 (due to its fibrous nature) helped to prevent capping of the tablets. Analysis of the disintegration tests of tablets containing Solka-Floc BW 40 and Dry-Flo were statistically different than cornstarch at the 1 per cent probability level.

Cornstarch has been used in compressed tablets as a disintegrant for decades and is still the disintegrant of choice. Dried corn or potato starch is sometimes used with the object of shortening the disintegration time of the tablet, when moisture is detrimental to the stability of the active ingredients (*i.e.*, in aspirin tablets), and in tablets containing medicinals that are incompatible with each other. Even though cornstarch is quite satisfactory, the search continues for faster and more efficient disintegrants. The quicker the release of the medicament after ingestion, the greater the efficiency of the tablet. In this study, two disintegrants are compared with cornstarch and the results are analyzed statistically.

Two compounds were chosen to be tested as tablet disintegrants: Solka Floc BW 40[1] (a purified wood cellulose) and Dry-Flo[2] (a starch ester containing a hydrophobic group). As a control disintegrant, 10% cornstarch was used.

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[1] Solka-Floc BW 40, The Brown Co., Berlin, N. H.
[2] Dry-Flo, National Starch Products, Inc., 270 Madison Avenue, New York 16, N. Y.

This differentiated the spots *E* and *F* due to bimolecular anthranols from the spots *D* and *H(1)* due to monomolecular anthraquinones. Hence it was clear that while benzene concentrate *G* contained chrysophanol, the spot *H(1)* most probably was due to rhein; the spot *H(2)*, however, did not correspond to any of the aglycones chromatographed.

Chromatography with a second solvent system: lower phase of water:acetone:benzene (2:1:4) gave the following R_f values: chrysophanol, 0.00; rhein, 0.74; benzene solution, 0.00; alcohol solution, (*1*) 0.74, (*2*) 0.89; *C* + *G*, 0.00; *D* + *H*, (*1*) 0.74 and (*2*) 0.89. All the spots were colored pink with 1 *N* sodium hydroxide.

In the above paragraph *H(1)* is evidently due to rhein as indicated by R_f values of rhein and *D* + *H* (*1*). Similarly, comparison of chrysophanol, benzene solution, and *C* + *G* indicated that the second aglycone was chrysophanol.

Identification of Sugars

The aqueous acidic layer (mentioned in *Identification of the New Aglycones*) was cooled, neutralized with sodium hydroxide pellets, and concentrated to dryness using high vacuum. The 1:3 filtered aqueous pyridine extract of the residue was used for spotting on Whatman No. 1 paper. For comparison, a glucose solution and a mixture of the two were spotted simultaneously.

The chromatograms were developed with *n*-butanol:glacial acetic acid:water (4:1:5), by radial chromatographic techniques, and sprayed with Partridge's reagent (5). The average of four R_f values were found to be—glucose, 0.32; aldose from the acid precipitate, 0.30; and a mixture of glucose and the aldose, 0.33. This clearly indicated that the sugar of the glycosides present in the acid precipitate was glucose.

DISCUSSION

In this study the drug was extracted with water instead of alcohol. This not only made the process more economical, but also permitted better separation of the acid precipitate. Since the glucosides present in the drug were thermolabile, all concentrations by heat even under reduced pressure were avoided, and a concentrated extract was obtained by following the procedure of repercolation.

Precipitation of the aqueous extract at different pH, from 1.0 to 7.0, showed that the highest yield of the so-called acid precipitate was obtained at pH 3. This precipitate showed the presence of a laxative principle when tested on albino mice. Tested similarly, the second fraction obtained from the filtrate on addition of calcium chloride and ammonium chloride at pH 7 was inactive, but the third fraction obtained on addition of sufficient alcohol to the mother liquor from the second fraction was again active. A detailed report on the biological assay of the different fraction will be published separately. The fraction II (*C*) contained sennoside A and B, the presence of which is already reported by Stoll (1).

The glucosides present in the acid precipitates could not be crystallized, but they have been shown to be different from sennosides A and B by chromatographic techniques. In addition, the new glycosides have been shown to be those of rhein and chrysophanic acid by cochromatography from two different solvent systems. The sugar part of the glycosides is glucose in both the cases.

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Two Disintegrating Agents *versus* Cornstarch for Compressed Tablets

By G. HECHT† and C. L. HUYCK‡

Cornstarch, Dry-Flo, and Solka-Floc BW 40, were tested for suitability as disintegrating agents. The Dry-Flo and the cornstarch proved favorable for the sodium bicarbonate and the acetylsalicylic acid tablets. It was noted that Solka-Floc BW 40 (due to its fibrous nature) helped to prevent capping of the tablets. Analysis of the disintegration tests of tablets containing Solka-Floc BW 40 and Dry-Flo were statistically different than cornstarch at the 1 per cent probability level.

Cornstarch has been used in compressed tablets as a disintegrant for decades and is still the disintegrant of choice. Dried corn or potato starch is sometimes used with the object of shortening the disintegration time of the tablet, when moisture is detrimental to the stability of the active ingredients (*i.e.*, in aspirin tablets), and in tablets containing medicinals that are incompatible with each other. Even though cornstarch is quite satisfactory, the search continues for faster and more efficient disintegrants. The quicker the release of the medicament after ingestion, the greater the efficiency of the tablet. In this study, two disintegrants are compared with cornstarch and the results are analyzed statistically.

Two compounds were chosen to be tested as tablet disintegrants: Solka Floc BW 40[1] (a purified wood cellulose) and Dry-Flo[2] (a starch ester containing a hydrophobic group). As a control disintegrant, 10% cornstarch was used.

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‡ Present address: Eaton Laboratories, Division of The Norwich Pharmacal Co., Norwich, N. Y.

[1] Solka-Floc BW 40, The Brown Co., Berlin, N. H.
[2] Dry-Flo, National Starch Products, Inc., 270 Madison Avenue, New York 16, N. Y.

EXPERIMENTAL

The disintegrants were tested with relatively soluble and insoluble medicinals. Sodium bicarbonate was selected as the soluble medicinal agent, while acetylsalicylic acid was selected as the insoluble medicinal. The tablets of sodium bicarbonate were tested for disintegration (1) in both simulated gastric juice U.S.P. and distilled water to determine the extent of disintegration caused by effervescence of the sodium bicarbonate.

As an aid to disintegration into fine particles one-half or 5% of the disintegrant was added to the medicament before granulation; the other 5% was added to the dry granulation before compression. Ten per cent acacia mucilage was used as a binder.

Granulation.—The sodium bicarbonate employed was furnished from one company and met the standards of the "United States Pharmacopeia XV" (1). Since 5% of the disintegrating agent was to be placed in the granulation, it was necessary to make three separate granulations. The proper weight of sodium bicarbonate for 1000 tablets was placed in a stainless steel container and mixed with 5% disintegrant. Each batch was mixed for 30 minutes in a small laboratory Hobart mixer. To insure a uniform hardness of granules, an attempt was made to keep the amount of acacia mucilage as equal as possible for each of the three batches. To achieve the proper consistency, an average 215 Gm. ± 8 Gm. of acacia mucilage was used. The three batches were then granulated and passed through a No. 10 screen on the Stokes oscillating granulator. The wet granules were spread uniformly on paper covered trays and air dried for 42 hours. The dry granules were then passed through a No. 16 screen. One per cent magnesium stearate, previously screened through a No. 80, along with 5% disintegrator was added to each granulation. Each batch was then mixed for 5 minutes in the Patterson–Kelly twin shell blender.

Compression.—The three batches were compressed into tablets on a Stokes model E single punch tableting machine at a speed of 80 tablets per minute using $^{13}/_{32}$-in. punches of standard concavity. The tablet weight was kept constant at 0.380 Gm., and the tablet hardness kept as nearly to 10 Kg. as possible by the Strong Cobb hardness tester. Sampling was done on a 10% time basis; *i.e.*, 13 minutes was required to compress 1000 tablets at a rate of 80 tablets per minute. Therefore, a sample of 10 tablets was taken every 80 seconds. As nearly constant as possible with all three granulations were the following: (*a*) hopper feed by the same hopper at the same initial feed level, (*b*) speed of the machine at 80 tablets per minute, (*c*) weight, size, and hardness of the tablets.

Difficulty in flow was encountered with Solka-Floc BW 40 granulation. A constant tablet weight and hardness was difficult to maintain with this granulation. Flow characteristics better than cornstarch were noted for Dry-Flo.

Disintegration.—The U.S.P. (1) apparatus and method was used to determine disintegration. Thirty tablets from each batch were tested in five trials using six tablets per trial. The average time per trial was calculated in seconds. The mean and mean deviation were calculated for the five trials, and from this the standard deviation for the population was calculated.

The disintegration time of 30 tablets of each of three granulations of sodium bicarbonate plus a disintegrant also was tested in distilled water at 37°. Table III represents the data compiled from this experiment.

In the second part of the experiment, disintegration time of tablets containing the relatively insoluble acetylsalicylic acid with each of the three disintegrants was compared. Five per cent disintegrant was incorporated before granulation, and 5% was incorporated with the granules after granulation and just before compression. Granulation was by the precompression method, using $^{5}/_{16}$-in. punches and die for the slugs. The slugs were passed through a No. 8 mesh screen on the Stokes oscillating granulator. This process was repeated in order to eliminate "fines." The final granulation was passed through a No. 16 mesh screen. The granules were then mixed with 5% disintegrant to be tested and 1% magnesium stearate was added as lubricant. The tablets were compressed on $^{13}/_{32}$-in. punches at a weight of 0.380 Gm. with a hardness of approximately 10 Kg. as measured by the Strong Cobb hardness tester.

TABLE I.—FORMULAS OF THE SODIUM BICARBONATE TABLETS CONTAINING CORNSTARCH, SOLKA-FLOC, AND DRY-FLO AS DISINTEGRANTS

	Solka-Floc		
	Starch	BW 40	Dry-Flo
Sodium bicarbonate	324.0	324.0	324.0
Cornstarch	32.4	..	..
Solka-Floc BW 40	..	32.4	..
Dry-Flo	..	..	32.4
Magnesium stearate	3.24	3.24	3.24
Acacia mucilage 10%	*q.s.*	*q.s.*	*q.s.*

TABLE II.—DISINTEGRATION TIME IN MINUTES OF SODIUM BICARBONATE TABLETS CONTAINING DRY-FLO, CORNSTARCH, AND SOLKA-FLOC AS DISINTEGRANTS IN SIMULATED GASTRIC JUICE

	Trials				
Disintegrant	1st	2nd	3rd	4th	5th
Dry-Flo	2.8	2.1	2.2	1.9	1.8
Cornstarch	2.7	2.1	2.8	4.8	4.0
Solka-Floc BW 40	11.6	11.3	10.3	15.6	17.3

DISCUSSION

Using equation $d = \Sigma(X_1 - \bar{X})/N$ for calculation of mean deviation in which $\Sigma(X_1 - \bar{X})$ is the sum

TABLE III.—DISINTEGRATION TIME IN MINUTES OF SODIUM BICARBONATE TABLETS CONTAINING DRY-FLO, CORNSTARCH, AND SOLKA-FLOC DISINTEGRANTS IN WATER

	Trials				
Disintegrant	1st	2nd	3rd	4th	5th
Dry-Flo	2.83	3.50	2.33	2.66	2.50
Cornstarch	6.66	5.50	5.66	7.00	7.16
Solka-Floc BW 40	28.00	29.33	28.83	28.83	29.66

TABLE IV.—FORMULAS FOR THE ACETYLSALICYLIC ACID TABLETS CONTAINING CORNSTARCH, SOLKA-FLOC, AND DRY-FLO AS DISINTEGRANTS

	Starch	Dry-Flo	Solka-Floc BW 40
Acetylsalicylic acid	324.0	324.0	324.0
Cornstarch	32.4	..	..
Dry-Flo	..	32.4	..
Solka-Floc BW 40	..	..	32.4
Magnesium stearate	3.24	3.24	3.24

With Dry-Flo, a starch ester containing hydrophobic groups, no lubricant was necessary because of the splendid flow characteristics of this compound. Solka-Floc BW 40 is a white, purified, wood cellulose of a purity of 99.5% and an average particle size of 90 μ. This grade of Solka-Floc exhibited poor flow characteristics, and a lubricant was necessary.

Tablets of acetylsalicylic acid containing Dry-Flo as the disintegrating agent had a mean disintegration of 38.99 minutes, compared to 47.57 minutes for cornstarch and 54.56 minutes for Solka-Floc BW 40.

TABLE V.—DISINTEGRATION TIME IN MINUTES OF ACETYLSALICYLIC ACID TABLETS CONTAINING DRY-FLO, CORNSTARCH, AND SOLKA-FLOC AS DISINTEGRANTS IN SIMULATED GASTRIC JUICE

Disintegrant	Trials 1st	2nd	3rd	4th	5th
Dry-Flo	35.16	38.16	40.66	37.66	40.33
Cornstarch	36.33	54.00	54.23	46.16	47.16
Solka-Floc BW 40	50.16	57.33	54.83	55.50	55.00

of the absolute deviations from the mean and $(N - 1)$ is the number of degrees of freedom for a small sample, then $s = (X_1 - \bar{X})^2/N - 1$ (2).

The results of the disintegration tests of the tablets were analyzed statistically by the students "t" test and were found to be statistically[3] different from cornstarch at the 1% probability level.

Tablets of sodium bicarbonate containing Dry-Flo as the disintegrating agent had a mean disintegration time in artificial gastric juice of 2.16 minutes, compared with 3.28 minutes for tablets containing cornstarch as the disintegrant and 13.22 minutes for tablets containing Solka-Floc BW 40 as the disintegrant. All disintegrants yielded tablets which were white and smooth in appearance.

The disintegration of the sodium bicarbonate tablets was tested in simulated gastric juice U.S.P. and in distilled water to determine how much of the disintegration was due to the decomposition of the sodium bicarbonate by the acid media. In distilled water the tablets containing the Dry-Flo disintegrated in 2.76 minutes or 27% longer than in simulated gastric juice U.S.P. Sodium bicarbonate tablets containing cornstarch disintegrated in water in 6.40 minutes or 94% longer than in simulated gastric juice and the tablets containing Solka-Floc BW 40 disintegrated in 28.92 minutes in water or 111% longer than in simulated gastric juice U.S.P.

[3] In the statistical study the authors acknowledge the help of Mr. George O'Bleness, Director of the Computer and Statistical Division, Eaton Laboratories, Division of The Norwich Pharmacal Co., Norwich, N. Y.

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Books

REVIEWS

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Cornstarch	32.4	..	..
Dry-Flo	..	32.4	..
Solka-Floc BW 40	..	..	32.4
Magnesium stearate	3.24	3.24	3.24

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February 1964 volume 53, number 2

Review Article

Thiophene Compounds of Biological Interest

By W. LEWIS NOBLES and C. DEWITT BLANTON, JR.

THE CONCEPT OF preparing thiophene analogs of biologically active compounds has stimulated workers in pharmaceutical chemistry so that it now appears that at least one thiophene analog has been prepared for every important therapeutic group containing a benzene nucleus. It has been hoped that the thiophene analog would possibly be as active as the parent compound or that the thiophene analog would, by virtue of its similar chemical structure, combine with the receptor and—if not eliciting a response of its own—serve effectively as a competitive inhibitor. Thus, when a thiophene analog is prepared, it is conceivable that such an agent may either intensify, mimic, or antagonize the physiological activity of the parent substance. Many of the thiophene compounds are more toxic than their benzene analogs and, consequently, less effective medicinal agents. There are, however, some exceptions: several thiophene derivatives are employed clinically.

Excellent summaries of the literature up to 1959 are available (1–5). In this review particular emphasis will be placed on knowledge gained since 1959. To present a more comprehensive understanding of the role of thiophene in pharmaceuticals, mention of some of the earlier work will be included.

The electronic structure of thiophene must be very similar to that of benzene, pyrrole, and furan. Each carbon atom must be in an approximately trigonal state of hybridization with a $2p$ orbital perpendicular to the plane of the ring. These orbitals interact laterally with the two $3p$ electrons provided by the sulfur atom to form the π-bonds. As the energy difference between $3p$ and $3d$ is not great, it has been suggested (6) that the sulfur-carbon bonds in thiophene do not consist of pure p-orbitals, but that some of the $3d$ orbitals of the sulfur atom were used. When pd hybridization of the sulfur orbitals occurs, three pd (2) hybrid orbitals are formed, two of which have the correct symmetry and energy to conjugate with the carbon atoms. The third hybrid orbital is high in energy and is unoccupied in the ground state (7). No agreement has been reached, however, on the importance of pd hybridization of the sulfur atom. This hybridization is considered by some workers as an essential feature in the electronic structure of thiophene, making the sulfur atom similar to a CH=CH group, and being responsible for some of the differences between thiophene, furan, and pyrrole; whereas others have suggested that most of the properties of thiophenes may be accounted for without invoking pd hybridization at all (8). Mangini and Zauli (8*b*) even doubt the basic validity of the Longuet-Higgins approach (7*a*).

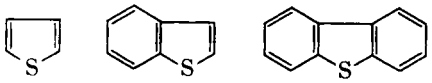
The structural similarity between thiophene and benzene is also reflected in that both form "sandwich" compounds with certain metals. For example, thiophene forms thiophene-chromium-tricarbonyl (9), and numerous similar compounds are known in the benzene series.

In addition to the established use as anti-

Received from the School of Pharmacy, University of Mississippi, University.

histamines (10), certain thiophene derivatives have shown (1, 5, 11–16) substantial activity as pressor compounds, local anesthetics, hypnotics, analgesics, anticholinergics, antispasmodics, anticonvulsants, tuberculostats, antibacterials, antimalarials, and germicides. Many of these products have biological and pharmacological properties markedly different from the benzene analogs.

Both biochemical and pharmacological studies have shown that the "theoretical equivalence" of a thiophene and a benzene nucleus—which is easy to establish if only similarities are mentioned and dissimilarities are disregarded—is by no means universal. The general conclusion to be drawn on determination of the biological properties of thiophene (I) and its higher ring homologs, thionaphthene (II) and dibenzothiophene (III), is that these compounds usually possess activities similar to those of benzene, naphthalene, and anthracene but less pronounced and often more toxic (1, 5). Comprehensive studies of the



I II III

biological properties of thiophene and its higher ring homologs have been limited by the low solubility of such compounds in aqueous media. However, excellent historical reviews of the biological properties of the simpler thiophene derivatives have been compiled by Blicke (1) and Martin-Smith (5).

THIOPHENE DERIVATIVES AS BIOLOGICAL ANTAGONISTS

The best known of the thiophene antimetabolites are the two isomeric β-thienylalanines which are antimetabolites of the essential amino acid, β-phenylalanine. The "antiphenylalanine" properties of thienylalanine have been investigated with *Saccharomycea cerevisiae*, *Escherichia coli*, *Streptococcus faecalis*, and *Lactobacillus arabinosus*. It was observed (17) that only the L isomer, and not the D, inhibited the growth of *S. cerevisiae*, *E. coli*, and *L. delbrueckii*, and that the inhibition of growth of rats produced by the DL, D, or L isomer is counteracted by phenylalanine (18–20). Of the two isomers, the 3-thienyl isomer appears to be a more active antagonist than the 2-thienyl isomer (21–23). For *S. cerevisiae*, strain 139, the growth inhibitory properties of the 3-thienyl isomer were about twice those of β-2-thienylalanine, and for *E. coli* the 3 isomer was about one-third more effective. The activity was reversed by phenylalanine.

Since no systematic study had been made of the tissue changes brought about by feeding 2-thienylalanine, Kaufman, *et al.* (24), studied the pathologic changes induced by β-2-thienylalanine. A purified synthetic diet of the 2 isomer in amounts of 1 and 2% was fed to rats for 4 weeks. Marked loss of weight was noted in all these animals. In addition to the expected loss of weight, the pyloric glands of the stomach and Brunner's glands of the duodenum were shrunken, and the cytoplasm was vacuolated. The acini of the submaxillary glands were atrophied; kidneys were enlarged and the proximal convoluted tubules were damaged; there was spermatogenic arrest at the spermatid level. The ductus epididymis was narrowed, the epithelium was atrophic, and the glands contained a decreased amount of secretion. There was a decrease in the number of mature lymphocytes of the spleen and of the thymus; the skin was hyperkeratotic, and the hair follicles were atrophic. Thinning of the epiphyseal plate was observed, with a decrease in growth of bone. Less marked changes were noted in the heart, pituitary gland, thyroid, adrenals, and the liver. Hruban and Wissler (25), using β-3-thienylalanine, reported changes in the pancreas, liver, spleen, and thymus which resembled in some respects the changes described by Kaufman for β-2-thienylalanine.

More recently, work has been directed toward the study of the biological activity of simple peptides (26) formed from the β-thienylalanines, and there has been interest in the simple amino acids themselves as potential antiviral (27) and anticancer agents (28). Generally, the toxicity for rats (29) and the growth stimulation and inhibition for *E. coli* (26*a*, *b*, *d*) are no more pronounced than with the corresponding simple amino acids; in some cases the thienyl peptides are less effective. Dipeptides of β-2-thienylalanine competitively inhibited the utilization of dipeptides of phenylalanine (26*d*, 30). Such inhibition studies have contributed additional evidence to previous proposals of a special role of peptides in the growth of microorganisms (31, 32) and indicate the possibility of specific sites of utilization of dipeptides.

Growth studies with *Lactobacillus arabinosus* (33) showed that L-leucyl-L-phenylalanylglycine was more effective than phenylalanine or the related dipeptide in reversing the toxicity of L-leucyl-β-2-thienyl-L-alanylglycine; this was accepted as evidence for possible utilization of tripeptides without previous hydrolysis to the free amino acids or the intermediate dipeptides. To pursue studies of the role of tripeptides in

metabolism, Dunn (34) prepared four tripeptide analogs containing β-2-thienylalanine. Tripeptides of phenylalanine were more active than phenylalanine in reversing the toxicity of β-2-thienylalanine in *E. coli*, strain 9723. Tripeptides of β-2-thienylalanine were comparable to free β-2-thienylalanine as growth inhibitors for *E. coli* when tested in the salt-glucose medium; and when tested in the presence of analogous tripeptides of phenylalanine, they were many times as toxic as free β-2-thienylalanine. The toxicity of the tripeptides of β-2-thienylalanine was reversed noncompetitively by phenylalanine and its dipeptides. The results are interpreted as indicating that tripeptides of β-2-thienylalanine compete with tripeptides of phenylalanine in a specific way and that their route of utilization is independent of the route followed by the possible hydrolytic products.

It has been shown that although structural analogs of amino acids added to a culture in the exponential growth phase inhibit normal multiplication of the organism, they do not cause an immediate cessation of growth and protein synthesis (35). Incorporation of structural analogs of amino acids, *e.g.*, *p*-fluorophenylalanine, β-2-thienylalanine, and norleucine, in bacterial proteins during linear growth of the organism has been observed (36). It is suggested that structural analogs of amino acids which can be incorporated into bacterial proteins during the linear growth phase do not act by inhibiting protein biosynthesis, but rather by promoting the synthesis of proteins which are abnormal with respect to structure and activity.

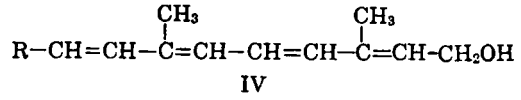
Comparison (37) of isotopic thienylalanine and fluorophenylalanine with respect to their participation in peptide and in protein synthesis reveals that the former amino acid was incorporated about 20 times more readily into gramicidin S by *Bacillus brevi* than the fluoro compound. In protein synthesis, fluorophenylalanine was more than five times more extensively utilized than was the thienyl derivative. Wolfe and Hahn (38) reported that β-2-thienylalanine was strongly activated by an *E. coli* extract but was not utilized for net protein synthesis by this organism.

3-Thienylalanine has also been reported (39) to have an effect on protein formation. The incorporation of labeled amino acids into serum and tissue proteins of β-3-thienylalanine–treated rats was compared with control rats. Suppression of the labeled amino acid uptake was found in all of the proteins analyzed, with a significant difference between the degrees of suppression. The γ-globulin and albumin among the serum proteins were less affected than the β- and α-globulins. Intestinal proteins showed the least suppression.

Moderate growth inhibition has been observed in several transplanted tumors (Murphy-Storm lymphosarcoma) by feeding β-3-thienylalanine in diets lacking phenylalanine (40). Diets of β-3-thienylalanine fed to male albino rats have also been noted to inhibit the growth of transplanted Jensen and Walker tumors, but no regression of the tumors occurred (41). These authors report that β-3-thienylalanine potentiates the inhibitory action of triethylene amine on tumor growth.

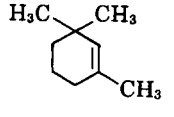
When β-3-thienylalanine is fed to rats for an appropriate time before and after antigen injection, it markedly inhibits antibody response (42). Added to antibody-synthesizing tissue culture system, it did not inhibit antibody synthesis. It appears that unless β-3-thienylalanine is fed during the induction period, its inhibitory effect will not be manifested. The activity is probably in inhibiting the setting up of the antibody-forming mechanism.

Thiophene derivatives as antimetabolites are not solely limited to the antiamino acids. Acheson, *et al.* (43), have synthesized some thiophenes (IV) related to vitamin A, although no thiophene derivative closely analogous to vitamin A appears to have been achieved.

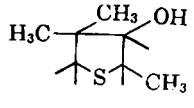


IV

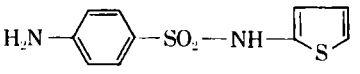
R (vitamin A) =



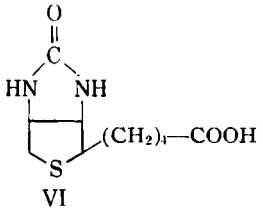
R (thiophene relative) =



Many modifications of the sulfanilamide molecule, an antimetabolite to folic acid-requiring bacteria, have been synthesized; among these are compounds containing a thiophene ring (5). 2-Thiophenesulfonamide (V), a carbonic anhydrase inhibitor, has been used to determine the role of carbonic anhydrase in the bicarbonate excretion from salivary glands and the mechanism of ionic excretion (44). There is also a considerable amount of literature on biotin (VI) (vitamin H) and structurally related compounds such as norbiotin, homobiotin, and biotin sulfone, which act as antimetabolites (5, 45).



V



VI

ANTIHISTAMINES

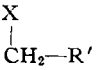
Thiophenes are most extensively used as antihistaminics. The literature on these compounds is so voluminous that only a brief consideration can be given herein. Blicke (1) records 26 thiophene derivatives which are analogous in structure to tripelennamine, while Martin-Smith (5) has observed that there are over 100 publications dealing with 2-thienyl isosteres of tripelennamine. In addition to the Blicke and Martin-Smith articles, reviews of antihistaminic drugs, in which the relationship of the various thiophene derivatives to other drugs of this class is shown, are available (10, 46). Some of the most important antihistamines possessing thiophene rings are included in Table I (5). Chlorothenylpyramine (No. XI, Table I) is also analogous to chlorprophenpyridamine. Being twice as active and possessing one-half the acute toxicity (54, 56), the 5-halogenated thiophenes (No. XI and XII, Table I) were more active than tripelennamine. When equal doses of the drugs were administered, chlorothenylpyramine protected against histamine shock twice as long as tripelennamine (61). Thiophene isosteres of other well established antihistamine drugs have also been prepared (50, 58, 62–66). However, the thiophene compounds have not shown marked superiority as antihistamines to compounds of other chemical groups, and the relative potency of the isosteres has varied according to the biological test system employed.

Campaigne and co-workers (67) studied the synthesis and reactions of 3-substituted thiophenes and prepared four *N*-substituted dimethylaminoethyl pyridines containing the 3-thienyl and halogen-substituted 3-thienyl nucleus. The 3-thienyl compounds all possessed activities about equal to those of the 2-thienyl analog (methapyrilene) and tripelennamine. The unsubstituted derivative (thenyldiamine; compound X, Table I) is more potent than the chloro and bromo substituted analogs (48, 53). This difference between the increased activity on halogenation in the 2-thienyl series and the lack of this in the 3-thienyl analogs is attributed to the difference in the position of the halogen atoms, being 5 in the former and 2 in the 3-thienyl series (48).

In common with other antihistamines, the thiophene derivatives show local anesthetic activity (68), possess antifungal action (69), prolong the blood clotting time (70), exert cardiodepressant activity (71), possess antipyretic activity in some species (72), possess some oxytocic activity, and demonstrate, to a greater or lesser degree, atropine-like activity.

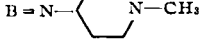
Methapyrilene is reported to possess hypnotic activity approximately equal to that of phenobarbital (73) and to potentiate the effect of other hypnotic drugs (74). The nicotinates of methapyrilene and thenyldiamine have been reported to be useful sedatives (75). Methapyrilene was found to possess quinidine-like activity in experimental cardiac arrhythmias in dogs (76). In atrial arrhythmias, it was much stronger than quinidine. For ventricular tachycardia resulting from acute mycocardial infarction, it was more effective than quinidine in suppressing the ectopic activity. In addition, methapyrilene was found to possess anti-

TABLE I.—ANTIHISTAMINIC THIOPHENE COMPOUNDS



No.		R	R′	X[a]	Ref.
VII	(Antergan)	Phenyl	Phenyl	A	...
VIII	(Tripelennamine)	2-Pyridyl	Phenyl	A	...
IX	(Methapyrilene)	2-Pyridyl	2-Thienyl	A	(47–51)
X	(Thenfadil)	2-Pyridyl	3-Thienyl	A	(48, 52, 53)
XI	(Chloromethapyrilene) (chlorothen)	2-Pyridyl	2-(5-Chlorothienyl)	A	(54, 55)
XII	(Bromothen)	2-Pyridyl	2-(5-Bromothienyl)	A	(56)
XIII	(Methaphenilene) (Diatrin)	Phenyl	2-Thienyl	A	(50, 57)
XIV	...	2-Pyrimidyl	2-Thienyl	A	(58)
XV	...	2-Lepidyl	2-Thienyl	A	(59)
XVI	...	2-Lepidyl	2-(5-Chlorothienyl)	A	(59)
XVII	...	Phenyl	2-Thienyl	B	(60)

[a] A = $N—(CH_2)_2—N(CH_3)_2$.



veratrinic activity. Results are available which indicate that antiarrhythmic activity is common to a large number of different antihistaminic compounds and is not restricted to specific structural types (77). However, only diphenhydramine and methapyrilene exhibit high activity against both types of arrhythmias. In a study of the effect of antihistamines on experimental cardiac arrhythmia, Agotsuma (78) reports that tripelennamine was most effective, followed by methapyrilene, diphenhydramine, and chlorprophenpyridamine. Methapyrilene and its chloro derivative (chlorothenylpyramine) have also been reported (79) to be more effective than quinidine when tested for ability to protect the canine heart from spontaneous ventricular fibrillation during progressive hypothermia. The isomeric carboxy methapyrilenes of 2- and 3-thiophene have been prepared, and the observed antihistaminic activity and the soporific effects are described (80).

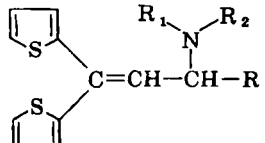
Finally, methapyrilene has also been employed in ophthalmology (81).

ANALGESICS

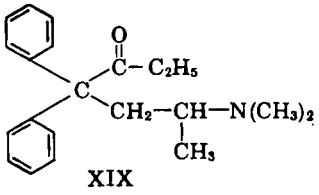
In the course of an investigation of the biological properties of a series of 3-tertiaryamino-1,1-di-(2-thienyl)but-1-enes prepared as analogs of the 3,3-diphenylallylamines which were known to have atropine-like, antihistaminic, and local anesthetic properties (82), the important discovery was made that the thiophene compounds—in addition to having similar activity to the compounds on which they were modeled—also possessed pronounced analgesic activity (83).

Unfortunately, the compounds of this series are addictive as are other potent analgesics.

The potent analgesics derived from 3-tertiary amino-1,1-di-(2-thienyl)-1-butene (XVIII; thiambutene) have been studied especially by



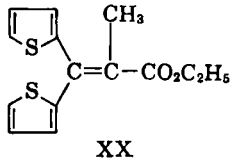
XVIII



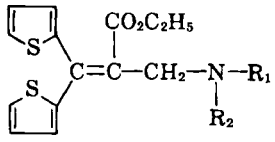
XIX

Japanese workers (85–88). Thiambutenes can be represented by the general formula (XVIII) and can thus be regarded as cogeners of methadone (XIX), in which the ketone side chain has been omitted, with the introduction of a double bond and the benzene rings replaced by thiophene rings. The double bond is not necessary for analgesic activity, and the corresponding saturated 1,1-di-(2-thienyl)-3-tertiaryaminobutanes are also potent analgesics, although somewhat weaker than the unsaturated compounds (89). It has been shown that it is the enantiomorph related to D-alanine which is the analgesically active optical isomer (90).

The thiambutene analgesics have attracted considerable attention in Japan where modified syntheses have appeared (91). Methods for the detection of thiambutenes have been described (92). The Reformatsky reaction between di-2-thienyl ketone and ethyl α-bromopropionate, followed by dehydration, gave compound XX. Bromination of the methyl group of XX with *N*-bromosuccinimide, followed by reaction with excess secondary amide, gave XXI which shows combined analgesic and antitussive properties (93). It is claimed that 3-piperidyl-1,1-di-(2-



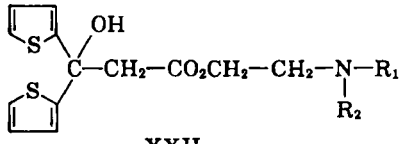
XX



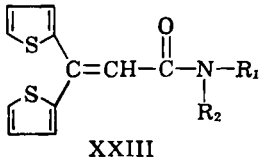
XXI

thienyl)but-1-ene is a potent antitussive as measured on the dog (94). The same compound was later reported to be very effective by oral administration to adults (95).

The Reformatsky reaction has also been used for the preparation of 2-aminoethyl 3,3-diaryl-3-hydroxypropanoates XXII and their dehydrated products (96). The propenamides (XXIII) have also been prepared for pharmacological evaluation (97). In 1-methyl-3-bis(2-thienyl)-



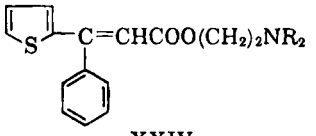
XXII



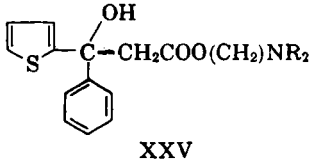
XXIII

methylenepiperidine, a potent nonnarcotic antitussive has been found (98). It is claimed to be more potent than codeine and shows no analgesic activity. 3-Piperidino-1,1-bis(2-thienyl)-1-butene has been tested clinically as an antitussive (99).

In a study of structure-activity relationships in antitussive agents, a working hypothesis—that the introduction of a piperidino group into a compound showing any action on the central nervous system can produce antitussive activity if the activity has been latent, or strength if such activity is already manifest—has been presented (100). For compounds of type XXI, XXIV, and XXV, some of which show thiambutene-like



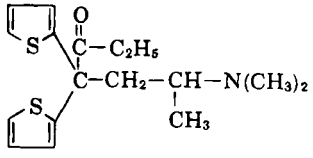
XXIV



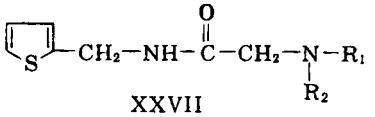
XXV

structure and weak analgesic activity, the piperidino compounds are definitely superior to the other antitussive activity (101).

The thiophene analogs of methadone XXVI and isomethadone have been prepared and shown to be active analgesics (102). Heterocyclic acetamides



XXVI

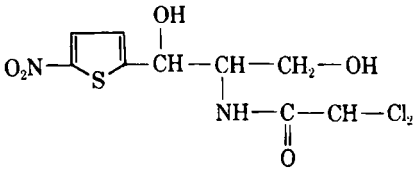


XXVII

of the type XXVII have been prepared for evaluation of their analgesics and antipyretic activity (103). Luts and Nobles have prepared the 3-thienyl analog of *d*-propoxyphene (104). Preliminary studies indicate that some of the 3-thienyl analogs combined sedative and stimulating properties in an unusual manner.

ANTIMICROBIAL AGENTS

Because of the complex chemical structure of many of the antibiotics, the synthesis of antibiotic isosteres has been somewhat limited. In the case of the penicillins, the important discovery that addition of a variety of organic acids or their derivatives to the culture medium resulted in their incorporation into new "biosynthetic" penicillins (105) provided a route for the easy production of several thiophene-containing penicillins (106). The thiophene analog of the naturally occurring antibiotic chloramphenicol (XXVIII) has been synthesized (107), as have been similar structures (5). The antibacterial



XXVIII

activity of all was much lower than that of the natural antibiotic (108).

An observation that nitrofurans possessed antibacterial activity encouraged the preparation of the thiophene isosteres. Thus 3-(5-nitro-2-thienyl) acrolein and 2-bromo-3-(5-nitro-2-thienyl) acrolein have been reported to be very active against *S. aureus* and *E. coli* (109) and to have a low toxicity to the tissues of the host. Cyanonitrovinylthiophenes have been reported to inhibit growth of bacteria and fungi (110).

Of the many nitrothiophenes prepared (111), it has been determined that the presence of the double bond as in the acrolein derivatives appears necessary for pronounced antibacterial activity and that the presence of a nitro group is not essential for effective antibacterial activity. However, it has been observed (111*a*) that the optimum position for the nitro group in the thiophene ring was the 5-position. Many chalcone derivatives of nitrothiophene carboxaldehydes (112) as well as hydantoin, thiohydantoin, and piperidine derivatives (113) have been tested, but these compounds exhibited little or no activity as bactericides. In addition, certain 5-chloro-2-thienyl-β-dialkylaminoethyl ketones have been reported to exhibit antibacterial activity (114).

In a comparative study of *in vitro* cellular toxicity of some isosteric derivatives with benzene and thiophene rings, Aurousseau (115) has noted that derivatives of thiophene have generally higher bacteriostatic potency than analogs of benzene. Only sodium thenoylacrylate and the sodium and ammonium salts of thenoic acid are slightly less active. The salt of thenoic acid with a bactericidal cation, 8-quinolinol, is more powerful than its analog. Synergism was observed in the cases of sodium thenoylacrylate, nitrothiophene, nitroacetothienone, and their homologs. Similarly, it has been demonstrated that some compounds, *e.g.*, DL-2-thienylalanine, with little or no antimicrobial activity when used

alone, act as synergists when mixed with certain antibiotics (116). Such synergism might make feasible increased therapeutic ranges of the more toxic antibiotics.

The main chemical classes of drugs showing high antitubercular activity are the sulfones, thiosemicarbazones, and certain acid hydrazides. Thiophene derivatives of each group have been prepared. The sulfone isosteres (117) possess slight antitubercular and antistreptococcal activity. All the thiosemicarbazone thiophene isosteres are reported to be active against *Mycobacterium tuberculosis* (118–123). The thiosemicarbazone of 4-(2-thienyl)-3-buten-2-one has been reported capable of completely inhibiting the *in vitro* growth of *M. tuberculosis* in relatively low concentration (124). Preliminary experiments with the 3 isomer have indicated that it has even higher activity. The hydrazine derivatives of the thiophene isosteres have been found to have an order of activity somewhat less than that of isonicotinylhydrazide (11, 125–128). The condensation products of these hydrazides with a large number of aldehydes and ketones have been investigated for the possibility of their blocking of the free amino group (11, 128). Also prepared for testing as potential tuberculostatic compounds were the condensation products of isonicotinylhydrazide and various aldehydes and ketones of the thiophene series (129).

Other compounds found to have antitubercular action were 1-*p*-thenoylphenyl-3-methyl-2-thiourea (130), which was reported to be very active against tuberculosis in mice, 6-(2-thienyl)-3-mercapto-1,2,4-triazin-5-one (131), and the thioamide of 2-thenoic acid (132). 2-Thiothenamide did not surpass thioisonicotinamide in antitubercular activity.

Recently a technique which employs a thiophene derivative has been developed for differentiation of *M. tuberculosis* into human and bovine types (133). By means of niacin test and tests of resistance against 2-thenoylhydrazine and 2-furoylhydrazine, *M. tuberculosis* strains can be divided into human and bovine types.

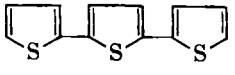
Many of those compounds exhibiting antibacterial activity are reported to possess fungicidal activity (134). Antifungal activity has also been reported in many other types of thiophene derivatives, especially those containing mercury (135). The antimicrobial activities of $(RC{\equiv}C)_2Hg$ and $RC{\equiv}CHgCl$ (R = 2-thienyl) were recently studied and found effective against both fungi and bacteria (136). 2-Thiophenemercurichloride has also found use as a plant bactericide by inhibiting the growth of imochibyo (a disease of rice) bacteria (137). Several 3-thienylrhodamines (138) and a thienyl benzimidazole derivative (139) are reported to exhibit fungistatic activity. 2-(2-Thienylthio)-ethylpyridine is reported (140) to have no insecticidal or bactericidal properties, but has mild fungicidal activity. 3,3,4,4-Tetrachlorohydrothiophene-1,1-dioxide is used as a fungicide (141). Fuerst and Higgins (142) have presented data on the inhibition of *Neurospora crassa* by a group of thiophenes. The phenomenon of colonization induction of *Neurospora* was observed with L-sorbose and by the thiophene derivatives. Some thiophenes were fungicidal while others were fungistatic.

Thiosemicarbazones of many substituted thiophene carboxaldehydes were synthesized as potential antiviral agents (143), while relatively few compounds containing thiophene rings have been prepared and tested for their toxicity to protozoan parasites. 2-Thiopheneglyoxal has been found only moderately active against Newcastle disease virus and influenza virus in embryonated eggs (144). Recently, certain β-amino ketones (Mannich bases) have been prepared (145) for which 2-acetyl thiophene was the ketone employed and 3-azabicyclo[3.2.2.]nonane served as the amine moiety. Results of preliminary screening (145*b*) indicated that significant antimicrobial activity against certain Gram-positive and Gram-negative bacteria, fungi, and protozoa is possessed by these compounds. Indications are that the thiophene isosteres are not as active as the benzene analogs.

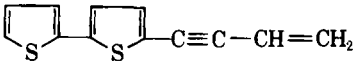
PESTICIDAL AGENTS

Thiophene compounds related to DDT have been examined for insecticidal properties (1, 5). For effectiveness against house flies, the thiophene isosteres and related thiophene derivatives have been disappointing, but they are reported effective against cockroaches and mosquito larvae, and a variety of other insects.

The interesting nematicidal activity of 2,2′-5′,2″-terthienyl (XXIX) (146) (also called α-terthienyl) and of 5-(3-buten-1-ynyl)-2,2′-bithienyl (XXX) (147), prompted Uhlenbroek and Bijloo (148) to investigate more systematically the nematicidal properties of polythienyls and related compounds. The compounds exhibiting highest activity are recorded in Table II.

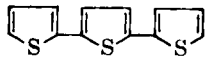
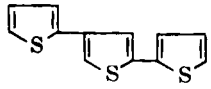
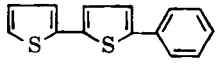
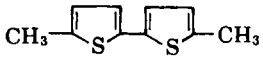
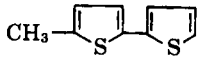
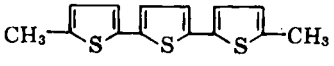
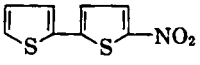
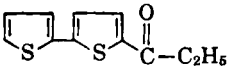


XXIX



XXX

TABLE II.—NEMATICIDAL THIOPHENE COMPOUNDS

Compd. No.		Structure
XXIX	2,2′-5′,2″-Terthienyl	
XXX	2,2′-4′,2″-Terthienyl	
XXXI	5-Phenyl-2,2′-bithienyl	
XXXII	5,5′-Dimethyl-2,2′-bithienyl	
XXXIII	5-Methyl-2,2′-bithienyl	
XXXIV	5,5″-Dimethyl-2,2′-5′,2″-terthienyl	
XXXV	5,5′-Dichloro-2,2′-bithienyl	
XXXVI	5-Nitro-2,2′-bithienyl	
XXXVII	5-Propionyl-2,2′-bithienyl	

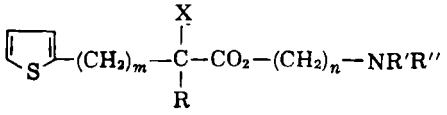
The consequences of the following structural variation were studied in particular: (*a*) isomeric changes, (*b*) substitution of one or more thiophene rings in polythienyls by benzene rings, (*c*) introduction of methyl groups into polythienyls, and (*d*) introduction of other substituents into polythienyls. The conclusion is drawn that any compound with very high nematicidal activity can be considered to be a derivative of 2,2′-bithienyl. However, 2,2′-bithienyl itself and a number of derivatives show little or no nematicidal activity. Therefore, proper substitution may also play an important part. In addition to their effectiveness against nematodes, the polythienyls are effective herbicidal agents against many noxious plants (149).

Several of the halogenated tetrahydrothiophene-1,1-dioxide compounds may be employed in nematicidal and other pesticidal application (150). An advantage is their ease of use, for no water seal is required. They may be used as dry powder with filler or as a liquid when wetting agents are added.

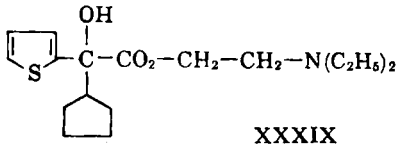
The nematicidal action of thenyl isothiocyanate has been demonstrated by its action against *Panagrellus redivivis* in water and *Meloidogyne incognita* var. *acrita* in soil (151).

ANTISPASMODICS

Earlier investigations have shown that powerful antispasmodic activity occurs in various basic esters of α-substituted thienylglycolic acids,



XXXVIII



XXXIX

thienylacetic acids, and thienylpropionic acids of the general type XXXVIII (5). One such compound, the 2-diethylaminoethyl ester of α-cyclopentyl-(2-thienyl)glycolic acid (XXXIX), is employed clinically in the form of its salt. The most recent work is concerned with the basic alkyl esters of α-(2-cycloalkenyl)-2-thienylacetic acids, which have been pharmacologically evaluated in the form of their acid addition and quaternary ammonium salts (152, 153). Several have been found to possess anticholinergic activity of a high order.

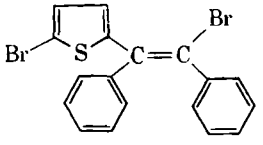
β-(α-Phenyl-α-3-thenylacetoxy)ethyldimethylsulfonium bromide was synthesized from 3-methylthiophene in an effort to determine the potentiality of this isostere of thiospasmin as an

antispasmodic agent (154). This agent was markedly less effective as an antispasmodic than is the cyclohexyl analog of thiospasmin. The compound appeared to potentiate the action of serotonin on the isolated rat uterus.

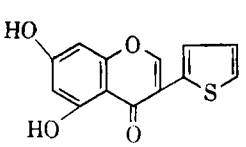
The main choleretic principle of the plant *Curuma domestica* was identified (155) as *p*-tolylmethylcarbinol. The isosteric alkyl-2-thienylcarbinols and alkyl(5-methyl-2-thienyl)-carbinols have been prepared (156) and reported to display choleretic activity in rats.

ESTROGENIC AGENTS

Many stilbene-like thiophene compounds have been prepared for a study of estrogenic activity, especially by Buu-Hoi, *et al.* Thiophene derivatives of nonhydroxylated stilbene types showed no significant activity (157), whereas weak estrogenic activity was found in 5-acetyl, 5-propionyl- and 5-benzoyl-2-(-stilbenzyl)thiophene (158). 1-Bromo-1,2-diphenyl-2-(5-bromo-2-thienyl)ethylene (XL) was found to inhibit body growth and to produce extensive testicular atrophy in male rats (159). It has been suggested that this compound deserves clinical trial in mammary cancer. 1 - Bromo - 1 - phenyl - 2 - (*p* - methoxyphenyl)-2-(5-bromo-2-thienyl)ethylene and 1-bromo-1-(*p* - chlorophenyl) - 2 - (*p* - methoxyphenyl - 2-(5-bromo-2-thienyl)-ethylene were reported to



XL



XLI

exert a true estrogenic action in rats (160), 1-Bromo-1,2-diphenyl-2-(5-bromo-2-thienyl)ethylene did not exhibit true estrogenic activity, although it did inhibit release or action of the follicle-stimulating hormone.

A thiophene analog of an isoflavone (XLI) showed no activity (161).

THIOSTERS OF CARCINOGENIC HYDROCARBONS

In the review article by Martin-Smith (5), the interest in compounds containing thiophene rings is reported to be derived largely from the ideas inherent in the receptor theory of drug action, the theory of biological antagonism, and the concept of bioisoterism. A new theory, which has gained favor in recent years and which also incorporates many of the principles of the three concepts mentioned above, is based on quantum mechanics. For example, although one would not ordinarily think of diagnosing diseases in a human patient by means of quantum mechanics, such a technique has been developed for differentiation between obstructive jaundice and nonobstructive jaundice.

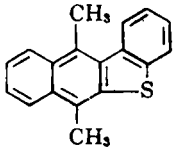
Schmidt (163) was the first to use wave mechanical methods to study the mechanism of the action of carcinogenic substances. He concluded that in order for an aromatic hydrocarbon to be carcinogenic, it was necessary that there exist in the molecule a region rich in π electrons, the density of which exceeded a certain threshold value. Svartholm (164) proposed to associate the carcinogenic properties of hydrocarbons with the reactivity of addition across one of the bonds. To explain an antagonism phenomenon which they had observed, Lacassagne, Buu-Hoi, Daudel, and Radali (165) imagined that carcinogenic hydrocarbons must be able to form an addition complex with the substrate which controls cellular division. This addition complex was assumed to be effective in producing a cancer only if it could take place at certain well defined points in the cell.

Several theoretical studies, of which summaries are available in recent literature (166), have shown that hydrocarbons or derivatives that are carcinogenic have a bond with high bond order. As this property determines the rate of certain addition reactions to this bond, it is tempting to assume that the addition of the hydrocarbon through one of its bonds to certain cellular constituents is an important step in the production of cancer. A more recent report states that it seems improbable that there was a clear relation between the total amount of a substance which was fixed to the cellular proteins and its carcinogenic power (167). However, it remained probable that a necessary condition for a substance to be carcinogenic was that a substantial amount was fixed by one of its bonds to the protein.

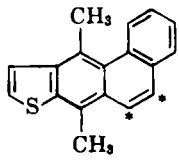
Many of the polycyclic hydrocarbons that have proved to be carcinogenic are phenanthrene derivatives suitably substituted by alkyl groups or with fused aryl rings attached to phenanthrene. Some heterocyclic analogs of these carcinogenic hydrocarbons, such as acridine, carbazole, and thiophene derivatives, have also proved to be carcinogenic (168). From these facts, Robinson proposed that the essential structural features for carcinogenicity in the polycyclic hydrocarbons may be an activated phenanthrene bridge (the 9,10 double bond in phenanthrene shown by asterisks in formula), which needs to be unsubstituted (169).

In an attempt to determine what effect, if any,

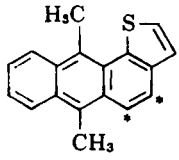
the 9,10 double bond might contribute towards carcinogenic activity, Robinson and Tilak have synthesized thiophene isosteres of carcinogenic hydrocarbons, in which the sulfur atom in the thiophene ring replaces the key phenanthrene bridge. A review of the work in this field, which contains a considerable amount of unpublished data, has been presented by Tilak (170). Interestingly, compound XLII was only slightly active



XLII

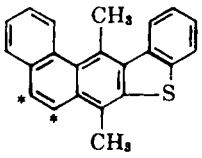


XLIII

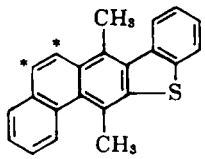


XLIV

when painted on mice and inactive by subcutaneous injection when compared to the carcinogenic compounds XLIII and XLIV. The



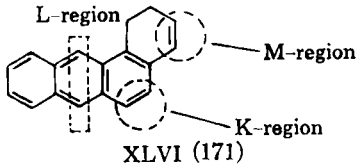
XLV



XLV*a*

inactivity of XLII was attributed to the absence of the phenanthrene bridge, since high activity again emerged in the case of XLV and XLV*a*.

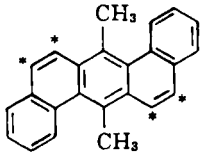
Since the first step in carcinogenesis involves a reaction of the carcinogen and a cellular receiver probably of an electrophilic nature, Pullman (166*a*) postulated that the "K-region" should be sufficiently active and that the "L-region" (*meso* position in anthracene residue) should be inactive (by substitution, *e.g.*, by methyl groups). The more carcinogenic hydrocarbons are generally



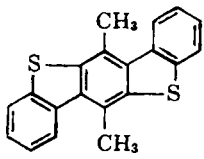
XLVI (171)

more strongly bound to proteins by the "K-region," while weaker carcinogenic or inactive hydrocarbons were bound through the "L-region" or by neither. For example, the electronic charge of the "K-region" of a series of benzoacridines was reported (172) to correlate directly with a tumor index (per cent of animals developing tumors per time in days for tumor appearance). There are exceptions which are difficult to explain on the basis of the Pullman hypothesis.

The synthesis of thiosters of carcinogenic hydrocarbons in which the "K-region" is replaced by isosteric substitution with thiophene has been continued (173). Many of the thiosters remain to be tested. Some unexpected results were observed among those which have been tested. The two phenanthrene bridges (PB's) in 9,10-dimethyl-1:2,5:6-dibenzanthracene (XLVII) have been replaced stepwise as in XLV*a* (173, 174) and XLVII*a* (175). Compound XLV*a* is a much more powerful carcinogen than XLVII (168*b*), and the high activity

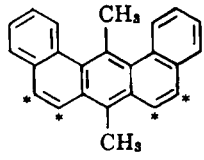


XLVII

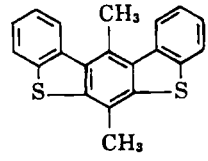


XLVII*a*

is attributed to a single activated PB instead of two competitive PB's as in XLVII (173). The key thioster XLVII*a*, wherein both the PB's are removed, proved highly carcinogenic, contrary to the expectation that it would be inactive (176). When one of the two PB's in 9,10-dimethyl-1:2,7:8-dibenzanthracene (XLVIII) was replaced, a highly active carcinogen XLV resulted as expected (177). However, the key thioster

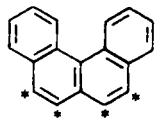


XLVIII

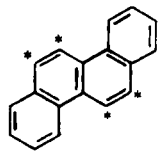


XLVIII*a*

XLVIII*a*, wherein both the PB's were removed (178), also proved to be highly carcinogenic contrary to its expected inactivity (176). The thioster of 3:4-benzphenanthrene (XLIX) and chrysene (L) have proved inactive (168*b*, 176), although the thiosters may contain one or two of the original phenanthrene bridges. The key thiosters of XLIX and L, in which both PB's

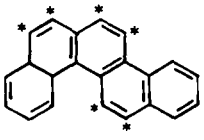


XLIX

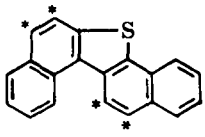


L

are replaced by sulfur, have also proved non-carcinogenic. The thioster (LI*a*) of the weakly carcinogenic 1:2,5:6-dibenzphenanthrene (LI) has given a highly toxic initial reaction when given subcutaneously to mice (178).



LI



LI*a*

Tilak (170) has observed that the interrelation between carcinogenicity and the structure of polycyclic hydrocarbons and their thiosters does not appear to be simple. The carcinogenicity of some of the thiosters which do not contain a phenanthrene bridge cannot be fitted into the "K-region" hypothesis, and it seems likely that the sulfur atoms in the condensed thiophenes have their own characteristic reactivity towards a biological substrate.

Metabolic studies (179) of dibenzothiophene have provided experimental support for the suggestion that the sulfur atom is implicated in metabolism. When fed to rats through a standard diet, 1-hydroxydibenzothiophene-5,5-dioxide[1] was isolated. The sulfur atom in thiosters, like the "K-region" in the corresponding hydrocarbon, is oxidized *in vivo* and is responsible for the carcinogenicity of these compounds.

As noted earlier for the β-thienylalanine, some thiophene derivatives possess the ability to retard the growth of various tumors. Thiophene-2,5-carboxylic acid in repeated doses inhibited the growth of Yoshida sarcoma in rats and the growth of a fibrosarcoma in mice (180). *In vitro* studies with a C^{14}-labeled glucose suggested that certain thiophene compounds interfered with the hexose monophosphate pathway. β-Chloroethylamino derivatives of thiophene were found quite toxic and inhibited the growth of tumors (sarcoma "45," Ehrlich's tumor) only slightly (181). Uptake and incorporation of glycine-1-C^{14} by Ehrlich ascites carcinoma cells was inhibited by α-amino-2-thiophene acetic acid (thienylglycine) (182).

MISCELLANEOUS

The earlier literature of thiophene chemistry indicates a potential use of thiophene isosteres as anticonvulsants (1, 5). A large series of 5-(2-thienyl) substituted hydantoins, including some bearing 3-alkyl substituents and 1,3-dialkyl substituents have been prepared (183), and some have been found to possess the same order of activity as 5,5-diphenylhydantoin. The anticonvulsant properties of 5-phenyl-5-(2-thienyl) hydantoin have been well established and compare directly with those of 5,5-diphenylhydantoin (184). It was clinically used for a time (185) on account of its favorable therapeutic ratio, since although less active than 5,5-diphenylhydantoin, it is also less toxic. 5,5-Di(2-thienyl) hydantoin is claimed to be active as an anticonvulsant (186).

Thiophene derivatives have been studied quite extensively for local anesthetic activity (1, 5). When a series of dialkylaminoalkyl esters of 3-thenoic acid were compared with the 2-isomers (67*c*), only γ-di-*n*-butylaminopropyl-3-thenoate was found to have significant local anesthetic activity. The activity of this compound was slightly less than that of the corresponding 2 isomer. A study of the influence of isosterism of acids on activity of salts and esters endowed with local anesthetic or analgesic properties has recently appeared (187). For procaine salts, the 2-thenoate was less potent, quicker acting, and of shorter duration of action than the benzoate, but both were more potent local anesthetics than the hydrochloride salts. The benzylurethan of glycol and the 2-thenylurethan of glycol were both local anesthetics, but much weaker than procaine hydrochloride. The thenyl isostere was more potent and also more toxic than the benzyl isostere. For morphine salts, the 2-thenoate acted more slowly, but the action was of longer duration than that of the benzoate. The (2-thenoyl)-acrylic acid salt of morphine was slightly more potent than the benzoylacrylic acid salt.

The discovery that certain β-haloethylamines possessed powerful adrenergic blocking action inspired a mild interest in thiophene compounds of this type (188). Of the thiophenes studied *N*-thenyl-*N*-benzyl-2-chloroethylamine was the most active (189). The antiadrenaline and antinoradrenaline activity of *N*-benzoyl-*N*-2′-halogenoethyl 2-thenylamines has been studied (190).

The radiation protective action of *N*-phenylamidines of thiophene carboxylic acid in white rats has been studied (191). The radioprotective effect of the *N*-phenylamidine of 2-thiophenecarboxylic acid has been reported (192) dependent on the dose used. The radioprotective effect began to be manifest at doses of 60 mg./Kg. Below these doses there was a sharp decline in the radioprotective capacities. At 60 mg./Kg. the percentage of survivals among the female animals was 60, while that among the males was 35. The difference between the protected male and female

[1] Tilak (170) reported earlier that 2-hydroxydibenzothiophene-5,5-dioxide was isolated during the metabolic studies of dibenzothiophene. Undoubtedly a typographical error has been made in one of these reports.

rats indicates a possible significance of female sex hormones. The radiosensitivity of various transplanted tumors is claimed to be increased by pretreatment with 2,5-dicarbethoxy-3,4-dihydroxythiophene (193). This agent inhibited the uptake of oxygen by homogenates of various mouse tumor tissues and of normal liver, kidney and spleen, but not of brain.

The diuretic activity of some thiophene derivatives has been studied recently (194). 2,4-Disulfonamidothiophene, 2,4-disulfonamido-5-chlorothiophene, and 2,4-disulfonamido-5-methylthiophene showed notable diuretic effect. However, at doses above 250 mg./Kg. they showed neuromuscular effects characterized by a degree of hypotonicity and ataxia and accompanied by respiratory depression. The diuretic activity of oral doses of 12.5 and 50 mg./Kg. was determined after 5 hours; the effect of daily doses was studied at the end of 3 and 6 weeks. Marked increases over the levels for controls were observed in the volume of urine, sodium, potassium, and chlorine excreted 5 hours after ingestion.

N'-Substituted-*N*-thiophenesulfonyl ureas have been reported to cause a significant lowering of the blood sugar level when administered intravenously as determined on rabbits (195).

A series of compounds containing thiophene rings which are isosteres of the less important biological active compounds, or else which have had very little work done on them is summarized by Martin-Smith (5). This series includes agents which act as vasoconstrictors, choleretics, antithyroid agents, vasopressors, sedatives, antimitotic substances, vasodilators, muscle relaxants hypotensive agents, and heart and respiratory regulators.

Recently 2-[3-(2-methylbenzothienyl)methyl]-2-imidazoline has been used to increase arterial blood pressure (196).

CONCLUSIONS

In the search for drugs with a higher degree of potency and fewer toxic side effects, considerable attention has been devoted to compounds which possess thiophene rings. From a voluminous amount of work very few new drugs of clearly superior clinical desirability have been produced. As a result, one can notice a certain slackening interest in the preparation of thiophenes for pharmacological studies. However, these results should serve as a guide to future research, and not as a sign that negative conclusions invalidate future work. As noted earlier (5), the work on thiophenes has made valuable contributions to the study of antimetabolites: it has shown in general the biological similarity of the phenyl and thienyl groups; and it has shown that the position of substitution of the thiophene ring is important (3-thienyl derivatives usually being more active and less toxic than their 2 isomers). The data accumulated should contribute to the development of newer theories which may lead to better understanding of drug action and biochemical processes.

In this connection, one can mention the use of thiophene and its homologs as starting compounds for the preparation of aliphatic amino acids (197). Long chain amino acids, both straight and branched, with a variable number of carbon atoms between the amino and carboxyl groups were prepared by synthesis of the corresponding thiophene derivatives and subsequent Raney nickel reductive desulfurization. The significant importance of the aliphatic amino acids and the opportunities offered by this method should be obvious, particularly regarding metabolic studies, since it would appear feasible for desulfurization to occur under biochemical conditions, as well as oxidation of the thiophene molecule. Studies have shown that there was increase of neutral sulfur in the blood, but no increase in the sulfate ion concentration (198).

It would appear advantageous to study thiopene derivatives more as individuals and less in regard to their isosteric relationship to some known biologically active agent. For example, 2,2'-5',2''-terthienyl (148) is reported to exhibit very high nematicidal activity, while the phenyl isostere (*p*-terphenyl) gives negative results. Also, the thiosters of the carcinogenic polycyclic hydrocarbons, in many cases, exhibited carcinogenic activity considerably higher than that of the phenyl isomer (170). Thus, while the thiophene isosteres may not exhibit activity comparable to the benzene isostere from which they were modeled, they may display a unique action (biological activity) of their own which will be entirely different from that of the benzene isomer.

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Research Articles

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Three gastrointestinal factors influencing aspirin absorption have been selectively adjusted for quantitative measurement of their effect on the rate of salicylate absorption in humans and dogs. These are (a) gastric emptying, (b) aspirin dissolution rate, and (c) intragastric pH. Other factors such as tablet disintegration time, aspirin particle size, and intersubject variability have been eliminated or reduced where possible. The major portion of the salicylate in the blood during the first 20 minutes comes from the stomach. Impedance of gastric emptying by the use of atropine and placing the subjects in a left lateral position did not eliminate the higher plasma salicylate levels produced by the inclusion of the buffer antacids in aspirin tablets. The major effect of these antacids appears to be an acceleration of aspirin dissolution. This has been confirmed in this study by an *in vivo* demonstration of more rapid absorption from solutions of aspirin than from tablets. Aspirin in solution has a lower pH alone than with the inclusion of buffering antacids. Selective adjustment of the degree of aspirin and sodium salicylate ionization through the use of various buffers provided a method of study of the influence of intragastric pH on the rate of aspirin absorption. Support was obtained from dog and human studies in favor of the pH partition hypothesis that salicylates are absorbed more rapidly at low pH values.

MANY INVESTIGATIONS of salicylate absorption have been made because of the ease with which salicylic acid can be measured in body fluids. Despite this, some factors regulating this membranous transfer have only recently been measured under physiologic conditions in man. One such factor is the direct transfer of salicylate from the stomach into the blood circulation (1, 2).

The claim of Paul, *et al.* (3), that certain antacids increased the gastrointestinal absorption rate of aspirin has received considerable examination. Despite a number of studies showing no significant differences (4–7), quite a few tests using adequate numbers and crossover design to reduce intersubject variability have shown clearly

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The claim of Paul, *et al.* (3), that certain antacids increased the gastrointestinal absorption rate of aspirin has received considerable examination. Despite a number of studies showing no significant differences (4–7), quite a few tests using adequate numbers and crossover design to reduce intersubject variability have shown clearly

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significant acceleration of aspirin tablet absorption rate (8–10). In addition, claims of accelerated gastric salicylate absorption have been reported for solutions of sodium acetylsalicylate (11), calcium aspirin (8), and for combinations of salicylates with magnesium aluminum hydroxide (12), mephenesin (13), methocarbamol (14), glucosamine (15), and an effervescent mixture (16).

Those factors which are theoretically involved in controlling the rate of absorption of oral aspirin tablets include: (*a*) tablet disintegration time, (*b*) aspirin particle size, (*c*) aggregation between aspirin particles and other tablet components such as disintegrators, binders, excipients, etc. (a function of tablet compression force), (*d*) aspirin dissolution time, (*e*) the gastric pH (by affecting the ratio of ionized to unionized drug), (*f*) the rate of gastric emptying, and (*g*) the presence of interfering factors such as gastric mucus, food, and other adsorbents and diluents Some of these factors can be experimentally controlled in human subjects to a degree that permits an evaluation of their quantitative function in the absorptive process. It is the purpose of this study to investigate the role of three factors—(*a*) gastric emptying, (*b*) aspirin dissolution rate, and (*c*) gastric pH—on the early absorption rate of aspirin. By testing these factors with and without the addition of the buffer antacids, magnesium carbonate, and dihydroxyaluminum aminoacetate, some evidence can be deduced concerning the mechanism by which they accelerate aspirin absorption.

METHODS AND MATERIALS

Twenty healthy subjects were tested following an overnight fast in a crossover comparison of a plain[1] and a buffered aspirin.[2] Each subject received 0.4 mg. of atropine sulfate orally 30 minutes before the salicylate drugs. After a control blood sample was withdrawn, each subject swallowed (without chewing) two tablets of one of the salicylate products, with 100 ml. of cool tap water. Each tablet contained 0.32 Gm. of acetylsalicylic acid. The subjects were then placed in the left lateral position. Blood samples were drawn at 5, 10, and 20 minutes with the subjects remaining in the recumbent position and were analyzed for total plasma salicylate by the Routh and Dryer modification of the Brodie method (17). After a 72-hour interval the experiment was completed.

Dissolved Aspirin Test.—A second panel of 20 fasted subjects (differing from the first group by six replacements) were tested using solutions of freshly dissolved aspirin with and without the buffer antacids. Each subject drank 250 ml. of water containing either (*a*) 648 mg. of aspirin (U.S.P. Monsanto brand) or (*b*) 648 mg. of aspirin

[1] Marketed as Bayer aspirin, lot No. L0228, by Winthrop Laboratories, New York, N. Y.
[2] Marketed as Bufferin, lot No. 1B08, by the Bristol-Myers Co., New York, N. Y.

TABLE I.—PLASMA SALICYLATE LEVELS (mg./100 ml.) IN 20 SUBJECTS WITH AND WITHOUT 0.4 mg. ATROPINE SULFATE AND L.L.P. 30 MINUTES BEFORE BUFFERED ASPIRIN ADMINISTRATION (0.648 Gm.)

Subject	$AtSO_4$ and L.L.P. Time, min. 5	10	20	No $AtSO_4$ Time, min. 10	20
1	0.10	0.30	0.80	0.80	1.95
2[a]	0.05	0.68	2.20	0.95	3.85
3	0.15	0.40	0.50	0.10	2.60
4	0.20	1.05	3.05	1.10	4.40
5	0	0.38	0.92	0.68	1.95
6	0	0.30	0.68	0.40	1.40
7	0	0.10	0.70	...	...
8	0.25	0.40	1.48	0.10	2.70
9[a]	0.10	0.40	1.25	0.89	5.69
10[a]	0.30	...	1.10	...	2.58
11[a]	0	0.60	2.65	0.65	2.15
12	0.32	1.00	2.90	0.95	4.00
13	0.10	1.70	3.90	0.45	3.51
14	0.30	0.70	1.76	0.85	3.40
15[a]	0.20	2.62	3.32	1.98	6.45
16	0	0.10	0.70	0.79	1.90
17[a]	0.15	1.40	3.30	1.85	4.30
18[a]	0.10	0.70	1.85	...	...
19	0.30	0.40	1.38	1.30	3.75
20	0	1.65	3.40	1.25	2.30
Mean	0.131	0.78	1.89	0.84	3.27

[a] Female.

TABLE II.—PLASMA SALICYLATE LEVELS (mg./100 ml.) IN 20 SUBJECTS WITH AND WITHOUT 0.4 mg. ATROPINE SULFATE AND L.L.P. 30 MINUTES BEFORE ASPIRIN ADMINISTRATION (0.648 Gm.)

Subject	$AtSO_4$ and L.L.P. Time, min. 5	10	20	No $AtSO_4$ Time, min. 10	20
1	0.05	0.55	2.00	0.35	0.60
2[a]	0.10	0.60	1.80	1.15	1.20
3	0.10	0.30	0.40	0.68	2.00
4	0.20	0.70	2.26	0.68	1.75
5	0	0.30	0.32	0	0.50
6	0	0.70	1.85	1.76	4.80
7	0	0.32	0.92	...	...
8	0	0.05	0.68	0	0.35
9[a]	0.32	0.70	3.05	0.75	1.95
10	0	0.30	0.92	1.85	5.60
11[a]	0	0.10	0.70	0.63	2.65
12	0	0.20	0.90	0.20	1.20
13	0.30	0.38	1.62	0.85	1.78
14	0	0.15	0.68	0.35	1.85
15[a]	0	0.30	1.00	0.60	0.95
16	0.30	0.35	1.40	0.70	1.80
17[a]	0.30	1.00	1.78	1.09	1.09
18[a]	0	0.10	1.05	...	
19	0.30	0.70	1.60	1.40	3.50
20	0.60	0.90	1.75	0.20	1.30
Mean	0.129	0.44	1.33	0.74	1.94

[a] Female.

TABLE III.—AVERAGE PLASMA SALICYLATE LEVELS IN 20 FASTING SUBJECTS WITH IMPEDED GASTRIC EMPTYING OWING TO ATROPINE AND A LEFT LATERAL POSITION (L.L.P.)

Treatment	Av. Plasma Salicylate, mg./100 ml. 5 min.	10 min.	20 min.
Buffered aspirin	0.131	0.78	1.89
Aspirin	0.128	0.44	1.33
Mean individual difference	0.003	0.34	0.54
S.E. difference	0.005	0.15	0.27
p	>0.90	<0.05	<0.10
		>0.02	>0.05

TABLE IV.—PLASMA SALICYLATE LEVELS IN 20 FASTING SUBJECTS AFTER DRINKING 250 ml. OF ASPIRIN SOLUTION (648 mg.) WITH AND WITHOUT BUFFER ANTACIDS (MAGNESIUM CARBONATE, 194 mg., AND DIHYDROXYALUMINUM AMINOACETATE, 97 mg.)

	Buffered Aspirin Solution				Plain Aspirin Solution			
	Time, min.				Time, min.			
Subject	5	10	20	30	5	10	20	30
1	0.50	1.95	4.05	6.50	0	0.70	3.50	3.90
2	0.70	1.58	3.00	5.30	0.30	2.70	5.35	6.90
3	0.35	1.30	4.30	7.50	0.75	1.48	4.80	7.60
4	...	3.05	3.85	4.30	0.38	1.55	3.88	5.60
5	0.70	2.60	5.30	7.25	0.95	2.90	5.95	6.90
6[a]	0	1.70	6.80	8.30	2.40	3.75	7.10	8.20
7	0.70	4.35	5.40	6.45	1.75	3.88	6.45	7.20
8[a]	1.40	5.60	7.50	7.50	...	...	6.15	7.95
9[a]	0.10	0.40	4.00	6.00	0.55	1.05	2.86	5.00
10[a]	0.80	2.70	7.60	8.40	1.65	3.78	7.50	7.50
11	0.35	2.30	5.30	5.90	1.50	4.80	6.55	6.45
12	0	0.80	4.00	5.40	1.50	3.90	5.50	6.60
13[a]	0	0.65	1.80	3.60	0.30	2.38	6.00	7.10
14[a]	0.70	2.95	5.85	6.35	1.10	4.35	7.10	7.60
15	0.40	1.95	4.70	6.15	0.35	2.75	5.85	6.15
16	0.65	2.38	5.20	6.00	1.75	3.30	5.35	6.35
17	0.25	1.70	4.35	4.80	1.05	3.10	5.50	6.45
18[a]	1.80	3.85	7.10	7.80	1.15	4.20	6.00	7.50
19	0.50	1.65	6.40	6.00	1.65	3.60	5.45	6.50
20	0.80	3.40	6.00	7.10	0.50	2.80	6.90	6.95
Mean	0.56	2.34	5.13	6.33	1.03	3.00	5.69	6.69

[a] Female.

TABLE V.—MEAN PLASMA SALICYLATE LEVELS IN 20 FASTING SUBJECTS AFTER DRINKING ASPIRIN SOLUTIONS—BUFFERED AND PLAIN

	Av. Plasma Salicylate, mg./100 ml.			
Treatment	5 min.	10 min.	20 min.	30 min.
Buffered aspirin solution	0.56	2.34	5.13	6.33
Aspirin solution	1.03	3.00	5.69	6.69
Mean individual difference	−0.55	−0.68	−0.56	−0.36
S.E. difference	0.189	0.30	0.295	0.275
p	<0.01	<0.05	<0.10	<0.3
		>0.02	>0.05	>0.2

with 194 mg. of magnesium carbonate and 97 mg. of dihydroxyaluminum aminoacetate. These solutions were prepared immediately before drinking with the aspirin fully dissolved; however, the buffers were incompletely dissolved in this limited amount of fluid. After 72 hours the subjects received the opposite portion of the crossover design.

Buffered Aspirin Solution Tests.—*Dog Study.*—Six dogs were tested for intragastric pH and plasma salicylate levels after receiving each of five citrate-phosphate buffer (0.15 *M*) solutions containing a 50 mg./Kg. dose of aspirin or sodium salicylate. The solutions were adjusted to pH's 3.2, 4.4, 5.2, 6.0, and 8.0, and the volume given was 200 ml. A control blood sample was removed; 0.025 mg./Kg. of atropine sulfate was injected intravenously. The dog was then intubated with a double lumen catheter having either a glass or antimony electrode at the tip (glass was necessary for low pH values). The indifferent electrode (calomel) was placed in contact with the skin using a salt paste for contact. The dog was trained to lie quietly on its left side and the stomach tube was adjusted to the fundic region of the stomach by markings on the tube which had been previously calibrated by X-ray photographs. The buffer was instilled through the catheter, washed in, and blood samples were taken at 5 and 10 minutes with the animal remaining in the left lateral position. Each dog received two tests with each aspirin buffer solution and one test for each sodium salicylate buffer solution, with no more than two tests per week.

Human Study.—Ten fasted subjects (all except two from the first two panels) were tested for intragastric pH and plasma salicylate levels after receiving each of three citrate-phosphate buffer solutions containing an aspirin dose equivalent to two tablets. Intragastric pH was recorded using an antimony electrode in the catheter tip and was compared to a calomel reference electrode which made contact with the subject's hand through some salt-containing gelatin paste. The subjects were placed in the left lateral position and remained so throughout the test. After the positioning of the electrode, the catheter was taped to the subject's face to eliminate movement of the electrode into the intestine, etc. Three 0.15 *M* citrate-phosphate buffers of McIlvaine's series with pH values of 2.5, 4.5, and 6.5 were used to prepare solutions of aspirin containing 648 mg. in 250 ml. of buffer.

After the initial pH was measured and a control blood sample removed, the aspirin in buffer solution was injected through the catheter. With a buffer of pH 2.5 at 15°, it was not possible to dissolve more than about 80–90% of the aspirin and the rest was instilled as a mixture. Intragastric pH was recorded continuously and blood samples were taken at 10 and 20 minutes with the subject reclining. Total plasma salicylate concentration was measured as before.

RESULTS

Impeded Gastric Emptying Test.—The plasma salicylate levels were generally lower for both aspirin and buffered aspirin when emptying of the stomach was impeded than those values usually found with the subject in the upright position. These differences are shown in Tables I and II. The summarized values which were measured in this test are shown in Table III. Paired statistical analysis of individual differences showed that atropine combined with the left lateral position reduced salicylate levels significantly for aspirin at 10 minutes and buffered aspirin at 20 minutes.

It may be seen that the buffered aspirin tablets produce significantly higher plasma levels at 10 minutes and the difference is close to this level of significance at 20 minutes, but not at 5 minutes.

Dissolved Aspirin Test.—The plasma salicylate levels measured after administration of solutions of aspirin were the highest ever measured in this laboratory for this dose and absorption time. Tables IV and V show the levels of salicylic acid following each solution.

It is apparent from the data summarized in Table V that aspirin alone becomes the more rapidly absorbed product when the dissolution step (in the absorptive process) is accomplished before ingestion of the drug. The addition of the buffer antacids significantly slows absorption during the first 10 minutes, but not at later periods.

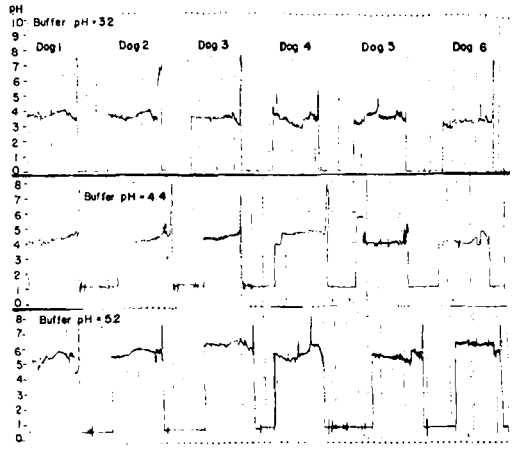


Fig. 1.—Dog intragastric pH after oral acetylsalicylic acid administration (50 mg./Kg.) in citrate-phosphate buffers.

Buffered Aspirin Solution Test.—*Dog Study.*—Figures 1 and 2 show the resulting 10-minute intragastric pH recordings for the six dogs at each of the five buffer pH values. It may be seen that the *in vivo* pH was stable and ranged about ±0.5 pH units of the value for the instilled buffer. Figures 3 and 4 show the plasma salicylate values for the test of sodium salicylate buffer solutions at the various recorded pH values and after 10 minutes of absorption. Although a large degree of individual variation in response occurred, there is a pronounced trend from high plasma levels at lower pH values to much lower levels at the high buffer pH values (*i.e.*, a suggestion that salicylate absorption is inversely correlated to intragastric pH). A similar relationship was found between intragastric pH and absorption rate for aspirin with these same buffers. The large interdog variation of these plasma salicylate levels prompted the larger study of this relationship in humans using a wider interval between buffer pH.

Human Study.—Generally the intragastric pH found after buffered aspirin instillation was close to the initial pH of the buffer *in vitro*. The magnitude of these differences are shown in Table VI.

These data suggest that the resultant intragastric pH following instillation of these buffers was within a reasonable range from the initial *in vitro* values. As expected, the two higher pH buffers were depressed, likely by gastric secretions. The lower value was raised, although the average initial pH measured in the stomach before administration of the buffer was 2.58 and quite uniform with a S.E. of ±0.13 pH units.

The rate of salicylate absorption may be expressed either in relation to the initial pH of the buffer *in vitro* or in terms of the resulting pH in the stomach. Table VII presents this relationship in correlation with the original pH of the instilled buffer.

An adequate test of the relationship between buffer pH and absorption rate was not completely possible, especially at the earlier time period, since the total amount of aspirin could not be fully dissolved in a pH 2.5 buffer. Thus, at 10 minutes the absorption of salicylate was greater for the pH 4.5 and 6.5 buffers, as expected. However, at 20 minutes the highest plasma levels occurred with the pH 4.5 buffer.

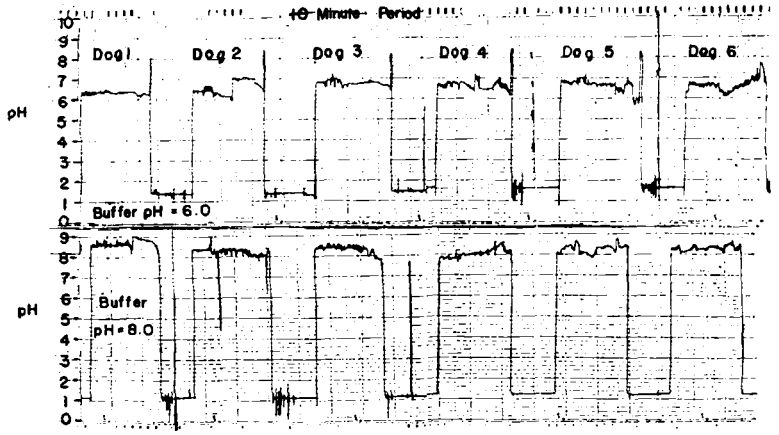


Fig. 2.—Dog intragastric pH after oral acetylsalicylic acid administration (50 mg./Kg.) in citrate-phosphate buffers.

If the plasma salicylate levels are compared instead to the actual pH measured from the intragastric electrode, a somewhat different pattern emerges. This is shown in Table VIII.

On this basis, an increased salicylate absorption is evident in two separate ranges, pH 3.0 to 3.9 and above pH 5.0. The peaks are better defined at the later time period, probably for the same reason of incomplete solubility for the lower pH buffer suggested before.

DISCUSSION

The well known accelerating action of antacids upon gastric emptying prompted the evaluation of this factor as the first possible explanation of the more rapid absorption of buffered aspirin. It was expected that if faster absorption by the buffered drug disappeared with the use of atropine and the left side position, it could be taken as evidence of a role for gastric emptying in its absorption. Roentgenographic evidence for the effectiveness of this procedure in delaying gastric emptying has been previously illustrated (2).

Impedance of gastric emptying produces a significant reduction in the total salicylate levels as shown in Table IV but did not eliminate the faster absorption of buffered aspirin as shown in Table V. This indicates that although a portion of the early absorption of each drug is dependent upon gastric emptying, the restriction of this factor does not eliminate the faster rise in blood salicylate with the buffered aspirin.

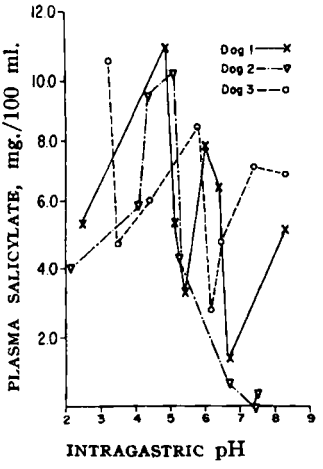


Fig. 3.—Salicylate absorption 10 minutes after 50 mg./Kg. sodium salicylate in citrate-phosphate buffers.

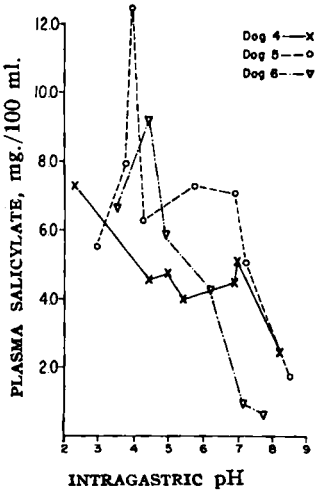


Fig. 4.—Salicylate absorption 10 minutes after 50 mg./Kg. sodium salicylate in citrate-phosphate buffers.

TABLE VI.—DEVIATIONS OF INTRAGASTRIC pH VALUES FROM INITIAL BUFFER pH *In Vitro* IN TEN FASTING SUBJECTS AFTER 250 ml. OF ASPIRIN-BUFFER SOLUTION

Initial Buffer, pH	Av. Deviations of Intragastric pH from Buffer pH 10 min.	S.E.	20 min.	S.E.
2.5	+0.35	0.24	+0.47	0.25
4.5	−0.50	0.19	−0.66	0.21
6.5	−0.54	0.26	−0.96	0.29

TABLE VII.—CORRELATION IN TEN SUBJECTS OF PLASMA SALICYLATE LEVEL WITH INITIAL pH *In Vitro* OF CITRATE-PHOSPHATE BUFFERS CONTAINING 648 mg. ASPIRIN

Initial Buffer, pH	Plasma Salicylate, mg./100 ml. 10 min. Av.	S.E.	20 min. Av.	S.E.
2.5[a]	0.68	0.10	2.32	0.28
4.5	0.95	0.09	2.73	0.21
6.5	1.02	0.15	2.56	0.42

[a] Aspirin not completely soluble at this pH and amount.

TABLE VIII.—CORRELATION OF PLASMA SALICYLATE LEVEL WITH INTRAGASTRIC pH AFTER INSTILLATION OF BUFFERS CONTAINING 648 mg. OF ASPIRIN

Intragastric pH Range	Plasma Salicylate Level, mg./100 ml. 10 min. No.	Av.	S.E.	20 min. No.	Av.	S.E.
2.0 to 2.9	9	0.68	0.12	8	2.26	0.19
3.0 to 3.9	3	1.01	0.19	7	3.07	0.33
4.0 to 4.9	8	0.76	0.17	7	2.27	0.21
over 5.0	9	1.01	0.17	8	2.56	0.39

Although it is frequently confused with tablet disintegration time, the rate of aspirin dissolution is probably the rate-limiting step in the absorptive process (18). Disintegration of the tablet may be rate limiting if prolonged, but was not significant here because it was less than 1 minute for each product. Dissolution time is a slow and quite variable process with different aspirin preparations (19). It is dependent upon aspirin particle size, aggregation between particles, pH, temperature, agitation, and other factors. Two of the products tested by Levy and Hayes (19) having the slowest disintegration times (calcium aspirin and buffered aspirin) showed the most rapid dissolution rates.

The rate limiting effect of dissolution time, suggested by Edwards (18), was confirmed *in vivo* by Nelson and Schaldemose (20), and by Levy and Hayes (19), but in both cases the evidence was accumulated using urinary excretion rates of salicylic acid rather than blood determinations as in this study. While both methods for estimating gastric absorption rates are indirect, blood levels are considered as more closely reflecting effective tissue levels of the drug than are urinary concentrations for the early absorption period as studied here. Although Leonards (11) showed that a solution of sodium acetylsalicylate was absorbed faster than an aspirin suspension, using a blood measurement, this comparison involved a difference in pH between a neutralized form of the drug in solution and an undissolved suspension of acetylsalicylic acid.

A comparison of the data in this study for aspirin solutions to previous data for aspirin tablets (10) showed that plasma SAL levels are 6.5 times higher at 10 minutes, 4.3 times at 20 minutes, and 3.3 times at 30 minutes. The ratios are much less for buffered aspirin solutions—2.7 at 10 minutes, 1.9 at 20 minutes, and 1.6 at 30 minutes. The essential difference between these two solutions is one of acidity. Aspirin in solution at this concentration gives a pH of about 3.0; whereas, when combined with magnesium carbonate and dihydroxyaluminum glycinate, the pH is 4.6. It was this difference in absorption of two aspirin solutions differing mainly in pH that prompted the last portion of the investigation with buffer adjustment of intragastric acidity.

The pKa of salicylic acid is 3.0 and that of acetylsalicylic acid is 3.5. According to a pH partition hypothesis for drug absorption (21), the rate of salicylate absorption from the solute state into the systemic circulation is dependent upon the concentration of unionized, lipid soluble molecules which increase at lower pH values. Schanker (22) showed that absorption of salicylates was more rapid from 0.1 *N* HCl than from bicarbonate solution in the rat, using ligation to assure gastric absorption. The tests of this hypothesis in dogs and in humans in this study generally agree with the idea that absorption is greater at lower pH values. However, in the human study and in some of the dog patterns, a second peak of absorption appears near neutrality. The optimum pH for absorption in this experiment was about pH 3.5 to 5.0 for dogs and 3.0 to 4.0 for humans. Owing to difficulties of dissolving aspirin at low pH values it cannot be said that the lower pH values represent the true lower limit of maximal absorption rates. Rubin, *et al.* (23), claimed that the amounts of antacid buffers included in buffered aspirin tablets were unable to alter gastric pH significantly. These results suggest that the environmental pH conferred upon aspirin particles by the presence in the same tablet of magnesium carbonate and dihydroxyaluminum glycinate with a buffer action at about pH 4.5 (3) do not markedly decrease aspirin absorption as shown in the dissolved aspirin test and by buffer adjustment of intragastric pH. Indeed, this pH of 4.5 may represent an optimum between high pH values which increase the rate of dissolution and low pH values which increase the rate of gastric absorption.

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Pharmacology of Alkylamino Ethanol Esters of *p*-Ethoxybenzoic Acid

By A. E. WADE and F. FORD MILLIKAN

A series of eight alkylaminoethanol esters of *p*-ethoxybenzoic acid were examined for local anesthetic activity on the rabbit cornea and frog sciatic nerve, for acute toxicity in mice, for irritancy on the rabbit cornea and by the trypan blue test in rabbits, and for their spasmolytic activity on the isolated rabbit ileum. In general, sciatic nerve block was hastened, irritancy was increased, acute toxicity increased, and duration of topical anesthesia decreased as the alkylamino portion of the molecule was enlarged.

THE SYNTHESIS and pharmacology of methylbenzyl monoethanolamine paraethoxybenzoate hydrochloride was previously reported by Millikan and Feurt (1). Although this compound appeared to have adequate spasmolytic and local anesthetic activities, its irritant properties were too pronounced for its acceptance as an injectable drug. The present work constitutes a study of

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A comparison of the data in this study for aspirin solutions to previous data for aspirin tablets (10) showed that plasma SAL levels are 6.5 times higher at 10 minutes, 4.3 times at 20 minutes, and 3.3 times at 30 minutes. The ratios are much less for buffered aspirin solutions—2.7 at 10 minutes, 1.9 at 20 minutes, and 1.6 at 30 minutes. The essential difference between these two solutions is one of acidity. Aspirin in solution at this concentration gives a pH of about 3.0; whereas, when combined with magnesium carbonate and dihydroxyaluminum glycinate, the pH is 4.6. It was this difference in absorption of two aspirin solutions differing mainly in pH that prompted the last portion of the investigation with buffer adjustment of intragastric acidity.

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Pharmacology of Alkylamino Ethanol Esters of *p*-Ethoxybenzoic Acid

By A. E. WADE and F. FORD MILLIKAN

A series of eight alkylaminoethanol esters of *p*-ethoxybenzoic acid were examined for local anesthetic activity on the rabbit cornea and frog sciatic nerve, for acute toxicity in mice, for irritancy on the rabbit cornea and by the trypan blue test in rabbits, and for their spasmolytic activity on the isolated rabbit ileum. In general, sciatic nerve block was hastened, irritancy was increased, acute toxicity increased, and duration of topical anesthesia decreased as the alkylamino portion of the molecule was enlarged.

THE SYNTHESIS and pharmacology of methylbenzyl monoethanolamine paraethoxybenzoate hydrochloride was previously reported by Millikan and Feurt (1). Although this compound appeared to have adequate spasmolytic and local anesthetic activities, its irritant properties were too pronounced for its acceptance as an injectable drug. The present work constitutes a study of

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structure-activity relationships of a series of compounds of this general type (2).

EXPERIMENTAL

Acute Toxicity.—Aqueous unbuffered solutions of each compound were administered intraperitoneally into randomly selected adult female albino Webster strain Swiss mice weighing 22 ± 4 Gm. The doses were spaced at 0.04 logarithmic intervals, and the volume of solution was maintained between 0.16 and 1.3 ml. Four to seven groups of ten mice were used for each LD_{50} determination. The LD_{50} and its standard error were calculated from the percentage of deaths occurring within 24 hours by the method of Miller and Tainter (3).

Topical Anesthesia.—The surface anesthetic activity was determined by the ability of each compound to cause loss of wink reflex when applied to the cornea of albino rabbits using a method similar to that described by Luduena and Hoppe (4). Three to five concentrations of each of the aqueous unbuffered solutions spaced at 0.3 logarithmic intervals were applied to the cornea of five to 11 rabbits each. The concentration of anesthetic necessary to produce corneal anesthesia for 10 minutes was estimated from dose-response curves according to the method of Luduena and Hoppe (5) and reported as threshold anesthetic $concentration_{10}$ (TAC_{10}).

Conduction Anesthesia.—The ability of these compounds to suppress nervous conduction through the frog sciatic nerve was determined on the grass frog *Rana pipiens* by a modification of the method of Rider (6). To avoid excessive trauma due to removing the viscera and to avoid the possibility of dilution of the anesthetic solution by blood and tissue fluids, the sciatic nerves were exposed by carefully dissecting the caudal portions of the urostyle. This bone needed only to be lifted to expose the sciatic nerves, its section from the upper vertebral column being unnecessary. The local anesthetic solution was then applied to pledgets of cotton carefully placed beneath both nerves. Another pledget saturated with the anesthetic solution was then placed on top of the nerves in juxtaposition to the first pledget to prevent the nerves from contacting cut muscle surfaces and to insure contact of the anesthetic on their circumference. Four to eight observations at each of four concentrations spaced at 0.3-logarithmic intervals were made for each compound. The potency of the anesthetics was determined from time of onset of anesthesia/log concentration of anesthetic curves and was reported as the concentration of anesthetic necessary to suppress reflex withdrawal in response to a pinch stimulus applied to the foot in 10 minutes (AC_{10}).

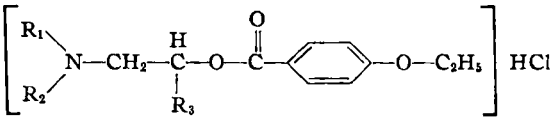
Irritancy.—The local tissue irritant properties of these compounds were estimated following the intradermal injection into the clipped abdominal skin of rabbits. The solutions were made isoosmotic with sodium chloride. A minimum of three concentrations of each compound spaced at 0.3-logarithmic intervals were used and each concentration was injected into six rabbits. The relative degree of irritation was estimated from the amount of local extravasation of intravenously administered trypan blue (7) and reported as the concentration resulting in an irritant score of four (threshold $irritant\ concentration_4$) (4).

Antispasmodic Activity.—The *in vitro* antispasmodic activity was determined by the ability of the drugs to overcome the spasmogenic action of acetylcholine on the isolated rabbit ileum (8). Each segment of ileum was used for no more than six tests. A minimum of three concentrations (spaced at intervals of 0.3 log) were used for each compound; five to eight tests on different ilea were used for each concentration.

Molar Procaine Ratios.—Molar procaine ratios of the results obtained in all of the above tests were calculated by dividing the mean response to the experimental compound into the mean response to procaine, thus giving the ratio of activity of the compound to that of procaine (9). All molar concentrations and procaine ratios are calculated on the basis of the hydrochloride of the compounds.

Correlation Coefficients.—*r* Values were obtained on standard I.B.M. electronic data processing equip-

TABLE I.—CHEMICAL STRUCTURE AND LOCAL ANESTHETIC ACTIVITY OF ALKYLAMINO ETHANOL ESTERS OF PARAETHOXYBENZOATE HCl



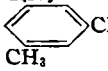
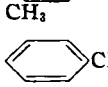
Compd. No.	Substituent Group R1	R2	R3	Local Anesthetic Activity: Rabbit Cornea, Threshold Anesthetic $Concn._{10}$ m*M*	Molar Procaine Ratio	Frog Sciatic Nerve, Anesthetic $Concn._{10}$ m*M*	Molar Procaine Ratio
I	CH_3	H	H	13.87	1.3	34.32	0.6
II	C_2H_5	H	H	17.18	1.1	26.57	0.8
III	iso C_3H_7	H	H	18.78	1.0	21.45	1.0
IV	iso C_4H_9	H	H	8.95	2.0	24.04	0.9
V	C_4H_9	H	H	25.86	0.7	7.96	2.7
VI		H	H	36.65	0.5	9.65	2.2
VII	CH_3	CH_3	CH_3	49.02	0.4	34.77	0.6
VIII		CH_3	H	43.18	0.4	49.76	0.4
Procaine HCl				17.96	1.0	21.19	1.0

TABLE II.—TOXICITY, IRRITANCY, AND ANTISPASMODIC ACTIVITY OF ALKYLAMINO ETHANOL ESTERS OF PARAETHOXYBENZOIC ACID

	Irritancy (Trypan Blue Test)		Intraperitoneal Toxicity in Mice			Antispasmodic Activity	
Compd. No.	Threshold Irritant Concn. mM	Molar Procaine Ratio	LD_{50} ± S.E. mg./Kg.	LD_{50} mM/Kg.	Molar Procaine Ratio	EC_{50} mM	Molar Procaine Ratio
I	38.5	4.95	319 ± 20	1.229	0.60	0.20	0.54
II	35.1	5.43	270 ± 10	0.987	0.75	0.39	0.28
III	31.6	6.03	229 ± 7	0.796	0.93	0.26	0.42
IV	29.5	6.46	285 ± 6	0.945	0.78	0.24	0.46
V	16.6	11.48	239 ± 10	0.792	0.93	0.14	0.76
VI	[a]	...	272 ± 11	0.810	0.91	0.14	0.79
VII	46.9	4.06	354 ± 8	1.231	0.60	0.25	0.44
VIII	17.27	11.04	627 ± 27	1.793	0.41	[a]	...
Procaine	190.6	1.0	202 ± 5	0.740	1.0	0.11	1.0

[a] Compounds too insoluble to acquire data for these tests.

ment using the statistic $r = Sx_1x_2/\sqrt{(Sx_1^2)(Sx_2^2)}$ with levels of significance determined using $n-2$ degrees of freedom (10).

RESULTS AND DISCUSSION

The chemical structures and physical properties of the compounds included in this series were reported by Millikan and Wade (2). The pharmacological activities are summarized in Tables I and II. The following structural-activity relationships appear to hold for the secondary amines of this series in which R_1 was varied. These compounds will subsequently be referred to as the homologous series.

The degree of irritancy as measured by the trypan blue test increased as the weight of the alkylamino substitution increased ($r = 0.8921$; $p < 5\%$). These data strongly suggest that there exists an inverse relationship between duration of corneal anesthesia and irritancy, since those compounds producing longer anesthetic activity on the cornea were least irritant ($r = 0.8966$; $p < 5\%$). Since irritation of the cornea increases lacrimation, it is possible that the decreased duration of action on the cornea is merely a reflection of the speed at which the anesthetic was washed out of the cornea. It appears also that the irritating qualities of these compounds were directly proportional to the speed of onset of action in the frog sciatic nerve ($r = 0.9263$; $p < 5\%$), and to their surface activity ($r = 0.9394$; $p < 5\%$). This would indicate that anesthetic compounds having the ability to lower surface tension would have greater penetrability through the nerve sheath. Luduena, *et al.* (11), found a highly significant positive correlation between surface activity and irritancy and postulated that the irritancy as measured by the trypan blue method might be due to a mechanism related to surface activity or to molecular interaction with protein receptors. Menkin (12) states, however, that tissue injury resulting from irritation causes leukotaxine to be liberated which results in increased capillary permeability and, in this test, increased extravasation of trypan blue.

Although secondary amines are generally considered more toxic and irritating than tertiary amines (13), it appears from this work that even tertiary nitrogen compounds having large alkyl substitutions on the nitrogen are highly irritant. Although the compound showing the least degree of irritancy by this test is a tertiary nitrogen compound, it does not approach procaine in this desireable quality.

As the lipophilic alkylamino portion of the secondary amines of the homologous series increased, the resulting compounds became more potent in inducing sciatic nerve blockade ($r = 0.8463$; $p < 5\%$) but tended to produce corneal anesthesia of a shorter duration ($r = 0.7555$). This finding indicates that lipoid solubility is relatively more important in the production of conduction block of intact sheathed nerves than it is in inducing blockade of the free nerve endings of the cornea. Luduena, Hoppe, and others (4, 5, 9, 14) in testing homologous series of local anesthetic compounds have repeatedly shown the positive correlation that exists between local anesthetic activity, toxicity, and irritancy with the length of the lipophilic side chain. Shaumann (15) concluded that there was a close relationship between conduction anesthesia and systemic toxicity but not between the latter and topical anesthesia.

Duration of anesthesia of secondary amino compounds is generally longer than the corresponding tertiary amines (13). These data confirm this since the concentration of anesthetic required to maintain corneal anesthesia 10 minutes in the rabbit was highest with the two tertiary amines of this series. The branched propanol derivative appears to be relatively less active in anesthetic potency than the ethanol derivatives. Suter (13) also refers to this relationship in local anesthetic compounds.

From the data acquired in these tests it appears that tertiary nitrogen compounds of this type are acutely less toxic than secondary amines (Table I). Although not statistically significant, there was a strong positive correlation between the systemic toxicity of the homologous secondary amines and the length of their alkylamino substituent ($r = 0.7567$). This finding agrees with previous reports (4, 5, 13).

No significant correlation appeared between molecular weight or other findings with the *in vitro* antispasmodic activities of these compounds. No compound of this series was of equal potency to procaine in this spasmolytic effect.

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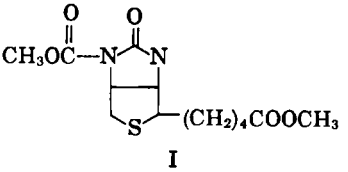
Chemical Reactivity of Models Related to a Proposed CO_2~Biotin Enzyme Complex II

By HOWARD J. SCHAEFFER and PARMATMA S. BHARGAVA

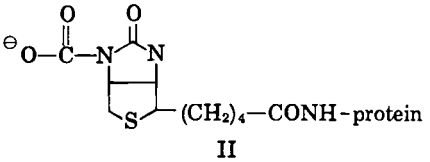
In order to gain more information about the mechanism by which biotin may function in biochemical carboxylations, a study of model compounds which are related to a proposed CO_2~biotin-enzyme complex was undertaken. The model compounds which were employed were the N-arylcarbamyl-2-imidazolidones and the N-alkyloxycarbonyl-2-imidazolidones. It has been shown that the carbonyl group of the model compounds is transferred to an attacking nucleophilic reagent. The significance of these reactions is discussed.

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A theory on the mechanism of biotin action has been proposed by Lynen and his co-workers from work on β-methylcrotonyl CoA carboxylase (2). These investigators found that this enzyme could not only carboxylate β-methylcrotonyl CoA but in addition could utilize free D-biotin as a substrate to give an unstable carboxybiotin. The unstable product was not isolated but after treatment with diazomethane gave the methyl ester of N-carbomethoxybiotin (I) (4). This result has led to the suggestion that the chemical structure of the CO_2~biotin enzyme complex may be represented by II.



I



II

Recently, Wakil and Waite have presented evidence that for acetyl CoA carboxylase the uriedo carbonyl group of the enzyme-bound biotin is the active carbon and that it is involved in the carboxylation reactions (5, 6). For example, it was found that in the presence of adenosine triphosphate and Mn^{2+}, acetyl CoA carboxylase incorporated C^{14}-bicarbonate to form a $C^{14}O_2$~biotin enzyme complex. Hydrolysis of this complex gave free C^{14}-biotin in which the carbon-14 was located in the ureido carbon atom (5, 6). In addition, it was also shown that growth of *Lactobacillus arabinosus* on limiting amounts of ureido-C^{14}-biotin resulted in the loss of most of the radioactivity from the biotin. Although this data is in conflict with that of Melville, Pierce, and Partridge (7), it was shown that the previous investigators had employed

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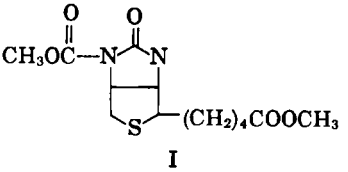
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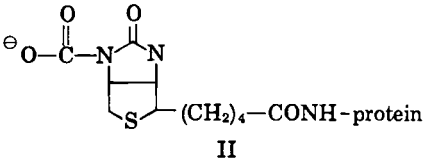
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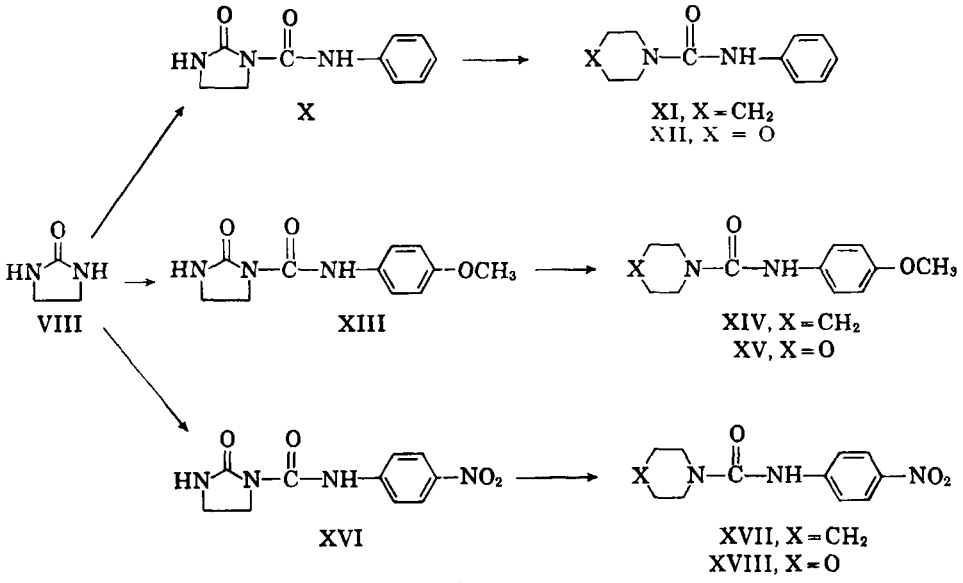
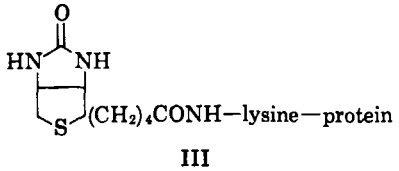
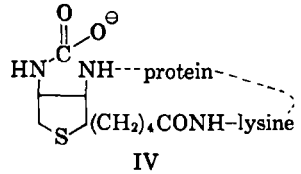


Chart I

ureido-C^{14}-biotin in an amount in excess needed for optimal growth of *L. arabinosus;* therefore, the C^{14}-biotin was stored within the cells (6). On these grounds, Wakil and Waite (5, 6) have suggested that the active form of the enzyme-biotin may be represented by either III or IV



III

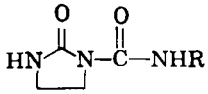


IV

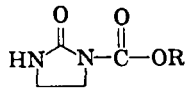
Compounds with structure  related to II or IV are known to be very unstable and undergo decarboxylation with extreme ease. It is quite possible that the actual structure of the CO_2~biotin enzyme complex is not one in which the carboxylate anion of biotin is present as such but rather one in which it has been partially stabilized by reaction with the associated protein. Thus, it appears that the carboxylation reactions may involve three steps: (*a*) formation of a CO_2~biotin enzyme complex, (*b*) partial stabilization of the complex by reaction of the carboxylate anion with a functional group of associated protein such as an amino or hydroxyl group, and (*c*) transfer of the carboxyl group to an attacking nucleophilic agent, *e.g.*, acetyl CoA which would produce the corresponding carboxylic acid and regenerate the biotin-containing enzyme.

To gain more insight into the mechanism of biotin action, a study of the transfer of a carbonyl group to an attacking nucleophilic reagent, using model compounds related to the proposed CO_2~biotin enzyme complex was undertaken. In particular, step (*c*) of the biochemical carboxylation reaction was studied.

Since it was essential to use model compounds which would not undergo spontaneous decarboxylation, certain model compounds containing an imidazolidone system were selected; the simplest model systems for such a study are the N-substituted derivatives of 2-imidazolidone (V and VI).



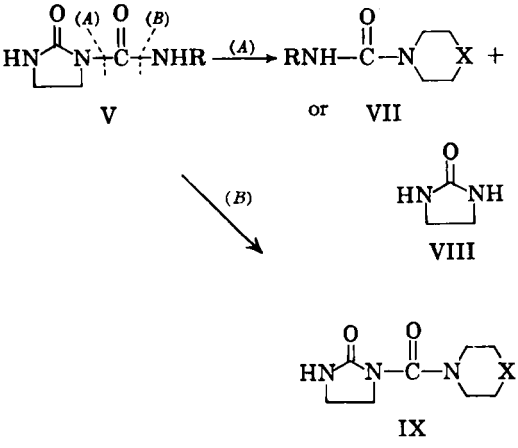
V



VI

In biochemical reactions, one of the common examples of a nucleophilic agent is acetyl CoA. Due to the presence of thiol ester group in acetyl CoA, a carbanion is generated at the α-carbon which attacks the CO_2~biotin enzyme complex to generate a carboxylic acid and the free enzyme. In our model studies morpholine and piperidine were used as the nucleophilic reagents to represent the attack of acetyl CoA on the CO_2~biotin enzyme complex.

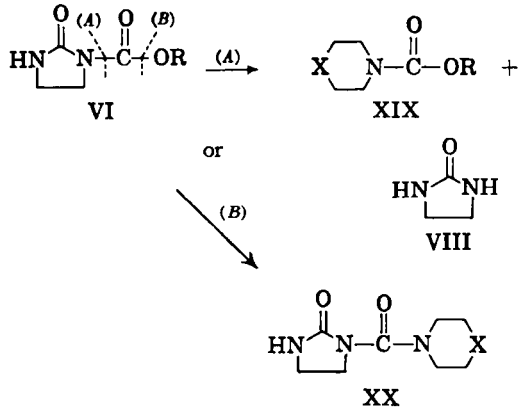
For those model compounds with structures related to V, *a priori* there are two pathways by which reaction may occur



For example, V could undergo reaction to give VII and VIII (path *A*) or by displacement to give IX (path *B*). To determine which pathway is preferred, several compounds related to V were prepared. When 2-imidazolidone was allowed to react with different isocyanates (phenyl, *p*-methoxyphenyl or *p*-nitrophenyl), moderate yields of N-phenylcarbamyl, N-*p*-methoxyphenylcarbamyl and N-*p*-nitrophenylcarbamyl-2-imidazolidone (X, XIII, XVI) were obtained (see Chart I). These model compounds had characteristic absorption in the 1735 and 1685 cm.$^{-1}$ region in the infrared spectrum indicative of carbonyl functions. When X, XIII, and XVI were allowed to react with either piperidine or morpholine at reflux temperatures, the carbonyl group was transferred to the attacking nucleophilic reagent with the formation of the corresponding ureas. Authentic samples of corresponding ureas XI, XII, XIV, XV, XVII, XVIII (see Chart I) were prepared by the reaction of different isocyanates with morpholine or piperidine.

Thus, it has been established that compounds related to a proposed CO_2~biotin enzyme complex are capable of transferring their carbonyl group. In our case, however, the reactivity of the model compounds is low and therefore cannot represent the *exact* structure of the complex which occurs in the enzymatic process; nevertheless, the principle of this type of transfer has been established.

In the second phase of our study of model compounds, we examine the reaction of compounds related to VI with nucleophilic reagents. Again, one can suggest several pathways by which these model compounds might undergo reaction, *e.g.*,



To assess the effect of stabilization of the carboxyl group *via* its ester, compounds XXI, XXV, and XXVIII were prepared by allowing 2-imidazolidone VIII to react with an equimolar concentration of the corresponding alkyl or aryl chloroformates (Chart II).

It seems likely that the model compounds of this group would also result in a similar type of product XIX as obtained from V. However, these model compounds on reaction with the nucleophilic reagents, piperidine, or morpholine, gave a variety of products. The variation in product formation was

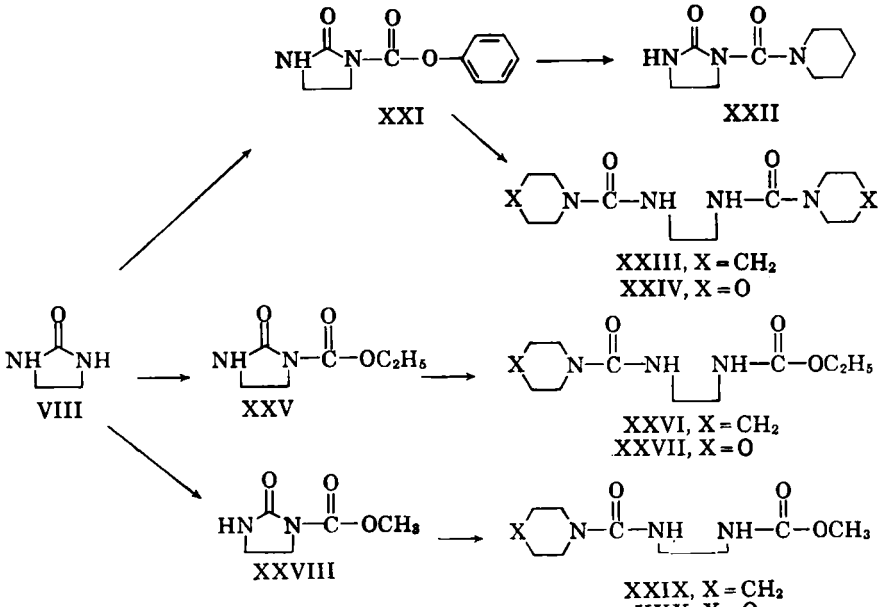


Chart II

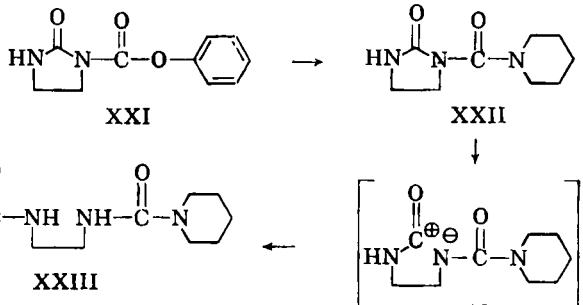
not dependent on the character of the nucleophilic reagent but rather on the ester employed.

Thus when XXI was allowed to react with piperidine or morpholine, opening of the ureido ring was observed, giving XXIII and XXIV as the major product. The reaction of compound XXI with piperidine in 1:2 molar ratio gave rise to XXII, identified as N(N-piperidinocarbonyl)-2-imidazolidone. When XXII was allowed to react with excess piperidine, XXIII was formed in good yields. However, when XXV or XXVIII was allowed to react with piperidine or morpholine, the products of reaction XXVI, XXVII, XXIX, and XXX were isolated in good yields. It was also shown that when XXVII was allowed to react with morpholine for extended times, it was converted into XXIV.

In general, the experiments were followed by a characteristic change in the infrared spectrum or by thin-layer chromatography and were found to be complete in less than 1 hour. Exceptions were noted in the case of reaction of XXI with piperidine in which it took 8 hours for complete reaction and in the case of product XXIII, 42 hours were required for its completion.

These observations suggest that the products are formed by different mechanisms. The peculiar activity of this group of model compounds indicated a second possible mechanism of action of biotin beside the N-activated carbon dioxide~biotin-enzyme complex. The opening of the ureido ring of 2-imidazolidone by nucleophilic attack suggests the possibility of transfer of ureido-carbonyl in the biological systems whenever the nucleophilic agent, *e.g.*, acetyl CoA attacks the complex.

If the following is assumed to be the mechanism of reaction of the formation of XXII and XXIII,



XXI XXII XXIII XXXI

it is possible to suggest that XXXI is the intermediate which would carry out the transfer of ureido carbonyl in different carboxylation reactions. There is a striking parallel between the result reported here from the studies of model compounds and the findings of Waite and Wakil (5, 6).

These reactions demonstrate that the transfer of a carbonyl group from model compounds, which are related to a proposed CO_2~biotin–enzyme complex, to a nucleophilic reagent can occur in two ways as shown by the model compounds V and VI. An extension of these findings is currently under investigation. The results of these studies will be the subject of a future paper.

EXPERIMENTAL[1]

N-Phenylcarbamyl-2-imidazolidone (X).—To a stirred solution of 2.58 Gm. (29.9 mmoles) of 2-imidazolidone (8) in 60 ml. of chloroform was added a 3.26-ml. (30.0 mmoles) quantity of phenyl isocyanate and 0.5 ml. of pyridine. The reaction mixture was heated under reflux for 24 hours; it was reduced to half volume *in vacuo* and allowed to crystallize. A 3.10-Gm. (50.4%) quantity of the white crystalline material, m.p. 168°, was obtained. Concentration of the mother liquor gave a second crop, 1.10 Gm. (17.9%), m.p. 168°. One recrystallization of the crude material from chloroform gave 2.80 Gm. (45.4%) of pure white crystalline product, m.p. 168–169°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3345 and 3160 (NH); 1740 and 1665 (C=O); 1625 and 1570 (NH); 1605 and 1490 (phenyl); $\lambda_{max.}^{chloroform}$ 246 mμ ($\epsilon \times 10^{-4}$) 1.40.

Anal.[2]—Calcd. for $C_{10}H_{11}N_3O_2$: C, 58.44; H, 5.35; N, 20.45. Found: C, 58.39; H, 5.30; N, 20.28.

N - p - Methoxyphenylcarbamyl - 2 - imidazolidone (XIII).—A mixture of 1.72 Gm. (19.9 mmoles) of 2-imidazolidone in 60 ml. of pyridine and 2.98 Gm. (19.9 mmoles) of *p*-methoxyphenyl isocyanate was heated under reflux for 24 hours. The reaction mixture was concentrated to half volume *in vacuo* and allowed to crystallize. A 0.840-Gm. (17.9%) quantity of the white crystalline material, m.p. 176°, was obtained. The mother liquor was evaporated *in vacuo* to dryness, and the residue on recrystallization from chloroform gave a white crystalline solid, 2.30 Gm. (49.1%), m.p. 175°. One recrystallization from chloroform gave the analytical sample, m.p. 176°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3355 and 3260 (NH); 1725 and 1685 (C=O); 1605 and 1560 (NH); 1510 (phenyl); $\lambda_{max.}^{ethanol}$ 274 mμ ($\epsilon \times 10^{-4}$) 1.20.

Anal.—Calcd. for $C_{11}H_{13}N_3O_3$: C, 56.11; H, 5.52; N, 17.86. Found: C, 56.46; H, 5.28; N, 17.86.

N - p - Nitrophenylcarbamyl - 2 - imidazolidone (XVI).—A 1.64-Gm. (9.99 mmoles) quantity of *p*-nitrophenyl isocyanate in 8 ml. of chloroform was added to a stirred solution of 0.860 Gm. (9.99 mmoles) of 2-imidazolidone in 7 ml. of chloroform. The reaction mixture was heated under reflux for 24 hours and on concentrating to half volume *in vacuo*, yellowish crystals appeared which were collected by filtration. A 1.60-Gm. (64.0%) quantity of yellowish crystalline material, m.p. 268°, was obtained. Concentration of the mother liquor gave a second crop, 0.312 Gm. (12.5%), m.p. 268–269°. The total yield was 76.5%. Two recrystallizations of the crude product from acetone gave the yellow

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 4000A spectrophotometer. The melting points were determined on a Kofler Heizbank and are corrected.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

crystalline analytical sample, m.p. 270°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3230 and 3120 (NH); 1735 and 1685 (C=O); 1610 and 1565 (NH); 1595 and 1505 (phenyl); $\lambda_{max.}^{ethanol}$ 317 mμ ($\epsilon \times 10^{-3}$) 13.4.

Anal.—Calcd. for $C_{10}H_{10}N_4O_4$: C, 48.00; H, 4.02; N, 22.39. Found: C, 48.10; H, 4.12; N, 22.00.

N-p-Methoxyphenylcarbamylpiperidine (XIV).—To 1.49 Gm. (9.99 mmoles) of *p*-methoxyphenyl isocyanate was added 0.85 Gm. (9.98 mmole) of piperidine with constant stirring. The reaction was quite exothermic, and an immediate white crystalline precipitate appeared which was collected by filtration; 1.70 Gm. (72.5%), m.p. 130°. Recrystallization of the crude product from methanol and water (1:1) gave the analytical sample, m.p. 130°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3350 and 3060 (NH); 1625 (C=O); 1515 (NH); $\lambda_{max.}^{ethanol}$ 244 mμ ($\epsilon \times 10^{-3}$) 30.0.

Anal.—Calcd. for $C_{13}H_{18}N_2O_2$: C, 66.68; H, 7.68; N, 11.95. Found: C, 66.79; H, 7.74; N, 11.92.

N-Phenylcarbamylpiperidine (XI).—This compound was prepared by the procedure of Crosby and Niemann in a 66% yield, m.p. 168°, in agreement with the literature value (9).

N-p-Nitrophenylcarbamylpiperidine (XVII).—This material was prepared in a 69% yield by a modification of the procedure of Taylor, m.p. 166°, in agreement with the literature value (10).

N-p-Nitrophenylcarbamylmorpholine (XVIII).—A solution of 1.64-Gm. (10.0 mmoles) of *p*-nitrophenyl isocyanate in 10 ml. of chloroform was added to 0.871 Gm. (10.0 mmoles) of morpholine in 5 ml. of chloroform. The reaction mixture was stirred for 3 hours, then was evaporated to dryness *in vacuo*. A 2.30-Gm. (91.3%) quantity of yellow product, m.p. 216°, was obtained. Two recrystallizations of the crude product from an acetone and methanol mixture gave the analytical sample, m.p. 219°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3400 (NH); 1670 (C=O); 1590 and 1540 (phenyl); $\lambda_{max.}^{ethanol}$ 326 mμ ($\epsilon \times 10^{-3}$) 58.4.

Anal.—Calcd. for $C_{11}H_{13}N_3O_4$: C, 52.48; H, 5.21; N, 16.68. Found: C, 52.81; H, 5.27; N, 16.67.

N-Phenylcarbamylmorpholine (XII).—This product was prepared in a 96% yield by the reaction of phenyl isocyanate with morpholine, m.p. 162°. The reported melting point for this compound is 156–159° (11).

N-p-Methoxyphenylcarbamylmorpholine (XV).—This material was prepared by the literature procedure in an 81% yield, m.p. 125°, in agreement with the literature value (12).

Reaction of N-Phenylcarbamyl-2-imidazolidone and Piperidine (Excess).—Piperidine (10.0 ml.) was added to 0.410 Gm. (1.99 mmoles) of N-phenylcarbamyl-2-imidazolidone (X). The reaction mixture was heated under reflux for 36 hours and then evaporated *in vacuo* to dryness. Recrystallization from a methanol and water mixture gave 0.200 Gm. (48.9%) of N-phenylcarbamylpiperidine (XI), m.p 168°. The identity of this product was confirmed by thin-layer chromatography (silica gel) R_f (chloroform 92.3%; acetone 7.7%) 0.80 and by mixed melting point with an authentic sample. Concentration of mother liquor gave an additional crop of crystals 0.033 Gm. (8.10%), m.p. 168°. The total yield was 57.0%.

Reaction of N-p-Methoxyphenylcarbamyl-2-imidazolidone and Piperidine (Excess).—A mixture of 0.47 Gm. (1.98 mmoles) of N-*p*-methoxyphenylcarbamyl-2-imidazolidone (XIII) and 15 ml. of piperidine was heated under reflux for 48 hours. The reaction mixture was evaporated to dryness *in vacuo*, and the residue recrystallized from a methanol and water mixture. There was obtained 0.190 Gm. (40.6%) of N-*p*-methoxyphenylcarbamyl piperidine (XIV), m.p. 130°. The identity of this product was confirmed by thin-layer chromatography (silica gel), R_f (chloroform 92.3%; acetone 7.7%) 0.65; and by mixed melting point with an authentic sample. The mother liquor on evaporating to dryness *in vacuo* and recrystallization from a methanol and water mixture gave another crop of 0.090 Gm. (19.2%), m.p. 128°. The total yield was 0.280 Gm. (59.8%).

Reaction of N-p-Nitrophenylcarbamyl-2-imidazolidone and Piperidine (Excess).—A mixture of 0.500 Gm. (2.00 mmoles) of N-*p*-nitrophenylcarbamyl-2-imidazolidone (XVI) and 15 ml. of piperidine was heated under reflux for 24 hours. The reaction mixture was evaporated to dryness *in vacuo*, and the residue after recrystallization from acetone gave 0.135 Gm. of unchanged starting material (XVI), m.p. 224°. Evaporation to dryness of the mother liquor and recrystallization of the residue from a mixture of acetone and water gave 0.248 Gm. of N-*p*-nitrophenylcarbamylpiperidine (XVII), m.p. 165°. The yield was 53.4% (based on unrecovered starting material), m.p. 165°.

Reaction of N-Phenylcarbamyl-2-imidazolidone and Morpholine (Excess).—To 0.205 Gm. (1.00 mmole) of N-phenylcarbamyl-2-imidazolidone (X) was added 7.7 ml. of morpholine. The reaction mixture was heated under reflux for 70 hours and then evaporated to dryness *in vacuo*. Recrystallization of the residue from a methanol and water mixture gave 70.0 mg. (33.9%), of N-phenylcarbamylmorpholine, m.p. 159°. The identity of this product was confirmed by thin-layer chromatography (silica gel), R_f (chloroform 92.3%; acetone 7.7%) 0.35 and by mixed melting point with an authentic sample. Concentration of mother liquor gave an additional crop of 43.0 mg. (20.8%), m.p. 159°. The total yield of XII was 113 mg. (54.8%).

Reaction of N-p-Methoxyphenylcarbamyl-2-imidazolidone and Morpholine (Excess).—To 0.117 Gm. (0.490 mmole) of N-*p*-methoxyphenylcarbamyl-2-imidazolidone (XIII) was added a 3.85-ml. quantity of morpholine. The reaction mixture was heated under reflux for 22 hours. Concentration of the reaction mixture to half volume *in vacuo* gave white crystals which were collected by filtration; 56.5 mg. (47.8%), m.p. 124°, of N-*p*-methoxyphenylcarbamylmorpholine (XV) was obtained. Evaporation of the mother liquor to dryness and recrystallization from a mixture of methanol and water gave another crop of 10.5 mg. (8.9%), m.p. 125°. The total yield of XV was 67.0 mg. (56.7%).

Reaction of N-p-Nitrophenylcarbamyl-2-imidazolidone and Morpholine (Excess).—To 0.125 Gm. (0.500 mmole) of N-*p*-nitrophenylcarbamyl-2-imidazolidone (XVI) was added 3.85 ml. of morpholine; the mixture was heated under reflux for 32 hours.

The reaction mixture was evaporated to dryness *in vacuo* and recrystallized from a methanol and acetone mixture. A 0.055-Gm. (44.0%) quantity of N-*p*-nitrophenylcarbamylmorpholine (XVIII), m.p. 216°, was obtained. The identity of the product was confirmed by thin-layer chromatography (silica gel), R_f (chloroform 92.3%; acetone 7.7%) 0.23 and by mixed melting point with an authentic sample. Concentration of mother liquor gave an additional crop of crystals, 0.008 Gm. (6.40%), m.p. 216°. The total yield was 0.063 Gm. (50.4%).

N-Phenoxycarbonyl-2-imidazolidone (XXI).—A 1.56-Gm. (9.96 mmoles) quantity of phenyl chloroformate was added to a stirred solution of 0.860 Gm. (9.99 mmoles) of 2-imidazolidone in 12 ml. of chloroform. The reaction mixture was heated under reflux until the evolution of hydrogen chloride ceased (45 hours). Concentration of the reaction mixture to half volume *in vacuo* gave white crystals which were collected by filtration. A 1.10-Gm. (53.5%) quantity of white crystalline material, m.p. 186–187°, was obtained. Concentration of the mother liquor gave a second crop of 0.400 Gm. (19.5%) of the desired product, m.p. 189°; total yield: 73%. One recrystallization of the crude product from chloroform gave the analytical sample, m.p. 189°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (NH); 1795 and 1695 (C=O); 1595 and 1490 (phenyl); $\lambda_{max.}^{ethanol}$ 274 mμ ($\epsilon \times 10^{-3}$) 5.80.

Anal.—Calcd. for $C_{10}H_{10}N_2O_3$: C, 58.24; H, 4.88; N, 13.58. Found: C, 58.33; H, 5.14; N, 13.60.

N-Ethoxycarbonyl-2-imidazolidone (XXV).—To a stirred solution of 1.72 Gm. (19.9 mmoles) of 2-imidazolidone in 25 ml. of chloroform was added 2.17 Gm. (20 mmoles) of ethyl chloroformate. The reaction mixture was heated under reflux for 96 hours and evaporated to dryness *in vacuo*. The residue was crystallized with water which gave the white crystalline product, 1.10 Gm. (34.9%), m.p. 130°. Concentration of the mother liquor gave an additional 0.540 Gm. (17.1%) of white crystalline material, m.p. 130° total yield: 52.0%. Two recrystallizations of the crude product from water gave the analytical sample, m.p. 130°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3255 and 3020 (NH); 1800 and 1680 (C=O).

Anal.—Calcd. for $C_6H_{10}N_2O_3$: C, 45.56; H 6.32; N, 17.71. Found: C, 45.77; H, 6.37. N, 17.67.

N-Methoxycarbonyl-2-imidazolidone (XXVIII).—A 0.945-Gm. (10 mmoles) quantity of methyl chloroformate was added to a stirred solution of 0.860 Gm. (9.99 mmoles) of 2-imidazolidone in 10 ml. of chloroform. The reaction mixture was heated under reflux until the evolution of hydrogen chloride ceased (42 hours); on concentrating to half volume *in vacuo* a white crystalline material appeared which was collected by filtration; yield: 0.500 Gm. (34.8%), m.p. 180°. Concentration of mother liquor gave a second crop of 0.400 Gm. (27.8%) of the desired product, m.p. 178°. The total yield was 62.6%. One recrystallization of the crude product from water gave the analytical sample, m.p. 180°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3380 (NH), 1760 and 1670 (C=O).

Anal.—Calcd. for $C_5H_8N_2O_3$; C, 41.66; H, 5.56; N, 19.44. Found: C, 41.85, H, 5.40; N, 19.49.

Reaction of N-Phenoxycarbonyl-2-imidazolidone and Piperidine (2 mmoles).—A mixture of 0.206 Gm. (1.00 mmole) of N-phenoxycarbonyl-2-imidazolidone (XXI) in 4 ml. of chloroform and 0.085 Gm. (1 mmole) of piperidine in 3.7 ml. of chloroform was heated under reflux for 6.45 hours after which time 1 mmole of piperidine was added to the reaction mixture. Heating of the reaction mixture was continued for another 8 hours, after which time the peak at 1795 cm.$^{-1}$ in the infrared spectrum had disappeared. The reaction mixture was evaporated to dryness *in vacuo*, and the yellow solid which was obtained (0.170 Gm., m.p. 156°) was recrystallized from water; yield: 0.092 Gm. (46.5%) of N-piperidinocarbonyl-2-imidazolidone (XXII), m.p. 181°. Two recrystallizations of the product from water gave the analytical sample. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3350 (NH); 1730 and 1680 (C=O); 1655 (NH).

Anal.—Calcd. for $C_8H_{15}N_3O_2$: C, 54.90; H, 7.56; N, 21.65. Found: C, 54.96; H, 7.35; N, 21.29.

Reaction of N-Phenoxycarbonyl-2-imidazolidone and Piperidine (Excess).—To 1.23 Gm. (6.00 mmoles) of N-phenoxycarbonyl-2-imidazolidone (XXI) was added 45 ml. of piperidine; the mixture was heated under reflux for 42 hours. On keeping the reaction mixture overnight at 0°, white crystals appeared which were collected by filtration. A 0.440-Gm. (26.0%) quantity of N,N'-bis-(N-piperidinocarbonyl)ethylenediamine (XXIII), m.p. 221°, was obtained. Evaporation of the mother liquor to dryness *in vacuo* and recrystallization of the residue from a methanol and water mixture gave 0.320 Gm. (18.9%), m.p. 221° of the product (XXIII). Three recrystallizations from a methanol and water mixture gave the analytical sample, m.p. 224°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3350 (NH); 1620 (C=O).

Anal.—Calcd. for $C_{14}H_{26}N_4O_2$; C, 59.54; H, 9.28; N, 19.84. Found: C, 59.36; H, 9.45; N, 20.01.

Reaction of N-Phenoxycarbonyl-2-imidazolidone and Morpholine (Excess).—A mixture of 0.206 Gm. (1.00 mmole) of N-phenoxycarbonyl-2-imidazolidone (XXI) and 7.70 ml. of morpholine was heated under reflux for 30 minutes, after which time the peak at 1795 cm.$^{-1}$ had disappeared. The reaction mixture was evaporated to dryness; the white residue on recrystallization from a mixture of chloroform and hexane gave 0.117 Gm. (41.0%) of N,N' - bis - (N - morpholinocarbonyl)ethylenediamine (XXIV), m.p. 215°. Concentration of the mother liquor gave another crop of crystals, 0.042 Gm. (14.7%), m.p. 214°. Two recrystallizations of the crude product from a mixture of chloroform and hexane gave the analytical sample, m.p. 214°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3310 (NH); 1620 (C=O); 1550 (NH).

Anal.—Calcd. for $C_{12}H_{22}N_4O_4$: C, 50.05; H, 7.75; N, 19.60. Found: C, 50.12; H, 7.86; N, 19.77.

Reaction of N-Ethoxycarbonyl-2-imidazolidone and Piperidine (Excess).—A mixture of 0.158 Gm. (1.00 mmole) of N-ethoxycarbonyl-2-imidazolidone (XXV) and 7.70 ml. of piperidine was heated under reflux for 25 minutes, after which time the peak at 1800 cm.$^{-1}$ had disappeared. The reaction mixture was evaporated to dryness *in vacuo;* the residue after recrystallization from a hexane and chloroform mixture gave the white crystalline material 0.180 Gm. (74.1%), m.p. 153°, identified as

N - (N - piperidinocarbonyl) - N' - (ethoxycarbonyl) ethylenediamine (XXVI). One recrystallization from a mixture of methanol and water and another from a hexane and chloroform mixture gave the analytical sample, m.p. 149–150°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3440 and 3300 (NH); 1695 and 1620 (C=O); 1520 (NH).

Anal.—Calcd. for $C_{11}H_{21}N_3O_3$: C, 54.29; H, 8.70; N, 17.27. Found: C, 54.35; H, 8.70; N, 17.28.

Reaction of N-Ethoxycarbonyl-2-imidazolidone and Morpholine (Excess).—A mixture of 0.158 Gm. (1.00 mmole) of N-ethoxycarbonyl-2-imidazolidone (XXV) and 7.70 ml. of morpholine was heated under reflux for 25 minutes, after which time the peak at 1800 cm.$^{-1}$ in infrared spectrum had disappeared. The reaction mixture was evaporated to dryness *in vacuo;* the residue after recrystallization from a mixture of chloroform and hexane gave 0.110 Gm. (44.8%) of N-(N-morpholinocarbonyl)-N'-(ethoxycarbonyl) ethylenediamine (XXVII), m.p 156°. Concentration of the mother liquor gave another crop of crystals, 0.075 Gm. (30.3%), m.p. 156°. Recrystallization of the crude product from a mixture of methanol and water gave the analytical sample, m.p. 156°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3420 and 3300 (NH); 1705 and 1635 (C=O).

Anal.—Calcd. for $C_{10}H_{19}N_3O_4$: C, 48.96; H, 7.80; N, 17.13. Found: C, 49.25; H, 7.62; N, 17.15.

Reaction of N-Methoxycarbonyl-2-imidazolidone and Piperidine (Excess).—A mixture of 0.144 Gm. (1.00 mmole) N-methoxycarbonyl-2-imidazolidone (XXVIII) and 7.70 ml. of piperidine was heated under reflux for 20 minutes, after which time the peak at 1760 cm.$^{-1}$ had disappeared. The reaction mixture was evaporated to dryness *in vacuo,* and the residue on recrystallization from a mixture of chloroform and hexane gave 0.150 Gm. (65.7%) of N-(N-piperidinocarbonyl)-N'-(methoxycarbonyl)-ethylenediamine (XXIX), m.p. 134–136°. One recrystallization from a chloroform and hexane mixture and another from a mixture of water and methanol gave the analytical sample, m.p. 136°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3450, 3305, and 3100 (NH); 1710 and 1630 (C=O); 1560 and 1540 (NH).

Anal.—Calcd. for $C_{10}H_{19}N_3O_3$: C, 52.38; H, 8.35; N, 18.32. Found: C, 52.46; H, 7.93; N, 18.28.

Reaction of N-Methoxycarbonyl-2-imidazolidone and Morpholine (Excess).—Morpholine (7.70 ml.) was added to 0.144 Gm. (1.00 mmole) N-carbomethoxy-2-imidazolidone (XXVIII), and the mixture heated under reflux for 45 minutes, after which time the peak at 1760 cm.$^{-1}$ in infrared spectrum had disappeared. The reaction mixture was evaporated to dryness *in vacuo;* the residue on recrystallization from a chloroform and hexane mixture gave 0.150 Gm. (64.9%) of N-(N-morpholinocarbonyl) - N' - (methoxycarbonyl) - ethylenediamine (XXX), m.p. 149–152°. One recrystallization of the product gave the analytical sample, $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3505, 3390, and 3175 (NH); 1725 and 1650 (C=O); 1555 (NH).

Anal.—Calcd. for $C_9H_{17}N_3O_4$: C, 46.74; H, 7.41; N, 18.17. Found: C, 46.39; H, 7.56; N, 17.90.

Reaction of N-(N-Piperidinocarbonyl)-2-imidazolidone (XXII) and Piperidine (Excess).—A mixture of 0.098 Gm. (0.50 mmole) of N-(N-piperidinocarbonyl-2-imidazolidone and 5.70 ml. of piperidine was heated under reflux for 6.5 hours, after which time the peak at 1730 cm.$^{-1}$ in infrared spectrum had disappeared.

The reaction mixture on cooling to room temperature gave 0.069 Gm. (42.5%) of N,N'-bis-(N-piperidinocarbonyl)ethylenediamine (XXIII), m.p. 222°. Concentration of the mother liquor gave an additional 0.03 Gm. (21.2%), m.p. 222–223° of the product. The total yield was 0.09 Gm. (63.7%).

Reaction of N - (N - Morpholinocarbonyl) - N' - (ethoxycarbonyl) - ethylenediamine (XXVII) and Morpholine (Excess).—A 7.80-ml. quantity of morpholine was added to 0.07 Gm. (0.28 mmole) of N-(N-morpholinocarbonyl)-N'-(ethoxycarbonyl)-ethylenediamine (XXVII). The reaction mixture was heated under reflux for 46 hours, after which time the peak 1705 cm.$^{-1}$ in infrared spectrum had disappeared. Evaporation to dryness *in vacuo* of the reaction mixture and recrystallization from a mixture of chloroform and hexane gave 0.03 Gm. (31.2%) of N,N'-bis-(N-morpholinocarbonyl)ethylenediamine (XXIV), m.p. 214°.

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Investigation of the Stereochemistry of Cycloheximide

By HOWARD J. SCHAEFFER and VIJAY K. JAIN

Cycloheximide has been converted by a series of reactions into a dideoxycycloheximide which was found to be optically active. This observation establishes that the methyl groups in dideoxycycloheximide are oriented in a *trans* manner. The significance of this result and an interpretation of the stereochemistry of certain other products are presented.

CYCLOHEXIMIDE, a glutarimide antibiotic which was isolated from *Streptomyces griseus*, exhibits antifungal and antitumor activity. Since the initial discovery of cycloheximide (1), several reports have appeared which describe the isolation of certain stereoisomers of this antibiotic. Okuda and co-workers have isolated naramycin A, naramycin B, and α-epi-isocycloheximide from *Streptomyces naraenses novo;* it has been established that naramycin A is identical with cycloheximide and that naramycin B and α-epi-isocycloheximide are stereoisomers of cycloheximide (2–4). Later, Lemin and Ford described the isolation of isocycloheximide from *S. griseus* and showed that isocycloheximide could be formed by the isomerization of cycloheximide with acid-deactivated alumina (5). Thus, there are four known antibiotics which are stereoisomeric to each other.

We became interested in determining the stereochemistry of cycloheximide and have reported certain preliminary results of our study (see *footnote*); this paper gives the complete details of this work and discusses the stereochemistry of certain degradation products of cycloheximide.

The method which we selected for the determination of the stereochemistry of the methyl groups involved the removal of the keto and hydroxyl groups of cycloheximide to form a dideoxy compound. Since the dideoxy compound would have methyl groups at the 1,3-positions and the glutarimide side chain at the 5-position, it could exist in a D (or L) form or in a *meso*-configuration. Therefore, the determination of the optical activity of the dideoxy compound would determine the relative configuration of the methyl groups because the compound with *cis*-methyl groups has a plane of symmetry and must be optically inactive, whereas the compound with *trans*-methyl groups does not possess a plane of symmetry and would be optically active. To prepare dideoxycycloheximide (VIII), cycloheximide (I) was dehydrated with phosphorus pentoxide in benzene solution and gave anhydrocycloheximide (II) (6). Hydrogenation of anhydrocycloheximide (II) with a palladium-on-charcoal catalyst gave deoxycycloheximide (III, m.p. 114–115°) in a 75% yield. Further hydrogenation of III with a platinum catalyst gave in good yield dihydrodeoxycycloheximide (VI) which upon treatment with thionyl chloride gave the corresponding chloro derivative (VII). When VII was allowed to react with zinc and acetic acid, the chlorine atom was removed, and the desired dideoxycycloheximide (VIII) was obtained. The structure of VIII was established by elemental analysis and by its infrared spectrum. Furthermore, the N.M.R. spectrum[1] of VIII revealed that it was free of absorption in the olefinic hydrogen region, thereby eliminating the possibility of contamination by the dehydrohalogenation product. Determination of the optical rotation of VIII gave $[\alpha]_D^{26}$ −19.0°.[2] For the reasons previously discussed, this observation establishes that the methyl groups of VIII are *trans*. This result leads to the conclusion that the methyl groups in cycloheximide are also *trans*. An identical stereochemical assignment for cycloheximide has been made by Lawes, who showed that pyrolysis of I gave (+)-*trans*-2,4-dimethylcyclohexanone (7).

Since it has been established that the methyl groups in VIII are *trans*, it is logical to assign a *trans* configuration to the methyl groups in III, VI, and VII. To prove that an isomerization had not occurred during the preparation of VI by catalytic hydrogenation of III, dihydrodeoxycycloheximide (VI) was oxidized with chromium trioxide in acetic acid, and a good yield of III was obtained. In addition, an attempt was made to isomerize III with *p*-toluenesulfonic acid, but only unchanged starting material was recovered. Because it is difficult to

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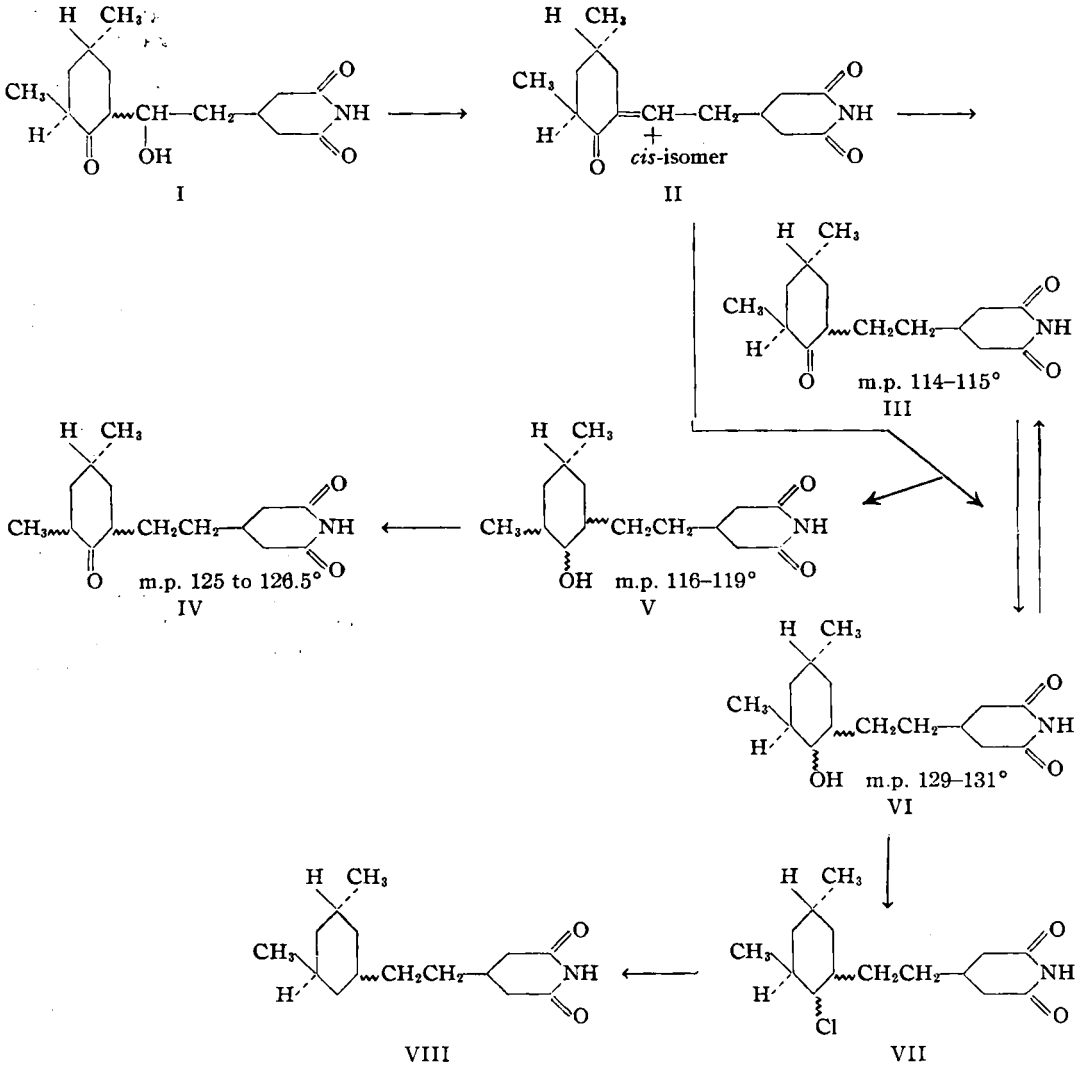
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Abstracted from the Doctor of Philosophy dissertation submitted by V. K. Jain.

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[1] The authors are indebted to Professor E. Wenkert who determined and interpreted the N.M.R. spectra.

[2] This value was erroneously reported in the communication as $[\alpha]_D^{26}$ −9.07°.



visualize an isomerization occurring in the subsequent steps, we believe the assignment of *trans* methyl groups in III, VI, and VII is most probably justified.

It was also found that VI could be prepared directly by hydrogenation of anhydrocycloheximide with a platinum catalyst. However, in this reaction, two dihydrodeoxycycloheximides (V and VI) were obtained. The new dihydrodeoxycycloheximide (V) on oxidation with chromium trioxide in acetic acid gave a new deoxycycloheximide (IV). Okuda has made a similar observation concerning the hydrogenation of III but has reported that on oxidation V gave III and VI gave IV. In addition, on the basis of optical rotatory dispersion studies on III, he has assigned the *cis* configuration to the methyl groups in III and V (8). These results are in direct contrast to our findings.

Several comments concerning the stereochemistry of anhydrocycloheximide (II) are necessary. It is now apparent that anhydrocycloheximide, whether obtained by synthesis (9) or by dehydration of cycloheximide (6), naramycin B (3) or isocycloheximide (5) must be a mixture of stereoisomers. For example, in the synthesis of anhydrocycloheximide (9), the 2,4-dimethylcyclohexanone which was employed was a mixture of *cis* and *trans* compounds in the ratio of 9:1. The initial product which was obtained in an analytically pure form had a m.p. of 130–133° and $[\alpha]_D^{25}$ −12.7°. These data are in excellent agreement for II which is obtained from the antibiotics. Lawes further states (9) that exhaustive recrystallizations of the synthetic anhydrocycloheximide or that prepared by dehydration of cycloheximide afforded a single isomer of cycloheximide with a m.p. 139–141° and $[\alpha]_D^{25}$ −33.2°. This pure isomer of anhydrocycloheximide may have either *cis* or *trans* methyl groups.

A number of attempts were made to convert dihydrodeoxycycloheximide (V) into a dideoxycycloheximide so that information could be obtained concerning the stereochemistry of this series of products. When an attempt was made to replace the hydroxyl function of V by a bromo or chloro group, the reaction proceeded with dehydration and the desired product was not obtained. Therefore, at the present time, we prefer to leave the stereo chemistry of IV and V unassigned.

EXPERIMENTAL[3]

Preparation of Deoxycycloheximide (III).—A solution of anhydrocycloheximide (6) (7.52 Gm, 28.6 mmoles) in 200 ml. of absolute ethanol was added to 750 mg. of 5% palladium-on-charcoal catalyst. The mixture was hydrogenated at room temperature and at an initial pressure of 48.8 p.s.i. Reduction was completed after 24.8 mmoles of hydrogen had been absorbed. After filtration, the solvent was removed *in vacuo*, and the semisolid residue on crystallization from methanol and water gave 5.76 Gm. (75.9%) of deoxycycloheximide (III), m.p. 114–115°, $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3200 and 3100 (NH); 1710 and 1700 (C=O).

Anal.[4]—Calcd. for $C_{15}H_{23}NO_3$: C, 67.89; H, 8.74. Found: C, 67.90; H, 9.03.

Hydrogenation of Anhydrocycloheximide with a Platinum Catalyst.—A solution of 7.50 Gm. (28.4 mmoles) of anhydrocycloheximide (6) in 200 ml. of glacial acetic acid was added to a pre-reduced platinum oxide catalyst (1.50 Gm.). The mixture was hydrogenated at room temperature at an initial pressure of 59.0 p.s.i. Reduction was complete after 54.2 mmoles of hydrogen had been absorbed. The catalyst was removed by filtration; the filtrate was concentrated *in vacuo* to a semisolid residue. The fractional crystallization of the residue from benzene and hexane gave two products: dihydrocycloheximide (VI), 1.82 Gm. (24.1%), m.p. 124–126°, and isodihydrodeoxycycloheximide (V), yield, 2.44 Gm. (32.2%), m.p. 112°. One recrystallization of VI from ethanol and water gave the pure product 129–131°. $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3500 (OH); 3200 and 3100 (NH); 1710 and 1690 (C=O). Recrystallization of V from benzene and hexane gave the pure sample, m.p. 115–116°. $\bar{\nu}$ cm.$^{-1}$ (KBr); 3500 (OH); 3200 and 3100 (NH); 1710 and 1690 (C=O). The reported melting point for VI is 130–131° and for V 117–118° (8).

Hydrogenation of Deoxycycloheximide.—A solution of 4.78 Gm. (18.0 mmoles) of deoxycycloheximide (III) in 200 ml. of glacial acetic acid was added to 500 mg. of pre-reduced platinum oxide catalyst. The mixture was hydrogenated at room temperature until the absorption of hydrogen stopped. After filtration, the solvent was removed *in vacuo;* the semisolid residue on crystallization from ethanol and water gave 3.60 Gm. (75.0%) of dihydrodeoxycycloheximide (VI), m.p. 123–125°. One recrystallization of the crude product from ethanol and water gave the pure sample, m.p. 128–131°, mixed m.p. 130–132° with an authentic sample of dihydrodeoxycycloheximide; $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3500 (OH); 3200 and 3100 (NH); 1710 and 1690 (C=O).

Preparation of Dihydrocycloheximide Chloride (VII).—A solution of thionyl chloride (6 ml.) in *p*-dioxane (30 ml.) was cooled in an ice bath for 15 minutes; a solution of dihydrodeoxycycloheximide (VI), (1.58 Gm., 5.87 mmoles) in 30 ml. of *p*-dioxane was added. The reaction mixture was allowed to stand overnight at room temperature, concentrated *in vacuo* and the semisolid residue on crystallization from methanol and water gave 1.05 Gm. (62.5%) of dihydrodeoxycycloheximide chloride (VII), m.p. 131–132°. Three recrystallizations of the crude product from methanol and water gave the analytical sample, m.p. 136–138°. $[\alpha]_D^{25} = -6.38$ (4.23%) in methanol; $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3300 and 3200 (NH); 1750 and 1700 (C=O).

Anal.—Calcd. for $C_{15}H_{24}NO_2Cl$: C, 63.03; H, 8.46; N, 4.90; Cl, 12.40. Found: C, 63.29; H, 8.29; N, 5.05; Cl, 12.14.

Preparation of Dideoxycycloheximide (VIII).—Zinc dust (0.6 Gm.) was added to a solution of dihydrodeoxycycloheximide chloride (0.362 Gm., 1.26 mmoles) in 6 ml. of glacial acetic acid, water (1.8 ml.), and the reaction mixture was stirred for 1.5 hours at 80°. After filtration, water was added to the filtrate which caused crystallization of the crude product; yield, 0.251 Gm. (79.4%) of dideoxycycloheximide (VIII), m.p. 135–137°. Two recrystallizations from acetic acid and water gave the analytical sample, m.p. 135–137°; $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3290 and 3180 (NH); 1740 and 1690 (C=O). $[\alpha]_D^{25} = -19.0$ ($c = 0.63$ methanol).

Anal.—Calcd. for $C_{15}H_{25}NO_2$: C, 71.67; H, 10.02; N, 5.57. Found: C, 71.90; H, 9.84; N, 5.76.

Oxidation of Dihydrodeoxycycloheximide (VI).—To a solution of 96 mg. (0.36 mmoles) of dihydrodeoxycycloheximide (VI), in 2.5 ml. of glacial acetic acid was added a solution of chromium trioxide (32 mg.) in 0.4 ml. of water. The reaction solution was heated on steam bath for 10 minutes and then was allowed to stand for 3.5 hours at room temperature. After dilution with water (10 ml.), the solution was extracted with chloroform (4 × 15 ml.); the organic extract was washed with a saturated solution of sodium bicarbonate (3 × 20 ml.), water (25 ml.), dried with anhydrous magnesium sulfate, and then filtered. The volatile materials were removed *in vacuo* from the filtrate, and the residue on crystallization from methanol and water gave 66 mg. (70%) of deoxycycloheximide (III), m.p. 114.5 to 115.5°, mixed m.p. 112.5 to 115°, with the authentic sample of deoxycycloheximide (III); $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3200 and 3100 (NH); 1730, 1700, and 1680 (C=O). The deoxycycloheximide was further identified by thin-layer chromatography on silica gel when compared with the authentic sample of deoxycycloheximide (R_f 0.17 chloroform containing 1% methanol).

Oxidation of Isodihydrodeoxycycloheximide (V).—A solution of chromium trioxide (140 mg.) in 2 ml. of water was added to a solution of 314 mg. (1.17 mmoles) of isodihydrodeoxycycloheximide (V) in 7.5 ml. of glacial acetic acid. The reaction mixture was heated on steam bath for 10 minutes, then was allowed to stand at room temperature for 3 hours. After dilution with water (40 ml.), the solution was extracted with chloroform (4 × 3 ml.); the organic extract was washed with a saturated solution of sodium bicarbonate (3 × 30 ml.), water (30 ml.), dried with anhydrous magnesium sulfate, then filtered. The volatile materials were removed *in vacuo* from the filtrate, and the semisolid residue on crystallization from methanol and water gave 150 mg. (48.4%) of isodeoxycycloheximide (IV), m.p. 120–122°. Two recrystallizations of the crude product from methanol and water gave the analytical sample, m.p. 125 to 126.5°; $\bar{\nu}$ in cm.$^{-1}$ (KBr); 3200 and 3100 (NH); 1730, 1700, and 1680 (C=O).

[3] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 4000A spectrophotometer. The melting points were determined on a Kofer Heizbank and are corrected.

[4] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Anal.—Calcd. for $C_{15}H_{23}NO_3$: C, 67.89; H, 8.74; N, 5.27. Found: C, 68.09; H, 8.92; N, 5.62.

Attempted Isomerization of Deoxycycloheximide (III).—A solution 0.10 Gm. (0.38 mmoles) of deoxycycloheximide (III) and 80 mg. (0.41 mmoles) of *p*-toluenesulfonic acid was refluxed for 18 hours. The reaction mixture was allowed to cool to room temperature, then poured onto ice. After the ice had melted, the mixture was filtered, and the filtrate was extracted with chloroform (4 × 30 ml.). The organic layer was washed with saturated solution of sodium bicarbonate (2 × 25 ml.), water (25 ml.), and dried with anhydrous magnesium sulfate. After filtration the volatile materials were removed *in vacuo;* the semisolid residue on crystallization from methanol and water gave 0.05 Gm. (50%) of deoxycycloheximide, m.p. 110–112°; $\bar{\nu}$ in cm.$^{-1}$ (KBr), 3200 and 3100 (NH); 1730, 1700, and 1680 (C=O). One recrystallization of the crude material from methanol and water gave a pure sample of deoxycycloheximide, m.p. 114–115°, mixed m.p. 112–114°, with an authentic sample of deoxycycloheximide.

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Segregation Kinetics of Particulate Solids Systems I

Influence of Particle Size and Particle Size Distribution

By JAMES L. OLSEN† and EDWARD G. RIPPIE

Rates of segregation of initially randomly distributed mixtures of steel spheres of various sizes have been studied. Extent of segregation was determined by sampling the entire system and expressed as the standard deviation of the sample compositions from the mean composition of the system. The several idealized systems studied showed apparent first-order kinetics, and rate constants were determined as a function of particle size and particle size distribution. Segregation in these studies proceeded to an equilibrium state in which mixing and unmixing effects were balanced.

MIXTURES OF SOLIDS are involved in many processes of industrial and technical importance. The glass, ceramic, paint, powder metallurgy, cement, and pharmaceutical industries are among those in which control of systems of solid particles is essential for the production of an acceptable end product. Such general characteristics as particle size distribution, "average shape," pore size, tendency to flow, and uniformity of composition are of prime importance (1).

Since the manufacture of tablets is generally accomplished by the compression of granulated or powdered solids, uniformity of dosage depends to a large extent on the uniformity of mixing of the granulation. The problem of maintaining a uniform mixture is particularly acute where many powdered ingredients having different size distributions, densities, and shapes are to be combined in a single tablet.

Solids mixing as a unit operation has received most attention in the literature in connection with specific types of mixers. Rate equations allowing approximate calculations of the time of mixing necessary to achieve a complete mix have been derived by several workers (2–5). Again, these studies primarily reflect the character of a specific mixer rather than the nature of the mechanisms of mixing and unmixing in general. It is thought, however, that mixing proceeds to a state wherein processes (convection, shear, and diffusion) leading to a random mix balance the tendency to segregate (5). Mixers are adjusted so that this segregation effect is minimized; however, subsequent handling of the mix may render it unsatis-

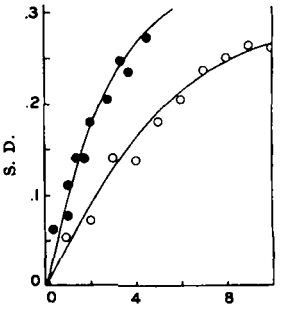


Fig. 1.—Plot of the standard deviation of a binary system *vs.* time of agitation. Solid points are at a 1:1 weight ratio of 8/32 *vs.* 3/32 in. steel balls. Open points are at a 1:1 weight ratio of 6/32 *vs.* 3/32 in. steel balls.

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Solids mixing as a unit operation has received most attention in the literature in connection with specific types of mixers. Rate equations allowing approximate calculations of the time of mixing necessary to achieve a complete mix have been derived by several workers (2–5). Again, these studies primarily reflect the character of a specific mixer rather than the nature of the mechanisms of mixing and unmixing in general. It is thought, however, that mixing proceeds to a state wherein processes (convection, shear, and diffusion) leading to a random mix balance the tendency to segregate (5). Mixers are adjusted so that this segregation effect is minimized; however, subsequent handling of the mix may render it unsatis-

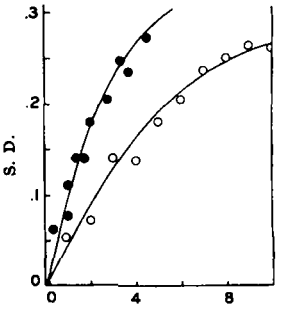


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factory for the next step in the process, due to induced unmixing.

Since, to our knowledge, no fundamental studies have been undertaken to determine the mechanisms of segregation or the factors which influence it, we have carried out preliminary work in this area. Results indicate that valuable information can be obtained on batch unmixing as well as on the nature of interparticulate interactions in a solids system.

THEORY

It is necessary in a study of this kind to determine the extent to which segregation has occurred so that a meaningful determination of unmixing rate can be made. Several methods of quantitatively assigning a "degree of mixing" to a particulate solids system have been used (6). While no single system is exclusively employed, we have found the standard deviation, as used by Weidenbaum (3), to be most satisfactory, since it is directly related to the relative numbers of particles which can be considered mixed or unmixed.

Consider a system consisting of two types of particles differing from each other only in size. The same treatment applies to particles differing by any other variable or combination of variables, such as density and shape. The standard deviation of the system equals

$$S = \sqrt{\frac{\Sigma f_i (u - X_i)^2}{n}}$$

where f_i is the weight of the i sample, u is the weight fraction in the system of the size particle being followed, X_i is the weight fraction in the i sample of the size particle being followed, and $n = \Sigma f_i$ which is the total weight of the system. For computational purposes this may be converted to

$$S = \sqrt{\frac{\Sigma f_i X_i^2}{\Sigma f_i} - \frac{(\Sigma f_i X_i)^2}{(\Sigma f_i)^2}}$$

In such binary systems, either component may be analyzed for without changing the calculated standard deviation. However, with systems containing more than two components, the standard deviation obtained will depend on the component followed and will reflect the degree of segregation of that component relative to the remainder of the system. Standard deviation has been selected as an index since it depends only on the extent to which the system deviates from a perfectly random state and on the size of the samples into which the system is divided. If proper selection is made of the sample size, the standard deviation is a reliable measure of the relative number of "mixed" and "unmixed" particles in the system and can serve as a basis for the study undertaken here.

EXPERIMENTAL

General Considerations.—Steel spheres of various sizes have been used as model systems in these studies so that the factors contributing to segregation can be determined without the complicating shape effects expected with nonspherical particles. The studies were carried out by a general procedure developed in the course of preliminary work.

The particulate systems under investigation were loaded into a cylindrical container $^3/_4$ in. in diameter and 6 in. in length. Loading was carried out by alternately adding the components in small increments to insure a random mixing initially. Samples taken at zero time showed standard deviations not significantly different from zero, indicating random mixing initially. The spheres contained in the cylinder were then subjected to a vertical sine wave motion at a frequency of 1025 c.p.m. and an amplitude of 0.100 in. for a predetermined time.

Vertical agitation was used since it was found to minimize segregation effects due to contact of the spheres with the cylinder walls. The shaking apparatus used was constructed to allow precise adjustment of both the rate and amplitude. Direct rate measurements were made with a Pioneer Photo-Tach model 12 tachometer to an accuracy of 5 c.p.m. In all cases the total weight of the systems was 171 Gm. which allowed sufficient space within the cylinder for expansion during shaking.

Sampling was accomplished by pushing the contents out of the cylinder with a plunger located in the bottom of the cylinder. This was done to divide the particles into six samples of approximately equal volume, allowing analysis of particle size distribution as a function of depth in the tube. Particle size analysis of the samples in these model systems was carried out by screening and weighing.

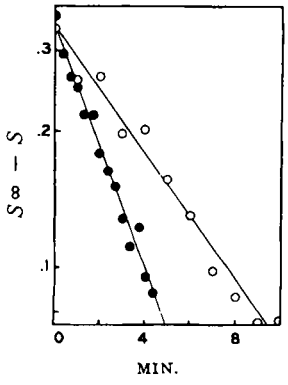


Fig. 2.—Semilog plot of equilibrium standard deviation minus standard deviation at time t *vs.* time of agitation, indicating apparent first-order approach to equilibrium. Solid points are at a 1:1 weight ratio of 8/32 *vs.* 3/32 in. steel balls. Open points are at a 1:1 weight ratio of 6/32 *vs.* 3/32 in. steel balls.

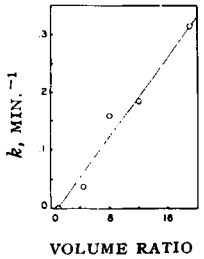


Fig. 3.—First-order rate constant k *vs.* the ratio of the particulate volumes of the spheres in the binary system. 3/32 in. steel balls were used in all runs at a 1:1 weight ratio with 5/32, 6/32, 7/32, and 8/32 in. steel balls, respectively.

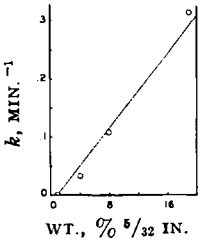


Fig. 4.—First-order rate constant k *vs.* the ratio of the particulate volumes of the spheres in the binary system. 8/32 in. steel balls were used in all runs at a 1:1 weight ratio with 6/32, 5/32, 4/32, and 3/32 in. steel balls, respectively.

TABLE I.—SEGREGATION RATE CONSTANTS IN BINARY SYSTEMS

Wt. Ratio in System	Size Ratio, 32nd in.	Particulate Volume Ratio	k, min.$^{-1}$
1:1	5:3	4.63	0.036 ± 0.003
1:1	6:3	8.00	0.161 ± 0.017
1:1	7:3	12.18	0.183 ± 0.025
1:1	8:3	18.96	0.314 ± 0.023
1:1	8:6	2.37	~0
1:1	8:5	4.10	0.033 ± 0.005
1:1	8:4	8.00	0.107 ± 0.007
1.2:1	7:3	12.18	0.136 ± 0.037
1.4:1	7:3	12.18	0.112 ± 0.016
1.6:1	7:3	12.18	0.103 ± 0.016

These data were used in calculating the standard deviation of the composition of the sample from the mean.

As previously mentioned, measurements of the standard deviation of particle size distributions made in this way are dependent on the size of the samples relative to the size of the particles. In the extremes, a sample consisting of the entire system will show no deviation from the mean composition, whereas samples containing one particle only will show complete segregation independent of the true state of the system. In the present work, samples of approximately one-sixth the weight of the total system were found to give a satisfactory estimate of the standard deviation.

Since the particulate system is disrupted by the sampling, it is necessary to carry out several experiments with each system. Repeated runs on a given system differing in duration of agitation are made so that the standard deviation is determined as a function of time of shaking.

RESULTS AND DISCUSSION

Time Dependence.—The results of two typical runs on systems containing equal weights of different size balls are shown in Fig. 1. When runs of extended duration are made it is found that segregation does not proceed to completion, which would correspond to a standard deviation of 0.500 in this case. Further, the same standard deviation is obtained from an initially unmixed system upon prolonged agitation as one which is initially randomly mixed, substantiating the existence of an equilibrium between mixing and unmixing. A typical system, $^{3}/_{16}$ in. *versus* $^{3}/_{32}$ in., was loaded with the large balls on top and the small balls at the bottom. Several standard deviations of this system after prolonged shaking were found not to differ significantly (using the "t" test) for those obtained from an initially randomly mixed system.

Figure 2 is a plot of log ($S_\infty - S$) *versus* time for the same runs as Fig. 1. S_∞ is the equilibrium standard deviation and S is the standard deviation at time t. The equilibrium standard deviation was taken as the average of three runs for times in excess of seven "half-lives." An initial estimate of half-life was obtained by treating the data by the method of Guggenheim (7) so that subsequent measurements of S_∞ could be made. It can be seen that the approach to equilibrium, of segregation in these systems, is an apparent first-order process.

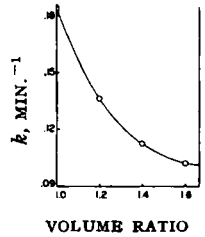


Fig. 5.—First-order rate constant k *vs.* the ratio by weight of 7/32 to 3/32 in. steel balls in the entire system.

TABLE II.—SEGREGATION RATE CONSTANTS IN TERNARY SYSTEMS

Wt. Ratio in System $^{3}/_{32}$ in.:$^{5}/_{32}$ in:$^{7}/_{32}$ in.	k (min.$^{-1}$) for Segregation of $^{7}/_{32}$ in. Balls
50 : 0 :50	0.183 ± 0.025
37.5:12.5:50	0.108 ± 0.028
25 :25 :50	0.061 ± 0.013
12.5:37.5:50	0.034 ± 0.008
0 :50 :50	0.015 ± 0.004
45 :10 :45	0.115 ± 0.038
40.5:19 :40.5	0.066 ± 0.009
30.5:39 :30.5	0.066 ± 0.038
20.5:59 :20.5	0.061 ± 0.111

In these and subsequent determinations of segregation rate, rate constants were determined by the method of least squares from the kinetic data. Accuracy of the rate constants is expressed as a 95% confidence interval based on the student's "t" distribution. In these calculations, n-2 degrees of freedom were assumed, where n is the number of points on the curve. It was further assumed that the points were normally distributed, with constant variance about the regression line.

Particle Size Dependence.—Determinations of the effect of particle size on the segregation rate in binary systems indicate an apparent linear dependence on the volume ratio of the individual particles when the size of one component is held constant. Figure 3 represents determinations of rate constants of systems in which the size of the small spheres was held constant while that of the large spheres was varied. Values are for systems where $^{3}/_{32}$ in. steel balls are mixed at a 1:1 weight ratio with $^{5}/_{32}$, $^{3}/_{16}$, $^{7}/_{32}$, and $^{1}/_{4}$ in. steel balls, respectively. Figure 4 shows the relationship of rate to size when the size of the smaller component is varied. The slopes of the lines in Figs. 3 and 4 are similar, indicating a consistent correlation of

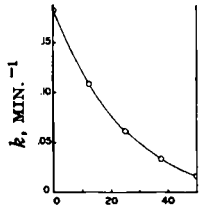


Fig. 6.—First-order rate constant k *vs.* weight per cent of 5/32 in. steel balls in a ternary system. The separation of the 7/32 in. steel balls was followed where the proportion of 7/32 in. steel balls was held at 50% by weight, and the proportion of 5/32 to 3/32 in. steel balls was varied within the balance of the system.

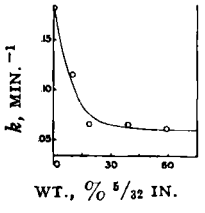


Fig. 7.—First-order rate constant k *vs.* weight per cent of 5/32 in. steel balls in a ternary system composed of a 1:1 weight ratio of 7/32 and 3/32 in. steel balls. Varying amounts of intermediate 5/32 in. steel balls were added, and segregation of the 7/32 in. steel balls was followed.

segregation rate *versus* particulate volume ratio. Values for $^1/_4$ in. steel balls mixed at a 1:1 weight ratio with $^3/_{16}$, $^5/_{32}$, $^1/_8$, and $^3/_{32}$ in. steel balls, respectively, are given. These results, summarized in Table I, suggest that segregation effects may in part be due to competition by the particles for void space created during agitation. In this case, small particles would be favored and would tend to displace those which are larger, at the time of upward acceleration in each shaking cycle.

From lines 2 and 7 of Table I, a reduction in segregation rate with an increase in absolute particle size is apparent in systems where the particles have the same volume ratio. This effect may be because a greater percentage of the total number of particles contact the cylinder walls in systems of larger particles.

Weight Fraction Dependence.—Results shown in Fig. 5 illustrate the effect of variations in the relative quantities of the components in a binary system. Determinations were made in which the relative quantities of $^7/_{32}$ and $^3/_{32}$ in. steel balls were varied while maintaining a total weight of 171 Gm. The resulting rate constants, presented in Table I, have been plotted against the proportion of large to small spheres. It is interesting that the rate of segregation decreases with a decreasing proportion of small particles throughout the range studied. In addition, it was found that in systems where the particle size difference was small, this effect was much less.

Effect of Third Component.—In these studies the effect of intermediate size particles on segregation rate was investigated, and data are given in Table II. Figure 6 shows the results of experiments in which the system (171 Gm. total) is made up of 50% by weight of $^7/_{32}$ in. spheres, and the remaining 50% consists of various ratios of $^5/_{32}$ and $^3/_{32}$ in. spheres. The rate of segregation of the $^7/_{32}$ in. spheres has been plotted and is not a linear function of the composition of the remainder of the system. These data, when plotted as a function of the ratio of the particulate volume of $^7/_{32}$ in. spheres to the weight average particulate volume of the mixture of $^5/_{32}$ and $^3/_{32}$ in. spheres, deviated somewhat from results such as those shown in Figs. 3 and 4. This can be expected, since the two small sized particles separate from each other as well as from the large particles.

The effect of the introduction of $^5/_{32}$ in. intermediate particles to a 1:1 mixture by weight of $^7/_{32}$ and $^3/_{32}$ in. on the segregation rate of the $^4/_{32}$ in. balls was also investigated. In this work, as before, the total weight of steel balls was held at 171 Gm. Results, shown in Fig. 7, indicate a marked reduction in unmixing tendencies with a relatively low proportion of $^5/_{32}$ in. balls. Increasing this beyond a 33% mixture, in this case, resulted in little further change.

SUMMARY

It would appear, as in many preliminary studies of this type, that many more questions have been raised than answered. Several conclusions may be drawn from these studies:

The standard deviation of a system from its mean composition, as used previously, serves as a useful index to the state of mixedness of particulate systems.

Segregation of binary mixtures of spheres measured by the standard deviation from the mean composition follows apparent first-order approach to equilibrium for over two half-lives.

Segregation approaches a true equilibrium since both mixed and unmixed systems reach the same state after prolonged shaking.

The rate of segregation of particles in a binary system is apparently in direct proportion to the ratio of the particulate volumes of the spheres.

The overall weight ratio of the components in a binary system markedly affects the rate of segregation.

The presence of particles of intermediate size greatly reduces the rate of segregation in the system.

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Production of Pyridoxine and Niacin by *Chlorella vulgaris* and *C. pyrenoidosa*

By ROBERTSON PRATT and EVELYN JOHNSON

Two species of Chlorella were compared with respect to their content of niacin and of the pyridoxine-pyridoxal-pyridoxamine complex (B_6) after different periods of incubation. Despite decreasing concentration of the vitamins (mmcg./mg. dry weight of cell material) in both species during the 3-week culture period, the total yield of each vitamin (mmcg./ml. of harvested culture) increased due to the substantial gain in total cell mass. The two species are roughly comparable in their ability to produce niacin but differ with respect to B_6. The niacin and B_6 content of both species compares favorably with that of conventional foods from the vegetable kingdom.

SERIOUS DOUBTS have been expressed that present agricultural methods and conventional sources can provide adequate food to support the world population in three or four generations if the present rate of human reproduction continues unabated. In several countries, including the United States, responsible scientists have considered mass culture of unicellular green algae as a means of coping with this problem, and experiments—both laboratory and pilot plant scale—have been conducted, generally with species of *Chlorella* (1).

The basal composition of *Chlorella* cells with respect to the ratio of protein/lipid/carbohydrate changes with the age of the culture, but not all species change equally. Comparison of *C. vulgaris* and of *C. pyrenoidosa* in a standard medium and in one reputed to favor production of lipid revealed that (*a*) both species grow poorly in the modified medium; (*b*) therefore, higher absolute yields of both protein and lipid are produced in the standard medium, despite the lower percentage yield; (*c*) the protein content (as per cent of dry weight) of both species decreases with age, although there is an increase in the absolute amount of protein, *i.e.*, mg./ml. of culture; and (*d*) at all ages and in both media *C. pyrenoidosa* excels *C. vulgaris* as a source of lipid, whether results are expressed in terms of percentage of dry weight or of absolute yield (2).

The present report deals with the content of two vitamins and their associated vitamers in cells of two species of *Chlorella* cultured in the standard medium. The vitamins studied were niacin and B_6 (pyridoxine-pyridoxal-pyridoxamine).

A few studies of the vitamin content of algae have been reported (3–6), and the view has been expressed that *Chlorella* and some other algae contain vitamins sufficient to give those plants "premium value. . .as human or animal food" although insufficient to render the plants suitable for recovery of vitamin concentrates (7). Morimura (6) studied variations in vitamin content at different times during the division and reproduction cycle of *C. ellipsoidea* grown in synchronous culture for short periods. However, no systematic evaluation of *Chlorella* growing nonsynchronously and normally over a period of several days or weeks has been published, especially with respect to changes in vitamin content that may occur with age of the culture. It should be emphasized that the data that follow deal exclusively with the vitamin content of the cells at the times of harvest and do not indicate total production of the vitamins by *Chlorella*. Designations of mmcg. of B_6 or of niacin per milliliter of culture refer to the amounts of the vitamins found in the cells harvested per milliliter of culture and do not include vitamins present extracellularly in the supernatant. In most harvests the niacin content of the supernatant was 4.5 to 6.5% of the amount found in the cells. The corresponding figures for B_6 were 18.5 to 25%.

EXPERIMENTAL

Organisms and Culture Conditions

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* used in this study; the method of maintaining stock cultures; the composition, preparation, and inoculation of stock and experimental culture solutions, and the details of cultivation, illumination, and harvesting of experimental cultures have been described previously (2).

Microbiologic Assays

Vitamin B_6 Activity.—The method of Atkin, *et al.* (8), using *Saccharomyces carlsbergensis* ATCC 4228 as the test organism, was found to yield satisfactorily reproducible results. The coefficients of variability (standard deviation expressed as a percentage of the mean) calculated for multiple cultures harvested at five different times during the culture period and assayed separately ranged from

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TABLE I.—VITAMIN B_6 AND NIACIN CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

Vitamin	*C. vulgaris* 6–8 days (1)	*C. vulgaris* 13–15 days (2)	*C. vulgaris* 20–22 days (3)	*C. pyrenoidosa* 6–8 days (4)	*C. pyrenoidosa* 13–15 days (5)	*C. pyrenoidosa* 20–22 days (6)
A Content Expressed as mmcg./mg. Dry Wt.						
B_6	16.05	13.51	11.51	25.28	20.55	12.80
Niacin	258.00	225.00	189.00	240.00	183.50	128.00
B Content Expressed as mmcg./ml. of Culture						
B_6	16.23	40.47	54.30	22.50	73.06	72.40
Niacin	253.00	645.98	850.69	213.60	652.41	723.97

0.5 to 7.7%. The actual values were: 8 days, 0.5%; 11 days, 4.6%; 15 days, 2.6%; 18 days, 7.6%; and 22 days, 7.7%.

Niacin.—Niacin was extracted from the cells according to the procedure recommended by Snell (9), and assays were performed with *Lactobacillus plantarum* ATCC 8014 in Difco niacin assay medium. Greater reproducibility was achieved in niacin assays than in those for B_6 activity, possibly because of the greater stability and greater ease of extraction of niacin. The maximum coefficient of variability calculated from the data for niacin content was 3%. Another factor contributing to greater uniformity of assays for niacin content of *Chlorella* cells undoubtedly was the greater retention of niacin than of pyridoxine by the cells during the culture period.

RESULTS

Table I presents averages of the results from experiments conducted under conditions that have been adopted as standard in this laboratory[1] in the columnar culture vessels described previously (2). Tables II–IV are derived from Table I.

It has been pointed out, with reference to the reporting of protein and lipid contents of *C. vulgaris* and *C. pyrenoidosa* (2), that values expressed in terms of percentage of dry weight sometimes provide only a partial picture and are, therefore, misleading with respect to the total or absolute yields that can be obtained from the two species at different times. Cultures with the highest percentage yields are not necessarily those with the largest cell population or cell mass. Frequently, therefore, the highest yields, in terms of absolute amount of protein or lipid recoverable, are obtained under conditions that produce abundant growth of organisms but a lower percentage content. The same principle applies with respect to vitamin content and yield.

In both species of *Chlorella*, B_6 and niacin content (expressed as mmcg./mg. dry weight, which may be considered analogous to percentage content referred to above) progressively decrease during the second and third weeks of growth (Table I *A*). However, despite the decline in concentration relative to dry weight, there is a continuous and substantial increase in the absolute amount of each of them because of the large increment in total mass of the cultures, especially during the second week of growth (Table I *B*). The magnitude of these changes can be more readily visualized when expressed as percentage increment or decrement, as in Table II.

TABLE II.—PER CENT CHANGE IN PROTEIN AND VITAMIN CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

	C. vulgaris 2nd wk. (1)	*C. vulgaris* 3rd wk. (2)	*C. pyrenoidosa* 2nd wk. (3)	*C. pyrenoidosa* 3rd wk. (4)
A % Change in Concn.				
Protein (% dry wt.)	− 12.7	− 7.7	− 7.8	−36.3
B_6 (mmcg./mg.)	− 15.8	−14.8	− 18.7	−37.7
Niacin (mmcg./mg.)	− 12.8	−16.0	− 23.5	−30.0
B % Change in Absolute Amount				
Protein (mg./ml.)	+247.4	+41.3	+226.5	+ 1.3
B_6 (mmcg./ml.)	+149.3	+34.2	+224.0	− 0.9
Niacin (mmcg./ml.)	+155.3	+31.6	+205.0	+11.0

TABLE III.—VALUE OF *C. vulgaris* RELATIVE TO *C. pyrenoidosa* AS A SOURCE OF VITAMIN B_6 AND NIACIN AT DIFFERENT HARVEST TIMES

Vitamin	Dry Wt., mmcg./ml. 2 wks. (1)	Dry Wt., mmcg./ml. 3 wks. (2)	mmcg./ml. Culture 2 wks. (3)	mmcg./ml. Culture 3 wks. (4)
B_6	0.66	0.90	0.57	0.75
Niacin	1.06	1.18	0.93	1.07

The numbers in Table II indicate the percentage loss or gain in vitamin content during the second week with respect to the levels existing at the end of the first week (columns *1* and *3*) and during the third week relative to the values at the end of the second week (columns *2* and *4*). Figures for protein are included for comparison. The upper and lower parts of the table deal, respectively, with changes in the concentration of different components relative to dry weight and with changes in the absolute amounts of the components in the cells recovered from the cultures. (It is important to bear in mind that the numbers in Table II *A* indicate per cent change in values that, for protein, already are expressed in percentages and, for the vitamins, are essentially the equivalent of percentages).

The relative values of the two species of *Chlorella* as sources of the two vitamins at 2 and 3-week harvest times are shown in Table III. At both harvest times, *C. pyrenoidosa* excels *C. vulgaris* with respect to B_6 content, whether results are expressed relative to dry weight (columns *1* and *2*) or in terms of total yield per milliliter of culture (columns *3* and

[1] Inoculum: 100 cells/mm.^{3} (100,000/ml.) from 4-day cultures in liquid medium. Light: 600 f.-c. (Mazda source). Aeration: 5% CO_2 + 95% air continuously passed through culture in finely dispersed bubbles. Temperature: 20.5 ± 0.5° C. Culture medium: KNO_3, 0.025 *M*; $MgSO_4 \cdot 7H_2O$, 0.02 *M*; KH_2PO_4, 0.018 *M*; $FeSO_4 \cdot 7H_2O$, 5×10^{-5} *M*; potassium citrate, 5×10^{-5} *M*; Zn (as $ZnSO_4 \cdot 7H_2O$), 0.4 p.p.m.; Cu (as $CuSO_4 \cdot 5H_2O$), 0.004 p.p.m.; Mn (as $MnSO_4 \cdot 4H_2O$) 0.4 p.p.m.; B (as H_3BO_3), 0.02 p.p.m. See *Reference 2* for details.

TABLE IV.—INTERDEPENDENCE OF CONCENTRATIONS OF NIACIN, PYRIDOXINE, AND PROTEIN IN *Chlorella*[a]

		C. vulgaris			*C. pyrenoidosa*		
		1 wk. *1*	2 wks. *2*	3 wks. *3*	1 wk. *4*	2 wks. *5*	3 wks. *6*
A	Niacin/B_6	16.07	16.67	16.42	9.49	8.93	10.00
B	Protein/niacin	2,341	2,342	2,571	2,650	3,192	2,914
C	Protein/B_6	37,632	39,008	42,224	25,158	28,515	29,140

[a] Calculated in terms of dry weight, *i.e.*, mmcg. niacin/mg. dry weight of cells divided by mmcg. pyridoxine/mg., etc.

TABLE V.—VITAMIN B_6 AND NIACIN CONTENT OF *Chlorella* AND OF SELECTED CONVENTIONAL FOODS (MCG. VITAMIN/100 GM. OF EDIBLE PORTION)[a]

Food Item	B_6	Niacin
C. vulgaris (dried 6–8 day harvest)	1,605	25,800
C. vulgaris (dried 13–15 day harvest)	1,351	22,500
C. vulgaris (dried 20–22 day harvest)	1,151	18,900
C. pyrenoidosa (dried 6–8 day harvest)	2,528	24,000
C. pyrenoidosa (dried 13–15 day harvest)	2,055	18,300
C. pyrenoidosa (dried 20–22 day harvest)	1,280	12,800
Apple	26	200
Banana	320	700
Barley	320–560	3,100
Beef	230–320	2,900–5,500
Beet greens	37	400
Cabbage	120–290	300
Eggs, fresh	22–48	100
Lamb	250–370	4,300–5,600
Liver, beef	600–710	13,700
Liver, calves	300	16,100
Milk, whole	54–110	100
Peas, dried	160–330	3,100
Potato	160–250	1,200–1,400
Rice, whole	1,030	4,600[b]
Rice, white	340–450	1,600
Salmon, fresh	590	7,200
Soybeans, dried	710–1,200	2,300
Tomatoes, canned	710	700
Tuna, canned	440	12,800
Spinach	60	300–600
Wheat bran	1,380–1,570	8,700[c]
Wheat, germ	850–1,600	4,600
Yeast, bakers'	620–700	28,200
Yeast, brewers' (dried)	4,000–5,700	36,200

[a] Values for foods abstracted from Sebrell, W. H., and Harris, R. S., "The Vitamins," Vol. III, Academic Press, New York, N. Y., 1954, and from "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962. [b] Brown rice. [c] Bran flakes breakfast cereal.

4). However, the disparity between the two species diminishes during the third week. This is not surprising, since growth is more rapid in cultures of *C. pyrenoidosa* than in those of *C. vulgaris* during the first 2 weeks after inoculation, while the reverse is true during the third week. Despite the different growth patterns of the two species and contrary to the findings regarding B_6, there appears to be little difference between the two species with respect to niacin content.

It has been pointed out that in both species of algae the content of B_6 and of niacin, relative to dry weight, decreases continuously throughout the 3-week culture period (Table I *A*) The data in Table IV indicate that the decreases in concentration of the two entities are not independent and that each of the two organisms tends to maintain a different but characteristic ratio of niacin to B_6 (line *A*). The niacin/B_6 ratio characteristic for *C. vulgaris* under the standard culture conditions mentioned above is approximately 16; for *C. pyrenoidosa* it is about 9 or 10.

Despite the potential sources of variability in the raw data, the values calculated from them relative to *C. vulgaris* and recorded in line *B* (columns *1–3*) of Table IV are remarkably uniform. A similar approach to uniformity is seen in the results for *C. pyrenoidosa* (columns *4–6* of the same line). The numbers (representing mmcg. protein/mg. dry weight ÷ mmcg. niacin/mg. dry weight) can be interpreted as indicating that the protein/niacin ratio tends to remain uniform during the 3-week growth period—approximately 2,400:1 in *C. vulgaris* and perhaps slightly higher (about 2,700:1) in *C. pyrenoidosa*. However, the difference recorded is too small to be considered significant at this time.

Protein/pyridoxine ratios calculated in the same way (Table IV, line *C*) appear to be less uniform than the protein/niacin ratios (line *B*) and do not seem to show random variation but instead to show increase with the age of the cultures. However, if there is a constant and uniform relation between the concentration of niacin and of B_6 and between protein and niacin in *Chlorella*, there should also be a constant relationship between protein and B_6. Stated another way, when the niacin/B_6 and protein/niacin relationships just mentioned obtain, one can write the formulation

$$\frac{\text{niacin}}{B_6} \times \frac{\text{protein}}{\text{niacin}} = \frac{\text{protein}}{B_6}$$

Therefore, we consider the seeming progressive increase in protein/B_6 ratios an artifact of coincidence that arises despite the random variations in lines *A* and *B*. We propose that under the conditions of these experiments *C. vulgaris* tends to maintain a protein/B_6 ratio of about 39,000 or 40,000:1 during the active growth period and that *C. pyrenoidosa* is characterized by a somewhat lower ratio—the order of approximately 27,000 or 28,000:1.

Table V shows the B_6 and the niacin content of *C. vulgaris* and of *C. pyrenoidosa* harvested at different times and of representative conventional items of diet. In examining the data in the table, allowance must be made for the fact that figures for *Chlorella* are for dried cells, whereas values for most of the other items (except rice, wheat, brewers' yeast, peas, and soybeans) are for fresh material. The water content of *Chlorella* is about 90%. Thus values expressed in terms of the fresh weight of *Chlorella* would be about 10% of those recorded in the table. It is apparent that *Chlorella* compares favorably with conventional dietary vegetable sources of the two vitamins.

Experiments were also performed with *Chlorella*

cultured in a nitrogen-deficient medium—conditions under which the organisms grow very poorly but produce a higher percentage (but not absolute amount) of lipid (2). Yields were so low in this medium that neither niacin nor B_6 activity could be satisfactorily determined with the procedures employed.

SUMMARY

In *C. vulgaris* and *C. pyrenoidosa* there is a continuous decrease in the concentration of niacin and of vitamin B_6 (mmcg./mg. dry weight of cells) during the second and third weeks of a 3-week culture period. However, despite the decrease in *concentration* of the two vitamins in the cells, there is a substantial increase in the *absolute amount* present in the cultures because of the large increase in total mass of cells during the same period.

C. pyrenoidosa excels *C. vulgaris* as a source of B_6 activity whether results are expressed in terms of concentration in the cells or of total yield from the harvested cells, although the superiority of the former species diminishes with increasing age of the cultures. The two species are approximately equivalent with respect to niacin content.

The niacin/B_6 ratio remains constant in both species during the culture period, but the ratio maintained by *C. vulgaris* is higher than that maintained by *C. pyrenoidosa*. Similarly, *C. vulgaris* contains more protein relative to B_6 than does *C. pyrenoidosa*.

As a source of B_6 and niacin, both species of *Chlorella* compare favorably with dietary vegetable sources.

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Scope of Acetonitrile as a Solvent in the Nonaqueous Titration of Organic Medicinals

By C. A. MAINVILLE* and L. G. CHATTEN

Acetonitrile has been shown to be a useful solvent for the assay of a variety of salts of organic bases. Its scope as a solvent must be investigated for each individual compound, since solubility cannot be predicted on the basis of chemical similarity. This solvent has also been demonstrated to be particularly valuable in the assay of tablets and capsules because so few excipients cause interference in it.

ACETONITRILE has been shown to be a satisfactory solvent medium for the titration of a limited number of organic compounds (1–3). However, its use as a primary solvent for salts of medicinals had been previously applied on a small scale by Mizukami and Hirai (4). In other investigations the role of acetonitrile in the titration of organic medicinal agents has been restricted to that of a stabilizing solvent (5–7).

From the results of a recent publication (8), which dealt with the titratability of tablet excipients in a variety of organic solvents, it was decided to select those solvents in which minimal interference by excipients occurred and to determine the scope of each as a titration medium for the analysis of medicinals and their pharmaceutical forms. Acetonitrile was one of those solvents chosen for this investigation.

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EXPERIMENTAL

Apparatus

An A. C. titrometer, Precision Scientific Co., equipped with a glass-calomel electrode combination, 5-ml. microburets graduated to 0.01 ml., and electromagnetic stirrers were employed.

Reagents

Acetone A.C.S., acetonitrile A.C.S., chloroform A.C.S., glacial acetic acid A.C.S., anhydrous

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An A. C. titrometer, Precision Scientific Co., equipped with a glass-calomel electrode combination, 5-ml. microburets graduated to 0.01 ml., and electromagnetic stirrers were employed.

Reagents

Acetone A.C.S., acetonitrile A.C.S., chloroform A.C.S., glacial acetic acid A.C.S., anhydrous

TABLE I.—TITRATABILITY OF DRUGS IN ACETONITRILE

Drug	Mol. Wt.	% Purity[a]	% Purity in Acetonitrile[b]	ΔE, (Mv.)
Local Anaesthetics				
Diperodon HCl	433.93	99.1 (*a*)	99.9	
		99.4	99.3	
			100.5[c]	[49]
Piperocaine HCl	297.83	100.6 (*a*)	100.5	
		100.2	100.1	
			100.7[c]	[49]
Pramoxine HCl	329.88	101.1 (*a*)	100.9	
		100.7	100.8	
			101.0[c]	[42]
Procaine HCl	272.77	100.0 (*a*)	100.6	
		99.5	100.2	
			100.4[c]	[15]
Sympathomimetics				
Ephedrine HCl	201.70	100.4 (*b*)	Insoluble	
		100.4		
Methamphetamine HCl	185.69	99.7 (*b*)	100.6	
		100.4	100.4	
			100.2[c]	[46]
Methoxamine (base)	211.24	99.8 (*b*)	98.6	
		99.1	98.2	
			99.5[c]	[120]
Methoxyphenamine HCl	215.72	100.1 (*b*)	100.1	
		99.9	99.9	
			99.8[c]	[48]
Antihistamines				
Chlorcyclizine HCl	337.30	99.2 (*e*)	99.4	
		99.5	99.4	
			100.1[c]	[11]
Diphenhydramine HCl	291.8	100.5 (*b*)	100.3	
		100.2	100.0	
			100.3[c]	[49]
Doxylamine succinate	388.45	98.9 (*c*)	98.8	
		99.6	98.8	
			98.6[c]	[44]
Isothipendyl HCl	321.88	100.7 (*d*)	100.3	
		100.8	99.8	
			100.3[c]	[37]
Tripelennamine HCl	291.83	100.3 (*b*)	100.4[c]	[40]
		100.4	100.2[c]	[40]
			100.5[c]	[40[
Phenothiazines				
Chlorpromazine HCl	355.53	100.4 (*f*)	100.2	
		100.2	100.0	
			100.8[c]	[24]
Mepazine HCl	364.92	100.6 (*f*)	100.1	
		100.9	100.4	
			100.4[c]	[41]
Prochlorperazine dimaleate	303.04	99.9 (*c*)	Insoluble	
		99.3		
Promazine HCl	320.88	99.8 (*f*)	100.1	
		99.2	100.5	
			101.6[c]	[33]
Promethazine HCl	320.88	99.9	99.7	
		99.7	99.7	
			100.6[c]	[46]
Pyrathiazine HCl	332.89	100.7	99.7	
		100.5	99.5	
			100.1[c]	[32]
Thiopropazate HCl	518.94	100.1 (*b*)	Insoluble	
		99.4		
Thioridazine HCl	407.11	100.0 (*f*)	100.5	
		100.9	100.4	
			100.9[c]	[18]
Trifluprom azine HCl	388.90	100.7 (*f*)	99.4	
		100.4	99.4	
			100.4[c]	[40]

[a] (*a*), (*b*), (*c*), (*d*), (*e*), and (*f*) indicate the procedure employed to establish purity of the drug. [b] *V* was 0.01 ml. [c] Determined potentiometrically.

methanol A.C.S., 6% mercuric acetate in glacial acetic acid, and 0.1 *N* perchloric acid in dioxane. The following indicators were utilized: (*a*) 0.5% crystal violet in glacial acetic acid, (*b*) 0.1% methyl red in glacial acetic acid, (*c*) 0.1% methyl red in anhydrous methanol, (*d*) 0.25% methyl red in phenol-chloroform, and (*e*) 0.5% thymol blue in anhydrous methanol.

Procedures

Determination of Drug Purity.—An accurately weighed quantity of the crystalline salt was dissolved in 50 ml. of solvent by stirring electromagnetically for 15 minutes. A suitable indicator was added, and the solution was titrated with 0.1 *N* perchloric acid in dioxane. A blank was determined on the solvent. All assays were performed in duplicate by one of the following techniques. (*a*) Dissolve sample in 25 ml. of phenol-chloroform (5:25) and 25 ml. of acetonitrile; add 2 ml. of mercuric acetate solution and 2 drops of methyl red in phenol-chloroform. (*b*) Dissolve sample in 50 ml. of glacial acetic acid; add 2 ml. of mercuric acetate solution and 1 drop of crystal violet in glacial acetic acid (9). (*c*) The same as (*b*), except mercuric acetate solution was not added. (*d*) Dissolve sample in chloroform-acetonitrile (1:1), add 2 ml. of mercuric acetate solution and 2 drops of methyl red in glacial acetic acid (7). (*e*) Dissolve sample in 50 ml. of chloroform-glacial acetic acid (1:1); add 1 drop of crystal violet in glacial acetic acid (7). (*f*) Dissolve sample in 50 ml. of acetone; add 2 ml. of mercuric acetate solution and 2 drops of methyl red in glacial acetic acid (10).

Methods of Assay of Crystalline Salts in Acetonitrile.—A sample of the salt was accurately weighed and dissolved in 50 ml. of acetonitrile by stirring electromagnetically for 15 minutes. A suitable indicator was used, and the solution was titrated with 0.1 *N* perchloric acid in dioxane.

All assays were performed in duplicate by this technique; the results were compared with that of a potentiometric titration. A blank was determined on the solvent system.

(*a*) Dissolve sample in 50 ml. of acetonitrile; add 2 ml. of mercuric acetate solution and 3 to 4 drops of methyl red indicator in glacial acetic acid. Titrate to red end point. (*b*) Dissolve sample in 50 ml. of acetonitrile; add 1 drop of crystal violet indicator in glacial acetic acid. Titrate to blue end point.

Method of Assay of Pharmaceuticals Using Acetonitrile as Solvent.—Weigh and powder 20 tablets or empty the contents of 20 capsules. Accurately weigh a sample of the powder which is equivalent to 50 mg. of the active ingredient. Add 50 ml. of acetonitrile and stir electromagnetically for 30 minutes; suction filter through a sintered-glass funnel of medium porosity, and wash beaker and funnel with 10 ml. acetonitrile. Add 4 drops of 0.5% methyl red indicator in glacial acetic acid and 2 ml. of 6% mercuric acetate solution if salt is a halide. The filtrate is titrated to a deep red with 0.1 *N* perchloric acid in dioxane.

RESULTS AND DISCUSSION

Because a number of chemically different compounds were investigated, it was necessary to use a variety of established nonaqueous procedures to determine the purity of each substance. The duplicate results of such assays appear in Table I, together with the appropriate letter which indicates the method employed.

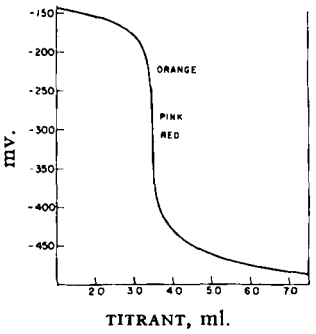


Fig. 1.—Titration of diphenhydramine HCl in acetonitrile.

Where solubility was adequate, triplicate determinations were performed on each drug in acetonitrile, and the results are reported in Table I. To check the validity of the visual end points, one of the triplicate assays was performed potentiometrically on the Precision-Shell titrometer, and the result appears in *footnote c* of Table I. An excellent titration curve was obtained in each instance; to demonstrate the magnitude of potential change at the end point, the ΔE is included in Table I for each drug. The ΔV, in the vicinity of the end point, was 0.01 ml. Figure 1 illustrates the titration curve for diphenhydramine hydrochloride in acetonitrile and is typical of the behavior of the stronger bases in this solvent.

Examination of the data in Table I shows that there is satisfactory agreement between the assay results obtained by standard or previously reported methods and the proposed procedure employing acetonitrile as primary solvent. Ephedrine was included in Table I because of its chemical similarity to methamphetamine. Although the latter compound is readily soluble, the former is not. For a similar reason, prochlorperazine and thiopropazate are included with those phenothiazine derivatives which are soluble. Such evidence indicates that chemical similarity is insufficient to predict solubility in this solvent. Each compound must be checked for solubility on an individual basis.

In addition to those compounds which are reported, the solubility of several sodium salts of barbiturates, some alkaloidal salts, and sodium *p*-aminosalicylate were investigated in acetonitrile. All had a solubility which was too low for practical value; hence no data are reported for them.

Several pharmaceutical preparations were assayed by the described procedure, and comparative analysis were obtained either by the official method (9) or by the control technique exercised by the manufacturer of the product. In the majority of instances, satisfactory agreement occurred as shown in Table II. The pharmacopeial method could not be applied to Abbott's chlorcyclizine hydrochloride because the orange color of the tablets appeared to interfere with the result and caused serious overestimation. No method of comparison was available from the manufacturer for the promazine tablets.

For the analyses of commercial preparations, a 30-minute stirring time is generally recommended. In

TABLE II.—ASSAY OF PHARMACEUTICALS IN ACETONITRILE

Drug	Mfr.	Official or Mfg. % Recovery	% Recovery, in Acetonitrile
Chlorcyclizine HCl U.S.P.	Burroughs Wellcome	97.9	97.8
		99.0	98.3
			98.4
Chlorcyclizine HCl U.S.P.	Abbott	...	103.0
			103.4
			101.3
Chlorpromazine HCl U.S.P.	Poulenc	98.8	98.1
		97.5	97.4
			97.8
Diphenhydramine HCl U.S.P.	Parke Davis	105.1	101.9
		106.2	101.5
			102.5
Doxylamine succinate U.S.P.	Merrell	98.0	97.3
		98.2	97.4
			97.3
Isothipendyl HCl	Ayerst	102.5	98.5
		103.2	99.0
			98.2
Methoxyphenamine HCl	Upjohn	97.3	100.3
		98.2	99.8
			99.6
Promazine HCl	Empire	No method	105.6
			105.6
			104.7
Promethazine HCl U.S.P.	Poulenc	99.8	88.0
		98.8	88.0
			87.2
Trifluprom azine HCl	Squibb	99.9	97.1
		100.0	97.1
			96.8
Thioridazine HCl	Sandoz	102.5	97.8
		102.5	97.3
			97.2
Tripelennamine HCl U.S.P.	Ciba	96.6	88.5
		97.6	87.1
			88.0

certain instances, it was necessary to increase this to 1 hour to extract the maximum amount of active constituent. This was particularly true of isothipendyl HCl tablets and trifluprom azine HCl capsules, where the ratio of drug was small compared to that of the excipients. For these products, a 1-hour stirring time is recommended. The results obtained for tripelennamine tablets and those for promethazine were low. No significant difference was obtained between a 30-minute stirring time and 1 hour. Since the crystalline salt of tripelennamine is quite soluble in acetonitrile, no satisfactory explanation can be offered for the values which are markedly below those obtained by the official procedure. For promethazine, it is obvious that the proportion of active constituent to that of tablet excipients is too small to permit complete extraction.

The present report should not be construed as representing the complete scope of acetonitrile as a solvent for the salts of organic medicinals. It does, however, give an indication of the utility of the solvent. Undoubtedly further investigation might reveal many more organic salts that could be titrated in acetonitrile.

A previous report (8) revealed that both isopropanol and *n*-hexane might be useful solvents for pharmaceutical analysis because of the small number of excipients that are titratable in them. Preliminary investigation shows that both of these solvents could also be useful in extending the applicability of nonaqueous titrimetry to the analysis of pharmaceuticals. Work is being continued in this direction.

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Spectrophotometric Determination of Poldine Methylsulfate and Application to Pharmaceutical Preparations

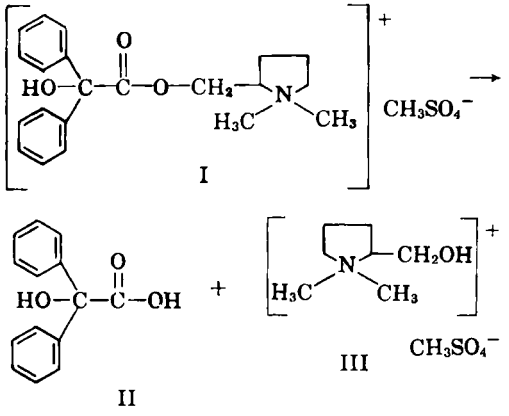
By JOHN W. POOLE and ALEXANDER A. MONTE

A spectrophotometric method for the determination of poldine methylsulfate is presented. The ultraviolet absorbance of methanol solutions of the compound is the analytical measurement utilized in the procedure. The quantitative separation of the drug from interfering substances and degradation products is accomplished through the formation of a reineckate derivative with the subsequent regeneration of the conjugate base by ion-exchange chromatography. The method is shown to be applicable to various pharmaceutical preparations and is useful for studying the hydrolytic degradation of the molecule.

PREVIOUSLY PUBLISHED METHODS for the determination of poldine methylsulfate[1] have employed infrared spectroscopy and colorimetric procedures. The infrared procedure of Rapson, *et al.* (1), was designed primarily for the examination of samples taken from bulk supplies of the pure material. Langley and his co-workers (2) employed a methyl orange technique for the determination of poldine methylsulfate in biological fluids; Singleton and Wells (3) utilized the ammonium cobaltothiocyanate complex for the colorimetric determination of this compound in pharmaceutical dosage forms. However, the colorimetric procedures cannot be applied to pharmaceutical formulations in many instances because of the presence of interfering substances.

The spectrophotometric assay described here might be expected to offer an advantage in that the absorption bands utilized correspond to the drug molecule being analyzed. The assay has been developed for the determination of the drug molecule in various pharmaceutical formulations alone and in combination with other compounds. In addition, it has been employed to follow the hydrolysis of this compound in aqueous systems under varying conditions of pH and temperature.

Poldine methylsulfate (2-benziloyloxymethyl-1,1-dimethylpyrrolidinium methylsulfate) (I) can hydrolyze to benzilic acid (II) and 2(α-hydroxymethyl) - 1,1 - dimethylpyrrolidinium methylsulfate (III). The ultraviolet absorption spectrum of poldine methylsulfate, shown in Fig. 1, is due almost entirely to the benzilic acid moiety. The development of a spectrophotometric assay for this compound resolved itself to the quantitative separation of the drug from interfering substances and its degradation product, benzilic acid.



Many organic bases react with ammonium reineckate to form derivatives which are useful in their identification and determination. Reineckate derivatives have been quantitatively determined gravimetrically, colorimetrically, and by titration procedures (4–8). However, in many instances, these methods suffer from a lack of specificity which may be overcome by regeneration of the base with the subsequent analysis of the pure material. Kapfhammer and Eck (9) utilized the treatment of acetone solutions of the reineckate derivative with silver sulfate, then with barium chloride as a means of regeneration of the basic compounds. More recently, Kum-Tatt (10) accomplished this regeneration by the use of a strong anion-exchange resin with acetone solutions of the reineckate derivatives.

In the present study, the formation of a reineckate derivative of the drug with the subsequent regeneration of the conjugate base by means of ion-exchange chromatography was the method used to accomplish the necessary quantitative separations. The ultraviolet absorption at 257 mμ of methanol solutions of the regenerated

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[1] Marketed as Nacton by McNeil Laboratories, Inc., Fort Washington, Pa.

compound was the analytical measurement employed in this procedure.

The purpose of this study was to develop an assay procedure for poldine methylsulfate useful for the determination of this drug in pharmaceutical preparations and in the presence of its hydrolysis products.

EXPERIMENTAL

A number of cationic and anionic ion-exchange resins were investigated as a means of regeneration of the conjugate base from the reineckate derivative of poldine methylsulfate. The only system studied which was found to give quantitative recovery of the basic compound with methanol solutions of the reineckate derivative was a weak anionic exchange resin. In the case of the strong cationic exchange resins, complete elution of the drug from the resin was not possible, while the intermediate and weak cationic exchange resins did not retain the drug quantitatively. The strong anionic exchange resins were efficient in removing the reineckate ion from the methanol solutions but also retained a considerable quantity of the drug molecule.

The use of varying amounts of reagents and filtration of the insoluble reineckate derivative over a wide range of time showed that critical control of conditions was not necessary to obtain reliable and accurate results with this procedure. The requirements for quantitative formation of the reineckate derivative were found to be sufficiently acidic sample solutions and an excess of reineckate reagent. No variance in results was noted when the samples were stored at 5° for 24 hours or in an ice bath for 2 hours. The details of the procedure are stated below.

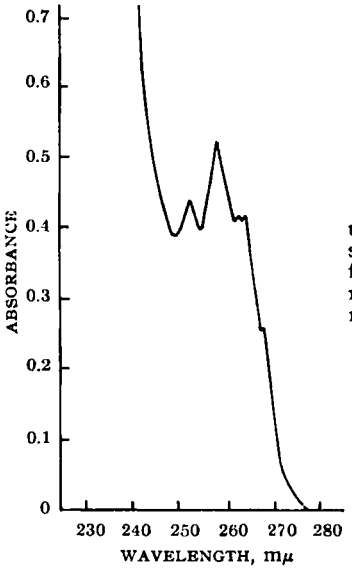


Fig. 1.—The ultraviolet absorption curve for poldine methylsulfate in methanol.

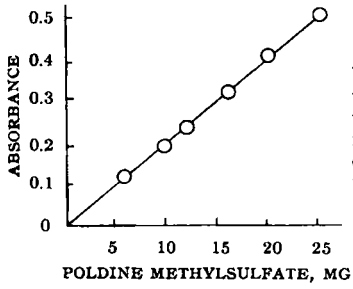


Fig. 2.—Calibration curve for the spectrophotometric determination of poldine methylsulfate.

Reagents.—*Ammonium Reineckate Solution.*—Approximately a 1% solution is prepared by dissolving 1 Gm. of ammonium reineckate in 100 ml. of distilled water. Shake for 10 minutes and filter through filter paper.

Ammonium Reineckate Wash Solution.—Add 2 ml. of ammonium reineckate solution to 1 L. of distilled water.

Methanol.—Analytical reagent grade is utilized.

Amberlite IR-4B Anionic Resin.—The resin is allowed to stand overnight in a 5% aqueous sodium hydroxide solution, then washed with distilled water until the effluent is neutral.

Procedure.—Add 10 ml. of ammonium reineckate solution to 20 ml. of a 5% sulfuric acid solution containing 10 to 50 mg. of poldine methylsulfate, and place this mixture in an ice bath for 2 hours. Filter the precipitate formed through a sintered-glass crucible, wash with 30 ml. of ammonium reineckate wash solution. Dry the precipitate in a vacuum oven for 1 hour at 50°, then dissolve the reineckate derivative in 30 ml. of absolute methanol. Prepare an ion-exchange column with a pledget of glass wool and 2 to 3 Gm. of Amberlite IR-4B anionic resin, previously treated as described above, and wash with 50 to 75 ml. of methanol. The methanol solution of the reineckate derivative is passed through the column at a rate of about 1 ml. per minute and collected in a 50-ml. volumetric flask. If color is present in the effluent, it is returned to the column or passed through a second column of the same type. The resin column is washed with sufficient methanol

Table I.—Assay of Poldine Methylsulfate in Aqueous Solutions Corresponding to a 10% Hydrolytic Degradation of the Drug

Sample	Poldine Methylsulfate, mg.	Benzilic Acid, mg.	2(α-Hydroxymethyl)-1,1-dimethylpyrrolidinium Iodide, mg.	Poldine Methylsulfate Found, mg.
1	45.0	2.5	2.5	45.7
2	45.0	2.5	2.5	45.5
3	45.0	2.5	2.5	44.3
4	45.0	2.5	2.5	45.6

Table II.—Determination of Poldine Methylsulfate in Tablets Containing a Combination of Poldine Methylsulfate and Butabarbital Sodium

Batch No.	Age, Mo.	Poldine Methylsulfate, mg./tablet— Declared	Found
271:43	3	4.0	4.10
	6	4.0	4.11
	7	4.0	4.01
278:31	3	4.0	4.00
278:35	2	4.0	3.81
	4	4.0	3.94
R1013	0	4.0	4.06
	2	4.0	3.96
R1017	2	4.0	4.05
	3	4.0	4.04

to bring the final volume to 50 ml. The absorbance of the methanol solution is measured at 257 mμ in a 1-cm. cell with a suitable spectrophotometer. The methanol wash from 2 to 3 Gm. of the Amberlite IR-4B anionic resin is used as a blank and the poldine methylsulfate content is determined from a standard curve. A Cary model 14 spectrophotometer was used for all absorbance measurements in this study.

Various preliminary manipulations were employed for the determination of poldine methylsulfate in the several pharmaceutical dosage forms investigated. Solutions such as parenterals and syrups were appropriately diluted with sulfuric acid and analyzed as described. For the analysis of tablets, 20 tablets were accurately weighed and reduced to a powder in a mortar and pestle. A suitable weight of powder equivalent to 50 mg. of poldine methylsulfate is extracted with 50 ml. of a 5% sulfuric acid solution, filtered, and a 20-ml. aliquot of the filtrate used for the assay.

TABLE III.—DETERMINATION OF POLDINE METHYLSULFATE IN SYRUPS

Sample	Poldine Methylsulfate, mg./5 ml. Declared	Found
A	4.0	4.22
B	4.0	4.25
C[a]	4.0	3.91
D[a]	4.0	3.84

[a] These samples contained butabarbital sodium, 15 mg./5 ml.

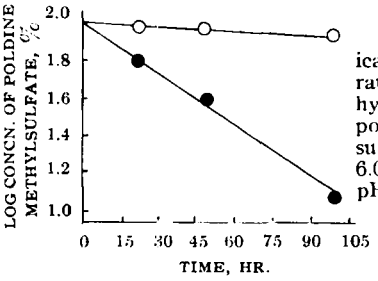


Fig. 3.—Typical first-order rate plots for the hydrolysis of poldine methylsulfate at pH 6.0 ○ and at pH 7.0 ●.

RESULTS AND DISCUSSION

Figure 2 illustrates a standard curve obtained with a series of aqueous poldine methylsulfate solutions carried through the complete analytical procedure. The precision of the analytical method was tested on a number of simple aqueous solutions of the drug, and the standard deviation in a series of 27 assays was found to be 1.84%.

Aqueous solutions of benzilic acid and 2(α-hydroxymethyl)-1,1-dimethylpyrrolidinium iodide alone and in combination showed no absorbance at 257 mμ when carried through the entire analytical procedure. To determine the reliability of the method for poldine methylsulfate in the presence of these products, synthetic mixtures, corresponding to a 10% hydrolytic breakdown of the drug, were prepared and analyzed. The results of this study are shown in Table I.

Table II shows the results of assays on compressed tablets containing a combination of poldine methylsulfate and butabarbital sodium.[2] The results obtained on several batches of syrups containing the drug alone and in combination with the barbiturate are given in Table III. None of the ingredients normally used in the formulation of such products showed interference in the assay procedure.

The stability of aqueous solutions of poldine methylsulfate under varying conditions of pH and temperature were studied employing the assay procedure described. Standard solutions containing 2.0 mg./ml. of the drug were prepared utilizing 0.2 *M* biphthalate or 0.2 *M* phosphate buffers, and the pH adjusted between 3.0 and 8.0 with hydrochloric acid or sodium hydroxide. These solutions were maintained at a constant temperature of 40°, 60°, or 80° and aliquots removed and assayed at various times. Some typical apparent first-order plots for the hydrolysis of poldine methylsulfate are shown in Fig. 3. The temperature dependency of the hydrolysis reaction, under the conditions of the study, is illustrated by the Arrhenius plots of Fig. 4. The heat of activation was determined from this data to be 20 Kcal./mole. Table IV summarizes the apparent first-order rate constants observed in this study. No hydrolytic degradation was detected in solutions buffered at pH 3.0 or 3.5 after 80 days at 40°.

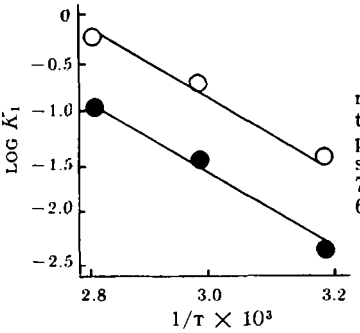


Fig. 4.—Arrhenius plots for the hydrolysis of poldine methylsulfate at pH 7.0 ○ and at pH 6.0 ●.

TABLE IV.—APPARENT FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF POLDINE METHYLSULFATE UNDER VARIOUS CONDITIONS OF pH AND TEMPERATURE

pH	Temp., °C.	Apparent First-Order Rate Constant (K_1) (hr.$^{-1}$ × 10²)
4.0	40	0.01
4.5	40	0.02
5.0	40	0.04
	60	0.33
	80	3.00
6.0	40	0.21
	60	2.80
	80	15.00
7.0	40	2.20
	60	9.20
	80	46.00
8.0	40	14.00

In the course of this investigation, it was noted that the amine formed by the hydrolysis of poldine methylsulfate forms a reineckate derivative which is insoluble in methanol. This suggests the possibility of the separation of amines by selective solution of derivatives of this type.

The disadvantages of the analytical method reported here are that it is rather time consuming and relatively large quantities of drug are required for the assay. However, it is applicable to a wide

[2] Marketed as Nactisol by McNeil Laboratories, Inc., Fort Washington, Pa.

variety of complex samples and unusual techniques or critical control of the conditions are not necessary to obtain reliable and accurate results.

SUMMARY

A spectrophotometric method is described for the determination of the quaternary ammonium compound, poldine methylsulfate. The method is based on the ultraviolet absorbance of methanol solutions of the drug after separation of interfering substances by a reineckate derivative and regeneration of the conjugate base through ion-exchange chromatography. The method has been shown to be applicable to the determination of the compound in pharmaceutical preparations and in the presence of its hydrolytic degradation products. The stability of the compound in buffered aqueous systems under various conditions of pH and temperature was investigated utilizing the spectrophotometric procedure presented.

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Methandrostenolone

Mechanism of Hydrochloric Acid Induced Fluorescence

By F. TISHLER and S. M. BRODY

The main products formed when methandrostenolone (17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one) is heated with methanolic hydrochloric acid have been isolated by preparative thin-layer chromatography and identified by application of infrared, ultraviolet, and nuclear magnetic resonance spectra. A mechanism for the hydrochloric acid induced fluorescence is postulated.

IN A PREVIOUS PAPER by Tishler, *et al.* (1), a fluorometric procedure was described for the determination of methandrostenolone[1] (Compound I, Fig. 1) based on the fluorogen formed when the steroid was heated with a methanolic solution of hydrochloric acid at 100°. A number of related steroids were studied to determine the selectivity of the reaction. Under the conditions employed, the reaction appeared to be selective for $\Delta^{1,4}$-dien-3-one or $\Delta^{1,3,5(10)}$-trien-3-ol steroids which had both a 17β-hydroxy and a 17α-alkyl or alkyne substitution.

With the aid of preparative thin-layer chromatography it has been possible to isolate the main products formed during the reaction. Based upon the identification of these products, a mechanism for hydrochloric acid-induced fluorescence with methnadrostenolone and structurally related steroids is postulated.

DISCUSSION

A preparative thin-layer technique as described by Korzun, *et al.* (2), was used to isolate the desired compounds. Figure 2 shows a typical separation after the entire plate has been sprayed with a modified LeRosen reagent (3). A summary of the isolated fractions is given in Table I.

The total recovery was approximately 80%. Material was lost due to the development of a guide strip on the plates and due to strongly adsorbed material which was not eluted. The three major components, Compounds IV, V, and VIII (Fig. 1), were further purified by chromatographing them separately on a small alumina column. Figure 3 shows the purified steroids as chromaotgraphed on a standard thin-layer plate together with the reaction mixture.

It was evident from the ultraviolet spectrum (Fig. 4) of the reaction mixture that methandrostenolone had undergone a change in its structure. Since the reaction was selective for those steroids which had the structure previously described above, one could predict that the changes had occurred solely in ring A or D or in both rings.

Infrared and nuclear magnetic resonance (N.M.R.) data of Compounds IV, V, and VIII showed the loss of the hydroxyl group originally present in ring D of methandrostenolone. The N.M.R. spectra for the above compounds showed one or two bands between 55 and 61 cycles per second, a region characteristic of the $C_{17,17'}$ dimethyls (4). The integrated area of the bands indicated the presence of six hydrogens which is consistant for two methyl groups. It was apparent that the loss of the 17-hydroxyl group was followed by the migration of

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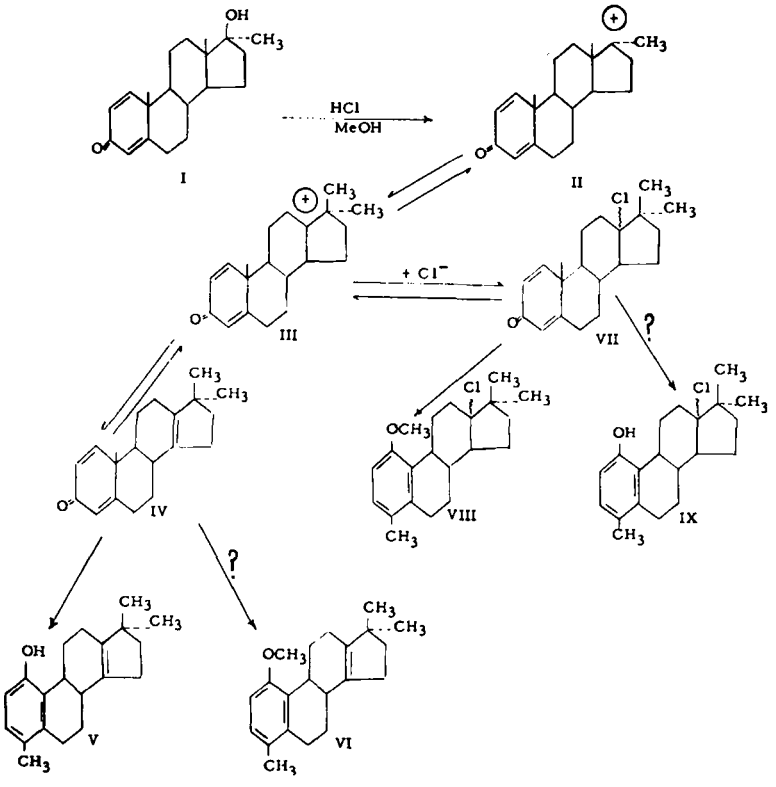
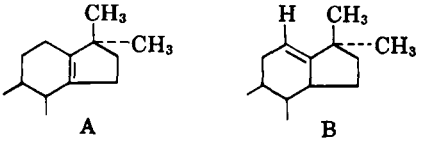


Fig. 1.—Postulated mechanism for hydrochloric acid-induced fluorescence of methandrostenolone.

the C_{18} methyl group from position 13 to 17 giving Species III (Fig. 3).

One pathway that Species III could follow is the loss of a proton at position 12 or 14 with the subsequent formation of a double bond either at position 13–14 (*A*) or 12–13 (*B*)



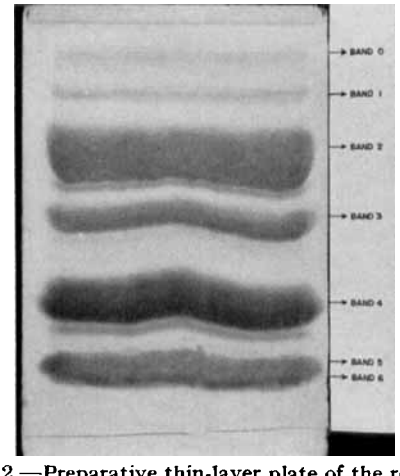


Fig. 2.—Preparative thin-layer plate of the reaction mixture after spraying with LeRosen reagent.

N. M. R. data for Compounds V and VIII showed that no olefinic hydrogens were present, while data for Compound IV showed no additional olefinic hydrogens were present besides those due to ring A which appear further down field in the spectrum, thus ruling out structure B (4, 5).

It was shown by spectral data that Compound IV was unchanged in ring A, *i.e.*, the $\Delta^{1,4}$-dien-3-one system was still present. In addition, the N.M.R. indicated that the C_{19} methyl group, which appears at about 75 cycles per second in structures of this type, was still at position 10 (5). From the above reasoning it can be inferred that Compound IV has the structure assigned in Fig. 1.

The data obtained for Compounds V and VIII

Table I.—Summary of Isolated Fractions

Band	Description	Wt., Gm.	R_f[b]	% Recovery
0	Origin	0.52	0.00	6.0
1	Mixture	0.24	0.00, 0.10	2.8
2	Compound IV	2.31	0.10	26.5
3	Unknown fraction	0.59	0.20	6.8
4	Compound V	1.97	0.35	22.6
5	Compound VIII	1.16	0.85	13.3
6	Unknown fraction	0.25[a]	0.90	2.9

[a] This weight includes unknown compound and Compound VIII. [b] These R_f values are those obtained from standard thin layer results.

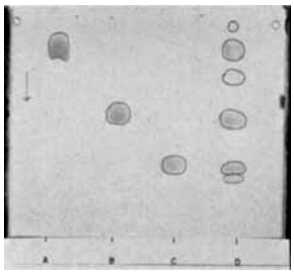


Fig. 3.—Standard thin-layer plate. Key: A, Compound IV; B, Compound V; C, Compound VIII; D, reaction mixture.

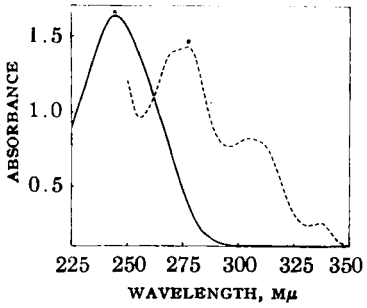


Fig. 4.—Ultraviolet absorption spectra of methandrostenolone *A*, 0.015 mg. per ml., and the reaction mixture *B*, 0.03 mg. per ml., in methanol.

indicated that there was no longer a ketone group, conjugated or unconjugated, present in the molecule. The N.M.R. spectra for these compounds in the aromatic region showed the splitting of the hydrogens to be 8.5 cycles per second which is indicative of ortho splitting (6). Since the N.M.R. indicated the loss of the 75 cycles per second band and I.R. and N.M.R. data indicated the presence of a hydroxyl group (phenolic) in Compound V, it was evident that Compound IV had undergone a dienone-phenol rearrangement (7–11) with the resulting structure assigned in Fig. 1.

Elemental analysis of Compound VIII showed that chlorine (Cl^-) had added to the steroid, thereby indicating that Species III could follow a second pathway as seen in Fig. 3. Although no chemical evdience is available to fix the location and configuration of the chlorine, a 13α-chloro is a most likely assignment (12). From I.R. and N.M.R. data it was shown that Compound VIII was a 1-methoxy-4-methyl steroid instead of a 1-hydroxy-4-methyl steroid. Elks and his co-workers (13) have reported the direct preparation of phenolic ethers by a dienone-phenol rearrangement in alcohol. This same mechanism can be applied to Compound VII with the subsequent formation of Compound VIII.

Further heating of Compounds IV, V, and VIII with methanoic hydrochloric acid showed by thin-layer chromatography that Compounds V and VIII were not changed, while Compound IV was converted, in large part, to Compounds V and VIII. Compounds V and VIII (10 mcg./25 ml. of methanol) possess native fluorescence at 325 mμ when activated at 280 mμ which is typical of estrogenic steroids; Compound IV exhibited no native fluorescence.

When Compounds V and VIII (10 mcg./25 ml.) were treated according to Tishler, *et al.* (1), for the fluorescent determination of methandrostenolone, the fluorescent readings at 345 mμ were increased 10–15-fold when the solution was activated at

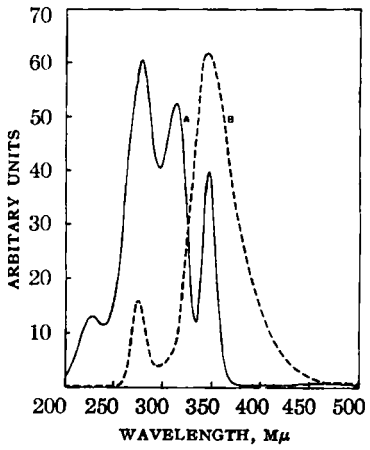
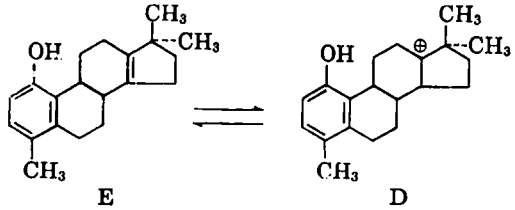
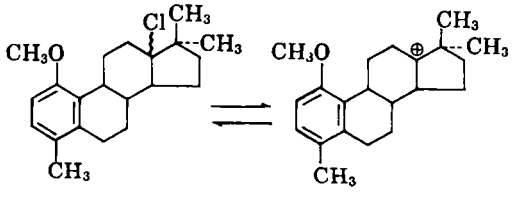


Fig. 5.—Excitation and fluorescent spectra of methandrostenolone, Compounds IV, V, and VIII Key: A, excitation scan—fluorescence held at 345 mμ; B, fluorescent scan—excitation held at 280 mμ.

280 mμ. Compound IV under the same conditions gave a fluorescent reading of the same increased intensity. The activation and fluorescent spectra which were obtained with Compounds IV, V, and VIII after heating with methanolic hydrochloric acid are the same as that obtained with methandrostenolone and appear in Fig. 5.

Based upon the above results, it is suggested that Species D and F are present in the methanolic hydrochloric acid solution after heating and are responsible for the fluorescent characteristic of methandrostenolone.





No explanation is available at present regarding why Compounds V and VIII rather than VI and IX are the predominant products formed. Further work is being carried out on structurally related steroids to learn more of hydrochloric acid-induced fluorescence.

EXPERIMENTAL

Apparatus

N.M.R. spectra were obtained in $CDCl_3$ on a Varian A-60 using tetramethylsilane as an internal reference standard. U.V. curves were made on a

Cary recording spectrophotometer, I.R. spectra were obtained with a Beckman I.R.-5. Excitation and fluorescent spectra were recorded on an Electro model 101 XY recorder in conjunction with an Aminco-Bowman spectrophotofluorometer.

Procedure

Methandrostenolone (1.0 Gm.) was dissolved in 15 ml. of methanol in a 50-ml. volumetric flask. Ten milliliters of concentrated hydrochloric acid was added and the mixture was placed in a 100° oven for 40 minutes. After cooling, the viscous oil (0.95 Gm.) formed during the reaction was separated from the remainder of the mixture by decantation.

Approximately 1.0 Gm. of the reacted material, dissolved in a minimum amount of chloroform-methanol (1:1), was applied with a pipet as a narrow band 8 in. long and at a point 1 in. from the end of the plate to eight glass plates (10 × 15 in.) coated to a thickness of 1 mm. with Silica Gel G (E. Merck, Darmstadt, Germany). The plates were placed at an angle in 12 × 12 × 24-in. rectangular chromatographic chambers and the solvent (benzene-chloroform [80:20]) was allowed to ascend to about 1 in. from the top of the plates. To obtain a satisfactory resolution of the mixture, the plates were removed, air dried, and then redeveloped once again by the above procedure. The two developments took from 6 to 8 hours. A guide strip 1 in. wide located along one edge of the plate was sprayed with a modified LeRosen reagent. The bands thus revealed were marked, scraped off the glass plates with a porcelain spatula, and eluted from the adsorbent with a 1:1 mixture of chloroform-methanol. Evaporation of the solvent afforded oils in all cases. The compounds were further purified by chromatographing them separately on a small alumina column.

Compound IV.—Isolation of this compound afforded a colorless oil which was resistant to crystallization; $\lambda_{max.}^{MeOH}$ 243 mμ, log ε 4.12; $\lambda_{max.}^{Nujol}$ 6.04 μ (ring ketone with αβ-α′β′ conjugation.

Anal.—Based on the 2,4-dinitrophenylhydrazone of Compound IV. Calcd. for $C_{26}H_{30}N_4O_4$: C, 67.53; H, 6.49; N, 12.12. Found: C, 67.49; H, 6.43; N, 11.88.

Compound V.—This material was recrystallized from aqueous methanol and yielded fine white needles; m.p. 106–108°; $\lambda_{max.}^{MeOH}$ 279 mμ, log ε 3.35; $\lambda_{max.}^{Nujol}$ 2.86 μ (⁻OH); 12.44 μ (*para*-type).

Anal.—Calcd. for $C_{20}H_{26}O$: C, 85.10; H, 9.22. Found: C, 84.96; H 9.22.

Compound VIII.—This material was recrystallized from methanol and yielded fine white needles; m.p. 53 to 54.5°; $\lambda_{max.}^{MeOH}$ 278 mμ, log ε 3.35 and $\lambda_{max.}^{MeOH}$ 284, log ε 3.32; $\lambda_{max.}^{Nujol}$ 8.00 and 9.56 μ (aromatic ether); 12.26 μ (*para*-type).

Anal.—Calcd. for $C_{21}H_{29}ClO$: C, 75.79; H, 8.72; Cl, 10.68. Found: C, 75.43; H, 8.77; Cl, 10.50.

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Synthesis and Pharmacology of Some Basic Esters of 3,4,5-Trimethoxybenzoic Acid

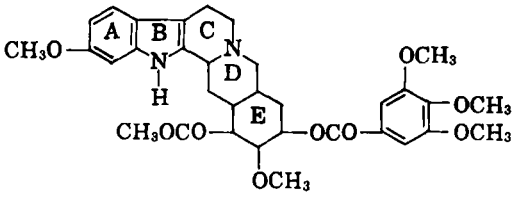
By A. J. VAZAKAS* and JAMES T. DOLUISIO‡

A series of basic esters of 3,4,5-trimethoxybenzoic acid has been synthesized and the compounds evaluated for antihypertensive and local anesthetic activity. One of the compounds, a diamine ester prepared from N-methyl-N-phenyl-N′-propylethylenediamine via a three-step synthesis, exhibited hypotensive activity following intravenous administration to normotensive dogs. No hypotensive activity was demonstrated following intraperitoneal administration to renal hypertensive rats. All but one of the compounds tested exhibited some degree of local anesthetic activity.

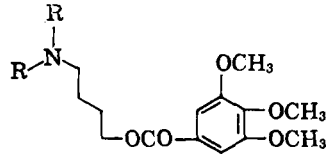
THE ISOLATION of reserpine (I) in crystalline form (1) and the subsequent developments which led to the elucidation of the structure (2–4) and total synthesis (5) of this alkaloid have stimulated many workers to prepare simple analogs of this molecule in search of compounds with reserpine-like activity (6–20).

In our laboratories, a similar objective was approached with the preparation of (*a*) a series of 4-dialkylaminobutyl 3,4,5-trimethoxybenzoates (II), all members of the series possessing portions of rings C, D, and E of the reserpine molecule; (*b*) two 4-(4-substituted piperazino)butyl 3,4,5-trimethoxybenzoates (III) which not only possess portions of rings C, D, and E but, with the inclusion of a second nitrogen atom in a position approximating that of the indole nitrogen of reserpine, might also be considered to possess a portion of ring B; (*c*) N-methyl-N-phenyl-N′-propyl-N′-[4-(3,4,5-trimethoxybenzoyloxy)butyl]-ethylenediamine (IV) which possesses all of ring A and portions of rings B–E.

The compounds of structures II and III were prepared by alkylation of the appropriate secondary amines with the previously unreported 4-chlorobutyl 3,4,5-trimethoxybenzoate (V). For the preparation of IV, N-propylchloroacetamide (VI) was converted by use of N-methylaniline to 2-(N-methylanilino)-N-propylacetamide (VII) which was then reduced with lithium aluminum hydride to N-methyl-N-phenyl-N′-propylethylenediamine (VIII). Acylation of VIII with β-carbethoxypropionyl chloride (IX) afforded the amide ester (X) which, on reduction with lithium aluminum hydride, yielded N-methyl-N-phenyl-N′-propyl-N′-(4-hydroxybutyl)ethylenediamine (XI). The latter was then esterified with 3,4,5-trimethoxybenzoyl chloride in ether and in the presence of triethylamine to produce IV.

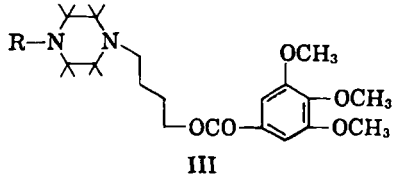


I



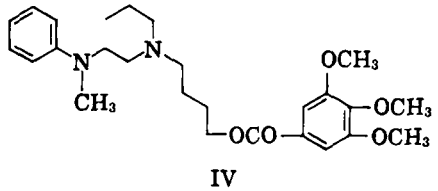
II

IIa, R = CH_3
IIb, R = C_2H_5
IIc, R = n-C_3H_7



III

IIIa, R = CH_3
IIIb, R = $HOCH_2CH_2$



IV

While this work was in progress, a publication

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Presented to the American Association for the Advancement of Science, Philadelphia meeting, December 1962.
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appeared (7) describing the preparation of the compounds of structure II by use of a different synthetic procedure. The compounds were reported to be "devoid of any reserpine-like activity as was indicated by their failure to prolong the hexobarbital sleeping time in mice and their failure to affect the fall-off time of rats from a rotating rod." In our evaluation, the compounds of both structures II and III did not significantly affect the blood pressure of anesthetized normotensive dogs.

Because of the nature of the structures of II and III, it was of interest to examine these compounds also for local anesthetic action. The procedure used consisted of the topical application of 3 drops of a 1% solution of the compound in the rabbit eye and measurement of the duration of anesthesia in minutes. It was found that all of these compounds except III*a* were quite active in this evaluation procedure. The anesthesia produced was accompanied by lacrimation and pitting with each compound except III*b*, which was found to be completely free of these side effects. Structure III*b* also proved to be the most active of the compounds tested. The 87-minute average duration of anesthesia found with III*b* compared favorably with the average durations of action observed for cocaine and procaine of like concentration, these being 54 and 36 minutes, respectively. Another standard, dibucaine, had an average duration of action of 94 minutes at a concentration of 0.5%.

unanesthetized renal hypertensive rats, the compound had no activity when blood pressures were recorded at 1, 2, and 4 hours post treatment at doses of 10 and 20 mg./Kg., i.p. Lack of activity in this experiment may have been due to the short duration of action demonstrated in the dog experiments.

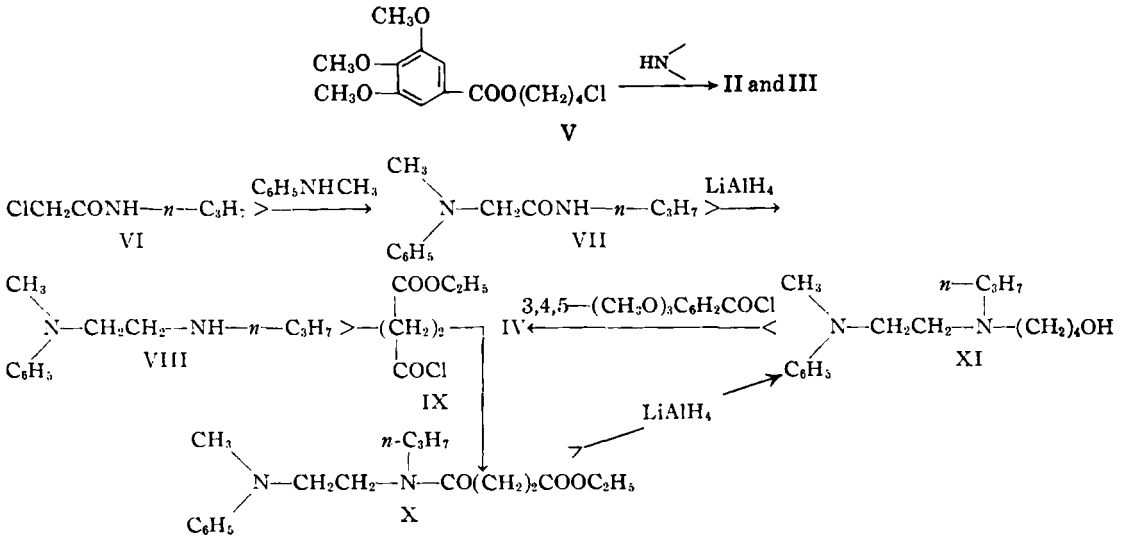
EXPERIMENTAL[1]

3,4,5-Trimethoxybenzoyl Chloride.—This compound, prepared according to the method of Lasslo and Jordan (21), was further purified by distillation *in vacuo*, b.p. 112–115°/0.8 mm. It crystallized in the receiver, m.p. 79.5 to 81°; reported (22) m.p. 79–82°.

4-Chlorobutyl 3,4,5-Trimethoxybenzoate (V).—A mixture of 50.5 Gm. (0.22 mole) of 3,4,5-trimethoxybenzoyl chloride and 29.3 Gm. (0.27 mole) of 4-chloro-1-butanol in 270 ml. of anhydrous benzene was refluxed for 8 hours, hydrogen chloride being evolved. After removal of the solvent, the residue was distilled under reduced pressure. A 42.1-Gm. quantity (63.5%) of pale yellow oil, b.p. 185–189°/1.1 mm. was obtained; n_D^{24} 1.5315.

Anal.—Calcd. for $C_{14}H_{19}ClO_5$: C, 55.54; H, 6.33; Cl, 11.71. Found: C, 55.32; H, 6.33; Cl, 11.71.

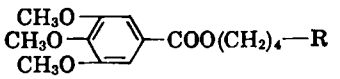
4 - [4 - (2 - Hydroxyethyl)piperazino]butyl 3,4,5-Trimethoxybenzoate Dihydrochloride (IIIb).—A mixture of 5.0 Gm. (0.016 mole) of 4-chlorobutyl 3,4,5-trimethoxybenzoate, 2.1 Gm. (0.016 mole) of N-(2-hydroxyethyl)piperazine, 1.7 Gm. of anhydrous sodium carbonate, and 3 Gm. of powdered potassium iodide in 200 ml. of methyl ethyl ketone was stirred and refluxed for 16 hours. The solvent was removed at reduced pressure, the residue treated with a concentrated solution of sodium carbonate, and the alkaline mixture extracted with ether. The dihydrochloride precipitated upon the addition of anhydrous hydrogen chloride to the dried ether solution. The white solid was collected, washed with ether, and recrystallized from ethanol to

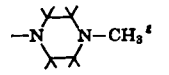
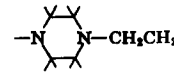


Compound IV, which was evaluated only with regard to its effect on blood pressure, had hypotensive activity when administered to anesthetized normotensive dogs at a dose of 8 mg./Kg., i.v. This dose caused an immediate fall in blood pressure ranging from 41 to 68 mm. of mercury in three dogs. The average duration of activity was approximately 17 minutes, the range being from 5 to 30 minutes. The immediate decrease in blood pressure observed with this compound is not typical of reserpine activity. The compound did not appear to possess adrenergic or ganglionic blocking activity. In

[1] Melting points were determined by the open capillary tube method and are uncorrected. The microanalyses were done by Dr. Alfred Bernhardt, Mülheim (Ruhr), Germany. Spectra of the oils were obtained on a Perkin-Elmer model 21A double beam infrared spectrophotometer on samples between salt disks.

TABLE I.—BASIC ESTERS OF 3,4,5-TRIMETHOXYBENZOIC ACID



No.	R	M.p., °C.	Yield, %	Formula	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	Cl, % Calcd.	Cl, % Found	N, % Calcd.	N, % Found
1	$-N(CH_3)_2$[a]	121.5 to 122[b]	39	$C_{18}H_{25}NO_5 \cdot HCl$	55.24	55.24	7.53	7.47	...	...	4.03	4.22
2	$-N(C_2H_5)_2$[c]	138.5 to 139.5[d]	35	$C_{18}H_{29}NO_5 \cdot HCl$	57.51	57.24	8.04	8.16	...	...	3.73	3.97
3	$-N(n-C_3H_7)_2$[e]	118–119[f]	25	$C_{20}H_{33}NO_5 \cdot HCl$	59.46	59.36	8.48	8.13	...	...	3.47	3.66
4		207[h]	45	$C_{19}H_{30}N_2O_5 \cdot 2HCl$	...	...	...	...	16.14	15.99	6.38	6.28
5		190[h]	56	$C_{20}H_{32}N_2O_6 \cdot 2HCl$	...	...	...	...	15.11	14.90	5.97	5.92

[a] Clusters of small white needles from benzene. [b] Reported (7) m.p. 122–124°. [c] White needles from acetone-ether. [d] Reported (7) m.p. 140–141°. [e] Rosettes of white needles from acetone-ether. [f] Reported (7) m.p. 118–119°. [g] Mats of white needles from ethanol or from a large volume of acetone. [h] With decomposition. [i] Rhombs from ethanol.

yield 4.2 Gm. (56%) of white crystalline product, m.p. 190° dec.

Anal.—Calcd. for $C_{20}H_{32}N_2O_6 \cdot 2HCl$: Cl, 15.11; N, 5.97. Found: Cl, 14.90; N, 5.92.

The compounds listed in Table I were prepared essentially as above, the only variations in the procedure being (*a*) the use of excess amine, rather than sodium carbonate, as the acid acceptor in the preparation of Nos. *1–3*, and (*b*) the preparation of No. *1* in a pressure bottle, rather than under reflux, at a temperature of 60° for 30 hours.

N-Propylchloroacetamide (VI).—This compound, prepared by the method reported for the synthesis of the N-ethyl homolog (23), was obtained as a colorless oil in 75% yield, b.p. 91–94°/4.5 mm.; reported (24) b.p. 90–91°/4 mm.

2-(N-Methylanilino)-N-propylacetamide (VII).—A mixture of 102 Gm. (0.75 mole) of N-propylchloroacetamide and 181 Gm. (1.7 moles) of N-methylaniline in 700 ml. of toluene was stirred and refluxed for 8 hours. The mixture, after having been allowed to stand overnight, was filtered to remove the precipitated N-methylaniline hydrochloride, the cake was washed with benzene, and the benzene-toluene filtrate was evaporated to dryness under vacuum with the aid of steam bath heat. The dark oily residue was dissolved in chloroform, the chloroform solution was washed with water, then dried over sodium sulfate. The mixture was filtered, the chloroform removed, and the residual oil distilled *in vacuo*. After a small forerun of N-methylaniline, 131 Gm. of oily product was obtained, b.p. 135–138°/0.4 to 0.5 mm., which gradually solidified in the receiver, forming rosettes of glistening pale-yellow needles. The product was further purified by recrystallization from 2 L. of skelly B. It crystallized in large shiny white plates. The yield was 118 Gm. (76%), m.p. 52–53°.

Anal.—Calcd. for $C_{12}H_{18}N_2O$: N, 13.58. Found: N, 13.64.

N-Methyl-N-phenyl-N′-propylethylenediamine (VIII).—A 20.0 Gm. (0.526 mole) quantity of lithium aluminum hydride was added, portionwise and with stirring, to 500 ml. of tetrahydrofuran under nitrogen. After addition was complete, the mixture was stirred for 30 minutes. A solution of 36.0 Gm. (0.175 mole) of 2-(N-methylanilino)-N-propylacetamide in 150 ml. of tetrahydrofuran was then added, dropwise, to the stirred mixture, the addition requiring approximately 30 minutes for completion. The reaction mixture became warm but did not reflux. The mixture was stirred for 30 minutes, then heat was applied; it was refluxed with stirring for 38 hours.[a] A 100-ml. quantity of a 20% solution of potassium sodium tartrate was then added, dropwise and with stirring, to the cold (ice bath) mixture. The resulting white precipitate was removed by suction filtration, the cake was washed with 200 ml. of hot tetrahydrofuran, and the

[a] This 38-hour period of reflux was selected arbitrarily and should not be considered a minimum requirement for this reaction. It has been observed (25) that the reduction of N-monosubstituted amides requires not only a considerable excess of hydride but also a fairly long period of reflux after the addition of the amide has been completed if good yields are to be obtained. We repeated this preparation with reactants, solvent, and procedure remaining the same, but with the reflux period limited to 2.5 hours, a reflux period which we have found adequate for the reduction of N-unsubstituted amides in good yield. In these experiments, although the mole ratio of hydride:amide was varied between 2:1 and 4:1, a 2.5 hour reflux period gave, in all cases, yields between 18 and 20% of VIII.

combined pale-yellow filtrates were dried over sodium sulfate and filtered. The solvent was removed at reduced pressure and, after the last traces of moisture had been removed by co-distillation with benzene, the residue was fractionated through a short Vigreux column to yield 24.4 Gm. (72.6%) of very fluid, pale greenish-yellow oil, b.p. 107–109°/1.0 mm.; n_D^{25} 1.5331.

Anal.—Calcd. for $C_{12}H_{20}N_2$: C, 74.95; H, 10.48; N, 14.57. Found: C, 74.87; H, 10.63; N, 14.51.

β-Carbethoxypropionyl Chloride (IX).—This compound was prepared from β-carbethoxypropionic acid by the procedure of Riegel and Lilienfeld (26) in 78% yield, b.p. 109–110°/28 mm.; reported (26) b.p. 110–115°/30 mm. The β-carbethoxypropionic acid, prepared from succinic anhydride and ethanol as reported (26) and further purified by distillation, was obtained in 47% yield, b.p. 118–120°/3 mm.; reported (27) b.p. 119°/3 mm.

N - (2 - N - Methylanilinoethyl) - N - propyl - β-carbethoxypropionamide (X).—A solution of 9.87 Gm. (0.060 mole) of β-carbethoxypropionyl chloride in 50 ml. of benzene was added, dropwise and with stirring, to a well cooled solution of 23.8 Gm. (0.124 mole) of N-methyl-N-phenyl-N′-propylethylenediamine in 100 ml. of benzene. When addition was complete, the reaction mixture was stirred at room temperature for 1 hour, then refluxed for 2 hours. The mixture was cooled, water was added with stirring to dissolve the copious white crystalline precipitate which had formed, and, while being stirred vigorously, the two-phase system was acidified with dilute hydrochloric acid. The aqueous phase was separated, washed well with ether, then made basic with a saturated solution of sodium carbonate. The basic mixture was extracted with ether, the ether solution was dried (sodium sulfate), filtered, and the ether removed by distillation under vacuum. The residual oil, after the last traces of moisture had been removed by co-distillation with benzene, was fractionated through a short Vigreux column. After removal of 11.8 Gm. of the starting diamine, which had also served as acid acceptor, in the forerun, 11.6 Gm. (60%) of viscous, pale-yellow oil was obtained, b.p. 166–168°/0.1 mm.; n_D^{25} 1.5240.

Infrared spectrum: ester, 1740 cm.$^{-1}$, 1200 cm.$^{-1}$ (broad); amide, 1640 cm.$^{-1}$

Anal.—Calcd. for $C_{18}H_{28}N_2O_3$: C, 67.47; H, 8.81; N, 8.74. Found: C, 67.22; H, 8.69; N, 8.89.

N - Methyl - N - phenyl - N′ - propyl - N′ - (4-hydroxybutyl)ethylenediamine (XI).—This compound was prepared by the lithium aluminum hydride reduction of N-(2-N-methylanilinoethyl)-N-propyl-β-carbethoxypropionamide (X) by the same method used for the preparation of N-methyl-N-phenyl-N′-propylethylenediamine (VIII). In this reduction, a solution of 10.0 Gm. (0.031 mole) of the amide-ester (X) in 40 ml. of tetrahydrofuran was added to a mixture of 4.72 Gm. (0.124 mole) of lithium aluminum hydride in 100 ml. of tetrahydrofuran under nitrogen. The duration of reflux was 11 hours, and 35 ml. of 20% potassium sodium tartrate was used for the subsequent hydrolysis of complex and of excess hydride. Fractionation gave 7.13 Gm. (86%) of pale-yellow oil, b.p. 132–133°/0.1 mm.; n_D^{25} 1.5301.

Infrared spectrum: O—H stretch, 3330 cm.$^{-1}$; primary alcohol, 1070 cm.$^{-1}$; ester and amide, absent.

Anal.—Calcd. for $C_{16}H_{28}N_2O$: C, 72.68; H, 10.67; N, 10.60. Found: C, 72.57; H, 10.42; N, 10.52.

N - Methyl - N - phenyl - N′ - propyl - N′ - [4 - (3,4,5 - trimethoxybenzoyloxy)butyl]ethylenediamine (IV).—To a solution of 5.53 Gm. (0.024 mole) of 3,4,5-trimethoxybenzoyl chloride in 100 ml. of anhydrous ether, a solution of 6.0 Gm. (0.06 mole) of triethylamine in 20 ml. of anhydrous ether was added dropwise and with stirring. To the white silky suspension of the complex which had formed, a solution of 5.28 Gm. (0.020 mole) of N-methyl-N-phenyl-N′-propyl-N′-(4-hydroxybutyl)ethylenediamine (XI) in 50 ml. of anhydrous ether was added, dropwise and with stirring. The mixture was stirred at room temperature for 2 hours, then stirred and refluxed for 3 hours. The copious white crystalline precipitate of triethylamine hydrochloride which had formed was removed by suction filtration, the cake washed with ether, and the ether and excess triethylamine removed from the combined filtrates by vacuum distillation to leave a pale-yellow oily residue. The oily product was dissolved in ether and extracted with dilute hydrochloric acid. The acidic aqueous solution was washed twice with ether, then made basic with a concentrated solution of sodium carbonate. The insoluble oily product was taken up in ether, the ether solution dried (sodium sulfate), filtered, the solvent removed to dryness, and traces of moisture remaining removed by co-distillation with benzene. The product, which began to decompose on attempted distillation *in vacuo*, was distilled successfully by use of an Asco "50" rota-film molecular still at a jacket temperature of 240–242°/27–30μ to yield 5.28 Gm. (57.6%) of very viscous pale-yellow oil; n_D^{25} 1.5495.

Infrared spectrum: ester, 1717 cm.$^{-1}$, 1222 cm.$^{-1}$; ether, 1123 cm.$^{-1}$

Anal.—Calcd. for $C_{26}H_{38}N_2O_5$: C, 68.09; H, 8.35; N, 6.11. Found: C, 67.72; H, 8.16; N, 6.03.

Attempts to prepare the crystalline hydrochloride, maleate, and *d*-tartrate of this compound all proved unsuccessful.

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Hemolysis of Erythrocytes by Antibacterial Preservatives

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A study has been made of the hemolytic activity of antibacterial preservatives on human and rabbit erythrocytes in the presence of 0.9 per cent sodium chloride. The hemolytic potency of phenol increased with methyl and/or chlorine substitution. The order of activity was: *p*-chloro-*m*-cresol ⟩ *p*-chlorophenol ⟩ tricresol and *m*-cresol ⟩ phenol. Chlorinated alcohols were similarly more hemolytic than the non-halogenated ones: *p*-chloro-β-phenylethyl alcohol ⟩ chlorobutanol ⟩ phenylethyl alcohol ⟩ benzyl alcohol. Erythrocytes were highly sensitive to benzalkonium chloride, benzethonium chloride, and phenylmercuric nitrate. Thimerosal, butylparaben, and sodium formaldehyde sulfoxylate also induced hemolysis. The data indicate that the hemolytic and antimicrobial mechanisms of action may be identical for many compounds.

THE U.S.P. XVI (1) states that suitable substances may be added to preparations intended for parenteral administration to increase the stability of the product, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or the assay procedures. Limited amounts of various antibacterial preservatives are recommended for multiple-dose parenteral products regardless of the method of sterilization. A personal survey of currently marketed parenteral products indicated that a variety of antibacterial agents are used in preparations intended for each route of administration, including intravenous administration.

It has been shown, *in vitro* and *in vivo*, that certain chemicals in amounts calculated to be isoosmotic to the red blood cell according to physicochemical data may pass through the erythrocytic membrane and fail to prevent osmotic hemolysis (2–9). In addition, certain substances may be cytotoxic to the erythrocyte, thus altering the integrity of the membrane and resulting in the escape and/or denaturation of the cell contents.

It was the purpose of this investigation to study the hemolytic activity of various antibacterial preservatives on human and rabbit erythrocytes in the presence of isotonic media. By preparing the preservative solutions in 0.9% sodium chloride it was felt that any alteration in the integrity of the erythrocytes could be directly attributed to the action of the preservative and not to the original osmotic pressure of the external solution.

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EXPERIMENTAL

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Materials.—The following chemicals used in this study were supplied gratuitously by their respective manufacturers: chlorobutanol U.S.P. and benzyl alcohol N.F., Benzol Products Co.; methylparaben U.S.P., propylparaben U.S.P., ethylparaben, and butylparaben, Heyden Newport Chemical Corp.; phenylmercuric nitrate, Metalsalts Corp.; *p*-chlorophenol, Dow Chemical Co.; *p*-chloro-*m*-cresol, Burroughs Wellcome and Co.; thimerosal N.F., Eli Lilly and Co.; sodium formaldehyde sulfoxylate, Rohm and Haas; and *p*-chloro-β-phenylethyl

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alcohol, Ciba Pharmaceutical Co. Cresol U.S.P., *m*-cresol, phenol U.S.P., sodium chloride reagent, and phenylethyl alcohol were obtained commercially.

Preparation of Solutions.—The various solutions of each preservative contained the antibacterial agent and 0.9% sodium chloride in purified water. All solutions were prepared on a per cent w/v basis, except for benzyl alcohol, (tri)cresol, *m*-cresol, phenylethyl alcohol, and *p*-chloro-β-phenylethyl alcohol, which were per cent v/v.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis, essentially that of Hunter (10), is based on the fact that the quantity of oxyhemoglobin exuded from the erythrocytes of a blood sample is a direct function of the proportion of red blood corpuscles hemolyzed.

Five-milliliter volumes of each preservative-saline solution were pipeted into each of two colorimeter tubes. Similarly, 5-ml. portions of purified water, 0.9% sodium chloride, and sodium chloride solutions ranging in concentrations from 0.32 to 0.52% were each transferred into duplicate tubes. Exactly 0.05 ml. of fresh defibrinated and aereated blood was added to each tube and the contents mixed by gentle inversion. The tubes were then placed in a water bath at 37° for 45 minutes, then centrifuged for 4 minutes at approximately 2000 r.p.m. A Klett-Summerson photoelectric colorimeter with a No. 54 green filter was adjusted to read zero absorbance with the tubes containing the blood in 0.9% sodium chloride. Each of the other tubes was read for its absorbance and observed for signs of denaturation. The average colorimeter reading of the two tubes containing the blood in purified water was taken to be the total hemolysis value. The per cent hemolysis in each duplicate pair of tubes was calculated by dividing their average absorbance reading by the total hemolysis value and multiplying by 100%. Trace hemolysis was considered to be the first colorimetric detection of exuded hemoglobin in a preservative test series; in all cases it represented less than 10% hemolysis. A minimum of six preservative concentrations intermediate to those inducing trace and total hemolysis were employed in each test series.

The data obtained on the response of erythrocytes to the various concentrations of sodium chloride were used to plot the characteristic sigmoid curves (11) for the purpose of verifying the osmotic normalcy of each blood sample.

Colorimetric readings indicating that well over 100% hemolysis had occurred in certain test solutions (notably the cationic surfactants) were attributed to an alteration of the normal color of exuded cellular material. Procedural modifications were made according to the method previously described (8) for the quantitative determination of hemolysis in colored solutions.

DISCUSSION AND RESULTS

All of the preservatives studied, except for the parabens, sodium formaldehyde sulfoxylate, and thimerosal, caused total hemolysis of rabbit and human erythrocytes within concentrations normally employed in parenteral solutions (see Table I). It is interesting to compare the reported bactericidal concentrations of many of these agents with the amounts found to induce hemolysis. The antibacterial mechanism of action of a preservative which promotes bacteriolysis may be similar to that which is responsible for hemolysis.

A widely accepted conception is that the erythrocyte is bounded by a lipid-like membrane which is perforated with positively charged aqueous pores or channels of various diameters (12–14). Schanker, *et al.* (15), suggest that small lipid-soluble molecules might penetrate the cells by dissolving in the lipoid phase and by diffusing through the aqueous channels; larger lipid-soluble molecules would enter mainly through the lipoid phase. Lipid-insoluble molecules and anions would enter the cell if they were small enough to pass through the pores, whereas cations would be largely excluded because of their lipid insolubility and their inability to pass through the positively charged pores. Wintrobe (16) agrees that the ultrastructure may act as a preferential barrier and help to determine the internal contents of the cell, but he points out that some chemical agents which have been shown to cause hemolysis *in vitro* do not do so *in vivo*.

TABLE I.—PER CENT CONCENTRATION OF PRESERVATIVE CAUSING TRACE AND TOTAL HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES IN THE PRESENCE OF 0.9% SODIUM CHLORIDE

Preservative	Concn. of Preservative Causing Hemolysis, %[a]			
	Rabbit Erythrocytes		Human Erythrocytes	
	Trace	Total	Trace	Total
Phenol	0.43	0.48	0.49	0.54
Cresol	0.16	0.225	0.225	0.25
m-Cresol	0.20	0.225	0.26	0.30
p-Chlorophenol	0.11	0.125	0.11	0.15
p-Chloro-*m*-cresol	0.047	0.055	0.052	0.060
Chlorobutanol	0.26	0.32	0.37	0.50
Benzyl alcohol	0.81	0.94	1.05	1.20
Phenylethyl alcohol	0.52	0.62	0.65	0.80
p-Chloro-β-phenylethyl alcohol	0.10	0.14	0.12	0.18
Thimerosal	0.050	0.50	1.50	2.00[b]
Phenylmercuric NO_3	0.0010	0.0030	0.0040	...[c]
Benzethonium Cl	0.0013	0.0023	0.0015	0.0022
Benzalkonium Cl	0.0010	0.0025	0.0017	0.0027
Na-Formaldehyde sulfoxylate	1.0[d]	5.0[e]	...[f]	...[f]
Methylparaben	...[c]	...[c]	...[c]	...[c]
Ethylparaben	...[c]	...[c]	...[c]	...[c]
Propylparaben	...[c]	...[c]	...[c]	...[c]
Butylparaben	0.02	...[c]	...[c]	...[c]

[a] Average of a minimum of two experiments. [b] 98% hemolysis. [c] Data unobtainable because of solubility limitations. [d] Black-red denaturation of cells. [e] Black-red hemoglobin color. [f] Data unobtainable prior to denaturation.

Phenolics.—Phenol, a protoplasmic poison, is toxic to all types of cells; high concentrations precipitate proteins and low concentrations denature them without coagulation. This denaturation process does not bind phenol but leaves it free to penetrate the tissues (17).

The hemolytic activity of the phenolics in order of increasing effectiveness was: phenol, *m*-cresol and cresol, *p*-chlorophenol, and *p*-chloro-*m*-cresol. Hemolysis induced by these compounds probably resulted from membrane damage and/or penetration of the compounds into the cells' interiors with the subsequent influx of water and the hemolytic consequence. It is interesting that the cresols were about twice as hemolytic as phenol and the *p*-chloro-derivatives of *m*-cresol and phenol were each about four times as hemolytic as the parent compounds.

A direct relationship seems to exist between the antibacterial and hemolytic activities of the phenolic compounds studied. Recent studies (18) indicate phenolic antibacterial agents exert lethal action by physical damage to the bacterial cell membrane or to mechanisms which control the permeability of the cell membrane. Phenolic agents promote the leakage of bacterial cellular constituents (19).

A comparison of some literature-reported antibacterial phenol coefficients with a similarly calculated "hemolytic phenol coefficient" strongly indicates a like mechanism of action for the two activities. "Hemolytic phenol coefficient," a term hereby fabricated to facilitate the desired comparison, was calculated by dividing the lowest per cent concentration of phenol found to cause total hemolysis (rather than killing of microorganisms) by the lowest per cent concentration of another phenolic chemical also causing total hemolysis under the same conditions. The two coefficients are presented in Table II.

The data obtained indicate that the methyl and/or chlorine substitution of phenol intensifies its hemolytic activity just as it increases its antibacterial potency (20).

Alcohols.—The hemolytic activity of the alcohols in order of increasing effectiveness was: benzyl alcohol, phenylethyl alcohol, chlorobutanol, and *p*-chloro-*β*-phenylethyl alcohol. As in the case of the phenolics, the chlorinated molecules were the most hemolytic. Lachman, *et al.* (21), in reporting on the antibacterial activity of the same alcoholic preservatives, noted that the chloro compounds were more active than the nonchlorinated ones. The greater activity was attributed to the inherent ability of the halogenated molecules to distribute more readily into nonpolar solvents and thus to penetrate the lipid barrier of the microorganisms more readily. The same may apply to their activity on the erythrocyte. The similarity of Lachman's data on bactericidal activity with that on the hemolytic activity obtained in the present study is shown in Table III.

TABLE II.—COMPARISON OF THE MICROBICIDAL AND HEMOLYTIC ACTIVITIES OF PHENOLIC COMPOUNDS

Compd.	Phenol Coefficient	Hemolytic Phenol Coefficient[a] Human Blood	Rabbit Blood
Phenol	1.0	1.0	1.0
Cresol	2.5[b]	2.2	2.2
p-Chlorophenol	3.9[b]	3.6	3.8
p-Chloro-*m*-cresol	10.7[c]	9.0	8.7

[a] Calculated from data in Table I. [b] Data from Wilson and Gisvold (17) using *E. typhosa*. [c] Data from Klarmann and Wright (20) using *S. typhosa*.

Mercurials.—The maximum amount of a mercurial preservative allowed in an official parenteral product is 0.01%. There was no hemolysis of rabbit or human erythrocytes in saline solutions containing 0.01% thimerosal. Trace hemolysis of rabbit erythrocytes was detected in 0.05% thimerosal, with total hemolysis occurring in concentrations of 0.5%. Trace hemolysis of human erythrocytes was observed in 1.5% thimerosal solutions, and near-total hemolysis was effected in 2.0% solutions. Although there was no macroscopic evidence of protein denaturation in this study, the use of an electron microscope by another investigator (22) has shown mercury to produce changes in the human red blood cell membrane in the form of folds, granulation, and coagulation.

Phenylmercuric compounds and mersalyl were reported (23) to be hemolytic agents due to the interaction of the organic mercurials with the cellular sulfhydryl groups which are considered to be essential for the maintenance of the intact structure of the erythrocyte. The interaction of mercury and sulfhydryl groups is also the basis for many explanations of the antibacterial mechanism of action of the mercurials (24).

Complete hemolysis of human erythrocytes in solutions of phenylmercuric nitrate was not accomplished because of the aqueous solubility limitations of the chemical. It was shown, however, that hemolysis commenced in solutions of 0.004% concentration. Rabbit erythrocytes hemolyzed in lower concentrations of the mercurial; 0.001% induced trace hemolysis, and 0.003% caused total hemolysis.

Shukis and Tallman (25), working with a series of aliphatic mercurials, found better antibacterial action with the compounds having a partition coefficient in favor of the lipid phase. Hess and Speiser (26) reported phenylmercuric borate, with its higher solubility in lipids, to be much more active against microorganisms than thimerosal which is poorly lipid soluble. They further describe the action of mercurials as a two-stage process: (*a*) the adsorption of the mercurial onto the membrane, and (*b*) the penetration through the lipid barrier. Thimerosal has been found to enter the rabbit red blood cell after a subcutaneous injection (27).

Cationic Surfactants.—Benzalkonium and benzethonium chlorides exerted similar effects on both human and rabbit erythrocytes. In all experiments, hemolysis of red corpuscles was initiated in cationic concentrations of between 0.0010 and 0.0017%, with total hemolysis occurring in solutions of between 0.0022 and 0.0027% concentration (Table I).

TABLE III.—BACTERICIDAL AND HEMOLYTIC CONCENTRATIONS OF SOME ALCOHOLS

Alcohol	—Concn., %, of Alcohol Causing— Total Hemolysis of Erythrocytes[a] Human	Rabbit	Total Killing of *S. aureus*[b]
Benzyl	1.20	0.94	0.90
Phenylethyl	0.80	0.62	0.50
Chlorobutanol	0.50	0.32	0.28
p-Chloro-*β*-phenylethyl	0.18	0.14	0.10

[a] Data from Table I. [b] Data from Lachman, *et al.* (21).

The hemolysis of human erythrocytes by ionic detergents has been attributed to a lowering of the interfacial tension and the collapse of the membrane's cholesterol-phospholipid-lipoprotein complex, with the cholesterol monolayer being thought of as the main site of the attack (28). As shown in Table I, the erythrocytes showed a delicate sensitivity to the presence of the cationic surface-active agents. The cationic agents examined were about 300 times as effective in inducing trace hemolysis and about 200 times as active in causing total hemolysis as phenol. Reported phenol coefficients for benzalkonium chloride on *S. aureus* at 37° range from 293 to 407 (29).

Rabbit blood in 0.05% benzalkonium chloride became denatured into a voluminous brown sediment.

Parabens and Sodium Formaldehyde Sulfoxylate.—Solutions of the parabens were employed in concentrations up to their individual maximum solubilities, *i.e.*, 0.25% methylparaben, 0.17% ethylparaben, 0.05% propylparaben, and 0.02% butylparaben (30). No hemolysis was induced by the paraben solutions, except for a saturated butylparaben solution which lysed 12% of the rabbit and 6% of the human erythrocytes it contacted. Butylparaben has been reported (30) to be the most toxic of this relatively nontoxic group of preservatives.

Sodium formaldehyde sulfoxylate, in amounts normally used in parenterals (0.1%), had no hemolytic effects on human or rabbit red blood cells. In 1% concentrations the erythrocytes blackened but remained unhemolyzed; in 5% solutions, hemolysis was induced with the liberation of a red-black exudate.

General Considerations.—It appears that preservative agents which affect the permeability and/or membrane integrity of the erythrocyte may have a like mechanism of action when functioning as antibacterial agents. Antibacterial preservatives which are not hemolytic may act through the disruption of a specific biological process or on a site unknown to, and thereby not affecting, the erythrocyte.

Further studies on the hemolytic activities of these and other preservative agents are being conducted with emphasis on the relationship between chemical structure and hemolytic activity.

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Spectrophotometric Reflectance Method for Matching the Color of Solid Dosage Forms

By MARTIN E. EVERHARD†, DONALD A. DICKCIUS, and FRANK W. GOODHART

The chromaticity coordinates and brightness of nearly 50 pharmaceutical colorants have been determined. The coordinates were calculated by a weighted ordinate method from the spectrophotometric reflectance curve of tablets prepared using the colorant. The use of the coordinates and the reflectance spectra in selecting colorants to match a color is explained. The modified Kubelka-Munk equation is used to calculate the concentration of colorant required for the match. Several examples are given which demonstrate how to match the color of a tablet to another tablet, to a color chip, or to other colored material. The method can be adapted to other nontransparent dosage forms such as ointments.

THE COLOR OF a pharmaceutical product is usually given to the colorist by higher management or a committee which selects the color for new products. The colorist may be presented a piece of colored paper, cloth, a chip from a paint catalog, etc., as the color to be matched. The colorist will then proceed, on the basis of his experience, to match the color by trial and error methods. With luck, he may achieve a satisfactory match in a few tries, particularly if it is a familiar color. On the other hand, it may require months of work before a match can be obtained. It is particularly difficult if more than two dyes must be used; this is because of the great increase in color variations possible with three or more dyes.

Part of the problem is that the colorist cannot use the same dyes in pharmaceuticals that have been used in the sample that he is given to match. In these cases, a metameric match is usually the best that can be obtained; that is, the colors will not be a match under all illumination, *e.g.*, daylight and tungsten light.

The decertification of dyes and pigments by the government has increased the problems of the colorist. When a color in current use in a product is removed from the approved list, most companies would prefer to match the old color of the product as closely as possible using different dyes. In this case the colorist has a reference sample of the same material as the final product, but the color may be no easier to match by trial and error methods than a sample of paper.

However, the trial and error method is not the only method available. Spectrophotometric techniques have been successfully used in the paint, plastic, and other industries for years (1–3). The work described here adapts these techniques to the color problems met with solid dosage forms of pharmaceuticals. The method requires suitable sample preparation for correct measurement of the diffuse reflectance of the sample, transformation of the reflectance data to tristimulus (4) and Kubelka-Munk terms (5), mathematical treatment of the data (preferably by computer methods), and the preparation of the final dosage form according to the formula predicted by the method.

To demonstrate the application of the spectrophotometric method, the study was performed in two parts. First, a group of six dyes was chosen which would be used in various combinations on tablets in the preparation of a series of different colored unknowns. Although the dye composition of the tablets was not disclosed until after the completion of the experiments, it was known that an exact match could be achieved by using the original six dyes. The second part of the experiment was more difficult because the colors to be matched were not prepared with certified dyes or pigments but were colors selected from a paint catalog. Thus, the same pigments used to make the color could not be used in the match; whereas in the first part of this study, the same dyes used to make the unknowns could be used.

THEORY

The concentration of a dye in a solid or on a surface is not directly related to the reflectance of the material or to its logarithm. (In solutions which obey Beer's law, the logarithm of the transmission is inversely proportional to the concentration.) A more complicated series of equations relating concentration and reflectance was developed by Kubelka and Munk (6, 7) and others (8, 9). These equations have been applied to the kinetics of fading of dyes on solid or coated surfaces, and a discussion of the restrictions on the equations in relation to pharma-

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ceuticals has been given (10). The modified Kubelka-Munk equation is

$$\Theta = (1 - R)^2/2R = a_1C_1 + a_2C_2 + \ldots + a_nC_n \quad \text{(Eq. 1)}$$

where Θ is the ratio of the absorption coefficient to the scattering coefficient of the material, R is the reflectance at a given wavelength, the a's are proportionality constants, and the C's are concentrations, the subscripts referring to components, 1, 2, etc.

The tristimulus values, X, Y, and Z (4), of a color can be considered as the amounts of each of the three primary lights—red, green, and blue—which must be mixed to match the color in question. The tristimulus values are obtained by integration of the reflectance spectra

$$X \equiv \frac{\int_0^\infty E_c\bar{x}Rd\lambda}{\int_0^\infty E_c\bar{y}d\lambda} \quad \text{(Eq. 2)}$$

$$Y \equiv \frac{\int_0^\infty E_c\bar{y}Rd\lambda}{\int_0^\infty E_c\bar{y}d\lambda} \quad \text{(Eq. 3)}$$

$$Z \equiv \frac{\int_0^\infty E_c\bar{z}Rd\lambda}{\int_0^\infty E_c\bar{y}d\lambda} \quad \text{(Eq. 4)}$$

where E_c is the luminous efficiency of the spectral energy distribution of illuminant C,[1] $\bar{x}$, $\bar{y}$, and $\bar{z}$ are the tristimulus values of the equal-energy spectrum; R is the reflectance of the sample; and λ is the wavelength. The denominator in the definitions normalizes the tristimulus values. The Y function, in addition to being green, was chosen by the International Commission on Illumination to match the response of the eye to brightness; consequently, the magnitude of the Y value is a measure of the brightness of the sample.

The chromaticity coordinates, x and y, are defined

$$x \equiv \frac{X}{X + Y + Z} \quad \text{(Eq. 5)}$$

$$y \equiv \frac{Y}{X + Y + Z} \quad \text{(Eq. 6)}$$

z is redundant since

$$x + y + z = 1 \quad \text{(Eq. 7)}$$

that is, only two coordinates in Eq. 7 are independent. By convention, x and y are used as the chromaticity coordinates. The chromaticity coordinates x and y, and Y completely specify a color.

The chromaticity coordinates are normally plotted on a chromaticity diagram which has a number of useful properties. For example, the coordinates of the colors which result from mixing two dyes lie approximately on a line connecting the chromaticity coordinates of the dyes. For a mixture of three dyes, the resulting colors lie within a triangle with the coordinates for each dye at the apices. The brightnesses, Y, of colors resulting from mixtures of dyes will always be somewhat lower than those of the single dyes.

A more complete discussion of the above concepts can be obtained in the literature (4, 5, 11).

[1] Illuminant C is defined by the International Commission on Illumination as "average daylight," such as light obtained from an overcast or north sky (see *Reference* 11).

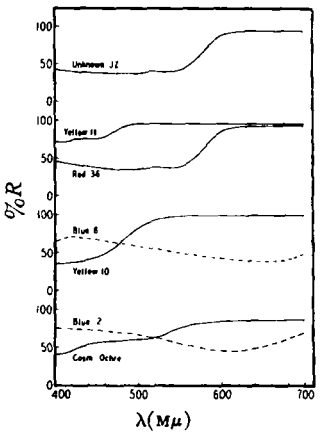


Fig. 1.—Reflectance spectra of the colorants used in the *Experimental* of this study and unknown JZ The concentrations of the colorants are those shown in Table III.

TABLE I.—COMPARISON OF THE ACTUAL AND CALCULATED DYE CONCENTRATION

Sample Identification	Dye	Actual wt. %	Calcd. wt. %
KA	Cosmetic ochre, C1624	0.5	0.4
	D&C Red No. 36	0.1	0.08
JZ	D&C Red No. 36	0.1	0.09
	D&C Yellow No. 11	0.1	0.1
JB	D&C Blue No. 6	0.01	0.008
	D&C Red No. 36	0.1	0.09
KC	Cosmetic ochre, C1624	0.1	0.08
	D&C Blue No. 6	0.005	0.005
	D&C Yellow No. 11	0.2	0.5
LC	FD&C Blue No. 2	0.02	0.02
	D&C Yellow No. 10	0.04	0.06

EXPERIMENTAL

Preparation of Tablets.—Standard tablets of each colorant were prepared at a concentration which gave a moderate color. The concentrations varied for each colorant, depending on their individual tinctorial strengths. Normally, pigment concentration ranged from about 0.1 weight % to 1.0 weight % except for Ebonine confectioners black,[2] which was used in a concentration of 0.01 weight %. The water and alcohol-chloroform soluble dyes, in general, were of higher tinctorial strength. Their concentration ranged from 0.01 weight % to 0.1 weight %.

The general formulas for the standard tablets and the color matching studies are: acacia U.S.P., 0–5 weight %; pigment, *q.s.*; dye, *q.s.*; water or alcohol-chloroform, *q.s.*; magnesium stearate U.S.P., 1.0 weight %; and lactose U.S.P. *q.s. ad.*, 100.0 weight %.

Tablets containing one or more pigments were prepared by mixing the pigments with lactose and magnesium stearate and pulverizing the blend. Tablets containing only the water or alcohol-chloroform soluble dyes were prepared by wetting the

[2] Product of H. Kohnstamm and Co., Inc., New York, N. Y.

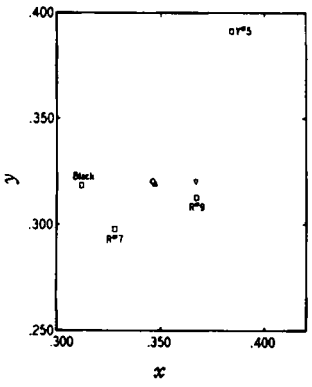


Fig. 2.—Chromaticity diagram for "camellia" match. Key: □Y#5, 0.1% FD&C Yellow No. 5; □ R #9, 0.2% D&C Red No. 9; □ R #7, 0.1% D&C Red No. 7; □ Black, 0.01% Ebonine confectioners black; ○ Spiffy "camellia"; ▽, first trial "camellia"; △, second trial "camellia."

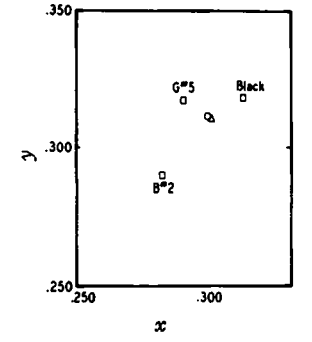


Fig. 3.—Chromaticity diagram for "moonglow" match. Key: □ Black, 0.01% Ebonine confectioners black; □ B #2, 0.04% D&C Blue No. 2; □ G #5, 0.04% D&C Green No. 5; ○, Spiffy "moonglow"; △, compressed tablet match for "moonglow."

lactose-acacia mixture with the dye solution. After drying at room temperature and in an oven at 37° for several hours, the powder was pulverized and the lubricant added. If a formula contained both pigment and dye, a combination of the above procedures was used.

One-inch tablets weighing 3 Gm. were pressed out on the Carver press at a pressure of 5000 p.s.i. One-inch compressed tablets were used for this study because of the ease of measuring the reflectance properties of this type of sample. Small, coated, capsule-shaped tablets have been measured to some degree of accuracy by mounting several tablets side by side in order to fill completely the beam of the spectrophotometer.

Reflectance Measurements and Calculations.—The diffuse reflectance measurements were made on the Bausch and Lomb spectrophotometer, model 505, using $BaSO_4$ as a white standard. Tristimulus values were calculated on a digital computer by a weighted-ordinate method using a wavelength interval of 10 mμ (4). The reflectance of the spectra used in matching "camellia" and "moonglow" were converted to θ at every 20 mμ by consulting a table (12). These θ values were entered into the COMIC analog computer[3] for the matching of the colors of "camellia" and "moonglow." The tristimulus difference computer (TDC), model 80,[3] was used to obtain a corrected formulation for the dyes in the matching of "camellia."

The Pierce Spiffy 116C decorator handbook[4] is a series of colors printed on paper strips about $^3/_4 \times 2$ in. This book has been used as a color library and is referred to as the Spiffy paint catalog and the individual paper strips as paint chips.

METHOD

To match a color, the chromaticity coordinates of the unknown color are plotted on a chromaticity diagram which also has the coefficients of the available dyes plotted on it. Then the coordinates of the dyes which form a small triangle about the unknown are selected. There may be several choices of triangles which can be made. The decision regarding which dyes to pick is made on the bases of the Y values (which should be close to the Y value of the unknown) and on the stabilities and costs of the dyes.

In an unusual case, the coordinates of the unknown may lie on a line between those of two dyes. The unknown could be matched by using these two dyes; however, a small off-color shift in a subsequent batch of the dye would result in an off-color product which could not be corrected by the other dye. Using a third dye in the match, a change in one of the dyes can be compensated by adjusting the concentration of the other two dyes. The use of more than four dyes would almost never be required.

After the dyes are selected from the chromaticity diagram, the reflectance spectra are examined to see that the gross features of the dyes conform to those of the unknown. It is most important that the spectral regions of rapidly changing slope correspond in both the dye and in the unknown. If they do not, one of the other dyes must compensate for the differences. The matching of the reflectance minimum is not as critical as matching the regions of rapidly changing slope. This will be pointed out below in the matching of "camellia."

If the spectra appear satisfactory, the modified Kubelka-Munk equation is used to calculate the concentration of dyes to use in the match. The exact method will be shown below in the examples. With experience, the use of the chromaticity diagram can be eliminated, and the matching can begin by examination of the spectra of the available dyes or tablets prepared from them.

EXAMPLES

In the *Experimental* of this study, five colored tablets were prepared using various combinations of six dyes in unknown amounts. The spectra of these dyes and one of the unknowns is shown in Fig. 1. In matching these tablets, it was not necessary to use the tristimulus data because, with only a limited number of dyes involved, it was possible to see which dyes had been used to prepare the unknowns by comparing the reflectance spectra of the unknown and each of the six dyes.

For example, in matching sample JZ (Fig. 1), the slope of the reflectance spectra from 550–600 mμ is compared to the slope of individual dyes. This slope

[3] Davidson and Hemmendinger, Easton, Pa.
[4] F. O. Pierce Co., Long Island City, N. Y.

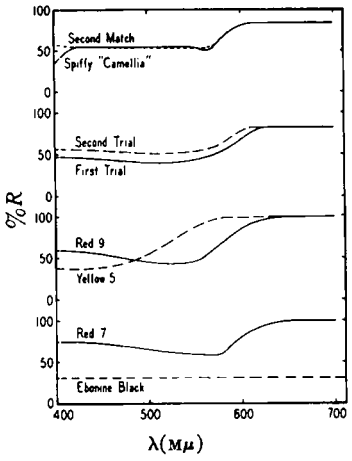


Fig. 4.—Reflectance spectra of the colorants used in the "camellia" match, the Spiffy "camellia" paint chip, the first trial match, and the second trial match.

could result only from D&C Red No. 36. The small slope of the unknown between 400–500 mμ, compared to D&C Red No. 36, indicates the presence of a yellow. Cosmetic ochre, C 1624,[5] can be ruled out immediately because it begins absorbing at about 580 mμ, and the yellow in the unknown does not contribute to the absorption until about 490 mμ. The choice between D&C Yellow No. 10 and No. 11 is a difficult one since their reflectance spectra are very similar. The chromaticity coordinates of D&C Yellow No. 10 and No. 11 are too similar to choose between them on that basis. However, careful examination of the spectra of D&C Yellow No. 10 shows that the slope between 450–500 mμ is too steep to be used in the match. The slope of D&C Yellow No. 11 between 450–500 mμ is more appropriate in this case. If D&C Yellow No. 10 had been used in attempting a match, it would probably be possible to get a suitable color by using a very small amount of the dye.

Having chosen the dyes to use in the match, the concentration of each dye is calculated using Eq. 1. The yellow dye does not contribute to the absorption above 500 mμ where the red dye has a maximum absorption. Therefore, the amount of red dye to use can be easily calculated at 535 mμ as

For the 0.1% Red No. 36 standard:

$$\Theta_{535} = (1 - R_{535})^2/2R_{535} = a_r C_r \quad \text{(Eq. 8)}$$

For the unknown, using primes:

$$\Theta'_{535} = (1 - R'_{535})^2/2R'_{535} = a_r C_r' \quad \text{(Eq. 9)}$$

where the 535 refers to 535 mμ and the subscript r refers to Red No. 36. Dividing Eq. 8 into Eq. 9 and solving for C_r' gives

$$C_r' = (C_r)(\Theta'/\Theta) \quad \text{(Eq. 10)}$$

Substituting the known values into Eq. 10, the Θ values can be read from a table which converts R to Θ (see *Reference* 12)

$$C_r' = (0.1\%)(0.4172/0.4660) = 0.090\% \quad \text{(Eq. 11)}$$

In Eqs. 8–11, the background absorption due to the inert ingredients was neglected because the inert materials were all white. The absorption due to this white material is constant from 400–700 mμ, but can only be detected beyond 620 mμ where the dyes are not absorbing. The correction is too small to affect the results of this calculation.

[5] Product of H. Kohnstamm Co., Inc., New York, N. Y.

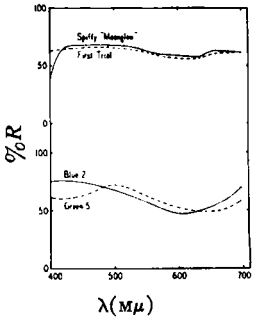


Fig. 5.—Reflectance spectra of the colorants used in the "moonglow" match, the Spiffy "moonglow" paint chip, and the match.

The amount of yellow dye cannot be calculated quite so easily as the red dye because wherever the yellow absorbs, the red absorbs also. However, the concentration of red dye has been calculated above, and the absorption of the red dye at 450 mμ can be calculated based on the absorption of the standard. This can then be subtracted from the absorption of the unknown, and the resulting absorption is due only to the absorption of the yellow dye. Application of Eq. 10 using the data at 450 mμ and solving for Θ_r' gives

$$\Theta_r' = (C_r'/C_r)(\Theta_r) = (0.090/0.10)(0.4660) = 0.4194 \quad \text{(Eq. 12)}$$

Θ_r' is subtracted from the measured Θ of the unknown at 450 mμ to obtain the Θ_y' due to the yellow dye. Then Eq. 10 is applied to solve for the concentration of yellow dye using the 0.1% D&C Yellow spectra as the standard.

$$C_y' = (C_y)(\Theta_y'/\Theta_y) = (0.1)(0.0391/0.0321) = 0.12\% \quad \text{(Eq. 13)}$$

A tablet was prepared according to the calculated amount of each dye. The spectra of this tablet was very close to that of the original unknown; the two tablets could not be distinguished by visual inspection (see Fig. 1.).

Table I shows the actual concentration of dye used in the unknowns and the calculated amount of dye using the above described technique. In each case the unknown tablet could not be distinguished visually from the tablet prepared by the calculated formula.

Table II.—Concentration of Dye to Match "Camellia" and "Moonglow"

Dye	"Camellia" Trial 1	"Camellia" Trial 2	"Moonglow" Trial 1
D&C Red No. 7	0.070	0.044	...
D&C Red No. 9	0.060	0.021	...
FD&C Yellow No. 5	0.012	0.008	...
Ebonine confectioners black	0.001	0.001	0.0070
FD&C Blue No. 2	...	...	0.0072
D&C Green No. 5	...	...	0.0056

TABLE III.—TRISTIMULUS DATA: CHROMATICITY COORDINATES AND BRIGHTNESS

	Concn.	x	y	Y
BLUES				
FD&C Blue No. 1	0.04	0.235	0.280	0.501
FD&C Blue No. 1 Lake	1	0.232	0.267	0.460
FD&C Blue No. 2	0.04	0.276	0.285	0.561
FD&C Blue No. 2 Lake	1	0.269	0.276	0.466
D&C Blue No. 4	0.04	0.235	0.280	0.508
BLACKS				
Ebonine confectioners black	0.01	0.312	0.318	0.651
BROWNS				
D&C Brown No. 1	0.04	0.385	0.369	0.688
GREENS				
FD&C Green No. 1	0.04	0.255	0.319	0.507
FD&C Green No. 1 Lake	1	0.264	0.318	0.565
FD&C Green No. 2	0.04	0.260	0.326	0.536
FD&C Green No. 3	0.04	0.252	0.314	0.478
D&C Green No. 5	0.04	0.290	0.317	0.618
D&C Green No. 7	0.04	0.247	0.297	0.547
IRON OXIDES[a]				
Cosmetic red oxide A6205	1	0.400	0.333	0.333
Cosmetic red oxide A6202	1	0.378	0.327	0.369
Cosmetic red oxide 3469	1	0.396	0.334	0.356
Lt. tab. brown 3228	1	0.379	0.337	0.387
Tab. brown 3330	1	0.348	0.324	0.302
Cosmetic umber	1	0.339	0.332	0.352
Cosmetic suntan	1	0.350	0.335	0.615
Cosmetic ochre 1624	1	0.339	0.346	0.832
ORANGES				
D&C Orange No. 10	0.04	0.368	0.316	0.656
ORANGES				
D&C Orange No. 11	0.04	0.379	0.329	0.687
REDS				
FD&C Red No. 2	0.04	0.328	0.287	0.514
FD&C Red No. 2 Lake	1	0.339	0.284	0.371
FD&C Red No. 3	0.05	0.359	0.269	0.524
FD&C Red No. 3 Lake	1	0.378	0.271	0.455
FD&C Red No. 4	0.1	0.381	0.317	0.509
FD&C Red No. 4 Lake	1	0.371	0.320	0.538
D&C Red No. 7	0.1	0.328	0.298	0.680
D&C Red No. 9	0.2	0.367	0.313	0.617
D&C Red No. 10	0.2	0.385	0.314	0.544
D&C Red No. 17	0.1	0.395	0.305	0.356
D&C Red No. 18	0.1	0.346	0.275	0.306
D&C Red No. 19	0.05	0.317	0.226	0.361
D&C Red No. 22	0.1	0.383	0.286	0.529
D&C Red No. 27	0.1	0.342	0.277	0.621
D&C Red No. 35	1	0.421	0.315	0.361
D&C Red No. 36	0.1	0.404	0.338	0.551
VIOLETS				
FD&C Violet No. 1	0.04	0.258	0.217	0.324
FD&C Violet No. 1 Lake	0.1	0.280	0.263	0.554
YELLOWS				
FD&C Yellow No. 5	0.1	0.384	0.391	0.852
FD&C Yellow No. 5 Lake	1	0.391	0.396	0.822
FD&C Yellow No. 6	0.1	0.411	0.358	0.636
FD&C Yellow No. 6 Lake	1	0.404	0.353	0.603
D&C Yellow No. 10	0.1	0.358	0.391	0.943
D&C Yellow No 11	0.1	0.351	0.384	0.928

[a] Products of H. Kohnstamm Co., Inc., New York, N.Y.

Although matches KC and LC contain too much yellow, θ changes only a small amount with large changes in concentration for these particular dyes and allows these matches to be accepted. Furthermore, the eye is not very sensitive to concentration changes of yellow, compared to blues and reds, which is shown by the color perception work of MacAdam as described by Judd (5). If a closer match were desired, the amount of dye needed for a match could be recalculated using the first trial tablet as a standard.

After the success in matching these tablets, the second part of the experiment was begun. In this half of the work, two colors from the Spiffy paint catalog were matched in tablets. In these matches it was necessary to use an analog computer to obtain a match because the same pigments used in the paint chip could not be used in tablets.[6] The output of the computer is fed to a cathode ray tube where the effect of various concentrations of dye on the resulting spectra can be displayed. A detailed discussion of the computer has been given (13), so only a brief summary will be given here.

The computer method is identical to the method described above, except that the computer solves for the sum of the θ values for all dyes at 16 different wavelengths simultaneously. The output is displayed on a cathode ray tube which allows the unknown curve to be compared to the curve which results from the dyes used in the match. By adjustment of potentiometer dials, the concentration of each of the dyes can be altered electronically until the mixture curve corresponds to the unknown curve. The settings of the potentiometers are directly related to the weight per cent of dye.

The choice of dyes to use in the match is made the same way described in the first part of this study. If there is a choice between two dyes, both can be entered into the computer; the dye which gives the closest match can be selected after viewing, on the cathode ray tube, the effect of both dyes on the mixture.

A tristimulus difference computer (TDC) can be used as an attachment to the basic computer. This indicates the difference in tristimulus values between the sample (standard) and the curve which results from the mixture of dyes used to match the sample. It is extremely useful when making a metameric match because it allows the choice of dye concentration which gives the closest tristimulus match. The best match cannot always be determined on the cathode ray tube because, in a metameric match, there is considerable overlapping of the spectra of the sample and of the mixture. The TDC shows

[6] Matches have been calculated using a desk calculator; however, because the matches are metameric, it is difficult to pick the best concentrations without calculating tristimulus values for each possible match. These calculations are ideally suited to an analog computer.

which combination of dyes gives the closest tristimulus values and, therefore, the best match.

If the trial tablet prepared according to the formula calculated by the computer is not a sufficiently close match, the θ values of this first trial tablet are determined and entered into the computer. The operator can then correct this first trial formula by adjustment of the concentration potentiometers and obtain a better match. Usually the second trial will be a satisfactory match.

The chromaticity diagram plots of the dyes used to match "camellia" and "moonglow" are shown in Figs. 2 and 3. These plots show that the color which results from a combination of dyes lies within the boundary of the triangle (Fig. 3) and quadrangle (Fig. 2), described by the chromaticity coordinates of the dyes used in the match.

Figure 4 shows the reflectance spectra of the dyes used in the "camellia" match. The first trial tablet was too dark (the chroma was too high),[7] but it was the correct hue.[8] The reflectance data from the first trial tablet were converted to θ values and entered into the computer; a satisfactory match was achieved with the corrections calculated on the second trial. The reflectance spectra of the second trial is shown next to the first trial and also next to the "camellia" for comparison.

The comparison of the reflectance curve of the second match of "camellia" to the Spiffy "camellia" shows two significant points. First, the minimum in the Spiffy "camellia" curve at about 565 mμ was not matched; second, the two curves cross over several times. The failure to match the minimum was not so important as matching the rapidly changing slope from 570–600 mμ, as was mentioned under *Method*. The sharp absorption from 400–415 mμ in the Spiffy "camellia" is caused by TiO_2 and is of no concern to the colormatcher. The crossing over of the spectra produces a metameric match.

Figure 5 shows the reflectance spectra of the dyes, other than black, used for the "moonglow" match. In this instance, the first trial was a satisfactory match. The ultimate dosage forms for these colors are sugar-coated, capsule-shaped tablets. No difficulties were encountered in changing from the compressed tablets used in obtaining the match to the capsule shaped tablets. The correct color was obtained by using 20 coats of a syrup solution containing one-half the calculated dye concentrations. Table II shows the concentrations of dyes used in the matching of "camellia" and "moonglow."

[7] Chroma is the degree of vividness of the hue or saturation.
[8] Hue is the attribute of a color which allows it to be described as red, blue, etc.

TRISTIMULUS DATA

The tristimulus data for nearly 50 colorants are given in Table III. The tablets were prepared according to the procedure described above, and the tristimulus data were calculated by the weighted ordinate method.

CONCLUSIONS

The problems in matching the color of a pharmaceutical product to a paint chip or any material which uses nonpharmaceutical colorants, have been mitigated by using a spectrophotometric approach. The ability to achieve a satisfactory color match in one or two tries is a significant improvement over a trial and error procedure. It can only be properly appraised by considering the number of man hours freed by this method for more productive tasks. The closeness of the matches achieved with the spectrophotometric technique establish this as the preferred color matching method. The technique should work equally well with other nontransparent dosage forms such as ointments.

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Kinetics and Mechanisms of Action of Antibiotics on Microorganisms I

Reproducibility of *Escherichia coli* Growth Curves and Dependence Upon Tetracycline Concentration

By M. R. W. BROWN† and EDWARD R. GARRETT

A method which minimizes the lag period of measuring growth rates of bacterial cultures using plate counts has been devised. The standard deviation, expressed as a percentage of the mean among replicate growth rate determinations for this strain of *Escherichia coli*, was 3.7 per cent. It has been shown that the apparent growth rate constants in the absence of antibiotic were independent of inoculum size. Growth rates and rates of kill have been obtained with cells in the constant growth phase in the presence of graded concentrations of tetracycline hydrochloride. Although low concentrations of antibiotic merely reduced the growth rate, increased concentrations had a definite bactericidal effect. After the initial kill with bactericidal concentrations of tetracycline, survivors multiplied at rates which appeared to be a function of the antibiotic concentration. The growth rate of the numbers of viables, X, can be quantitatively expressed as a function of tetracycline concentration, D, and the growth rate constant, k_o, so that $dx/dt = (k_o - kD^n)X$. For a net increase in viables, the rate is a function of the first power of the antibiotic concentration, whereas a dependence upon the concentration to the one-third power best describes the initial rate of change of numbers of viables when the bactericidal activity is experimentally manifested.

THE ELUCIDATION of the effect of antibiotics on the growth rate of microorganisms is complicated by the classical procedure of introducing organisms from steady state cultures into solutions of antibiotic in broth (1). The growth process undergoes an induction period when a degree of cell maturation precedes the logarithmic growth phase characterized by

$$X = X_o e^{kt} \qquad \text{(Eq. 1)}$$

where X_o is the cell concentration at some time $t = t_o$ during this growth phase, and X is the number of cells per unit volume at time t. The growth rate is characterized by the apparent first-order rate constant, k (2).

A systematic approach to the effect of an antibiotic in graded concentrations would be to analyze the induction and log growth phases separately. The latter can readily be studied by combining antibiotic with logarithmic phase cells. It can then be assumed that on a statistical basis each viable cell is reproducing at the same rate as the next (3, 4). There are two convenient procedures: (*a*) the introduction of cells from a master culture in the exponential growth phase into a broth solution of antibiotic, and (*b*) the introduction of antibiotic into a culture of log phase cells.

In these circumstances, any change in the net rate of growth characterized by the constant k can be assigned to the effect of the antibiotic. This can be deduced by the change in slope of the logarithmic transformation of Eq. 1 as a function of antibiotic concentration

$$\log X = kt/2.303 + \log X_o \qquad \text{(Eq. 2)}$$

It is apparent that for any net increase in the number of viables the diminution of the rate of growth compared to the rate in the absence of antibiotic can be assigned to actual "kill" of the microorganism and/or to a reduction in the generation rate. For any measured decrease in colony count from the time of antibiotic addition, the validity of the term "kill" is unquestioned, since it is operationally defined as a reduction in the numbers of cells capable of colony formation under the stated conditions.

It could be postulated *a priori* that if the decrease in the apparent growth rate constant is a function of antibiotic concentration for all log viable *versus* time plots for all antibiotic dosage, then "kill" by antibiotic as defined is valid, even for a positive but lessened growth rate. However, if the nature of the functional dependence of growth rate on antibiotic dosage varies with antibiotic dosage, then the interpretation is necessarily uncertain, and only when the apparent $k<0$ can "kill" be concluded.

It is difficult to deduce the relative contribu-

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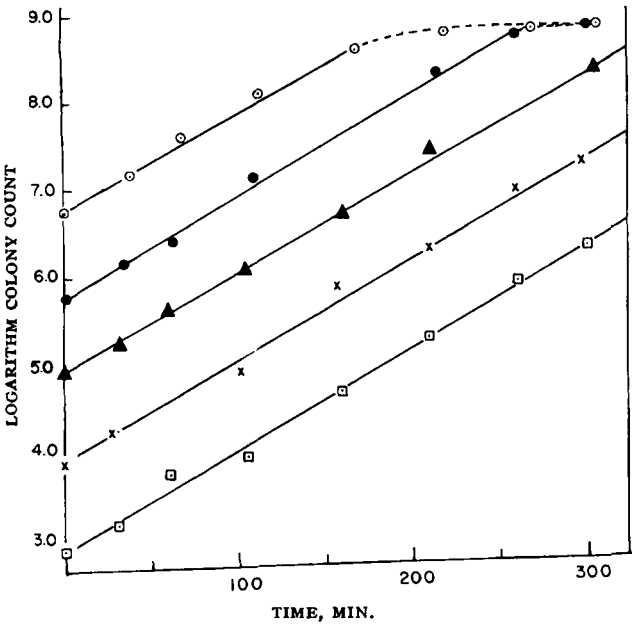


Fig. 1.—Growth curves for *E. coli* using graded inocula concentrations.

Table I.—Apparent First-Order Growth Rate Constants for Replicate *E. coli* Cultures on Different Days

Replicate Growth Rates		
Day 1	Day 2	Day 3
0.01202	0.01153	0.01178
0.01165	0.01110	0.01145
0.01193	0.01121	0.01147
0.01129	0.01113	0.01130
0.01124	0.01118	0.01169

EXPERIMENTAL

Methods.—The test organism was *Escherichia coli* strain E/r. A broth culture from an isolated colony was used to inoculate replicate agar slants using Bacto antibiotic medium 3;[1] these slants were then stored frozen. This medium was also used for broth cultures and plate counts. Dilutions for plate counts were made using distilled water containing 0.85% sodium chloride. Plates were incubated for at least 48 hours before counting, and subsequently re-examined for appearance of new colonies after further incubation. Assayed tetracycline hydrochloride (995 mcg./mg.) was supplied by courtesy of The Upjohn Co. Antibiotic solutions in distilled water containing 2 mg./milliliter were sterilized by filtration, then stored frozen. Fresh slants and antibiotic solution were used for each experiment.

Reproducibility of Growth Rate Measurements.—Growth rates were measured with a Klett-Summerson colorimeter and also by plate counts. The colorimeter was used mainly as a screening device prior to viable count experiments. A measured volume of an overnight broth culture was diluted into broth

Table II.—Analysis of Variance of Apparent First-Order Growth Rate Constants for Replicate *E. coli* Cultures Between and Within Days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Components of Variance	Variance Ratio (F)
Between days	2	5.0×10^{-7}	2.5×10^{-7}	$5\sigma_B^2 + \sigma_W^2$	4.3
Within days	12	7.0×10^{-7}	5.8×10^{-8}	σ_W^2	
Totals	14	12×10^{-7}			

σ_W^2 = variance within days = 5.8×10^{-8}
σ_B^2 = variance between days = 3.8×10^{-8}
$\sigma^2 = \sigma_W^2 + \sigma_B^2 = 9.6 \times 10^{-8}$
$\sigma = 3.1 \times 10^{-4}$

S.D.: % of Mean = 2.7

Tabulated value of (F) at 0.05 Significance level = 3.9

tions of cells whose generation rate has been reduced (perhaps to zero), cells which have lost the capacity to form colonies, and cells which continue to reproduce at an unchanged rate. All of these types of cells may contribute to an observed reduction in colony count with time.

This paper presents initial results of an investigation of these concepts. The effect of inoculum size upon the exponential growth rate and its reproducibility has been tested. Attempts have been made to fit a functional dependence of rate upon antibiotic dosage.

[1] Difco Laboratories, Detroit, Mich.

TABLE III.—APPARENT FIRST-ORDER GROWTH RATE CONSTANTS FOR *E. coli* USING INOCULA OF GRADED CONCENTRATION

Initial Concn., approx.	Rate Constant
10^3	0.0118
10^4	0.0115
10^5	0.0117
10^6	0.0115
10^7	0.0113

$$\sigma^2 = \Sigma \frac{(x - \bar{x})^2}{n - 1} = 5.4 \times 10^{-8}$$

at 37.5°, the growth rate followed turbidimetrically at 500–570 mμ and at a predetermined turbidity, so that the cells known to be in the logarithmic phase were diluted into five replicate volumes of broth at 37.5°. This procedure of diluting logarithmic phase cells into warm broth reduces any lag phase to a minimum (Fig. 1). Dilutions of the replicate cultures were plated out at intervals; these intervals were chosen after preliminary experiments had indicated which were suitable for determination of growth rates. These growth rate determinations for five replicate cultures were repeated on each of 3 days. The correlation coefficients were calculated for the logarithm of viable count against time for the part of the curves preceding th tionary phase. *In every* case the correlatior highly significant ($P > 0.99$); Table I lists th culated apparent first-order growth rate cons obtained on each of the 3 days. Table II pre the results of an analysis of variance of the gr rate constants between and within days. The minations of reproducibility of viable counts made with cultures in Klett-Summerson colori tubes since the reproducibility of the colorir determinations was established concurr Erlenmeyer flasks (250-ml.) were used conta 20 ml. of culture.

Effect of Inoculum Size.—Graded concentra of logarithmic phase cells were used to inoc broth previously warmed to 37.5°, and the gr curves of the resulting cultures were deter by plate count (Fig. 1). Table III lists the gr rate constants obtained for the cultures in Fig

Effect of Tetracycline Concentration.—Fo purposes of this study it is necessary to adc arithmic phase cells to solutions of the antibic prewarmed broth or to add antibiotic to logari phase cells (4). The former procedure was initially in this work, and the results are illust in Fig. 2. Subsequently, it was decided to add biotic to cells in the logarithmic phase as the s

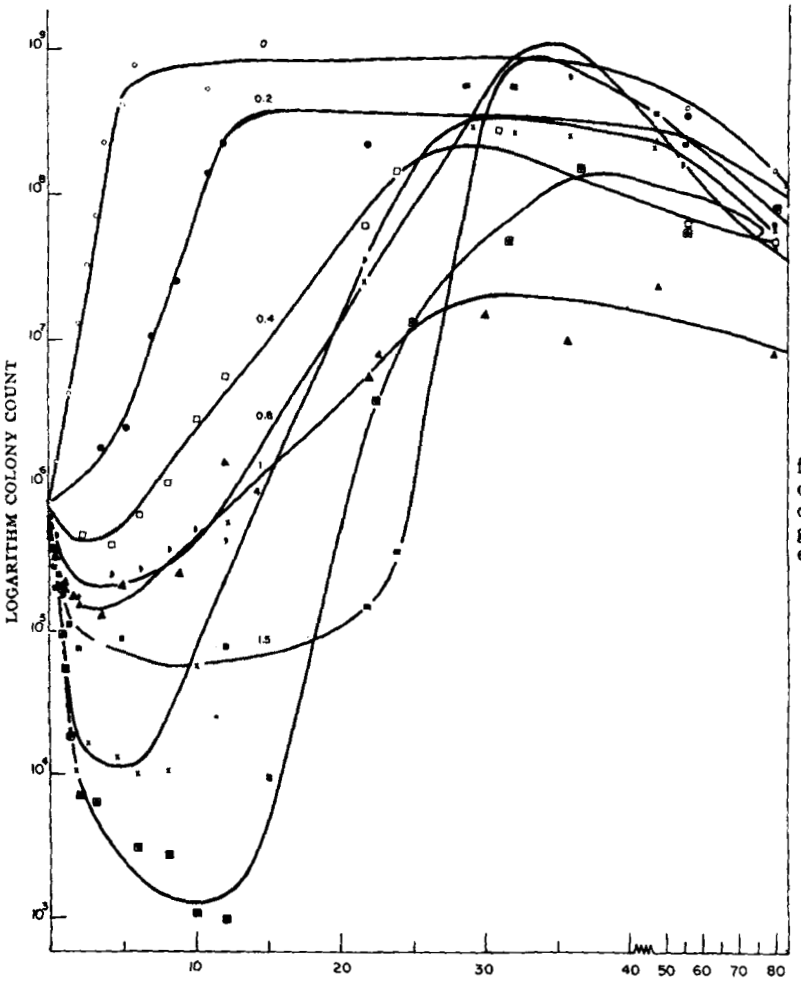


Fig. 2.—Growth for *E. coli* in the pr of graded concentr of tetracycline (grams per millilite extended periods.

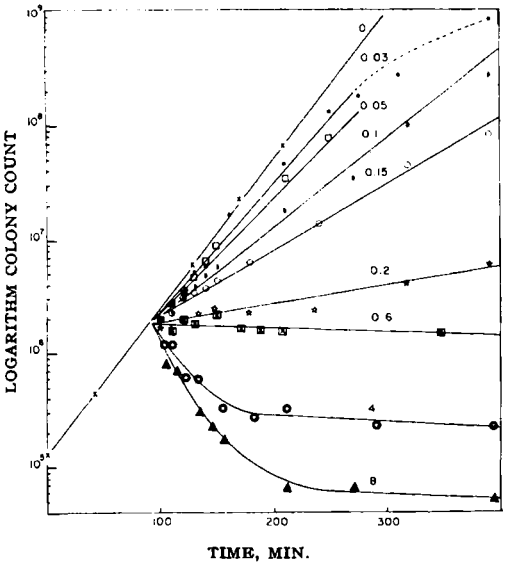


Fig. 3.—Initial growth curves for *E. coli* in the presence of graded concentrations of tetracycline (micrograms per milliliter).

ard method. The procedure was as follows. Nine replicate cultures of logarithmic phase cells were allowed to grow at 37.5° for a predetermined period so that the concentration increased from about 10^5 cells/ml. to about 10^6/ml.; eight of the cultures were then inoculated with tetracycline hydrochloride solution. The cultures then contained 0, 0.03, 0.05, 0.1, 0.15, 0.2, 0.6, 4, and 8 mcg./ml. of antibiotic. Viable counts were made at intervals; Fig. 3 illustrates the results. The growth rate constant was calculated for the initial straight line part of each curve.

DISCUSSION

The results of the analysis of variance of the apparent first-order growth rate constants for replicate *E. coli* cultures show that the between days variance is significantly greater than the within days variance (F test, Table II). The apparent rate constants that were used were uncorrected for the transformation to decadic logarithms, *i.e.*, $S = k/2.303$ for the slope of Eq. 2. The mean value for the rate constants, S, is 0.01146, and the standard deviation for the several studies within days $\sigma_W = 2.4 \times 10^{-4}$ and among days is $\sigma_D = 1.95 \times 10^{-4}$, so that the standard deviation of an S value among and within days is $\sigma = \sqrt{\sigma_W^2 + \sigma_D^2} = 3.1 \times 10^{-4}$ or 2.7% of the mean value.

The procedures used have resulted in an apparent elimination of the lag period of growth for each of the initial concentrations tested (Fig. 1). The variance, $\sigma^2 = 5.4 \times 10^{-8}$ (Table III and Fig. 1) among rate constants for the cultures with various initial inocula concentrations is not significantly different from the variance among rate constants within days in Table II ($\sigma^2 = 5.8 \times 10^{-8}$) for cultures with replicate inocula.

The rates of growth of *E. coli* in the presence of tetracycline are decreased as functions of the concentrations of the antibiotic but are still consistent with Eq. 1. The log X *versus* time plots of Fig. 3 in the presence of antibiotic are consistent with the expectation of Eq. 2, which describes the exponential growth of X_o microorganisms from the time of antibiotic addition. A more general logarithmic expression, in the presence of an ith concentration of tetracycline is

$$\log X = S_i t + \log X_o \qquad \text{(Eq. 3)}$$

where $S_i = k/2.303$, k in minutes^{-1}. The apparent first-order growth rate constant, S_i, is a linear function of the antibiotic concentration, D, in micrograms per milliliter for all positive values of S_i (Fig. 4)

$$S_i = S_o - KD \qquad \text{(Eq. 4)}$$

where $S_o = 1.20 \times 10^{-2}$ is 2.303 times the rate constant $k = k_o$ (minutes^{-1}) for the growth of *E. coli* in the absence of tetracycline, $D = 0$. The specific rate constant for tetracycline concentration effect is

$$K = 4.81 \times 10^{-2}$$

At $S_i = 0$ or less, after an initial period of reasonable linearity, a nonlinear relationship develops between log viables and time. This could be due to consumption or degradation of antibiotic, development of resistant organisms, and/or the presence of antibiotic-resistant strains in the original culture.

The rate of change of viable organisms with time may be a function of the growth rate and the antibiotic concentration (4)

$$1/2.303\ (dX/dt) = S_i X = (S_o - KD^n)X \qquad \text{(Eq. 5)}$$

which integrates to Eq. 3. Consequently, the apparent first-order growth rate to *E. coli* in the presence of tetracycline may be explained on the assumption that the rate constant, S_o, characterizing growth, is in competition with the removal of viable microorganisms. This removal is caused by a rate determining attack of antibiotic molecules on the microorganism. The dependence of the slope S_i on the first power of the antibiotic concentration (Fig. 4) for $S_i > 0$ implies that $n = 1$ in Eq. 5, and that one microorganism may be inactivated by one molecule of antibiotic.

One may conclude from Eq. 4 that if the rate dependency on a power n of antibiotic concentration is permitted

$$\log (S_o - S_i) = n \log D + \log K \qquad \text{(Eq. 6)}$$

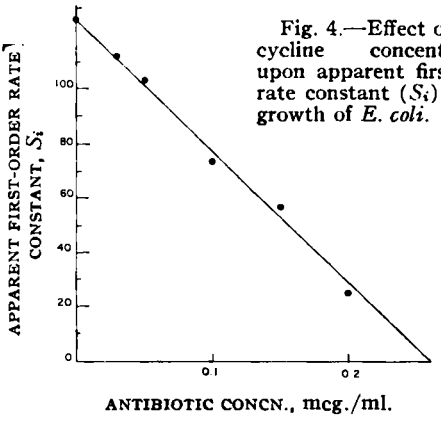


Fig. 4.—Effect of tetracycline concentrations upon apparent first-order rate constant (S_i) for the growth of *E. coli*.

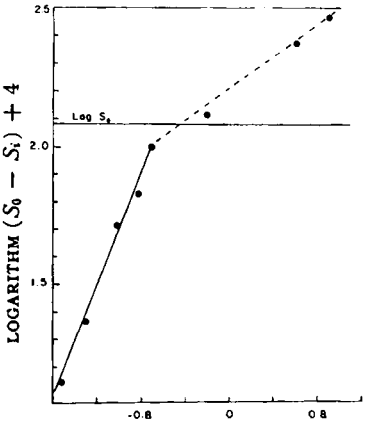


Fig. 5.—Relationship between log ($S_0 - S_i$) and log concentration of tetracycline for *E. coli*. S_0 and S_i are apparent growth rate constants in the absence and in the presence, respectively, of ith concentrations of antibiotic.

A plot of log ($S_o - S_i$) against log D should be linear and of slope positive n. Figure 5 shows that for tetracycline the decrease in growth rate with increased antibiotic concentration is a function of the first power of tetracycline concentration ($n = 1$) for $S_i > 0$. At concentrations of tetracycline sufficiently high to cause a reduction in the logarithm of colony count with time, the initial rates of "kill" are dependent on a fractional power of the antibiotic concentration. Estimates from Fig. 5 for log ($S_o - S_i$) > log S_o; $S_i < 0$ indicate that the rate dependence for "kill" is to the one-third power of antibiotic concentration. These two relationships for $S_i > 0$ and $S_i < 0$ have been reproduced by several replicate experiments made on different days.

The results in Fig. 2 indicate that after an initial "kill" at the higher antibiotic concentrations, the multiplication of the remaining viable organisms was sufficient to overcome the net decrease in colony count. The transition from a negative to a positive slope in Fig. 2 for the 8 mcg./ml. of tetracycline study is an example. On this premise, the survivors of the lower antibiotic concentrations (in the tetracycline concentration range that reduced the total number of viables) multiplied at slower rates than the survivors at the higher concentrations. The bacteria that survived concentrations of 4 and 8 mcg./ml. of tetracycline reproduced at rates approximately the same as those of cultures without antibiotic. The possible implications have been previously discussed, and experiments are in progress to evaluate the possible alternative explanations. A recent experiment with culture containing 8 mcg./ml. of tetracycline has shown more conclusively that the rate of growth of these survivors is not significantly different from that for an initial inoculum in the absence of antibiotic.

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Effect of Certain Drugs on Perfused Human Placenta III

Sympathomimetics, Acetylcholine, and Histamine

By HENRY P. CIUCHTA and RONALD F. GAUTIERI

Perfusion studies conducted on full term human placentas indicated that histamine and serotonin consistently cause marked increases in perfusion pressure. A moderate increase in pressure was observed in response to epinephrine and *l*-epinephrine. In addition, norepinephrine and acetylcholine produced only slight increases in the perfusion pressure. Maximum effects were recorded with the above agents when the preparation was composed of a long cord placenta. Iproniazid was incapable of enhancing the action of the sympathomimetic amines tested significantly.

IT IS WELL KNOWN that the neurohormones, either in normal homeostatic concentrations or in response to drug therapy, are the controlling factors in bringing about the overall integrity of many body functions. However, an upset in the balance of any of the naturally occurring mediators—epinephrine, norepinephrine, acetylcholine, and serotonin—as well as histamine production, may result in the occurrence of cardiovascular disease, metabolic disturbances, and even mental aberrations. When an imbalance of any of these neurohormones is incurred during pregnancy, even greater complications arise which endanger maternal and fetal life.

It has been postulated by Luschinsky and Singher (1) that increased levels of pressor amines, resulting from changes in monoamine oxidase activity in the placenta, may be a causative factor in the development of toxemia of late pregnancy. In a subsequent investigation (2) Luschinsky rejected this theory. Eliasson and Astrom (3) also proved this concept inadequate by *in vitro* placental studies that indicated epinephrine and norepinephrine concentrations were not enhanced to an appreciable degree by the monoamine oxidase inhibitors, propamidine and *l*-isonicotinyl-2-isopropyl hydrazine (IIH).

The effects of the sympathomimetic amines, acetylcholine, serotonin, and histamine, on vessels of the perfused human placenta have been investigated quite extensively. There is agreement regarding the vasoconstrictor activities of serotonin (3–5, 7–9) and histamine (3, 4, 6, 10, 11), but the same cannot be said for the effects of epinehrine, *l*-epinephrine, norepinephrine, and acetylcholine.

Early perfusion experiments conducted by Schmitt (10, 12) established that adrenaline lacked vasoconstrictor activity on isolated placental vessels because of the absence of sympathetic fibers in the vasculature. Subsequent placental perfusion experiments conducted by Kosakae (13), Budelmann (14), Ueda (15), Ordynsky (16), Eliasson and Astrom (3), Astrom and Samelius (9), and Panigel (6) demonstrated that adrenaline possessed vasoconstrictor activity. However, a great degree of variance existed quantitatively. von Euler (11) confirmed Schmitt's original finding that adrenaline did not act on individually isolated placental vessels, but also reported that perfusion of the whole placental preparation elicited vasoconstriction after adrenaline administration. These studies also demonstrated that acetylcholine had weak vasoconstrictor activity. However, previously it had been observed by Ueda (15) that low concentrations of acetylcholine caused dilatation, while higher concentrations produced vasoconstriction of placental vasculature. The inconsistency of acetylcholine's action has been confirmed by Eliasson and Astrom (3), Panigel (5, 6), and others.

Nyberg and Westin's experiments (17) with placental perfusions indicated that noradrenaline produced an increase in perfusion pressure. Eliasson and Astrom (3) observed similar activity with noradrenaline but noted, as did Panigel (5, 6), that a greater degree of constriction occurred in placental preparations with long umbilical cords (8–20 cm.) than in those with relatively short cords (0–3 cm.).

Although the actions of some neurohormones

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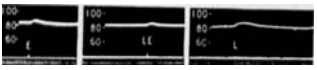


Fig. 1.—Typical pressure responses in short cord placental preparations. Key: E = epinephrine HCl (10–20 mcg.); LE = Levophed bitartrate (10–20 mcg.); L = *l*-epinephrine bitartrate (10–20 mcg.).

on placental vessels appear relatively weak, Dornhorst and Young reported (18), with *in situ* experiments on rabbit and guinea pig placentas, that epinephrine and norepinephrine may act upon maternal-placental circulation producing constriction and thus impairing placental blood flow with a subsequent production of fetal asphyxia. Although a correlation of these observations cannot be made with human placentas at this time, subsequent investigations may amplify this problem.

Since the placenta is devoid of innervation (10, 12, 15, 19), it serves as a useful preparation in ascertaining drug activity without the influence of neurotropic activity. Its application in determining the musculotropic action of drugs on vasculature has been indicated in a previous paper (8). Consequently, in view of the irregular responses obtained with epinephrine, *l*-epinephrine, levarterenol, and acetylcholine, an investigation was conducted to determine the effects of these agents and to confirm serotonin and histamine activity on isolated vessels of human placentas perfused at various pressures and having either long or short umbilical cords.

MATERIALS AND METHODS

Full term human placentas were obtained from the hospital 5–15 minutes after normal delivery. Each was transported to the laboratory in a container of preheated (38°) Tyrode's solution.

The preparation was flushed free of blood by injecting 2.3% sodium citrate solution into one of the umbilical arteries. Cannulae were inserted into the vein and one artery of the umbilical cord. The umbilical cord was either 2–3 cm. (short cord) or

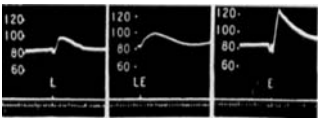


Fig. 2.—Typical pressure responses in long cord placental preparations. Key: L = *l*-epinephrine bitartrate (10–20 mcg.); LE = Levophed bitartrate (10–20 mcg.); E = epinephrine HCl (10–20 mcg.).

8–10 cm. (long cord) from the stalk of the placenta, depending on how much of the cord was free of clots and constrictions. The remaining umbilical artery was ligated. The whole preparation was then placed into a perfusion chamber containing aerated Tyrode's solution which had a pH range of 7.2 to 7.4 and was maintained at a constant 37° with a Bronwill thermoregulator. The arterial cannula was connected with a short piece of rubber tubing to polyethylene tubing which passed through a model TS-8 Sigmamotor pump attached to a 5-gal. polyethylene reservoir. The reservoir contained aerated Tyrode's solution which was modified by the addition of 0.525% polyvinylpyrrolidone (Plasdone C) and kept at a constant 40° by another Bronwill thermoregulator.

Adjustment of the Sigmamotor pump produced constant perfusion pressures which were set between 50–90 mm. Hg and were accompanied by volume inflow rates of 35–75 ml. per minute. Volume inflow was measured by a glass RGI flowmeter placed between the pump and arterial cannula; perfusion pressure was recorded by a mercury manometer located between the pump and flowmeter. Recordings of pressure fluctuations were

TABLE I.—RESPONSES OBTAINED WITH ISOLATED HUMAN PLACENTAS PERFUSED AT 50–90 mm. Hg PRESSURE

Drug Dose, mcg.	Cord Length[a]	Range of Pressure Changes, mm. Increase	(No. expt.)	Decrease	(No.) expt.	Av. Increase, mm.	Unresponsive Experiments, No.
Epinephrine, 10–20	S	2–5	3	2	1	3.3	...
	L	4–39	5	...	...	12.0	1
Epinephrine, 200–500	S	3	1	...	...	...	1
	L	4–6	2	1–2	2	...	...
Levarterenol, 10–20	S	1–5	5	...	...	2.8	...
	L	1–14	5	...	...	4.6	2
Levarterenol, 200–500	S	1–7	2	...	...	...	...
	L	1–4	3	...	...	2.3	...
l-Epinephrine, 2	S	4–12	2	...	...	...	...
	L	1–10	5	...	...	5.8	1
l-Epinephrine, 10–20	S	3–10	5	11	1	5.8	...
	L	3–16	4	...	...	10.8	...
Acetylcholine, 100	S	1–5	8	...	...	2.6	2
Histamine, 10–20	S	5–50	8	...	...	14.1	1
	L	5–192	9	5–8	2	46.1	...
Histamine, 100	L	28–100	3	...	...	65.3	...
Histamine, 1000	S	...	...	11	1	...	...
	L	52–136	4	...	...	92.5	...
Serotonin, 2–4	S	3–54	4	...	...	21.3	1
	L	6–168	6	...	...	90.0	...
Serotonin, 50	S	8–76	15	...	...	30.2	...
	L	17–180	4	...	...	76.5	...

S = small cord; L = long cord.

TABLE II.—COMPARATIVE RESPONSES BETWEEN NORMAL AND IPRONIAZID TREATED VESSELS OF ISOLATED HUMAN PLACENTAS WITH SHORT UMBILICAL CORDS AND PERFUSED AT 50–90 mm. Hg PRESSURE

Drug Dose, mcg.	Range of Pressure Changes, mm. Increase	(No. expt.)	Decrease	(No. expt.)	Av. Increase, mm.	Unresponsive Experiments, No.
Epinephrine, 20						
before iproniazid	2–5	3	2	1	3.3	...
after iproniazid	2–6	5	...	...	3.4	...
Levarterenol, 20						
before iproniazid	1–5	5	...	...	2.8	...
after iproniazid	2–4	3	...	...	3.0	2
l-Epinephrine, 20						
before iproniazid	3–10	4	11	1	5.8	...
after iproniazid	3–14	6	...	...	6.1	2
Acetylcholine, 100						
before iproniazid	1–5	8	...	...	2.6	2
after iproniazid	2–5	3	...	...	3.3	3
Histamine, 20						
before iproniazid	5–50	8	...	...	14.1	1
after iproniazid	15–60	6	2–5	2	25.8	1
Serotonin, 50						
before iproniazid	8–76	15	...	...	30.2	...
after iproniazid	12–66	10	...	...	32.6	...

made on the smoked drum of a Livingston long paper kymograph. The venous volume outflow, which was measured directly by a graduated glass cylinder and never reperfused, was determined at the onset of each experiment and at various intervals in order to compare it with the volume inflow. In 41 successful placental preparations, each lasting 1–4 hours, a total of 211 different experiments were performed. The results obtained from nine placentas were discarded because the volume inflow at the onset of the experiments was below average, or placental perfusion pressure could not be maintained at a constant level.

The following agents were injected into the polyethylene tubing prior to entering the pump in a volume of distilled water not exceeding 1 ml.: acetylcholine hydrobromide, 1.0%; histamine dihydrochloride, 0.1%; 5-hydroxytryptamine creatinine sulfate (serotonin), 0.01%; epinephrine hydrochloride, 0.1%; *l*-epinephrine bitartrate, 0.1%; levarterenol bitartrate, 0.2% (0.1% as base); and iproniazid, 1.0%.

RESULTS

The following results were obtained from placental preparations having umbilical cords 2–3 cm. long and perfused at a pressure of 50–90 mm. Hg and a volume inflow of 35–75 ml. per minute, unless stated otherwise.

Epinephrine.—Doses of 10–20 mcg. of epinephrine hydrochloride brought about an increase in perfusion pressure (Fig. 1*a*) in three cases, having a range of 2–5 mm. (av. 3.3 mm.). One experiment demonstrated a 2-mm. decrease in pressure. A dose range of 200–500 mcg. produced a 3-mm. increase in one experiment and no response in another (Table I).

Epinephrine, in doses of 10–20 mcg., produced a 4–39-mm. (av. 12.0 mm.) increase in pressure (Fig. 2*c*) in five preparations where the umbilical cord was 8–10 cm. long. No response occurred in one experiment (Table I); an increase in pressure followed by a decrease was observed in two cases. The administration of 200–500 mcg. of epinephrine caused an increase in pressure in two experiments and a decrease in pressure in two others (Table I).

The injection of 5 mg. iproniazid[1] prior to the administration of 20 mcg. epinephrine elicited an increase in perfusion pressure of 2–6 mm. (av. 3.4 mm.) in five experiments (Table II).

Levarterenol.—Levarterenol bitartrate, administered in doses of 10–20 mcg. in five placentas produced an increase in pressure (Fig. 1*b*), having a range of 1–5 mm. (av. 2.8 mm.). A dose range of 200–500 mcg. caused an increase of 1 and 7 mm. in two cases (Table I), while two experiments exhibited an increase in pressure followed by a decrease.

Employing placentas with cords 8–10 cm. long, the administration of 10–20 mcg. levarterenol caused a 1–14-mm. (av. 4.6 mm.) increase in pressure (Fig. 2*b*) in five preparations. No response was observed in two cases. Doses of 200–500 mcg. produced a 1–4-mm. (av. 2.3 mm.) increase in perfusion pressure in three cases (Table I).

In five experiments, pretreatment with 5 mg. of iproniazid, followed by 20 mcg. of levarterenol elicited an increase of 2–4 mm. (av. 3.0 mm.) in three cases. No response was obtained in the other two (Table II).

l-Epinephrine[2] Bitartrate.—A dose of 2 mcg. of *l*-epinephrine bitartrate caused an increase in perfusion pressure in two experiments. A dose range of 10–20 mcg. in five cases produced a 3–10-mm. (av. 5.8 mm.) increase (Fig. 1*c*), while a decrease of 11 mm. was recorded in another experiment (Table I).

In placental preparations with cords 8–10 cm. long, a dose of 2 mcg. of *l*-epinephrine caused a 1–10-mm. (av. 5.8 mm.) increase in perfusion pressure in five cases, while no response was observed in one experiment. Doses of 10–20 mcg. of *l*-epinephrine in four preparations produced an increase in pressure (Fig. 2*a*), having a range of 3–16 mm. (av. 10.8, Table I).

The prior administration of 5 mg. of iproniazid in six placentas receiving 10–20 mcg. *l*-epinephrine elicited a 3–14-mm. (av. 6.1 mm.) increase in per-

[1] Supplied through the courtesy of Hoffmann-LaRoche, Inc., Nutley, N. J.

[2] Supplied through the courtesy of Sterling-Winthrop Research Institute.

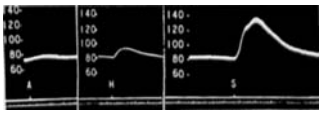


Fig. 3.—Typical pressure responses in short cord placental preparations. Key: A = acetylcholine HBr (100 mcg.); H = histamine dihydrochloride (10–20 mcg.); S = serotonin (50 mcg.).

fusion pressure. Two other placental preparations were unresponsive (Table II).

Acetylcholine.—In eight experiments the administration of 100 mcg. of acetylcholine hydrobromide evoked a 1–5-mm. (av. 2.6 mm.) increase in perfusion pressure (Fig. 3*a*), while two cases were unresponsive (Table I).

The injection of 5 mg. of iproniazid, followed by 100 mcg. of acetylcholine caused an increase in pressure in three cases, having a range of 2–5 mm. (av. 3.3 mm.). Three other preparations did not elicit a response (Table II).

Histamine.—Doses of 10–20 mcg. of histamine dihydrochloride in eight experiments produced an increase in pressure (Fig. 3*b*) having a range of 5–50 mm. (av. 14.1 mm.). One placenta did not respond. A 1000-mcg. dose of histamine produced a decrease in the only case tested (Table I).

The administration of 10–20 mcg. of histamine in nine experiments with long cords resulted in a 5–192-mm. (av. 46.1 mm.) increase in perfusion pressure (Fig. 4*b*). Pressure decreases of 5 and 8 mm. were observed in two cases. An increase in pressure followed by a decrease was noted in two experiments. A dose of 100 mcg. of histamine produced a 28–100-mm. (av. 65.3 mm.) increase in perfusion pressure in three experiments. Histamine, in a dose of 1000 mcg., caused an increase (Fig. 4*c*) in pressure in four cases, having a range of 52–136 mm. (av. 92.5 mm.) (Table I).

The administration of 5 mg. of iproniazid prior to histamine resulted in an increase in perfusion pressure in six cases. The range of the increase was 15–60 mm. (av. 25.8 mm.). One preparation did not respond, and two others demonstrated decreases in pressure (Table II).

Serotonin.[a]—The administration of 2–4 mcg. of serotonin in four experiments brought about a 3–54-mm. (av. 21.3 mm.) increase in pressure, while one other placenta was unresponsive. A dose of 50 mcg. of serotonin (15 cases) caused an increase in pressure (Fig. 3*c*), having a range of 8–76 mm. (av. 30.2 mm.) (Table I).

Serotonin, in doses of 2–4 mcg., caused a 6–168-mm. (av. 90 mm.) increase (Fig. 4*a*) in perfusion pressure in six experiments where the umbilical cord was 8–10 cm. long. A dose of 50 mcg. of serotonin in four experiments produced a 17–180-mm. (av. 76.5 mm.) increase in pressure (Table I).

The injection of 5 mg. of iproniazid preceding 50 mcg. of serotonin evoked a 12–66-mm. (av. 32.6 mm.) increase in pressure in ten preparations (Table II).

Perfusion experiments attempted with pressures less than 50 mm. Hg—hence with lower volume inflow rates—resulted in irregular and inconsistent responses with the above drugs in the same dose ranges.

[a] Supplied through the courtesy of Abbott Laboratories, North Chicago, Ill.

DISCUSSION

The results of this investigation have indicated that the administration of the sympathomimetic amines—epinephrine, *l*-epinephrine, and levarterenol—and acetylcholine, histamine, and serotonin, to vessels of perfused human placentas caused vasoconstriction in practically all preparations with short (2–3 cm.) or long (8–10 cm.) umbilical cords.

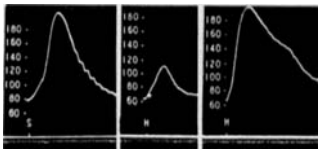


Fig. 4.—Typical pressure responses in long cord placental preparations. Key: S = serotonin (2–4 mcg.); H = histamine dihydrochloride (10–20 mcg.); H = histamine dihydrochloride (1000 mcg.).

In most cases the degree of vasoconstriction produced was greater in the preparations with long cords. This increased responsiveness of long cord compared to short cord preparations also has been reported by Eliasson and Astrom (3). Though direct evidence is lacking at this time, it appears likely that the vascular musculature present in the umbilical cord may have greater amounts of responsive effector cells than other placental vessels. Hence, when a relatively long cord is present in a placental perfusion, it contributes more to the overall vasoconstriction than the other placental vessels.

The responses obtained with the amines and acetylcholine in long and short cord preparations were not in direct proportion to the doses administered, since low (10–20 mcg.) or high (200–500 mcg.) doses usually elicited responses that did not vary quantitatively in the same perfusion. von Euler (11) has also pointed out that a dose-response relationship is seemingly nonexistent in placental perfusions. This fact was confirmed in these studies, for vascular responsiveness depended upon the presence of the agent and not its quantity. However, histamine was the exception in this respect, as larger doses based on average response produced greater increases in perfusion pressure.

Other investigations (3) have demonstrated that *l*-epinephrine and levarterenol produce consistently marked increases in perfusion pressure, although levarterenol is the least potent. This investigation did not corroborate the marked constrictor action of these amines, but did agree in finding levarterenol less effective than epinephrine or *l*-epinephrine in causing vasoconstriction of placental vessels.

The relative lack of activity of the sympathomimetic amines and acetylcholine on vessels of human placentas was expected, since the placenta is devoid of innervation, and its vessels probably have a correspondingly fewer effector cells than other vasculature.

Eliasson and Astrom (3) and von Euler (11) obtained inconsistent results with acetylcholine on placental vasculature, although slight vasoconstrictor activity usually was predominant. It was proposed, from von Euler's investigation (20) on pulmonary vessels of the rabbit, that a correlation may exist between the response of placental and pulmonary vessels because both constrict in response to acetyl-

choline, whereas other vascular beds are dilated. Results in this present investigation confirm that acetylcholine produces weak vasoconstriction in placental vessels—and sometimes even lacks this action.

Pretreatment of placental preparations with 5 mg. of iproniazid (only short cord placentas were tested) before the administration of the sympathomimetic amines, acetylcholine, or serotonin, did not produce an appreciable augmentation of perfusion pressure—except in the case of histamine—which at this time is inexplicable. However, increases in sympathomimetic amine activity would be expected since monoamine oxidase is one of the enzymes involved in the breakdown of the amines, and blockage of this placental enzyme sometimes can produce slight increased amine activity. In view of the results, it is possible that iproniazid is washed out too rapidly to tie up effectively the large quantity of monoamine oxidase present in the placenta. Also, it is possible that monoamine oxidase is so abundant that whatever quantity is blocked is still not sufficient to allow augmented responses to occur with these amines. These results are similar to studies conducted by Eliasson and Astrom (3), who showed that increased responses do not occur after administration of *l*-epinephrine or levarterenol to propamidine and *l*-isonicotinyl-2-isopropyl hydrazine (IIH) treated placental preparations.

Perfusion experiments attempted at lower pressures (under 50 mm. Hg), and consequently lower volume inflow but the same dose ranges, produced only slight changes in perfusion pressure with all the previously mentioned agents. Therefore, there appears to be a dependence upon volume inflow and perfusion pressure for the attainment of significant responses. Optimum results were recorded when the volume inflow rate was at least 70 ml. per minute, and the perfusion pressure was at least 60 mm. Hg, but preferably around 80 mm. Hg.

Although the results reported here are those occurring *in vitro*, the possibility of similar qualitative effects being manifested *in vivo* with the above drugs cannot be overlooked. With this in mind, it appears likely that the systemic release of excess quantities of sympathomimetic substances, serotonin, or even histamine in the pregnant state could, by directly constricting placental vessels, cause a decrease in the oxygenation of fetal blood flowing through such vessels—and hence contribute to fetal asphyxia.

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Properties of Fused Mannitol in Compressed Tablets

By JOSEPH L. KANIG

Among the carbohydrates used in compressed tablets, mannitol is the only one which possesses high heat stability. This substance melts at 167°, but does not decompose at temperatures up to 250°. Mannitol, alone and in combination with other carbohydrates, was fused and recrystallized or spray-congealed. Phase diagrams determined for these mixtures indicated that mannitol is eutectic with other carbohydrates. Selected drugs were soluble in the fused mixtures, and the crystallized or spray-congealed material obtained from these solutions possessed excellent flow and compression characteristics.

MANNITOL HAS BEEN used as a tablet diluent for more than a decade. This hexahydric alcohol, an isomer of sorbitol, is a white, odorless, crystalline powder and is the least hygroscopic of all known carbohydrate tablet diluents (1). It has a sweetness threshold of approximately the same value as glucose and a nutritive value of 2 cal. per gram (2).

In recent years, mannitol has been shown to exhibit a uniquely cooling and pleasant taste effect when used in formulations for tablets intended to be chewed or dissolved in the mouth. Several types of medicaments, including antacids, analgesics, multivitamins, and antihistamines are being marketed in the form of chewable tablets prepared in a mannitol base (3, 4). This diluent

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In recent years, mannitol has been shown to exhibit a uniquely cooling and pleasant taste effect when used in formulations for tablets intended to be chewed or dissolved in the mouth. Several types of medicaments, including antacids, analgesics, multivitamins, and antihistamines are being marketed in the form of chewable tablets prepared in a mannitol base (3, 4). This diluent

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has also been suggested as a base in compression-coated and multilayer tablets (5).

The advantages exhibited by mannitol in such tablet formulations have been offset by its inherent shortcomings in that it has poor flow and compression characteristics. As a result, it generally requires the addition of a granulating-binding agent employed in a wet granulation procedure for producing compressible granules. The added binding agents generally tend to detract from the desired effects expected from mannitol, and the wet granulation process does not lend itself to the modern trend toward labor saving, automated techniques. Consequently, a need exists for enhancing the tableting characteristics of mannitol without detracting from its otherwise useful properties.

It was suggested (6) that spray-congealing fused mannitol might result in improved flow and compression properties. Mannitol is one of the few carbohydrates that possesses an exceptionally high heat stability. It has a melting point of 166.0 to 167.0°, but unlike other carbohydrates, it can be heated to 250° without decomposition. When fused, mannitol becomes a clear, colorless low-viscosity fluid which crystallizes very rapidly when the source of heat is removed. It is not susceptible to anhydrization under ordinary conditions during the fusion process. It has been demonstrated (7–9) that anhydrization of mannitol may be accomplished only under such drastic conditions as prolonged refluxing with concentrated hydrochloric acid.

It was therefore of interest to investigate the possible advantages to be gained by utilizing fused mannitol as a means of altering its tableting characteristics. Concurrent with this approach was the equally interesting potential to be gained in exploring the ability of fused mannitol to function as a solvent or dispersant for other materials to produce solid-solid solutions or dispersions which might prove to be directly compressible into tablets.

PROCEDURES AND RESULTS

Fusion Apparatus.—Molten mannitol rapidly crystallizes into a hard mass if the temperature is permitted to drop only a few degrees below its melting point. Consequently, attempts to spray-congeal the hot liquid with the centrifugal wheel atomizer of a Nerco-Niro portable spray dryer resulted in rapid congealing within the feed-pipe and vaned-wheel of the atomizer. This clogged the mechanism and stopped the operation.

A specially designed apparatus was constructed to overcome this obstacle and to facilitate the incorporation of additional materials into the fused mannitol. A kitchen-type, electrically heated deep-fat fryer was converted into an apparatus which was used to fuse mannitol under controlled temperature and as a mixer and a feeder of the fused blends to the spray dryer. Figure 1 represents the apparatus and its essential components. An opening was drilled into the bottom of the electric pan (A), and this was fitted with an aluminum fitting (F) which served as the seat for a tapered Teflon needle valve attached to the bottom of a stainless steel threaded rod (E). The rod was positioned over the opening (F) with a stainless steel rod placed horizontally across the diameter of the pan and screwed into the walls on both sides (D). This rod also served to hold a thermometer (K) in position in the fused liquid. An electric overhead stirrer (G) was utilized to aid in the dispersion of materials added to the fused mixture.

A threaded nozzle was screwed into the aluminum fitting (F) and a $^1/_2$ in. I.D. Teflon tube was attached to the outlet nozzle. The tube was wrapped with a Nichrome heating element (H) which was controlled with a rheostat (J). The Teflon tube was utilized to connect the fusion apparatus with the feed-pipe of the atomizer in the spray dryer. It was necessary to use additional heating elements around the feed-pipe of the atomizer to prevent cold-spots and subsequent congealing of the fused mass within the atomizer.

The heating elements on the bottom of the pan (B) were rheostatically controlled, and the rheostat (C) was calibrated to correspond with centigrade units.

A heater was installed in the compressed air line which operates the atomizer of the Nerco-Niro spray dryer. This served to raise the temperature of the compressed air entering the atomizer so that the entire unit became hot by conduction; this was an additional safeguard against premature congealing within the atomizer.

All heating elements were turned on for about 30 minutes before each run. This was generally done while the solids in the fusion apparatus were being heated and fused.

Fused Mixtures.—Trial batches ranging in weight from several hundred grams to 2 kilos were fused and spray-congealed with the special apparatus and laboratory spray dryer. Mannitol, alone and in combination with other materials, was fused and spray-congealed after preliminary tests had indicated that the mixtures were miscible in the fused state and exhibited no signs of decomposition due to high heat. Subjective evidence of apparent decomposition among materials added to fused mannitol was noted by the production of an odor, change in color, or evolution of a gas.

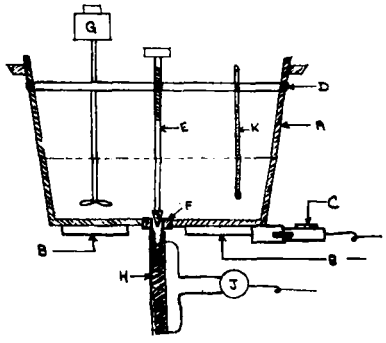


Fig. 1.—Fusion apparatus used in conjunction with Nerco-Niro portable spray dryer.

A number of randomly selected drugs and chemicals and inert materials were tested for their solubility or miscibility in fused mannitol. The screening procedure involved the addition of incremental quantities of the material under test to fused mannitol which was maintained at 175–180°. Observations were made after each addition to determine whether physical or chemical changes had taken place and whether the material being added was soluble in the fused mixture. Table I lists the materials which were thus tested. The drugs and chemicals which formed clear solutions in most proportions with fused mannitol and manifested no visible signs of decomposition were arbitrarily classified as being soluble without regard for the actual state of matter which resulted upon congealing. Additional tests and chemical analyses on these materials and other substances are being conducted to determine their stability in fused mannitol. The classification "insoluble-immiscible" is used to designate those mixtures which formed an insoluble dispersion of the solid in the fused mannitol or which melted into two immiscible liquid layers. Some materials, such as the metallic stearates and the celluloses, were not wetted by the fused mannitol and were not dispersible in the mixture.

Other materials which were tested and found to decompose readily included aspirin, *dl*-amphetamine sulfate, chlorobutanol, benzocaine, aminophylline, terpin hydrate, pyridium, pentabarbital sodium, and sulfaguanidine.

Melting Point–Composition Relationships.—To obtain additional information on the physical characteristics of several of the fused mixtures, phase diagrams were obtained with mixtures of mannitol and other sugars and with mannitol and several active ingredients.

TABLE I.—SOLUBILITY/MISCIBILITY OF RANDOMLY SELECTED DRUGS AND CHEMICALS WITH FUSED MANNITOL

Soluble	Insoluble-Immiscible
Amobarbital	Aluminum hydroxide
Barbital	Beeswax
Chlorpheniramine maleate	Butylaminobenzoate
Diphenhydramine HCl	Caffeine
Dulcitol	Hydrogenated castor oil[a]
Erythritol	Cetyl alcohol
Galactose	5,5-Diphenylhydantoin
Lactose	Glyceryl distearate
p-Hydroxy acetanilide	Glyceryl monostearate
Phenobarbital	Hydroxy propyl sucrose
Phenobarbital sodium	Magnesium stearate
Phenylephrine HCl	Magnesium trisilicate
Procaine HCl	Microcrystalline waxes
Sucrose	Phenolphthalein
Sulfacetamide	Polyethylene glycol 400
Sulfanilamide	Polyethylene glycol 4000
	Polyvinyl alcohol
	Prednisolone
	Prednisolone acetate
	Sodium carboxymethylcellulose
	Spermaceti
	Stearic acid
	Stearyl alcohol
	Sterotex[b]
	Succinylsulfathiazole
	Sulfamerazine

[a] Marketed as Castorwax by the Baker Castor Oil Co.
[b] Marketed by Capital City Products Co., Columbus, Ohio.

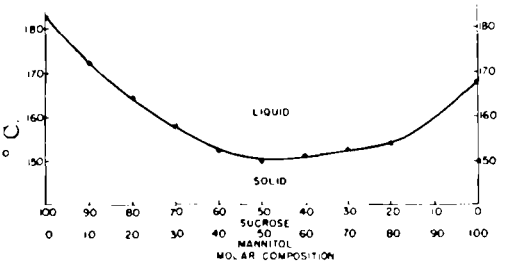


Fig. 2.—Mannitol-sucrose phase diagram

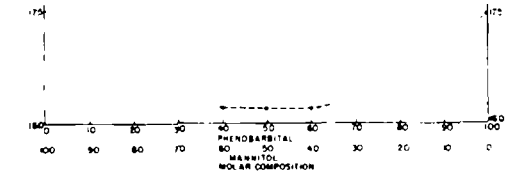


Fig. 3.—Phase diagram of fused mixtures of mannitol and phenobarbital.

A number of binary mixtures of mannitol and other materials were prepared with accurately weighed quantities ranging in overall composition from 100 molar parts of one to 100 molar parts of the other. Each mixture was fused in an oil bath at a temperature approximately 5° higher than the melting point of the component in the mixture which had the highest melting point. The fused mass was stirred, transferred to a watch glass, and allowed to crystallize. A portion was then ground to a fine powder in a small glass mortar, and the melting point of the powder was then obtained.

Phase diagrams were plotted on binary systems of mannitol with other carbohydrates such as sucrose, lactose, dulcitol, erythritol, and galactose. Three physiologically active materials were chosen from the list of soluble drugs, and binary mixtures of mannitol with diphenhydramine hydrochloride, with phenobarbital, and with sulfanilamide were prepared and phase diagrams obtained.

All blends of carbohydrates and mannitol resulted in eutectic mixtures. Figure 2 is illustrative of the phase diagrams obtained with the carbohydrates listed above. In each instance the mixture permitted melting of the carbohydrate involved at a point below its own melting point and thus prevented decomposition. Mannitol-sucrose binary systems manifested the lowest melting point at an equimolar composition. This mixture made possible a 32° decrease in the melting point of sucrose.

Figures 3 and 4 indicate the eutexias formed between mannitol and phenobarbital and mannitol and sulfanilamide. These curves are similar to the mannitol-carbohydrate curves, and all of them indicate solid state miscibility. Just as two liquids may dissolve in each other to form a liquid solution, so one solid may dissolve in another to form a solid solution. These solutions are homogeneous and may vary in composition within wide limits. This uniformity of distribution differentiates a solid solution from a mixture of solids, for in the latter instance each constituent preserves its own characteristic crystal structure. This is borne out by the existence of a minimum in the melting point curve (10).

When two pure components react to form a new

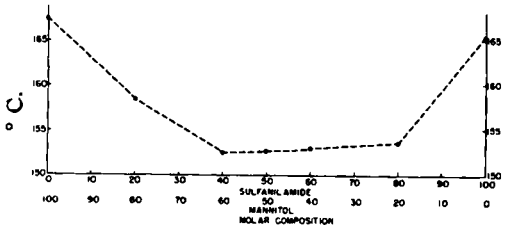


Fig. 4.—Phase diagram of fused mixtures of mannitol and sulfanilamide.

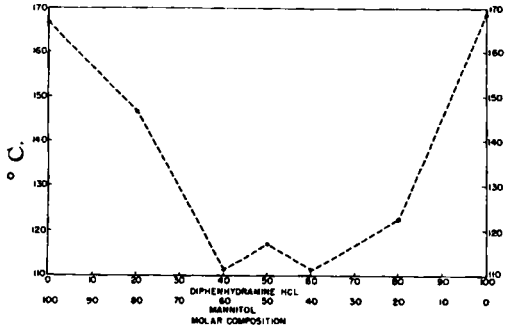


Fig. 5.—Phase diagram of fused mixtures of diphenhydramine hydrochloride and mannitol.

compound stable up to its melting point, the phase diagram takes on the typical appearance shown in the curve for the mannitol-diphenhydramine hydrochloride system (Fig. 5). The maximum observed at equimolar composition is indicative of 1:1 compound formation (10).

Tableting Characteristics.—To determine the tableting characteristics of fused mannitol, alone and in combination with other inert and active components, the following mixtures were prepared in 2-kilo batches and spray congealed.[1]

(*a*) Mannitol (100)
(*b*) Mannitol (95) + sucrose (5)
(*c*) Mannitol (50) + sucrose (50)
(*d*) Mannitol (50) + sucrose (40) + lactose (10)
(*e*) Mannitol (50) + lactose (50)
(*f*) Mannitol (80) + phenobarbital (20)
(*g*) Mannitol (80) + sulfanilamide (20)
(*h*) Mannitol (80) + diphenhydramine HCl (20)

No consideration was given to the usual dosages of the active ingredients in mixtures (*f*), (*g*), and (*h*). The ratios of drug to mannitol was selected only as a basis for comparison of the tableting characteristics of the resulting solid solutions.

Each of these mixtures was carefully fused in the fusion apparatus and spray-congealed. Additional batches of the same mixtures were prepared by fusion but were permitted to congeal in stainless steel vessels. These crystallized masses were removed and passed through a No. 20-mesh screen in a Stoke's oscillating granulator. This latter procedure was conducted so that a comparison could be made between granular material obtained in this manner and that obtained by spray-congealing.

Both single punch and rotary tablet machines (F. J. Stoke's model F and model B-2) were employed in the compression studies. Tablets weighing 0.5 Gm. each were compressed with $^{13}/_{32}$ in. S.C. or F.F. punches.

Flow Properties.—Fused mannitol and its mixtures, processed by either spray-congealing or screening, possessed excellent flow characteristics. All of the batches employed in this study presented no problem by flowing smoothly from the hoppers of the tablet compression machines, in the feed frames, and into the dies. Flow properties were decidedly superior to powdered mannitol, especially in the feed frames and in the dies. Uniform filling of the dies was evidenced by tablet weight deviations within U.S.P. limits.

Compressibility Factors.—In all cases it was found that, although the materials had excellent flow properties and required no glidant, they all needed a lubricant to assist in the removal of the compressed tablets from the dies. Calcium or magnesium stearate, 1 to 2%, blended into each batch before comrpression, was sufficient for this purpose.

A difference in lubricant requirements was observed between batches prepared by spray-congealing and identical mixtures processed by screening.

Tablets prepared from the screened materials exhibited a high degree of resistance to ejection from the dies. This difficulty was overcome by the further addition of 1% of talc to the lubricated mixture. This difference between the screened and spray-congealed material may be attributed to the shape of the particles. Microscopic examination of spray-congealed particles revealed them to be spherical in shape while those prepared by screening were irregular and angular. It is conceivable that the smooth, regular spheres are capable of producing tablets with edges that offer decreased frictional resistance during ejection.

Fused mannitol and the various mixtures prepared by either spray-congealing or screening possessed excellent tableting properties. Other than the lubrication problem previously cited, no additional difficulties were encountered in compressing tablets over a wide range of pressures. There was no evidence of capping, laminating, sticking, or other physical deterrents to the production of suitable compressed tablets. The surfaces of the tablets were hard and smooth with no indications of dusting or friability.

Taste Properties.—Tablets made with fused mannitol were found to have a moderate grittiness which is not found in tablets prepared with mannitol N.F. In addition, the "cooling" effect was noticeably reduced. Sweetness and flavoring efficiency were unimpaired by the fusion process. It was observed, however, that fused mixtures of mannitol and other carbohydrates more closely approximated the taste properties of unfused mannitol.

Dry Binder Qualities.—In view of the excellent tableting properties of the fused carbohydrate mixtures it was decided to study the dry binding applica-

Table II.—Strong-Cobb Hardness Values of Fused Mannitol Tablets

Pressure, p.s.i.	Fused Mannitol	
	No. 20 mesh	Spray-congealed
1000	6	...[a]
2000	14	6
3000	22	9
4000	...	12

[1] Numbers in parentheses indicate parts by weight.

[a] A tablet was not formed at this pressure.

tions of these materials. Dried aluminum hydroxide gel U.S.P. was selected as an example of a powdered material which cannot be compressed directly. Blends containing 25 and 50% by weight of the aluminum compound were prepared with fused mannitol, mannitol and sucrose (50:50), and mannitol and lactose (50:50) in a Twin Shell blender. Magnesium stearate (1%) was included as a lubricant. These blends continued to exhibit the excellent flow and compression characteristics which had been observed with the vehicles alone. Tablets weighing 0.5 Gm. prepared on a model F compression machine gave Strong-Cobb hardness tester values of up to 10–11 with no signs of capping. The tablets were satisfactory in all other respects.

Comparison of Fused Spray-Congealed and Screened Mannitol.—To compare the relative compressibility of the two physical states of mannitol obtained by either spray-congealing or screening, a Carver laboratory hydraulic press was employed. This press was modified to operate a specially constructed rig containing a set of 13/32 in. F.F. punches and a die. Accurately weighed 500-mg. portions of the different batches of mannitol were compressed at pressure levels of 1000, 2000, 3000, and 4000 p.s.i. Tablets produced at each level were tested for hardness. It was determined that the spray-congealed mannitol produced softer tablets at corresponding pressure levels than did the 20-mesh screened material (Table II).

SUMMARY AND CONCLUSIONS

The unusual heat stability of mannitol has led to the development of several new applications which possess a high degree of potential. Foremost among these is the discovery that fused mannitol, which is recrystallized and processed by either spray-congealing or screening, possesses exceptionally good tableting characteristics.

The liquid state of mannitol has been shown capable of dissolving or dispersing a number of pharmaceutical adjuvant or physiologically active drugs. Phase diagrams of several of these combinations have indicated that solid-solid solutions are obtained. In at least one instance (mannitol-diphenhydramine HCl) the phase diagram indicates the probable formation of a new compound. New compounds formed in this manner may conceivably possess different physical, chemical, and physiological properties.

In view of present day considerations of the effect of particle size on the biological availability of drugs, these solid-solid solutions or microcrystalline dispersions offer a unique opportunity of making available an extremely fine state of subdivision of active ingredients in tablet form. Moreover, these solid solutions, capable of being directly compressed into tablets, obviate the necessity for blending procedures and assure complete uniformity of dosage.

Additional investigations are under consideration which will explore more thoroughly the nature of the interaction involved in the solid-solid solution in fused mannitol and will quantify the extent of solubilities of various chemicals and drugs.

Fused mannitol has also been utilized to produce eutectic mixtures with other less costly carbohydrates, such as sucrose and lactose. These mixtures have also been found to possess excellent flow and compression properties when used either as solvents for active principles or when admixed with them as a dry tablet binder.

Fused mixtures of mannitol with other sugars advance the possibility of large-scale production of an inexpensive, efficient vehicle for the direct compression of tablets.

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4′,5,6,7-Oxygenated Flavones and Flavanones

By MASON G. STOUT, HANS REICH, and MAX N. HUFFMAN

The preparation of 4′-hydroxy-5,6,7-trimethoxyflavanone, 6-hydroxy-4′,5,7-trimethoxyflavanone, and the corresponding flavones is described as well as a new synthesis of 4′,6-dihydroxy-5,7-dimethoxyflavone. The results of the biological tests of these and other closely related bioflavonoids in our general endocrine screening assay are tabulated.

For biological experiments, the 4′,5,6,7-substituted flavanones X, XII, XIV, and XV and the corresponding flavones XVI, XVIII, XX, and XXII were required. Compounds X and XVI had been prepared previously (1) from the acetophenone derivative II and anisaldehyde *via* the chalcone IV, and the flavanone XV had been obtained from the acetophenone derivative I by condensation with *p*-hydroxybenzaldehyde (2). This flavanone was later used as starting material for the preparation of the flavone XXII

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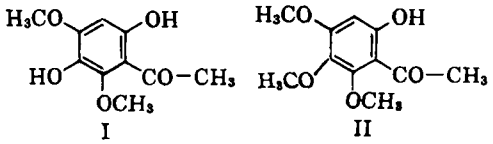
4′,5,6,7-Oxygenated Flavones and Flavanones

By MASON G. STOUT, HANS REICH, and MAX N. HUFFMAN

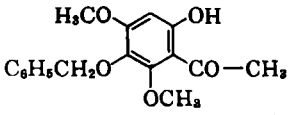
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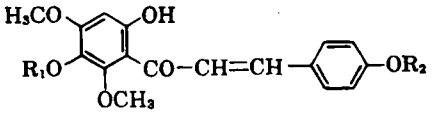
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I II



III



IV $R_1 = R_2 = CH_3$
V $R_1 = CH_3$; $R_2 = CH_2C_6H_5$
VI $R_1 = H$; $R_2 = CH_3$
VII $R_1 = CH_2C_6H_5$; $R_2 = CH_3$
VIII $R_1 = R_2 = H$
IX $R_1 = R_2 = CH_2C_6H_5$

which was accomplished by bromination and dehydrobromination with alkali (3). For the synthesis of this flavone XXII, we chose the condensation of the benzyloxyacetophenone derivative III with *p*-benzyloxybenzaldehyde, oxidation of the resulting chalcone IX with selenium dioxide to the flavone XXI, and debenzylation with hydrochloric acid in acetic acid.

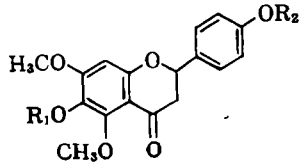
The flavanone XII and the flavone XVIII were synthesized in the following manner. The trimethoxyhydroxyacetophenone II was brought into reaction with *p*-benzyloxybenzaldehyde, and the chalcone V thus obtained was either cyclized or oxidized with selenium dioxide to give XI and XVII, respectively. Removal of the benzyloxy group in XI was achieved by hydrogenation and debenzylation of XVII with hydrochloric acid in acetic acid.

Condensation of III with anisaldehyde gave a mixture of the chalcone VII and the flavanone XIII which could be separated by crystallization. Oxidation of the chalcone with selenium dioxide led to the formation of flavone XIX and acidic debenzylation of the latter to the required flavone XX. Since the flavanone XIII could not be debenzylated by hydrogenation or with acids, it was necessary to condense the dihydroxydimethoxyacetophenone I with anisaldehyde. Also in this case, the reaction product was a mixture of the chalcone VI and the flavanone XIV which had to be separated by fractional crystallization.

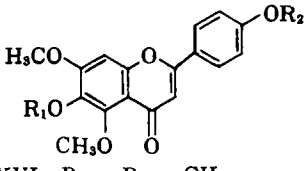
EXPERIMENTAL[1]

Aldehydes.—*p*-Hydroxybenzaldehyde and anisaldehyde were commercial preparations (Eastman).

[1] All melting points are uncorrected. The microanalyses were carried out by Huffman Microanalytical Laboratories, Wheatridge, Colo., and Galbraith Laboratories, Knoxville, Tenn.



X $R_1 = R_2 = CH_3$
XI $R_1 = CH_3$; $R_2 = CH_2C_6H_5$
XII $R_1 = CH_3$; $R_2 = H$
XIII $R_1 = CH_2C_6H_5$; $R_2 = CH_3$
XIV $R_1 = H$; $R_2 = CH_3$
XV $R_1 = R_2 = H$



XVI $R_1 = R_2 = CH_3$
XVII $R_1 = CH_3$; $R_2 = CH_2C_6H_5$
XVIII $R_1 = CH_3$; $R_2 = H$
XIX $R_1 = CH_2C_6H_5$; $R_2 = CH_3$
XX $R_1 = H$; $R_2 = CH_3$
XXI $R_1 = R_2 = CH_2C_6H_5$
XXII $R_1 = R_2 = H$

p-Benzyloxybenzaldehyde was prepared from *p*-hydroxybenzaldehyde according to the method of Bergmann and Sulzbacher (4).

3′,6′-Dihydroxy-2′,4′-dimethoxyacetophenone (I).—This compound was prepared from 2,6-dimethoxyhydroquinone diacetate (1) (m.p. 126–127°) in the following manner. A fairly rapid stream of boron trifluoride was introduced into a cooled solution of 75 Gm. of the diacetate in 500 ml. of glacial acetic acid, keeping the temperature below 40°. After 1.5 to 2 hours, the total uptake of boron trifluoride amounted to 393–425 Gm. The yellowish-green solution was allowed to stand at room temperature overnight and was poured into 2.25 L. of water which contained 750 Gm. of ice. After standing for 3 hours in the refrigerator, the yellow precipitate was filtered, washed with water, transferred to a 3-L. flask, and dissolved in 1 L. of 95% ethanol by heating. Then 525 ml. of 10% hydrochloric acid was added and the mixture refluxed for 2 hours. Upon concentration *in vacuo* and cooling overnight, yellow crystals were obtained which were filtered and dried. They weighed 58.7 Gm. (94%), melted at 158–159°, and did not depress the melting point of a sample prepared from 2,6-dimethoxyhydroquinone diacetate by a Fries rearrangement, reported m.p. 162–163° (1, 5), 164–165° (6).

When the hydrolysis with ethanolic hydrochloric acid was omitted, the reaction product proved to be 6′-hydroxy-3′-acetoxy-2′,4′-dimethoxyacetophenone, which, after recrystallization from aqueous methanol, melted at 107 to 107.5°.

Anal.—Calcd. for $C_{12}H_{14}O_6$: C, 56.59; H, 5.55. Found: C, 56.93; H, 5.61.

2′,3′,4′-Trimethoxy-6′-hydroxyacetophenone (II).—The methylation of I was carried out with dimethyl sulfate as described by Oliverio and Bargellini (1) and gave a crude product of m.p. 33–36°, reported m.p. 40–41° (1), which was sufficiently pure for the condensation with aldehydes.

2′,4′-Dimethoxy-3′-benzyloxy-6′-hydroxyacetophenone (III).—A 110-Gm. quantity of anhydrous potassium carbonate and 32 ml. of benzyl

TABLE I.—CHALCONES

	Yield, %	M.p.,[a] °C.	Crystallization Media[b]	Color	Empirical Formula	Analysis Calcd. C	Calcd. H	Found C	Found H
IV	90	142[c]	E	Red-orange	$C_{19}H_{20}O_6$	...	...	...	...
V	69	123 to 123.5	M	Yellow	$C_{25}H_{24}O_6$	71.42	5.75	71.19	5.58
VI	26[d]	141–142[e]	E	Dark red	$C_{18}H_{18}O_6$	65.45	5.49	65.49	5.76
VII	72	139 to 139.5	E	Red-orange	$C_{25}H_{24}O_6$	71.42	5.75	71.32	5.81
VIII	...	198–200[f]	M-W	Dark red	$C_{17}H_{16}O_6$	...	...	...	...
IX	71	121 to 121.5	E	Yellow	$C_{31}H_{28}O_6$	74.98	5.68	74.92	5.68

[a] Capillary, uncorrected. [b] E = ethanol; M = methanol; W = water. [c] Reported 140–141° (1); 141–142° (8). [d] An additional 29% of flavanone was obtained; see *Experimental*. [e] Reported 191° (8). [f] Reported 191° (2). This chalcone gave a yellow triacetate, m.p. 182.5 to 183° (from methanol). Calcd. for $C_{31}H_{22}O_9$: C, 62.44; H, 5.01. Found: C, 62.67, 62.65, 62.67; H, 5.16, 5.00, 5.21.

chloride were added to a solution of 27 Gm. of I and 20 Gm. of sodium iodide in 300 ml. of anhydrous acetone. The mixture was refluxed with stirring for 10 hours, kept an additional 6 hours at room temperature, poured into 400 ml. of water, and concentrated *in vacuo* to remove all the acetone. The aqueous solution was extracted with one 100-ml. and four 50-ml. portions of ether and the combined ether solutions with 20, 20, 10, and 10 ml. of 5% sodium hydroxide. The alkaline solutions were acidified and extracted again with one 50-ml. and five 15-ml. portions of ether. These solutions were dried and evaporated leaving 15.4 Gm. of a red oil which did not solidify and could not be distilled without decomposition. It therefore was used in this impure form. An additional amount of 6.5 Gm. (total yield 56.7%) was obtained by concentration of the first ether extracts and renewed extraction with 5% sodium hydroxide.

Chalcones IV–IX.—These compounds were prepared according to established methods (7) with 50% aqueous KOH as the condensing agent. As a rule, the mixture was allowed to stand at room temperature 3 to 4 days. Only in one case (chalcone VI), it was acidified with HCl after 3 days and kept at room temperature for 3 more days. Consequently, the reaction product was a mixture of chalcone VI and flavanone XIV. The melting points and analyses of six chalcones are listed in Table I.

4′,5,6,7-Tetramethoxyflavanone (X).—The conversion of chalcone IV to the corresponding flavanone was carried out according to the method of Seshadri (8). The yield was 33% and the flavanone melted at 119.5 to 120.5° reported m.p. 114–115° (1), 124–125° (8, 9).

Anal.—Calcd. for $C_{19}H_{20}O_6$: C, 66.27; H, 5.85. Found: C, 66.41; H, 5.98.

5,6,7-Trimethoxy-4′-benzyloxyflavanone (XI).—A solution of 18.2 Gm. of chalcone V in 830 ml. of ethanol was mixed with 163 ml. of 10% HCl, refluxed for 26 hours, diluted with 300 ml. of boiling water, and cooled slowly. The precipitate consisting of a mixture of chalcone and flavanone was filtered, dried (16.9 Gm.), and extracted three times with 100 ml. of boiling methanol. The combined methanol solutions, upon cooling, gave 6.0 Gm. of crystals, m.p. 152–154°. By further recrystallization from methanol, light yellow needles were obtained, m.p. 155–156°.

Anal.—Calcd. for $C_{25}H_{24}O_6$: C, 71.42; H, 5.75. Found: C, 71.57; H, 5.62.

4′-Hydroxy-5,6,7-trimethoxyflavanone (XII).—A mixture of 3.8 Gm. of XI, 145 ml. of glacial acetic acid, and 0.72 Gm. of 5% palladium-carbon catalyst was shaken under hydrogen at 24 lb. pressure for 1.5 hours. The suspension was filtered, diluted with 1.5 L. of water, and cooled overnight. The resulting precipitate was filtered, washed with water, and dried (2.7 Gm.; 90.3%). It was dissolved in 25 ml. of acetone, diluted with 50 ml. of benzene, and concentrated to approximately 10 ml. This procedure gave 2.2 Gm. of colorless needles, m.p. 185–187°, which were recrystallized once more from acetone-benzene. They turned red at 187° and melted at 190–192°.

Anal.—Calcd. for $C_{18}H_{18}O_6$: C, 65.45; H, 5.49. Found: C, 65.50; H, 5.42.

4′,5,7-Trimethoxy-6-benzyloxyflavanone (XIII).—Attempts to convert chalcone VII to the corresponding flavanone XIII proved unsuccessful. This flavanone was isolated, however, as a by-product in the preparation of VII. When the crude reaction product of III and anisaldehyde was dissolved in 1:2 acetone-ethanol, concentrated to half the volume, and cooled, the chalcone VII crystallized in 50% yield. After standing at room temperature overnight, the mother liquor contained faintly colored

TABLE II.—FLAVONES

	Yield, %	M.p.,[a] °C.	Crystallization Media[b]	Color	Empirical Formula	Analysis Calcd. C	Calcd. H	Found C	Found H
XVI	49	159–160[c]	M	Light yellow	$C_{19}H_{18}O_6$	66.66	5.30	66.68	5.41
XVII	77	156	M	Tan	$C_{25}H_{22}O_6$	71.76	5.30	71.72	5.36
XVIII	77.5	226–227	EA[d]	Tan	$C_{18}H_{16}O_6$	65.85	4.91	65.77	4.89
XIX	...	135 or 144 to 144.5	B-C	Light yellow	$C_{25}H_{22}O_6$	71.76	5.30	71.67	5.48
XX	44.5[e]	217–218	M-B	Yellow	$C_{18}H_{16}O_6$	65.85	4.91	65.98	4.78
XXI	86	155.5 to 156	B-C	Yellow	$C_{31}H_{26}O_6$	75.29	5.30	75.58	5.38
XXII	82.5	283.5 to 284[f]	M-B	Yellow	$C_{17}H_{14}O_6$	64.96	4.49	64.84	4.55

[a] Capillary, uncorrected. [b] M = Methanol; EA = ethyl acetate; B = benzene; C = cyclohexane. [c] Reported 158–160° (10); 162–163° (6, 1); 160° (3). [d] The mother liquor gave crystals, m.p. 243–246°, which were probably 4′,5-dihydroxy-6,7-dimethoxyflavone. Calcd. for $C_{17}H_{14}O_6$: C, 64.96; H, 4.49. Found: C, 64.93; H, 4.29. [e] From chalcone VII *via* XIX. [f] Reported 283° (3).

TABLE III.—GENERAL ENDOCRINE SCREENING ASSAY OF 4′,5,6,7-TETRAOXYGENATED FLAVANONES AND FLAVONES[a]

	Variation from Control, %[b]								
	Final Body	Vent. Prost.	Testes	Lev. Ani	Adrenals	Left Kid.	Liver	Spleen	Thymus
4′,5,6,7-Tetramethoxyflavanone (X)	− 4.9	− 5.9	−4.0	− 5.1	+12	−6.7	−3.4	+ 6.5	−12
4′,5,6,7-Tetramethoxyflavone (XVI)	− 7.6	+ 5.3	0	− 6.6	+ 3.0	−2.8	−8.0	+ 2.1	−14
4′-Hydroxy-5,6,7-trimethoxyflavanone (XII)	− 4.9	+12	+0.74	+ 4.0	+ 9.0	−1.3	−6.8	+ 9.6	− 9.5
4′-Hydroxy-5,6,7-trimethoxyflavone (XVIII)	−10	− 1.9	−6.0	− 3.7	+ 9.6	−4.0	−5.7	+26	−16
6-Hydroxy-4′,5,7-trimethoxyflavanone (XIV)	− 4.9	+ 5.3	−3.7	+ 2.6	+14	−0.90	−5.7	+14	− 2.7
6-Hydroxy-4′,5,7-trimethoxyflavone (XX)	− 4.9	− 1.3	−6.2	+ 0.37	+13	−3.3	−9.1	+ 6.7	− 3.5
4′,6-Dihydroxy-5,7-dimethoxyflavanone (XV)	− 5.6	+ 9.2	−1.6	+11	+18	−5.0	−9.1	+ 2.1	− 6.8
4′,6-Dihydroxy-5,7-dimethoxyflavone (XXII)	− 1.5	− 3.1	−0.23	+ 2.1	+ 2.4	+0.69	−2.2	+14	+ 3.9

[a] Twenty-one-day-old intact male Holtzman rats were used at the start of the experiment. Ten animals were in each group and the control group. Administration was at 2 mg./day, s.c., in CMC for 14 days with autopsy on 15th day. Controls given CMC diluent, and initial starting weights matched for the groups. [b] Percentage variation is calculated from the arithmetic mean of each group against the arithmetic mean for the control group.

crystals which were filtered (yield 10%) and recrystallized from benzene-cyclohexane to give colorless plates, m.p. 152 to 152.5°.

Anal.—Calcd. for $C_{25}H_{24}O_6$: C, 71.42; H, 5.75. Found: C, 71.56; H, 5.63.

6-Hydroxy-4′,5,7-trimethoxyflavanone (XIV).—As mentioned above under *Chalcones*, flavanone XIV was obtained in 29% yield, when the alkaline reaction mixture which contained chalcone VI was acidified with HCl and allowed to stand for 3 days. After the chalcone had been precipitated by addition of water (alcohol concentration 40%), the flavanone was obtained from the mother liquor by lowering the alcohol concentration to 15%. One recrystallization from benzene-cyclohexane gave light yellow needles, m.p. 123.5 to 124°.

Anal.—Calcd. for $C_{18}H_{18}O_6$: C, 65.45; H, 5.49. Found: C, 65.66; H, 5.38.

4′,6-Dihydroxy-5,7-dimethoxyflavanone (XV).—Although the directions given by Zemplen (2) were followed very closely, this flavanone could not be prepared according to this method. Usually it was obtained in small yield from the mother liquor after recrystallization of the chalcone VIII. A satisfactory conversion of the chalcone to the flavanone was accomplished by dissolving the chalcone in a mixture of 20 parts of methanol and 1 part of water and adding boiling water (30% by volume). Upon cooling and seeding, the flavanone separated and was recrystallized repeatedly from absolute ethanol. Although its color is reported to be ochre-yellow (2), our preparations were always red-orange, which would indicate that they still contained small amounts of chalcone (dark red). They melted consistently at 205°, reported m.p. 198° (2), and when admixed with the chalcone VIII (m.p. 198–200°), the melting point was strongly depressed (189–196°).

Anal.—Calcd. for $C_{17}H_{16}O_6$: C, 64.55; H, 5.10. Found: C, 64.23; H, 4.99.

Furthermore, the flavanone gave the same diacetate (m.p. 133–135°) as reported in the literature [137° (2)].

Anal.—Calcd. for $C_{21}H_{20}O_8$: C, 63.00; H, 5.04. Found: C, 63.20; H, 5.44.

In contrast, the chalcone VIII, when acetylated under identical conditions, gave an acetate m.p. 182.5 to 183°, which analyzed correctly for a triacetate (see Table I).

Flavones XVI, XVII, XIX, and XXI.—Equal amounts of chalcones IV, V, VII, and IX and selenium dioxide were dissolved in amyl alcohol (10–14 ml. per gram of chalcone) and refluxed for 12 hours. The solutions were cooled and filtered, the residue washed with ether, the filtrates washed with 20% NaOH and saturated NaCl solution, and the ether removed *in vacuo*. Flavones XVII and XXI crystallized directly from the amyl alcohol solutions, and additional amounts were obtained when the amyl alcohol was removed by steam distillation. This procedure had to be used for flavones XVI and XIX which are too soluble in amyl alcohol. Usually the crude flavones were recrystallized first from dilute methanol or dilute acetic acid, then from the solvents listed in Table II.

Flavones XVIII, XX, and XXII.—The benzyloxyflavones XVII, XIX, and XXI were dissolved in glacial acetic acid—concentrated HCl 3:1 (30–40 ml. per gram of flavone) and the solutions heated on the steam bath for 1.5 hours. After cooling, they were poured into five or more volumes of water, and the precipitates were filtered and recrystallized from appropriate solvents (Table II).

SUMMARY

The bioflavonoids X, XVI, XII, XVIII, XIV, XX, XV, and XXII were submitted to our general endocrine screening assay, with results as indicated in Table III. Flavone XVIII increased the weight of the spleen in the young intact male rat. The flavanone XV slightly increased the size of the adrenals, but this effect, if real, appeared to be a nonfunctional hypertrophy or hyperplasia. In general, these flavones and flavanones produced no significant endocrine effects.

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Mechanism of Action of Phenolic Disinfectants III

Uptake of Phenol-C-14, 2,4-Dichlorophenol-C-14, and *p-tert*-Amylphenol-C-14 by *Escherichia coli*

By JOSEPH JUDIS

Cell suspensions of *Escherichia coli* ATCC 11229 were incubated with phenol-C^{14} (P-C^{14}), 2,4-dichlorophenol-C^{14} (DCP-C^{14}), or *p-tert*-amylphenol-C^{14} (PTAP-C^{14}); radioactivity of the cells was determined. In general, the amount of a phenol associated with the cells increased directly with concentration of the phenol. Increasing the pH between 4.8 and 10 did not significantly affect the radioactivity of the cells incubated with P-C^{14} or PTAP-C^{14}, but decreased the activity of cells incubated with DCP-C^{14}. Temperature had no marked effect on labeling of the cells, and kinetic data indicated that the cells became labeled to the maximum extent very rapidly. The activity of P-C^{14} labeled cells was almost entirely extractable by saline-buffer at 22°. Activity from cells incubated with DCP-C^{14} and PTAP-C^{14} could be partially extracted with saline-buffer at 22°, more activity was extractable in a boiling water bath, and most of the activity was extractable with 70 per cent ethanol at 70°. Polysorbate 80 in a concentration of 1 per cent markedly reduced labeling of cells with DCP-C^{14} and PTAP-C^{14} and extracted much of the activity from labeled cells, the effect being greater with the latter phenol. These findings suggest that the phenols partition themselves between the aqueous medium and cellular lipids.

PREVIOUS FINDINGS (1, 2) in studies on the mechanism of action of phenolic germicides are consistent with the hypothesis that these compounds cause damage to the permeability barrier. The latter is generally associated with the cell membrane of microorganisms; this membrane contains a considerable amount of lipid (3–5). In addition, the cell walls of Gram-negative bacteria are rich in lipids unlike the mucopeptide nature of cell walls of Gram-positive organisms (6).

Phenol derivatives become less water soluble, more lipid soluble, and more bactericidal with increasing halogenation and/or alkylation (7, 8). If these compounds owe their biological activity to disruption or disorganization of membranes because of their ability to associate with lipids of the membranes, the proportion of the total amount of a phenol derivative associated with the bacteria in a suspension should increase as the bactericidal power of the derivatives increases. An approach to testing this hypothesis was made by choosing three phenolic germicides: phenol, 2,4-dichlorophenol, and *p-tert*-amylphenol with reported phenol coefficients of 1, 13, and 30, respectively (7, 8). These compounds labeled with carbon-14 were obtained or synthesized and the relative binding of the derivatives by *Escherichia coli* cells was determined.

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METHODS AND MATERIALS

Materials.—Phenol U-C^{14} (U indicates uniformly distributed label) was obtained from the New England Nuclear Corp. The 2,4-dichlorophenol-C^{14} was prepared by direct chlorination of phenol-C^{14}, and *p-tert*-amylphenol-C^{14} was synthesized by alkylation of phenol C^{14} by the Friedel-Crafts reaction. The phenol derivatives had approximately the following specific activities: phenol-C^{14}, 6×10^6 c.p.m./mg.; 2,4-dichlorophenol-C^{14} 1.9×10^5 c.p.m./mg.; and *p-tert*-amyphenol-C^{14}, 6×10^4 c.p.m./mg. Other chemicals were obtained commercially at the highest purity available.

Bacterial Suspensions.—The strain of *E. coli* used (ATCC 11229) and growth conditions were the same as previously described (1, 2). Washed cells were suspended in saline to a concentration of 4×10^{10} cells per milliliter, as determined by turbidity readings previously calibrated with plate counts. This concentration corresponds to 40 mg. wet weight or 10 mg. dry weight per milliliter or about 40 µl. of packed cells.

Reaction Mixtures and Measurement of Radioactivity.—As previously reported (1, 2), phenol derivatives (except phenol) were dissolved in 25% v/v ethanol. Reaction mixtures contained, in a total volume of 3.0 ml., 2.4×10^{10} cells, pH 7.2 (or as otherwise indicated) phosphate buffer in a final concentration of 0.02 *M*, the phenol derivative in a volume of 0.5 ml. (or made up to 0.5 ml. with 25% v/v ethanol), and enough saline to bring to volume. Reaction mixtures were incubated for 20 minutes at 22 ±2° and centrifuged for 10 minutes. Aliquots of the supernatants and cells, after resuspension, were placed on $1^1/_4$-in. concentric ring stainless steel planchets. The contents of each planchet were treated with 4 drops of bromine water, incubated for 10 minutes at room temperature, 0.075 ml. of 10% KOH was added, and the planchets were dried at 98°. This procedure was necessary because of the volatility of phenol and its derivatives; although self absorption was increased, it was constant for all

TABLE I.—BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14}, OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* CELLS

Compd. Added	Total c.p.m. Added	Total c.p.m. in Cells	Added Radioactivity Bound to Cells, %	Amount of Compd. Bound to Cells
Phenol-C^{14}, mg./ml.				
0.005	88,850	2,114	2.38	0.00036 mg.
4.17	88,850	1,997	2.25	0.28 mg.
8.33	88,850	2,013	2.27	0.566 mg.
12.5	88,850	1,972	2.22	0.832 mg.
2,4-Dichlorophenol-C^{14}, mcg./ml.				
86.7	58,195	5,314	9.13	23.72 mcg.
333.3	58,195	4,395	7.55	75.5 mcg.
500	58,195	4,248	7.30	109.5 mcg.
667	59,195	4,110	7.06	141.3 mcg.
p-tert-Amylphenol-C^{14}, mcg./ml.				
50	8,089	2,228	27.54	41.31 mcg.
100	16,178	4,008	24.77	74.32 mcg.
150	24,267	7,385	30.43	136.9 mcg.
200	32,356	9,612	29.71	178.2 mcg.
250	40,445	11,844	29.28	219.6 mcg.

planchets. Radioactivity was determined with a Nuclear-Chicago D-47 thin window gas flow counter and counting times were so as to give a counting error of 5% or less. In the experiments involving uptake of labeled compounds over different lengths of exposure, a number of replicate reaction mixtures was prepared and individual tubes removed at the indicated times for centrifugation (5 minutes at 15,000 r.p.m.). Solutions for determining extractability of labeled phenols from cells, except for 70% ethanol, contained phosphate buffer at a final concentration of 0.02 *M* and pH 7.15 unless otherwise stated.

RESULTS AND DISCUSSION

It would certainly be reasonable to expect that an antimicrobial agent active in very low concentrations is probably bound to susceptible cells, thus producing—at least in localized regions—a relatively high concentration. One would also suspect that perhaps one basis of resistance to an antimicrobial agent might be inability or development of inability to bind such agents. For example, Few and Schulman (9) found that cell walls from sensitive organisms take up about five to six times as much polymyxin as walls from resistant organisms; more recent evidence indicates the cell membrane as the site of binding of polymyxin (10). Newton (11) gives additional examples. Further, nystatin is bound by yeast (12, 13), streptomycin by *E. coli* and other organisms (14–17), hexylresorcinol by *E. coli* (18), and the penicillin-binding component of *Micrococcus pyogenes* has been studied (19).

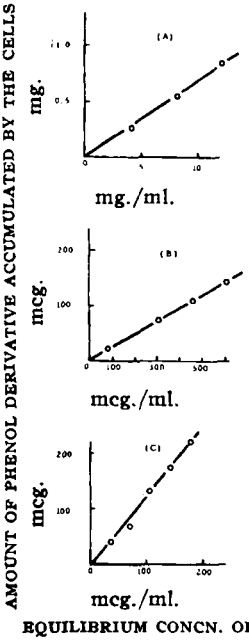


Fig. 1.—Accumulation of phenol-C^{14} (A), 2,4-dichlorophenol-C^{14} (B), and *p-tert*-amylphenol-C^{14} (C) as a function of concentration of the phenol derivatives in the reaction mixtures.

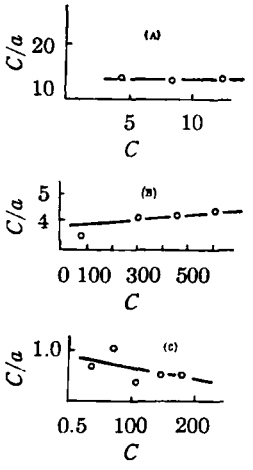


Fig. 2.—Langmuir adsorption isotherm plots of binding of phenol-C^{14}, 2,4-dichlorophenol-C^{14}, and *p-tert*-amylphenol-C^{14}. Key: *C*, equilibrium concentration of the phenol derivative in milligrams per milliliter in the case of phenol-C^{14} (A), or micrograms per milliliter for 2,4-dichlorophenol-C^{14} (B), and *p-tert*-amylphenol-C^{14} (C); *a*, amount of the phenol derivative accumulated by the cells as calculated from radioactivity. The units are milligrams for phenol and micrograms for the others.

Binding of an agent which is selectively toxic, *e.g.* an antibiotic, may involve specific chemical groups, and if the concentration of antibiotic is so low that it does not damage the permeability barriers, binding may resemble simple adsorption, following the Langmuir adsorption isotherm equations as was shown with streptomycin and *E. coli* (14). Phenol and its derivatives, however, are general protoplasmic poisons; their general toxicity may be explained on the basis of an ability to associate with compounds found in all microorganisms. Because toxic concentrations are too low to be protein denaturants (although this may be possible at local-

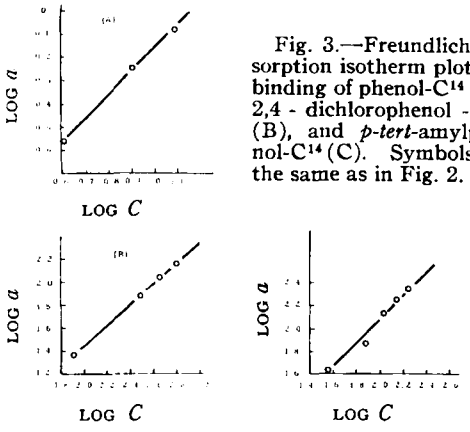


Fig. 3.—Freundlich adsorption isotherm plots of binding of phenol-C^{14} (A), 2,4 - dichlorophenol - C^{14} (B), and *p-tert*-amylphenol-C^{14} (C). Symbols are the same as in Fig. 2.

ized sites) and because of the low water solubility and high lipid solubility of phenols, it is likely that the latter associate primarily with cellular lipids. If this suggestion is true, then phenolic germicides should partition themselves between the aqueous medium and the lipid components of the bacterial cell. The more lipid soluble and water insoluble the derivative, the more antimicrobial it should be on a weight basis and the higher the fraction accumulating in the bacterial cells.

The three phenols studied here are different in water solubility and germicidal activity. Phenol is soluble in 15 parts of water, while 2,4-dichlorophenol and *p-tert*-amylphenol are not significantly soluble in water. It is possible to dissolve about 4 mg. of 2,4-dichlorophenol in 1 ml. of 25% v/v ethanol, but only about 1.5 mg. of *p-tert*-amylphenol. The phenol coefficients of the three compounds are also quite different (*see above*). One should expect, therefore, that if there is partition of the compounds between the bacterial lipids and the aqueous medium, the more biologically active a compound is on a weight basis, the higher the fraction of the compound associated with the cells. The data in Table I show that the per cent of each compound associated with the cells is roughly constant, independent of concentration, and increases directly with increasing bactericidal potency. The data also indicate that there is a direct proportionality between concentration of a phenol in the system and the amount of phenol associated with the cells. If equilibrium concentrations (initial concentrations minus the amount accumulated by the cells) are plotted against the amount of derivative accumulated by the cells, a straight line relationship seems to hold. Such a relationship might indicate either the type *Ln* or type *L* isotherm described by Giles and MacEwan (20), although enough data are not available to differentiate with certainty (see Fig. 1). The limited data seem to give a reasonable fit to a Langmuir plot (equilibrium concentration divided by the amount taken up by the cells *versus* amount taken up) or to a Freundlich plot (log equilibrium concentration *versus* log amount taken up) as illustrated in Figs. 2 and 3.

Oka (21–26), in his studies of the uptake of phenols

TABLE III.—EFFECT OF TEMPERATURE ON BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14}, OR *p-tert*-AMYLPHENOL BY *E. coli* CELLS

Compd. Added	Temp., °C.	Total c.p.m. Added	Total c.p.m. in Cells	Added c.p.m. Bound to Cells, %
Phenol-C^{14}, mg./ml.				
4.17	0	109,205	2,851	2.61
12.5	0	109,205	3,114	2.85
4.17	22	109,205	2,433	2.23
12.5	22	109,205	3,163	2.90
4.17	40	109,205	2,900	2.66
12.5	40	109,205	3,297	3.02
2,4-Dichlorophenol-C^{14}, mcg./ml.				
333	0	36,405	4,322	11.87
667	0	36,405	3,983	10.94
333	22	36,405	3,183	8.74
667	22	36,405	3,514	9.65
333	40	36,405	3,906	10.73
667	40	36,405	4,079	11.20
p-tert-Amylphenol-C^{14}, mcg./ml.				
125	0	19,470	5,045	25.91
250	0	38,940	14,138	36.31
125	22	19,470	4,674	24.00
250	22	38,940	10,724	27.53
125	40	19,470	5,069	26.03
250	40	38,940	10,293	26.43

TABLE IV.—RATE OF BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14} OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* CELLS

Time After Addition of Cells to Reaction Mixture, min.	Phenol-C^{14}	2,4-Dichlorophenol-C^{14}	*p-tert*-Amylphenol-C^{14}
	FINAL CONCN. IN REACTION MIXTURE		
	8.33 mg./ml.	667 mcg./ml.	250 mcg./ml.
	TOTAL C.P.M. ADDED		
	130,000	47,360	45,255
	TOTAL C.P.M. IN CELLS		
0	3,286	4,123	10,190
1	3,244	3,833	10,614
2	3,396	3,596	11,577
3	3,271	3,666	8,637
5	3,315	3,344	10,614
10	3,374	3,833	11,320
15	3,442	4,079	9,265
30	3,720	4,250	9,019
60	3,539	3,952	11,577
120	3,606	4,180	14,140

TABLE II.—EFFECT OF pH ON BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14}, OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* CELLS

Compd. Added	Total c.p.m. Added	Total c.p.m. in Cells at pH- 4.80	5.95	6.45	7.15	8.20	10.0
Phenol-C^{14}, 12.5 mg./ml.	75,000	1,896	1,970	2,232	1,986	1,802	1,855
2,4-Dichlorophenol-C^{14}, 667 mcg./ml.	37,770	7,459	5,770	4,497	3,779	2,576	2,638
p-tert-Amylphenol-C^{14}, 250 mcg./ml.	43,325	12,896	11,450	13,058	11,713	10,959	11,579

TABLE V.—EXTRACTABILITY OF PHENOL-C^{14} AND 2,4-DICHLOROPHENOL-C^{14} FROM *E. coli* CELLS

Labeled Phenol Bound to Cells	Extraction Treatment	Total c.p.m. Remaining in Cells	C.p.m. Extracted, %
Phenol-C^{14}	None	1669	...
	Saline-buffer at 22°C., 30 min.	135.7	91.9
	70% v/v ethanol at 70°C., 30 min.	25	98.5
	Saline-buffer in boiling water bath, 20 min.	48.7	97.1
2,4-Dichlorophenol-C^{14}	None	3727	...
	Saline-buffer at 22°C., 30 min.	1038	72.1
	70% v/v ethanol at 70°C., 30 min.	58	98.4
	Saline-buffer in boiling water bath, 20 min.	695.7	81.3

TABLE VI.—EXTRACTABILITY OF 2,4-DICHLOROPHENOL-C^{14} FROM *E. coli* CELLS

Extraction Procedure	Total c.p.m. Remaining in Cells	C.p.m. Extracted, %
None	4110	...
Polysorbate 80, 1% at 22°C. for 30 min.	523	87.3
BTC, 0.5% at 22°C. for 30 min.	1771	56.9
Hyamine 3500, 0.1% at 22°C. for 30 min.	1598	61.1
Polyethenoxy nonionic ether, 0.5% at 22°C. for 30 min.	569	86.2
Aerosol, 1% at 22°C. for 30 min.	4231	0
Aerosol MA, 1% at 22°C. for 30 min.	1676	59.2
n-Butanol, 6.7% v/v at 22°C. for 30 min.	1676	59.2
Ethanol, 70% v/v at 70°C. for 30 min.	133.4	96.8
Saline-buffer, pH 7.15 at 22°C. for 30 min.	897.2	78.2
Saline-buffer, pH 7.15 in boiling water bath for 20 min.	562.2	86.3

TABLE VII.—EXTRACTABILITY OF *p-tert*-AMYLPHENOL-C^{14} FROM *E. coli* CELLS

Extraction Procedure	Total c.p.m. Remaining in Cells	C.p.m. Ex-Extracted, %
None	8950	...
Polysorbate 80, 1% at 22°C. for 30 min.	1209	86.5
BTC, 0.5% at 22°C. for 30 min.	5670	36.6
Hyamine 3500, 0.1% at 22°C. for 30 min.	2362	73.6
Polyethenoxy nonionic ether, 0.5% at 22°C. for 30 min.	1028	88.5
Aerosol, 1% at 22°C. for 30 min.	5546	38.0
Aerosol MA, 1% at 22°C. for 30 min.	7077	20.9
n-Butanol, 6.7% v/v at 22°C. for 30 min.	4678	47.9
Ethanol, 70% v/v at 70°C. for 30 min.	174	98.1
Saline-buffer, pH 7.15 at 22°C. for 30 min.	5050	43.6
Saline-buffer, pH 7.15 in boiling water bath for 20 min.	3089	65.5

and other antiseptics by yeast and bacteria, found a linear relationship between the log of the equilibrium concentration of the antiseptics in the medium and the log of the transferred quantity, and concluded that the phenols were partitioned between the bacterial lipids and the aqueous medium. He substantiated his contention by measuring partition of the antiseptics he studied between soy bean oil and water and by estimating partition between lipid and nonlipid phases of *E. coli* and *Staphylococcus aureus*. Oka concludes, however, that the toxic effect can be better correlated with the total amount of antiseptic adsorbed on the cell rather than the amount of antiseptic dissolved in the lipid or aqueous phases of the microorganisms. This conclusion must be taken with caution because his estimation of the concentration of antiseptic in the microbial lipids is based on the assumption that partitioning between soybean oil and water approximates partitioning between microbial lipids and the rest of the cell. Bacterial lipid does not consist simply of glyceryl esters of fatty acids (27, 28) and varies in both composition and cell distribution with gram reaction and species (6, 28).

The solubility in nonpolar solvents of an organic compound with a polar group should be greater if the polar group is unionized. Lipoproteins are important components of biological membranes and are involved in regulation of permeability, especially of organic molecules. In general, one should expect that the more lipid soluble an organic compound, is the greater the permeability of biological membranes to the compound. For example, Simon and Beevers and Beevers, *et al.* (29, 30), found that the undissociated form of weakly ionized organic acids or their esters penetrated bacteria more effectively than the ionized form. Skou (31) reported that the minimum concentration of local anesthetics blocking nerve conduction was lower at pH values at which the anesthetic was unionized. Phenol having a K_a of 10^{-10} would not be ionized significantly below pH 9–10, and its lipid solubility should be unaffected by pH values below 9 or 10. Alkylation of phenol depresses the K_a (32–34), but halogenation increases phenol's acidic properties, *e.g.* 2,4-dichlorophenol has a K_a of 7.08×10^{-9} (35). As can be seen in Table II, the amount of 2,4-dichlorophenol bound by *E. coli* is affected by pH in the predicted fashion; *i.e.*, the higher the pH, the higher the proportion of ionized form. Thus, if this derivative is being dissolved in bacterial lipids, the less soluble it would be. Phenol and *p-tert*-amylphenol having low K_a values would exist primarily in unionized form even at pH 10 and thus not be affected in terms of lipid solubility by the pH values tested here.

In general, a rise in temperature is accompanied by an increase in solubility. In a system involving partition of a solute between two solvents, there may be an increase in solubility in each of the phases with an increase in temperature. Thus the fraction of solute associated with one of the phases may remain roughly constant. The latter situation

TABLE VIII.—EFFECT OF POLYSORBATE 80 ON BINDING OF 2,4-DICHLOROPHENOL-C^{14} OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* AND EXTRACTABILITY OF THESE COMPOUNDS

Treatment	Total c.p.m. Remaining, After Treatment, in Cells Incubated with 2,4-Dichlorophenol-C^{14}	Total c.p.m. Remaining, After Treatment, in Cells Incubated with *p-tert*-Amylphenol-C^{14}
(*a*) Cells incubated with C-14 phenol derivative	3266	11,617
(*b*) As in (*a*) plus 1% polysorbate 80	1691	1,124
(*c*) As in (*a*), followed by extraction of the cells with saline-buffer at pH 7.15 at 22°C. for 30 min.	1109	6,305
(*d*) As in (*a*), followed by extraction of the cells with saline-buffer containing 1% polysorbate 80, pH 7.15 at 22°C. for 30 min.	391	1,175

might hold at low temperatures also. The effect of temperature on uptake of the three phenolic compounds is given in Table III. Any effects of temperature on uptake could be explained by uneven effects on solubility in one of the phases.

The association of the phenol derivatives with *E. coli* cells occurred rapidly and essentially to the maximum extent in even the shortest time interval measured (Table IV). Since centrifugation required 5 minutes—and an additional 5 minutes were expended in separation of the supernatant from the cells—the earliest time period is more realistically 10 minutes.

Tables V, VI, and VII indicate the extractability of the phenol derivatives from *E. coli*. Phenol is easily removed by either aqueous systems or 70% ethanol as would be expected from its solubility. However, 2,4-dichlorophenol and *p-tert*-amylphenol are much more readily removed by ethanol than saline-buffer, and *p-tert*-amylphenol is less extractable by aqueous systems than 2,4-dichlorophenol. It would appear that the phenol derivatives are held by the cells even after the permeability barrier is completely broken with *n*-butanol treatment or heating the cells in a boiling water bath. Of the surfactant solutions used for extraction, polysorbate 80[1] and a polyethenoxy nonionic ether[2] nonionics, were the most effective solvents. The data in Table VIII show that polysorbate 80, if present in the reaction mixture, substantially reduced the amount of 2,4-dichlorophenol and *p-tert*-amylphenol associated with the cells or very effectively extracted these compounds from cells previously having bound them.

The effect of pH on extractability of phenol derivatives by aqueous media is given in Table IX. Saline-buffer at pH 10 extracted a higher percentage of 2,4-dichlorophenol than solutions of lower pH, while no differences were found with *p-tert*-amylphenol. One might assume that the higher extractability of 2,4-dichlorophenol at pH 10 could be due to its existence to a greater extent at that pH in the ionized form which is less soluble in lipids than the unionized form.

If toxic effects of a phenolic disinfectant are due to

[1] Marketed as Tween 80 by the Atlas Powder Co., Wilmington, Del.

[2] Marketed as Triton-X-100 by the Rohm and Haas Co., Philadelphia, Pa.

TABLE IX.—EFFECT OF pH ON EXTRACTABILITY OF 2,4-DICHLOROPHENOL-C^{14} AND *p-tert* AMYLPHENOL-C^{14} FROM *E. coli*

Phenol Derivative	Extraction Procedure	Total c.p.m. Remaining in Cells	C.p.m. Extracted, %
2,4-Dichlorophenol-C^{14}	None	2680	...
	Saline-buffer, pH 4.8	892	66.7
	Saline-buffer, pH 7.1	737	72.5
	Saline-buffer, pH 10.0	473	82.4
p-tert-Amylphenol-C^{14}	None	9903	...
	Saline-buffer, pH 4.8	4549	54.1
	Saline-buffer, pH 7.1	4210	57.5
	Saline-buffer, pH 10.0	4263	57.0

the presence of a certain minimum concentration in the bacterial cells, it is obvious that the more potent a derivative is, the lower the concentration needed in solution to reach that minimal cell concentration. Because of the high lipid solubility of phenols, especially those highly bactericidal at low concentration, it is reasonable to assume that the site of accumulation of these compounds is in the microbial lipids. Bacterial lipids are most generally located in the cell membrane (4, 28) and in the cell walls of Gram-negative bacteria. Thus, it is not surprising that phenol derivatives damage permeability barriers in microorganisms (1, 2, 36).

While damage to surface structures seems to be a likely explanation for the mechanism of action of phenolic disinfectants, it is also quite possible that these compounds inhibit respiratory enzymes and prevent cell division by preventing energy yielding reactions. A number of phenol derivatives have been shown to inhibit glucose oxidation at bactericidal concentrations (37). Since some respiratory enzymes are located at the cell surface (38–40), their inhibition either directly or by preventing transfer of their substrates through the permeability carrier must be considered in the total picture. Such possible effects are presently under study in our laboratory. Finally, all of the data presented above have involved only one organism, a Gram-negative one, with surface structures decidedly different from those of Gram-positive bacteria (41–43). The latter are well known to be more resistant to phenol than Gram-negative bacteria (8), and these studies should be extended to include Gram-positive bacteria for a more complete visualization of the mechanism by which phenols are bactericidal.

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Drug Standards

Assay of Vitamin E in Pharmaceutical Products

By ROBERT W. LEHMAN

Control assays for vitamin E may appear to be hopelessly complex to the pharmaceutical analyst. "The National Formulary" describes a different assay method for each of the six forms or sources of vitamin E listed. Alkaline saponification is used for some materials and acid hydrolysis for others. Ceric sulfate titration is used for some; reaction with ferric chloride and dipyridyl for others. The derivation of the factors used in the calculations is not readily apparent. The job of the analyst is further complicated because the N.F. methods do not automatically apply to these same forms or sources when they are used in finished pharmaceutical products. This review is intended to help the analyst who must modify the N.F. assay methods for vitamin E in order to apply them to finished pharmaceutical products not covered by the N.F. monographs. This review mentions some reasons for the differences between methods and suggests modifications that can be used in assaying vitamin E in pharmaceutical products.

ALPHA TOCOPHEROL can be easily oxidized by ferric chloride or by ceric sulfate to the "yellow quinone" as shown in Fig. 1. The N.F. assay methods depend on either measuring the amount of ferrous ions produced (they form a red complex with α,α'-dipyridyl) or titrating until ceric ions are left in solution to oxidize an indicator (diphenylamine).

α-Tocopheryl esters such as the acetate and acid succinate shown in Fig. 2 cannot be oxidized until the ester group has been removed by hydrolysis (either with potassium hydroxide or sulfuric acid).

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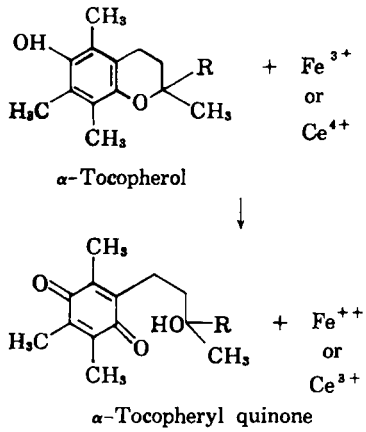


Fig. 1.—Oxidation of α-tocopherol.

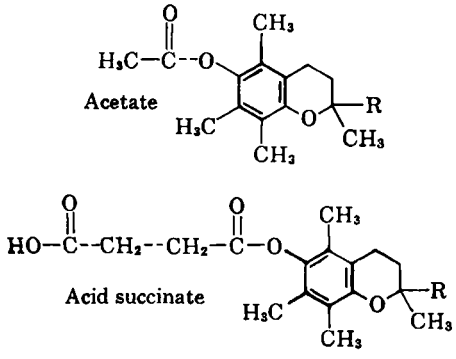


Fig. 2.—Esters of α-tocopherol.

chloride–dipyridyl procedure. Acid hydrolysis would have no advantage here, because it, too, would have to be worked up by the same procedure.

Ceric Sulfate versus Dipyridyl

Because it is more precise, ceric sulfate titration would usually be chosen over ferric chloride–dipyridyl colorimetry wherever it can be applied. Table II shows the relative precision of the N.F. assay methods which was obtained in a collaborative study of five samples among nine laboratories (2). Both the intra and the interlaboratory variation of the ceric sulfate titration are significantly less than those of the dipyridyl procedure.

Unfortunately the ceric sulfate titration can be applied only to tocopherol samples of high concentration. It fails on mixed tocopherols concentrate because of fading end points, which are a result of a solubility problem. Tocopherol is soluble in ethanol, but not in water; ceric sulfate is soluble in aqueous sulfuric acid, but not in ethanol. As the aqueous ceric sulfate solution is run into the ethanol solution of tocopherol, one reactant or the other tends to be thrown out of solution. When excess oil phase is present, this causes fading end points. Sharp end points exist only within very narrow solubility limits.

The ferric chloride–dipyridyl reaction can be applied to extremely dilute tocopherol solutions. In almost all pharmaceutical dosage forms, it would present fewer problems than the ceric sulfate procedure.

Derivation of Assay Factors

In the two N.F. monographs that specify the dipyridyl assay method, the derivation of the "factors," 28.2 and 32.0, is not presented. To obtain proper factors needed for accurate application to "pure" tocopherols or esters in pharmaceutical products, the analyst needs the following information.

The formulas follow the pattern

$$A \times \text{factor}/C \times L = \text{mg. per Gm.}$$

where A is absorbance, C is concentration of sample, and L is length of the cell.

This "factor" in the mixed tocopherols dipyridyl assay calculation is derived as

$$\text{Factor} = \frac{25 \times 1000}{2 \times 443.3} = 28.2$$

The 25, 1000, and 2 come from the dilutions made for the spectrophotometry, and 443.3 is the extinction coefficient (E, 1%, 1 cm.) calculated for the particular mixture of α-, β-, γ-, and δ-tocopherols present in the average lot of mixed tocopherols concentrate. These four tocopherols have extinction coefficients in the dipyridyl assay of 414, 437, 455, and 561, and they are present in the average proportions 52.0, 9.3, 28.0, and 10.7%, respectively. Thus, the average E, 1%, 1 cm., is (414 × 0.520) + (437 × 0.093) + (455 × 0.280) + (561 × 0.107) = 443.3.

For *d*-α-tocopheryl acetate concentrate, the proportions are 68, 22, 5, and 5%, respectively, and the calculated extinction coefficient is 428.5. However, these calculations are in terms of milligrams of unesterified tocopherols, while the assay result is to be expressed in milligrams of tocopheryl acetate. Thus, the ratio of molecular weights must also be introduced, and the factor is

$$\text{Factor} = \frac{25 \times 1000 \times 472.76}{2 \times 428.5 \times 430.72} = 32.0$$

Multicomponent pharmaceutical products might be assayed for vitamin E by the dipyridyl procedure rather than by the ceric sulfate titration, even when a pure form of vitamin E is used in its preparation. In such cases, the extinction coefficient, 414, for α-tocopherol should be used, and a molecular weight adjustment should be made to yield milligrams of the particular ester present. Thus, the proper factor for *d*- or *dl*-α-tocopheryl acetate is

Table I.—Assay Methods Specified by "The National Formulary"

N.F. Product	N.F. XI (1) Page	Purity	Hydrolysis	Analytical Method	Nitroso
dl-α-Tocopherol	376	High	(None)	Ceric SO_4	No
d-α-Tocopheryl acetate	377–378	High	Acid	Ceric SO_4	No
dl-α-Tocopheryl acetate	379–380	High	Acid	Ceric SO_4	No
d-α-Tocopheryl acid succinate	380	High	Saponification	Ceric SO_4	No
Mixed tocopherols concentrate	376–377	Low	(None)	Dipyridyl	Yes
d-α-Tocopheryl acetate concentrate	378–379	Low	Saponification	Dipyridyl	Yes

TABLE II.—PRECISION OF ASSAY METHODS

	Coefficient of Variation, % Intralab	Interlab
Ceric sulfate	0.45	0.70
Dipyridyl	1.52	2.80
Nitroso	5.45	17.50

$$\text{Factor} = \frac{25 \times 1000 \times 472.76}{2 \times 414 \times 430.72} = 33.1$$

and the one for *d*-α-tocopheryl acid succinate is

$$\text{Factor} = \frac{25 \times 1000 \times 530.80}{2 \times 414 \times 430.72} = 37.2$$

Colorimetry

A method using a spectrophotometer rather than one using a colorimeter was proposed to The National Formulary because the spectrophotometric method can be set up quickly in any laboratory without a need for reference standards. But since the spectrophotometer is a null-point instrument, and not direct reading, it is difficult to use where absorbance is changing rapidly with time. Therefore, the time interval of 10 minutes was recommended to The National Formulary.

Figure 3 shows how the rate of reaction and the degree of color development may vary with the tocopherol homolog. The problem caused by the different responses of the β, γ, and δ forms can be avoided by using a colorimeter instead of a spectrophotometer and by using a reaction time of 50 seconds instead of 10 minutes.

By using a direct reading colorimeter and a time interval of 2 minutes (see Fig. 3), it is less necessary to calibrate against all four tocopherols or to know the relative abundance of the four tocopherols in each sample for assay.

By changing the concentration of the reagents and the order of addition, these four reaction curves can be made to cross each other at a time interval of about 50 seconds. Quaife (4) developed a specific procedure for determining tocopherols in biological samples that could be very useful in a pharmaceutical control laboratory. The reaction rates are shown in Fig. 4.

Conditions needed to duplicate the relationships in Fig. 4 are fairly critical; directions for conducting the assay are as follows.

50-Second Assay for Total Tocopherol.—*Apparatus.*—An Evelyn colorimeter (Rubicon Instruments Division, Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.) or similar instrument having a constant, low intensity light source, means for filtering this source to a narrow wavelength region, and a direct reading, instantaneously responding photocell and galvanometer. For this determination it should be equipped with a 515 or a 520-mμ filter.

Reagents.—Ethanol (absolute ethyl alcohol); α,α′-dipyridyl solution. (dissolve 0.25 Gm. in 50 ml. of ethanol, protect from light); and ferric chloride solution (dissolve 0.10 Gm. of $FeCl_3 \cdot 6H_2O$ in 50 ml. of ethanol, protect from light) are utilized.

Procedure.—Place in the colorimeter tube 8 ml. of ethanol and 1 ml. of α,α′-dipyridyl solution. With the tube in the colorimeter, adjust the light intensity so that the galvanometer reads 100% transmittance. Add 1 ml. of ferric chloride solution. Shake well for 5 seconds, and record the per cent transmittance (G) exactly 50 seconds after adding the ferric chloride. Convert this to "L_B" (2 − log G).

Place in the colorimeter tube 8 ml. of ethanol containing sufficient sample to supply about 60 mcg. of tocopherol. Add 1 ml. of α,α′-dipyridyl solution and, with the tube in the colorimeter, adjust the light intensity so that the galvanometer reads 100% transmittance. Add 1 ml. of ferric chloride solution. Shake well for 5 seconds and record the per cent transmittance (G) exactly 50 seconds after adding the ferric chloride. Convert this to "L_S" (2 − log G). Subtract L_B, determined for the blank reagents, from L_S, determined for the sample and record the difference as "L_D."

Prepare a calibration curve, using solution of pure α-tocopherol in place of the sample. On linear graph paper, plot L_D *versus* milligrams of α-tocopherol. Draw the best fitting smooth line through the points and through zero. Do not necessarily attempt to draw a straight line.

From the calibration curve, find milligrams of tocopherol corresponding to L_D found for the sample. From the concentration of sample in the colorimeter tube, calculate milligrams of total tocopherol per gram of sample.

Nitroso Assay.—For the two "sources" of vitamin E in "The National Formulary," mixed tocopherols concentrate and *d*-α-tocopheryl acetate concentrate, a nitroso reaction is specified to determine the tocopherols "other than alpha-." The relatively poor precision of the nitroso assay procedure is shown in Table II. The supplier of these concentrates needs to use this assay routinely; but because of the assay uncertainty, the industrial analyst might use an average figure for the ratio of alpha to total tocopherol, relying on the dipyridyl assay for control purposes.

The ratio of alpha to total tocopherol will not

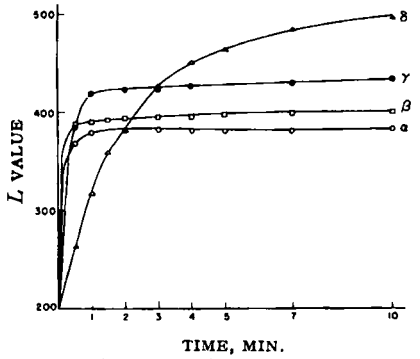


Fig. 3.—Reaction rates of tocopherols in Stern and Baxter (3) procedure.

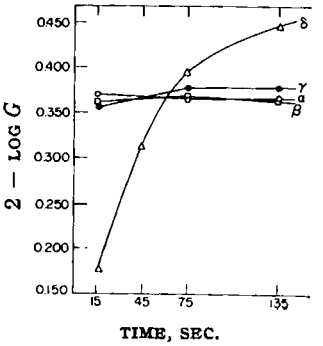


Fig. 4.—Reaction rates of tocopherols in Quaife (4) procedure.

change, except under conditions of severe destruction, and these would always be avoided in pharmaceutical practice. Once it has been established for a given lot of concentrate, that ratio can be applied to the total tocopherol content of finished products made from the known lot.

The N.F. monographs do not specify that the nitroso assay be used on the high purity "forms" of vitamin E. When it is applied to them it shows an absorption corresponding to 1 to 2% of *apparent* non-α-tocopherol. However, this occurs even with the synthetically prepared forms and with highly purified experimental preparations; thus it seems probable that the slight color observed is caused by a side-reaction between α-tocopherol and the reagent.

Detection of d-Isomers

Until recently, there was no accurate way to distinguish between the *d* epimer (from natural sources) and the *dl*-racemic mixture. It is important to develop methods for distinguishing because the *d* form is credited with 36% more "potency" than the *dl*. While the *d* epimer does exhibit optical rotation, the α_D is only a fraction of a degree, and impractical to use in control work.

Of course, the pharmaceutical control chemist will not usually need this type of test for work on his company's product. But he may wish to check on the raw materials. And law enforcement chemists may wish to examine products off the shelf.

A control procedure can probably be worked out, based on the method published in 1962 by Nelan and Robeson (5). They oxidized a sample of 400 mg. of tocopherol by shaking for 3 minutes in 50 ml. of ether with 10 ml. of 10% potassium ferricyanide in 0.2 *N* aqueous sodium hydroxide. After washing and drying over sodium sulfate, the solvent was removed, the sample taken up in iso-octane, and the optical rotation determined on a 5% solution using a Rudolf polarimeter (model 70). The crude oxidation product gave an $[\alpha]_D^{25}$ of +27.5.

By using only 3 ml. of 5% solution in a 20-cm. tube, a rotation of at least 0.25° (which can be reliably distinguished from zero) can be obtained with a sample of only 15 or 20 mg. of α-tocopherol. This can be applied directly to high purity forms of vitamin E. Finished dosage forms might require a clean-up by chromatography or other method modifications if other optically active ingredients are present.

SUMMARY

This review discusses limitations in acid hydrolysis and ceric sulfate procedures that are specified in some of "The National Formulary" monographs for forms and sources of vitamin E. It suggests that vitamin E in pharmaceutical products not covered by the N.F. monographs be assayed by saponification followed by the dipyridyl determination. It shows how the calculation factors in the dipyridyl assay can be modified to apply to any of the vitamin E sources. A more simple, colorimetric method is described for the dipyridyl assay. The nitroso assay and detection of *d* isomers are discussed briefly.

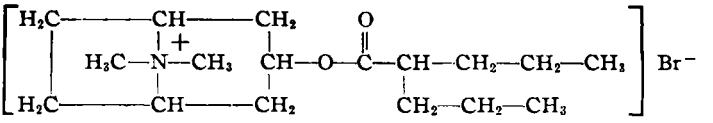
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Qualitative and Quantitative Tests for Anisotropine Methylbromide

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drugs concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

8-METHYLTROPINIUM BROMIDE 2-propylpentanoate; $C_{17}H_{32}BrNO_2$; mol. wt. 362.36. The structural formula of anisotropine methylbromide may be represented as



Physical Properties.—Anisotropine methylbromide occurs as a white, glistening, odorless, bitter hygroscopic powder. It is freely soluble in chloroform and alcohol, soluble in water, slightly soluble in acetone, and practically insoluble in ether. The pH of a solution of anisotropine methylbromide in carbon dioxide-free water (1 in 100) is between 6 and 7.

Identity Tests.—A solution of anisotropine methylbromide (1 in 20) responds to the U.S.P. XVI tests for bromide.

Transfer about 50 mg. of anisotropine methylbromide to a 150-ml. beaker, dissolve in 50 ml. of water, and add 5 ml. of 20% sulfuric acid. Add slowly, with stirring, 10 ml. of a freshly prepared and filtered solution of ammonium reineckate (1 in 50); allow to stand 1 hour. Filter the pink precipitate through a sintered-glass filter of medium porosity, and wash it with three 2-ml. portions of ice cold water. Allow to air dry and recrystallize from 70% alcohol at 55°. Dry the crystals in a vacuum desiccator over phosphorus pentoxide for 16 hours: the reineckate melts with decomposition at 195–199°, U.S.P. XVI Class Ia.

A 1 in 400 solution of anisotropine methylbromide in water does not exhibit an ultraviolet absorption maximum in the wavelength range of from 220 to 360 mμ.

The infrared spectrum of a 0.5% dispersion of anisotropine methylbromide in potassium bromide in a disk of about 0.82-mm. thickness is shown in Fig. 1.

Purity Tests.—Dry about 1 Gm. of anisotropine methylbromide, accurately weighed, at 105° for 3 hours: it loses not more than 1.0% of its weight.

Char about 1 Gm. of anisotropine methylbromide, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Determine the heavy metals content by the U.S.P. XVI heavy metals test, method I: the heavy metals limit for anisotropine methylbromide is 20 p.p.m.

Transfer 500.0 mg. of anisotropine methylbromide, previously dried at 105° for 3 hours and accurately weighed, to a glass-stoppered 125-ml. conical flask. Add 50.0 ml. of 0.05 *N* sodium hydroxide, stopper, and allow to stand for exactly 1 hour. Prepare a blank in the same manner, omitting the anisotropine methylbromide. To each flask add 50.0 ml. of 0.05 *N* hydrochloric acid, 2 drops of phenolphthalein T.S., and titrate with 0.05 *N* sodium hydroxide. Subtract the volume of alkali consumed by the blank to obtain the volume required to neutralize the excess acid in the sample solution: not more than 2.5 ml. of 0.05 *N* sodium hydroxide is required.

Determine the nitrogen content by the U.S.P. XVI nitrogen determination, method II, using about 325 mg. of anisotropine methylbromide (previously dried at 105° for 3 hours and accurately weighed) and 0.1 *N* sulfuric acid for the titration. Each milliliter of 0.1*N* sulfuric acid is equivalent to 1.401 mg. of nitrogen (N). The amount of nitrogen found is not less than 3.79% and not more than 3.95% of the weight of the sample taken.

Assay

Bromine.—Transfer about 600 mg. of anisotropine methylbromide, previously dried at 105° for 3 hours and accurately weighed, to a 250-ml. conical flask; dissolve in 50 ml. of water. Add 50 ml. of methanol, 10 ml. of glacial acetic acid, and 2 or 3 drops of eosin Y T.S. Titrate with 0.1 *N* silver nitrate until the color of the precipitated silver bromide changes to pink. Each milliliter of 0.1 *N* silver nitrate is equivalent to 7.991 mg. of bromine (Br). The amount of bromine found is not less than 21.6% and not more than 22.5% of the weight of the sample taken.

Anisotropine Methylbromide.—Transfer about 700 mg. of anisotropine methylbromide, previously dried at 105° for 3 hours and accurately weighed, to a tall form 200-ml. beaker; dissolve in 30 ml. of glacial acetic acid. Add 10 ml. of mercuric acetate T.S. and 2 drops of methylrosaniline chloride T.S., and titrate with 0.1 *N* acetous perchloric acid to a

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Endo Laboratories, Inc., Richmond Hill, N. Y., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

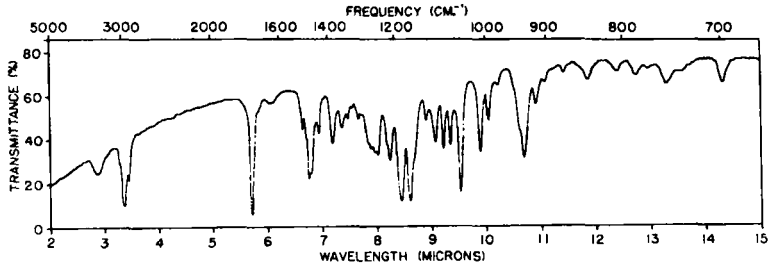


Fig. 1.—Infrared spectrum of anisotropine methylbromide in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

blue end point. Perform a blank titration and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 36.24 mg. of $C_{17}H_{32}BrNO_2$. The amount of anisotropine methylbromide found is not less than 98.0% and not more than 102.0% of the weight of the sample taken.

DOSAGE FORMS OF ANISOTROPINE METHYLBROMIDE

Anisotropine Methylbromide Tablets

Identity Test.—Finely powder a number of anisotropine methylbromide tablets; transfer an amount equivalent to 50 mg. of anisotropine methylbromide to a 125-ml. conical flask. Add 50 ml. of water, and shake mechanically for 30 minutes. Filter through paper, discarding the first few milliliters of filtrate, and collect the remainder of the filtrate in a 125-ml. separator. Add 5 ml. of a solution prepared by mixing 1 volume of chloroform with 2 volumes of carbon tetrachloride; shake for 5 minutes, and allow the phases to separate. Discard the organic phase and bubble air through the aqueous phase for 5 minutes. Transfer the aqueous phase to a 150-ml. beaker and continue as directed under *Identity Tests* in the monograph for anisotropine methylbromide, beginning with ". . . add 5 ml. of 20% sulfuric acid . . ." The reineckate melts with decomposition at 195–199°, U.S.P. XVI Class Ia.

Assay.—*Standard Preparation.*—Transfer about 100 mg. of anisotropine methylbromide reference standard, previously dried at 105° for 3 hours and accurately weighed, to a 100-ml. volumetric flask; dilute to volume with water and mix.

Procedure.—Weigh and finely powder not less than 20 anisotropine methylbromide tablets. Transfer to a glass-stoppered 125-ml. flask an amount of powdered tablets, accurately weighed, equivalent to about 50 mg. of anisotropine methylbromide. Pipet in 50 ml. of water, stopper the flask, and shake mechanically for 30 minutes. Filter through dry paper, discarding the first 10 ml. of filtrate; transfer the remainder of the filtrate to a 125-ml. separator. Add 5 ml. of a solution prepared by mixing 1 volume of chloroform with 2 volumes of carbon tetrachloride, shake for 5 minutes, and allow the phases to separate. Discard the organic phase and bubble air through the aqueous phase for 5 minutes. Pipet 10 ml. of the aqueous phase into a graduated 15-ml. conical centrifuge tube; pipet 10 ml. of the *Standard Preparation* into a similar tube. Add 1.0 ml. of 20% sulfuric acid and 2.0 ml. of a freshly prepared and filtered solution of ammonium reineckate (1 in 50) to each tube. Stopper the tubes, mix by inversion, allow to stand for 1 hour, and centrifuge. Decant the supernatant liquid, and wash each precipitate with two 1-ml. portions of ice cold water, centrifuging and decanting each time. Dissolve the precipitates in acetone and dilute to 10.0 ml. with acetone Allow the solutions to stand for 1 hour, centrifuge if they show a haze, and determine the absorbances in 1-cm. cells with a suitable spectrophotometer at 525 mμ using acetone as the blank. Record the absorbance of the solution from the *Standard Preparation* as A_s and that from the tablets as A_u. Calculate the weight (in milligrams) of anisotropine methylbromide in the portion of the powdered tablets taken by the formula $0.5W \times A_u/A_s$, where W is the weight (in milligrams) of anisotropine methylbromide reference standard taken. The amount of anisotropine methylbromide found is not less than 93.0% and not more than 107.0% of the labeled amount.

Anisotropine Methylbromide Elixir

Identity Test.—Transfer a volume of anisotropine methylbromide elixir, equivalent to 50 mg. of anisotropine methylbromide, to a 150-ml. beaker. Dilute to 50 ml. with water and continue as directed under *Identity Tests* in the monograph for anisotropine methylbromide, beginning with ". . . add 5 ml. of 20% sulfuric acid. . ." but allowing the precipitate to stand 4 hours before filtering: the reineckate melts with decomposition at 195–199°, U.S.P. XVI Class Ia.

Assay.—*Standard Preparation.*—Prepare as directed in the *Assay* in the monograph for anisotropine methylbromide tablets.

Procedure.—Pipet a volume of anisotropine methylbromide elixir, equivalent to 10 mg. of anisotropine methylbromide, into a graduated 15-ml. conical centrifuge tube; dilute to 10.0 ml. with water. Pipet 10 ml. of the *Standard Preparation* into a similar tube and proceed as directed in the *Assay* in the monograph for anisotropine methylbromide tablets, beginning with "To each tube add 1.0 ml. of 20% sulfuric acid . . ." but allowing the precipitate to stand 4 hours before centrifuging. Calculate the weight (in milligrams) of anisotropine methylbromide in the volume of elixir taken by the formula $0.1W \times A_u/A_s$, where W is the weight (in milligrams) of the anisotropine methylbromide reference standard taken. The amount of anisotropine methylbromide found is not less than 93.0% and not more than 107.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Anisotropine methylbromide[1] is a parasympatholytic agent which is used as a gastrointestinal

[1] Marketed as Valpin by Endo Laboratories, Inc., Richmond Hill, N. Y.

antispasmodic and visceral smooth muscle relaxant. It differs from atropine and most other ester antispasmodics in the substitution of an aliphatic for an aromatic side chain in the acid moiety of the ester. This substitution results in a compound which is highly resistant to hydrolysis. Under the conditions specified under *Purity Tests* in the monograph for anisotropine methylbromide, the volume of alkali consumed in the residual titration was zero so that the degree of saponification was nil. The melting range of the compound is not reproducible, but varies widely with the conditions of the test (initial temperature and rate of heating).

Identity Tests.—The inclusion of an ultraviolet absorption identity test is for the purpose of distinguishing anisotropine methylbromide from aromatic acid esters of tropine and its derivatives. The ultraviolet absorption spectra of atropine and homatropine derivatives show absorbance maxima in the vicinity of 251–252, 257–258, and 263–264 mμ due to the presence of tropate and mandelate moieties, respectively (1).

The identity test and the assay for anisotropine methylbromide in the tablets involve a similar procedure for the extraction and purification of the sample. If desired, the amounts called for in the assay may be increased proportionately so that the identity test and assay may be performed on the same treated tablet extract.

Quantitative Methods.—The assay for bromine (as bromide ion) determines the nonactive portion of the molecule but serves as a control on the purity of the bulk material. Analysis of anisotropine methylbromide by the indicator titration method gave an average value of 22.1 ± 0.0%[2] bromine (Br). Potentiometric titrations of anisotropine methylbromide (900 mg. in 50 ml. of water) by means of silver-calomel electrodes (the latter connected to the titration vessel with a saturated potassium nitrate–agar salt bridge), gave an average value of 22.1 ± 0.1%[2] bromine (Br). The calculated value for the bromine content is 22.05%.

The nonaqueous titration method for anisotropine methylbromide gave an average value of 100.0 ± 0.2%.[2] The indicator end point is very sharp and corresponds to a 200–225-mv. break in a potentiometric titration curve obtained under identical conditions.

The colorimetric reineckate method for the dosage forms is a variation of the assay in the U.S.P. XVI monograph for homatropine methylbromide tablets. Other variations of the method have appeared in the literature (2). The present method offers the convenience of carrying out precipitation, washing, and dilution in the same container. However, it suffers somewhat from loss of precision compared to the method in which the precipitate is transferred to a sintered-glass filter, washed, dissolved in acetone, and diluted to volume in a volumetric flask. The 15-ml. graduated centrifuge tubes were calibrated with water; only those found accurate at the 10.0-ml. mark were used. Analysis of commercial anisotropine methylbromide elixir by this method gave an average value of 95.3 ± 2.5%[2] of the labeled amount.

Prior to precipitating the reineckate the tablet assay requires the insertion of a wash step in order to remove interfering dye used to color the tablets. The red dye was found to be water-soluble in acid medium (absorbance maximum at about 550 mμ), but extractable from neutral medium with a chloroform–carbon tetrachloride mixture. A demonstration with fortified samples showed that the anisotropine methylbromide in the tablet samples survived the wash step completely. Analysis of commercial 10-mg. tablets gave an average value of 95.4 ± 2.4%[2] of the labeled amount.

[2] Maximum deviation from the mean value.

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Manufacture of Emulsions by Shock Cooling

By MORTON W. SCOTT*

A "shock cooling" technique was used in the preparation of five emulsion systems. The shock cooling was achieved by atomizing a heated blend of the emulsion phases in a laboratory model spray dryer operating with chilled inlet air. The test preparations included an o/w hand lotion, an o/w baby lotion, a w/o cold cream formulation, and an o/w cosmetic lotion containing suspended pigments and hydrophilic ointment U.S.P. The physical stability of the shock cooled products was equivalent to or improved over that of control materials processed by conventional slow cooling techniques. The results of this study suggest that the principle of shock cooling has value in large scale manufacture of emulsions.

EMULSION SYSTEMS containing waxes, fats, and other low melting solids are widely used as dermatological vehicles and in the formulation of numerous cosmetic creams and lotions. The products generally are prepared by the fusion process. Briefly, this involves emulsifying the molten oil phase with the aqueous phase at elevated temperature (approximately 65 to 75°), then slowly cooling the product to room temperature. During the cooling step, the emulsion is agitated continuously to insure uniform rates of congealing throughout the batch (1–4).

Slight variations in the conditions of manufacture can alter final emulsion characteristics significantly (1, 5). The rate at which the hot emulsion is cooled has been reported to be particularly critical (2, 6). Boylan, DeKay, and Banker have shown, for example, that products with increased thixotropy and decreased stability result as the rate of cooling is increased (7). Strianse noted that emulsion viscosity increased with increasing rates of cooling (8); others have reported that the reverse effects can occur (5).

It has been generally accepted that rapid cooling promotes the crystallization and separation of the higher melting components of the emulsion. This in turn results in products which are "grainy" and unstable (9, 10). Consequently, slow congealing techniques, although time consuming, laborious, and inefficient, are commonly employed in practice.

A reanalysis of the congealing process and of the results obtained with nonaqueous systems (11–14) suggested, however, that crystal growth and similar effects could not occur if the emulsions were subjected to instantaneous rates of cooling. The present study was undertaken, therefore, to evaluate the feasibility and potential applications of shock cooling techniques in the manufacture of emulsion systems.

EXPERIMENTAL

Materials.—Five emulsion products were studied in the present experiments. The test systems included hydrophilic ointment U.S.P., an o/w baby lotion, an o/w hand lotion, a o/w pigmented lotion (cosmetic make-up base), and a w/o cold cream formulation. The formulas for the latter four products are given in Table I.

All raw materials were U.S.P. or N.F. quality unless otherwise indicated.

Methods of Preparation.—Each emulsion was prepared by heating and melting the components of the oil phase together at approximately 65° and adding this phase to the preheated aqueous phase held at the same temperature.[1] The resulting emulsions were blended at this temperature for 5 minutes using a loop-type agitator rotating at low speed.

The hot emulsions were divided into two equal portions. The first portion was shock cooled. This was accomplished by spray congealing the emulsion in a Nerco-Niro laboratory model spray dryer[2] operating with the inlet air chilled to approximately 14°. To maintain the inlet air at this temperature, a supply of dry ice was placed at the mouth of the inlet duct and allowed to vaporize into the inlet air stream. The centrifugal wheel atomizer was run at full turbine air pressure (6 Kg. per cm.).[2] Liquid feed rates to the atomizer were maintained at 150–200 ml. per minute and resulted in outlet air temperatures of less than 20°. The products were collected by cleaning the walls of the spray dryer with a rubber spatula.

The remaining portion of each hot emulsion served as the control preparation and was congealed by the conventional procedures. This involved the slow

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[1] The order of addition was reversed for the baby lotion and cold cream.
[2] Nichols Engineering and Research, Inc., New York, N. Y.

TABLE I.—COMPOSITION OF TEST EMULSION SYSTEMS

Phase	Component	% by Wt. Baby Lotion	Hand Lotion	Pigmented Lotion	Cold Cream
Oil	Lanolin	1.00	...	...	...
	Cetyl alcohol	2.00	1.25	0.50	...
	Span 80[a]	2.10	...	...	...
	Tween 80[a]	4.90	...	...	...
	Liquid petrolatum, light	34.00	4.00	25.00	56.00
	Silicone 200 fluid[b]	5.00	...	...	...
	Glyceryl monostearate	...	0.50	0.50	...
	Stearic acid	...	1.00	...	...
	Atlas G-3694[a]	...	0.50	...	...
	Spermaceti	...	...	0.50	12.50
	White wax	...	...	...	12.00
Aqueous	Methylparaben	0.20	0.15	0.20	...
	Emcol E 607-S[c]	...	1.00	...	...
	Glycerin	...	1.00	...	...
	Propylparaben	...	...	0.05	...
	Sodium lauryl sulfate	...	...	0.63	...
	Titanium dioxide	...	...	12.00	...
	Cosmetic red oxide, A6205[d]	...	...	0.50	...
	Sodium borate	...	...	...	0.50
	Water *q.s.*	100.00	100.00	100.00	100.00

[a] Atlas Powder Co., Wilmington, Del. [b] 350 Centistoke viscosity, Dow Corning, Inc., Midland, Mich. [c] Emulsol Corp., Chicago, Ill. [d] Kohnstamm and Co., Inc., New York, N. Y.

cooling of the product to room temperature with continuous mixing applied by the loop-agitator. Approximately 1 hour was required to complete the preparation by this technique.

Evaluation.—Shock cooled products were compared with their controls immediately after preparation and periodically during a 2-month storage interval at 5, 37, 45°, and room temperature (27°). Samples were subjected also to freeze-thaw conditions for 1 week by cycling between −5° (16 hours) and 27° (8 hours). All products then were transferred to room temperature storage and observed during an additional 4-month interval.

Shock cooled materials and their controls were compared by microscopic, visual, and tactile evaluation. Overall physical characteristics, including phase separation, color, texture (graininess), and spreadability were observed routinely.

The rheological characteristics of each product when first prepared and after 6-month storage at room temperature were measured using an Epprecht viscometer.[3]

RESULTS AND DISCUSSION

Each of the five emulsion systems was prepared successfully by the shock cooling technique. No difficulties were experienced in congealing the emulsions in the spray dryer; the entire cooling operation was completed in less than 5 minutes.

Table II summarizes the characteristics of the shock cooled and control products. The shock cooled emulsions were smooth, free of graininess, lighter in color, and generally more uniform than the control materials.

Improved dispersion and a greater reduction in the particle size of the discontinuous phase was obtained by the shock cooling technique. This was particularly evident with the cold cream formulation. The majority of particles observed in the shock cooled product ranged between 2 and 7 μ in diameter. The emulsion prepared by the conventional slow cooling method showed particle sizes ranging from 10 to 40 μ. Since aliquots of the same hot emulsion were used in preparing both products, the changes in particle size necessarily occurred during the cooling operations. In the spray congealing technique, the hot emulsion was subjected to atomization and dispersed into droplets with small particle size. This, in effect, represented a form of homogenization and accounts for the reduced particle sizes found in the spray congealed preparation. The particles observed in the control product (cold cream) were not uniformly spherical, thereby suggesting that some size enlargement (due to crystallization or agglomeration) may have occurred during the slow cooling process. There were no microscopic or macroscopic indications that abnormal amounts of air had been trapped in the spray congealed products. Therefore, the lighter color of the shock cooled products was attributed to the decreased particle size.

In two of the five test systems (the hand lotion and the cold cream), the spray congealed products were less viscous than their controls. The situation was reversed with the pigmented lotion and the baby lotion. For the latter formulations, shock cooling also resulted in (qualitative) changes in the level of thixotropy. Hydrophilic ointment showed similar flow characteristics when made by either process.

These results are inconsistent with considerations of particle size and presently are not fully understood, particularly because of the rheological studies reported by others for similar products (7, 15). The short holding time involved in the shock cooling process led to a reduction in evaporation losses (of water) from the emulsions; this may account, in part, for the observed differences in product rheology.

Products prepared by spray congealing generally were more stable than their control preparations. This was particularly evident in the case of the three lotion products.[4] Except for the hand lotion (made by either process), all products were stable under freeze-thaw and 5°. Storage at 45° most clearly

[3] Manufactured by J. W. Fecker Division, American Optical Co., Pittsburgh, Pa.

[4] These preparations represented formula modifications of products with known and satisfactory stability. The emulsifier systems were altered deliberately to insure that the influences of shock cooling would be readily apparent.

TABLE II.—CHARACTERISTICS OF SHOCK COOLED AND CONTROL PREPARATIONS

Product	Cooling Procedure[a]	Particle Size,[b] μ	Color	Texture and Appearance[c]	Rheology[d]	Separation[e] 45°	37°	RT
Baby lotion	SC	2–5	Off-white	U	MV, NT	3	2[f]	0[f]
	C	5–10	Tan	NU	ST	3	3[f]	0[f]
Hand lotion	SC	2–5	White	U	LV, NT	1	1	1
	C	2–10	White, off-white	U	NT	2	2	2
Pigmented lotion	SC	2–10	Pink	U	MV, ST	2	1	0
	C	5–30	Dark pink	SA	NT	3	2	2
Cold cream	SC	2–7	White	U	LV, MT	1	0	0
	C	10–40	White, off-white	NU	MT	1	0	0
Hydrophilic ointment	SC	7–15	White	U	MT	1	0	0
	C	10–25	White	U	MT	1	0	0

[a] SC = Shock cooled; C = control (cooled slowly). [b] Particle size range included approximately 75% of total particles counted. [c] U = Uniform and smooth; NU = nonuniform; SA = "streaky" application. [d] MV = More viscous than control; LV = less viscous than control; NT = negligible thixotropy; ST = slight thixotropy; MT = marked thixotropy. [e] Phase separation observed after 2 months at 45 and 37° and after 6 months at room temperature, respectively. 0 = No phase separation; 1 = trace indications of separation; 2 = moderate separation; 3 = severe separation. [f] Mold growth occurred at these conditions.

demonstrated the comparative improvements in stability obtained in the shock cooled products. The degree of phase separation observed after long term storage at 45°, 37°, and room temperature served as the final measure of relative stability. These observations are summarized in Table II.

The rate of cooling established in the spray congealing process was approximately 13° per 0.1 second.[5] This rapidity of chilling precludes extensive crystal growth, and it is, therefore, not surprising that smooth and grain-free products were obtained. In fact, it appears that the slow cooling rates commonly used in preparing emulsion systems would promote rather than inhibit crystallization and the separation of components.[6] By applying sufficient agitation to the system, however, nucleation rather than crystal growth can be induced. The required rates of agitation clearly would be governed by the congealing rates employed and vice versa. These considerations help explain the critical relationships which exist in emulsion manufacture between the rates of cooling and the intensities of agitation (1, 5).

The results of the present study showed that stable and pharmaceutically elegant emulsions can be prepared by shock cooling. The spray congealing procedure used to demonstrate the feasibility of this approach was fast, simple to control, and reproducible; nevertheless, the equipment requires modification for large scale manufacturing operations. The necessity for modification is primarily because the conventional spray dryer, as used in this study, is not designed for use with liquid and semisolid (outlet) products. For example, it was difficult to clean the unit, and the accumulation of product on the chamber walls and in the outlet air duct work limited the amount of product that could be processed in a single run. The results suggest, however, that other spray congealing units (more appropriately designed for emulsion applications) or the use of other shock cooling techniques which minimize particle growth during the cooling step[7] will be valuable in commercial applications.

[5] Approximately: $\frac{175 \text{ Gm. (feed)}}{\text{minute}} \times \frac{(65° - 20°)}{\text{Gm.}} \times \frac{1 \text{ minute}}{60 \text{ seconds}} = \frac{130°}{\text{second}}$.

[6] It is recognized that controlled degrees of crystallization are desired in some cosmetics in order to impart "pearlescent" and other characteristics.

[7] Such as the use of chilled ointment mills operating at close clearance.

CONCLUSIONS

The results of this study demonstrate the feasibility of applying shock cooling techniques in the preparation of emulsified creams and lotions. The shock cooled products were prepared by spray congealing and compared favorably in all respects with the products made by conventional slow cooling procedures. The improved stability and texture characteristics and the absence of "graininess" in the spray congealed materials was noteworthy, particularly because of the generally accepted impression that rapid cooling is detrimental to emulsion systems. Although the spray congealing procedure used in the present experiments was simple to control, reproducible, and resulted in very high cooling rates, its usefulness for commercial applications is limited. These experiments do suggest, however, that other shock cooling procedures, more appropriately designed for large scale manufacturing operations, hold promise for future application.

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Lined and Unlined Rubber Stoppers for Multiple-Dose Vial Solutions I

Sorption of Preservatives and Leaching of Extractives

By LEON LACHMAN, PRAVIN B. SHETH, and TIBOR URBANYI

The protective action of epoxy lining on rubber stoppers of natural, neoprene, and butyl rubber was evaluated against unlined stoppers of the same composition for sorption of preservative from vial solutions and extractives leached from closures into the solution. For the preservatives tested—namely, *p*-chloro-β-phenylethyl alcohol and its nonchlorinated analog, phenylethyl alcohol—no protective action against sorption was exhibited by the lined closures. The solutions used in the extractives study were water, 10 per cent ethanol, 50 per cent polyethylene glycol 300, 50 per cent *N,N*-dimethylacetamide, and 2 per cent benzyl alcohol. The lining on the closures was found to afford protection against leaching. For both the sorption and extractives studies, the butyl rubber closures exhibited optimal stability.

RECENT PUBLICATIONS from this laboratory and others have shown that rubber closures used for multiple-dose vial solutions can be detrimental to the stability of the vial contents through sorption of materials from solution (1–7) or through the action of closure extractives on the solution (7–10). To alleviate these inadequacies, one approach used by certain rubber stopper manufacturers is to line the closure surface that will come in contact with the vial solution with a lacquer containing polymeric materials as its major component.

Lacquered stoppers have been in use as closures for multiple-dose vial solutions for about 5 years. However, reports relative to the effectiveness of the linings against sorption of materials from solution into the rubber and leaching of extractives from the rubber by the solution appear to be lacking.

Accordingly, this study was initiated to determine the degree of protection against sorption and leaching contributed to the rubber stopper by an epoxy lining. Lined and unlined natural, neoprene, and butyl rubber stoppers were used in the evaluation. The sorption characteristics were tested with aqueous solutions of the preservatives, phenylethyl alcohol and *p*-chloro-β-phenylethyl alcohol, stored in multiple-dose vials. The leaching tendencies of 10% ethanol, 50% polyethylene glycol 300, 50% *N,N*-dimethylacetamide, 2% benzyl alcohol, and water on the stoppers were evaluated. Identification of the extractives leached from the stoppers was performed.

EXPERIMENTAL

Materials.—*p*-Chloro-β-phenylethyl alcohol, Ciba, b.p. 80–83°; phenylethyl alcohol, Eastman Organic Chemicals; thiazole type accelerator; substituted butylphenol, diphenylamine, substituted carbamic acid, thiuram, thiazole dicarbamate reaction product, imidazoline type accelerator, from rubber manufacturer were employed.

Polyethylene glycol 300, Union Carbide; *N,N*-dimethylacetamide, E. I. du Pont; benzyl alcohol, reagent grade, Fisher Scientific Co., double distilled at Ciba; 0.275*M* citric acid-sodium phosphate buffer of pH 4.0; natural rubber, neoprene rubber, and butyl rubber stoppers, epoxy lined and unlined, 13 mm., West Co.; U.S.P. type 1, 10-ml. clear ampuls and vials, Kimble Glass Co.; three-piece aluminum caps for vials, West Co., No. 13–30 were also utilized.

Equipment.—Beckman model DU spectrophotometer, Cary model No. 11 recording spectrophotometer, Beckman model G pH meter, and microdistillation apparatus (as described in an earlier report) (7) were employed.

Preparation of Ampuls, Vials, and Stoppers.—These units were washed and dried in accordance with the methods described in a previous publication from our laboratories (6).

Sorption of Preservative from Solution.—To evaluate the effectiveness of the epoxy lining against sorption of antibacterial preservatives from vial solutions, lined and unlined closures of the same basic rubber formulations were used. The preservative solutions used in the evaluation were 0.3% *p*-chloro-β-phenylethyl alcohol and 0.5% phenylethyl alcohol prepared on a weight-to-volume basis with water for injection buffered to a pH of 4.0. Each preservative solution was filtered through a medium porosity sintered-glass filter before filling into 10-ml. clear glass ampuls and vials. The ampuls were closed by customary pull sealing technique with an oxygen-gas flame. The vials of each preservative solution were divided into six equal parts and stoppered with epoxy lined and unlined natural, neoprene, and butyl rubber stoppers. The stoppered vials were then sealed with three-piece aluminum caps at a constant head pressure of 50 p.s.i. with a hydraulic capper. The preservative solutions in the ampuls and vials were placed into constant temperature cabinets regulated at 25, 40, 50, and 60 ± 1.5°.

Half of the vials were stored upright and half

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TABLE I.—CLOSURE COMPOSITION

Natural Rubber	Neoprene Rubber	Butyl Rubber
Natural Rubber[a]	Neoprene rubber[b]	Butyl rubber[c]
Zinc oxide	Natural rubber	Aluminum silicate
Stearic acid	Aluminum silicate	Zinc oxide
Microcrystalline wax	Zinc sulfide	Iron oxide
Sulfur	Barium sulfide	Stearic acid
Thiazole-type accelerator	Iron oxide	Sulfur
Thiuram-type accelerator	Zinc oxide	Thiazole-type accelerator
Substituted butylphenol	Magnesium oxide	Substituted carbamic acid
...	Stearic acid	Butoxy phosphate
...	Light mineral oil	...
...	Diphenylamine	...
...	Imidazoline-type accelerator	...
...	Thiazole-type accelerator	...

[a] Rubber content: 95.8% of total composition. [b] Rubber content: 43% of total composition—38.3% neoprene and 4.6% natural. [c] Rubber content: 48.1% of total composition.

inverted. At designated time intervals, samples were withdrawn and evaluated for loss in preservative content.

Rubber Closure Extractives.—To determine the effectiveness of the epoxy lining on the three composition closures against leaching of unreacted materials and reaction products from the rubber stoppers into the vial solution, several solvents used in practice for the formulation of injectable preparations were employed. These include water, 10% ethanol, 50% polyethylene glycol 300, 50% *N,N*-dimethylacetamide, and 2% benzyl alcohol. These solvents were filled into 10-ml. clear glass vials; each set of vials for a particular solvent was divided into six parts. These were then stoppered with the unlined and epoxy-lined natural, neoprene, and butyl closures. The stoppered vials were then sealed with three-piece aluminum caps at a constant sealing head pressure of 50 p.s.i. with a hydraulic operated capper. Half of each of these vials were autoclaved in an inverted position at 115° for 6 hours at 10 p.s.i., the other half were autoclaved in an upright position at 115° for 30 minutes at 10 p.s.i. After treatment at these two conditions, the solutions in the vials were scanned spectrophotometrically to obtain the ultraviolet absorption curve of the rubber extractives.

Identification of Extractives.—In order to obtain a qualitative identification of the rubber closure extractives, the accelerators, activators, antioxidants, and possible reaction products for the three rubber compositions used in this study were obtained. These materials were dissolved in the solvents used for the extractives study and their ultraviolet absorption spectra determined.

Analytical Method.—*Phenylethyl Alcohol.*—The concentration of preservative was determined by pipeting 3 ml. of solution into a microsteam distillation apparatus and the sample steam distilled to 25 ml. The absorbance was measured at 257 mμ where A (1% 1 cm.) = 18.

p-Chloro-β-phenylethyl Alcohol.—Residual preservative was determined by pipeting 2 ml. of solution into the microsteam distillation apparatus and the sample steam distilled to 25 ml. Absorbance was measured at 267 mμ where A (1% 1 cm.) = 22.

TABLE II.—EFFECTIVENESS OF EPOXY LINING ON NEOPRENE RUBBER STOPPERS AGAINST SORPTION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time, Days	Temp., 25° C. Upright L[a]	Upright P[b]	Inverted L	Inverted P	A[c]	Temp., 40° C. Upright L	Upright P	Inverted L	Inverted P	A
0	..	..	..	..	100	..	..	..	..	...
2	96	96	91	91	100	96	96	87	87	98
7	..	..	..	..	...	..	..	..	..	...
9	95	93	81	81	...	91	89	74	75	97
11	95	95	81	79	97	91	92	74	75	97
14	93	91	79	79	99	89	89	74	73	99
28	91	92	76	73	101	85	85	68	68	99
43	92	91	73	74	101	79	80	65	63	99
56	88	89	83	72	99	79	79	61	62	99
84	86	87	69	70	101	79	79	61	62	99

Time, Days	Temp., 50° C. Upright L	Upright P	Inverted L	Inverted P	A	Temp., 60° C. Upright L	Upright P	Inverted L	Inverted P	A
2	96	96	85	86	99	96	96	85	85	98
7	..	..	..	..	...	..	..	..	..	...
9	86	88	72	75	...	83	83	74	72	...
11	86	88	70	72	99	83	83	70	70	98
14	82	84	72	71	99	81	81	71	70	99
28	80	78	63	62	99	72	72	61	61	99
43	71	72	58	59	98	65	66	56	57	101
56	70	69	56	57	100	61	62	53	53	101
84	..	61	54	54	102	56	56	50	51	104

[a] L = Epoxy lined. [b] P = Plain. [c] A = Ampul.

TABLE III.—EFFECTIVENESS OF EPOXY LINING ON NATURAL RUBBER STOPPERS AGAINST SORPTION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time, Days	Temp., 25° C. Upright L[a]	Upright P[b]	Inverted L	Inverted P	A[c]	Temp., 40° C. Upright L	Upright P	Inverted L	Inverted P	A
0	..	..	..	..	100	..	..	..	..	...
3	97	97	91	93	99	96	96	88	89	99
5	97	97	89	92	101	94	95	82	89	99
10	97	97	88	91	103	94	94	81	84	99
12	96	97	88	91	101	93	94	81	84	100
14	97	96	87	91	101	93	93	80	82	100
28	94	96	82	87	101	87	88	75	78	100
34	93	94	83	86	100	84	86	74	76	100
56	93	94	83	79	102	87	90	74	75	101
84	91	91	78	80	99	77	78	70	75	100

Time, Days	Temp., 50° C. Upright L	Upright P	Inverted L	Inverted P	A	Temp., 60° C. Upright L	Upright P	Inverted L	Inverted P	A
3	94	97	86	88	98	92	94	84	86	98
5	94	94	81	86	100	90	93	80	82	100
10	91	91	78	80	99	86	88	76	80	100
12	91	93	79	81	97	84	87	75	78	100
14	89	89	76	80	100	82	84	73	76	100
28	82	84	74	75	100	74	79	68	74	100
34	79	79	69	74	100	74	73	68	68	100
56	75	78	69	72	101	69	71	64	65	101
84	70	73	68	68	101	63	62	59	63	101

[a] L = Epoxy lined. [b] P = Plain. [c] A = Ampul.

RESULTS AND DISCUSSION

The effectiveness of the epoxy lining on natural, neoprene, and butyl rubber stoppers against sorption of antibacterial preservatives from solution and leaching of extractives from the stoppers by several solvents used in injectable formulations was investigated. The formulations for the three different rubber stoppers used in this investigation are not the same as used in our previous studies (6, 7) because of required modifications to permit the application of the epoxy lacquer. The composition and per cent rubber content of these stoppers is presented in Table I.

Sorption Studies.—The sorption tendencies of the epoxy lined and unlined rubber stoppers were evaluated with vial solutions of phenylethyl alcohol and *p*-chloro-β-phenylethyl alcohol buffered to a pH of 4.0. These two preservatives were chosen for this study since their partition tendencies between rubber and solution are substantially different, with the chlorinated analog favoring rubber to a much larger extent than phenylethyl alcohol (7).

TABLE IV.—EFFECTIVENESS OF EPOXY LINING ON BUTYL RUBBER STOPPERS AGAINST SORPTION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time, Days	Temp., 25° C. Upright L[a]	Upright P[b]	Inverted L	Inverted P	A[d]	Temp., 40° C. Upright L	Upright P	Inverted L	Inverted P	A
0	..	...	..	...	100	..	..	..	..	...
3	97	100	97	100	100	96	99	97	99	100
7	99	100	96	98	100	98	98	96	98	100
8	97	100	96	99	100	97	99	94	98	100
10	98	99	97	99	100	97	98	95	97	100
13	97	99	95	98	101	96	99	95	97	100
16	96	97	95	98	100	93	96	93	95	99
28	97	98	94	99	99	94	94	93	97	99
42	97	98	95	99	100	93	95	93	96	100
50	95	96	93	97	100	92	94	91	96	99
84	95	97	95	97	100	91	94	93	99	99

Time, Days	Temp., 50° C. Upright L	Upright P	Inverted L	Inverted P	PC[c]	A	Temp., 60° C. Upright L	Upright P	Inverted L	Inverted P	PC	A
3	96	99	94	99	...	101	96	97	96	97	..	100
7	97	97	95	97	...	100	95	96	95	96	..	100
8	95	97	94	97	...	99	95	96	93	96	..	98
10	96	97	95	97	...	100	95	96	95	96	..	100
13	95	96	93	97	...	100	95	96	93	96	..	99
16	94	95	93	97	...	99	93	96	92	95	..	97
28	94	94	93	98	...	99	92	94	92	97	..	100
42	93	96	93	100	98	99	93	95	92	100	97	100
50	94	91	91	103	97	100	90	93	91	107	95	99
84	93	94	91	103	96	100	90	93	91	104	96	101

[a] L = Epoxy lined. [b] P = Plain. [c] PC = Plain corrected. [d] A = Ampul.

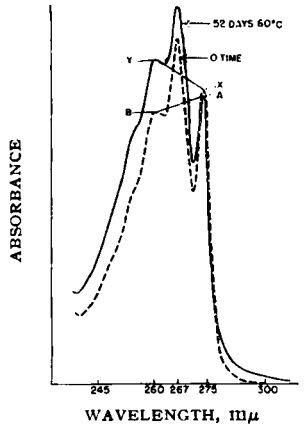


Fig. 1.—Plots showing the influence of butyl rubber extractives on the absorption characteristics of *p*-chloro-β-phenylethyl alcohol.

The data in Tables II–IV summarize the sorption tendencies of the lined and unlined closures for *p*-chloro-β-phenylethyl alcohol from vial solutions stored at several temperature conditions. It is evident from the results in these tables that the epoxy lining imparts *no* protective action against preservative sorption by the three different composition closures for vials stored upright or inverted. Only for the neoprene closures do the vial solutions stored in an inverted position show greater loss of preservative than for the vials stored upright. The vial solutions stoppered with the neoprene closures showed the greatest loss in preservative concentration, while the vials stoppered with the butyl closures showed the least. The vials stoppered with the natural rubber closures showed losses in preservative content between that found with the other two closures. Since there was no diminution of preservative concentration in the ampul solutions, the loss in the vial solutions must be attributed to closure effect.

The data for the butyl closures given in Table IV appear to show that the vials stoppered with unlined closures are increasing in preservative concentration with time, instead of decreasing or remaining at initial concentration. The same stoppers having the epoxy lining show the expected decrease in preservative concentration with storage. This would indicate that an extractive from the unlined closure is entering the vial solutions and influencing the analysis for residual *p*-chloro-β-phenylethyl alcohol. This is not surprising, in light of our recent report (7), where we had experienced this situation with benzyl alcohol.

The effect of closure extractive on the absorption characteristics of *p*-chloro-β-phenylethyl alcohol in a buffered solution stored in vials stoppered with the butyl rubber closures is shown in Fig. 1. By comparing the curve for zero time and storage at 60° for 52 days, it is obvious that the absorbance difference between 260 and 275 mμ changes greatly. This effect at 260 and 275 mμ causes a concurrent effect at 267 mμ, the absorption maximum used for the preservative analysis.

The equations developed to correct for the interference of rubber closure extractive on the analysis for residual *p*-chloro-β-phenylethyl alcohol were

$$C = D - [(X - A) + (Y - X) + K]$$

where X is lower than Y

$$C = D - [(X - A) - (X - Y) + K]$$

where X is higher than Y and C = corrected absorbance at 267 mμ; D = uncorrected absorbance at 267 mμ; A = absorbance at 275 mμ for standard solution; B = absorbance at 260 mμ for standard solution; X = absorbance at 275 mμ for test sample; Y = absorbance at 260 mμ for test sample; and $K = A - B = 0.09$.

Using the above equation, the data for the butyl rubber at the elevated temperatures were recalculated and are presented as corrected values in Table IV. These data now represent the true loss in concentration of preservative due to the closure effect.

TABLE V.—EFFECTIVENESS OF EPOXY LINING ON NEOPRENE RUBBER STOPPERS AGAINST SORPTION OF PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time, Days	Temp., 25° C. Upright L[a]	Upright P[b]	Inverted L	Inverted P	A[c]	Temp., 40° C. Upright L	Upright P	Inverted L	Inverted P	A
0	..	..	..	..	100	..	..	..	..	..
3	97	97	97	97	99	97	96	95	95	99
5	99	98	97	99	101	97	97	96	97	100
7	97	97	96	98	100	97	95	97	95	100
10	96	96	95	95	100	94	94	92	93	99
14	95	96	94	95	100	93	93	93	93	99
28	95	95	94	94	99	91	93	90	92	100
42	90	94	93	94	100	92	89	89	89	99
56	94	93	92	93	100	88	89	88	88	99
84	93	93	91	92	97	88	87	86	86	97

Time, Days	Temp., 50° C. Upright L	Upright P	Inverted L	Inverted P	A	Temp., 60° C. Upright L	Upright P	Inverted L	Inverted P	A
3	97	96	95	95	99	95	95	94	95	99
5	97	95	95	95	100	95	95	93	94	100
7	95	96	94	95	98	94	94	93	94	100
10	93	93	93	92	99	93	93	89	89	100
14	92	90	90	90	99	90	90	88	88	99
28	89	89	89	88	100	87	87	86	86	100
42	86	87	86	86	100	85	84	84	84	100
56	85	88	85	86	99	83	83	81	82	101
84	84	82	82	80	100	78	80	79	80	103

[a] L = Epoxy lined. [b] P = Plain. [c] A = Ampul.

TABLE VI.– EFFECTIVENESS OF EPOXY LINING ON NATURAL RUBBER STOPPERS AGAINST SORPTION OF PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time, Days	Temp., 25° C. Upright L[a]	Upright P[b]	Inverted L	Inverted P	A[c]	Temp., 40° C. Upright L	Upright P	Inverted L	Inverted P	A
0	..	..	..	..	100	..	..	..	..	...
3	98	98	98	98	100	97	97	97	97	99
7	98	98	98	98	100	97	97	98	96	99
10	98	98	97	97	100	96	97	95	96	100
14	98	98	98	98	100	96	96	96	96	100
28	97	96	96	97	100	94	94	94	95	99
42	96	96	95	96	99	94	92	90	93	98
56	96	96	95	96	100	93	91	92	91	99

Time, Days	Temp., 50° C. Upright L	Upright P	Inverted L	Inverted P	A	Temp., 60° C. Upright L	Upright P	Inverted L	Inverted P	A
3	98	96	96	96	99	96	98	95	96	98
7	96	96	95	96	99	94	94	94	95	98
10	95	95	94	96	100	94	94	93	94	100
14	96	96	94	95	100	94	94	94	94	101
28	93	96	92	92	100	89	89	88	89	100
42	89	90	87	88	98	84	85	84	83	99
56	88	88	86	87	102	81	82	81	83	101

[a] L = Epoxy lined. [b] P = Plain. [c] A = Ampul.

A comparison of phenylethyl alcohol loss from vial solutions stoppered with the epoxy lined and unlined closures is presented in Tables V–VII. It is evident from the results shown in the tables that, as in the case for the chlorinated analog, the epoxy lining on the closures affords no protection against sorption of phenylethyl alcohol from the vial solutions. For this preservative, both the natural and neoprene stoppers cause about the same loss of phenylethyl alcohol from solution and, again, the butyl rubber closure shows the least tendency to absorb the preservative. Compared to the chlorinated analog, phenylethyl alcohol is absorbed by the closures to a lesser degree. This can be explained by the relative tendencies of these two preservatives to distribute between rubber and water, the chlorinated analog favoring rubber to a greater extent than phenylethyl alcohol (7).

A situation was observed for this preservative similar to that which existed for *p*-chloro-β-phenylethyl alcohol with vials stoppered with butyl rubber closures. Table VII shows that the vial solutions (stored at the elevated temperature conditions and stoppered with the unlined butyl rubber closures) exhibited an increase in assay for residual preservative with storage, instead of the expected decrease or maintenance of initial concentration. This is indicated by absorption curves presented in Fig. 2. Here again the extractives being leached from the rubber stoppers by the preservative solution in the vials were interfering with the analysis for preservative content. Using the equations derived for *p*-chloro-β-phenylethyl alcohol, corrections were made for the influence of closure extractives on residual preservative assays.

For this preservative, *C* and *D* of the equation

TABLE VII.—EFFECTIVENESS OF EPOXY LINING ON BUTYL RUBBER STOPPERS AGAINST SORPTION OF PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time, Days	Temp., 25° C. Upright L[a]	Upright P[b]	Inverted L	Inverted P	A[d]	Temp., 40° C. Upright L	Upright P	Inverted L	Inverted P	PC[c]	A
0	..	..	...	..	100	..	..	..	...	..	..
2	98	98	98	99	98	98	98	98	98	..	99
5	99	99	99	99	99	98	99	98	98	..	99
8	97	99	97	98	99	98	99	97	98	..	97
13	99	99	99	99	99	99	99	98	99	..	99
15	99	98	99	99	99	99	98	98	99	..	99
28	99	99	99	99	100	99	99	98	99	..	99
42	98	99	99	99	100	98	99	98	101	98	99
56	98	99	101	99	99	99	99	99	99	..	99
84	..	..	...	..	...	..	..	..	...	..	..

Time, Days	Temp., 50° C. Upright L	Upright P	Inverted L	Inverted P	PC	A	Temp., 60° C. Upright L	Upright P	Inverted L	Inverted P	PC	A
2	98	98	97	98	..	99	98	99	98	99	..	98
5	99	100	98	99	..	99	97	99	98	97	..	99
8	97	97	97	98	..	100	97	99	97	98	..	99
13	99	99	98	99	..	99	99	99	97	102	98	99
15	99	99	99	99	..	99	97	99	98	101	99	99
28	97	98	98	101	98	99	97	98	97	112	95	99
42	98	98	97	110	96	99	98	99	97	110	94	99
56	98	98	99	99	..	99	99	99	99	115	95	100
84	..	...	..	...	..	...	..	..	..	...	..	...

[a] L = Epoxy lined. [b] P = Plain. [c] PC = Plain corrected. [d] A = Ampul.

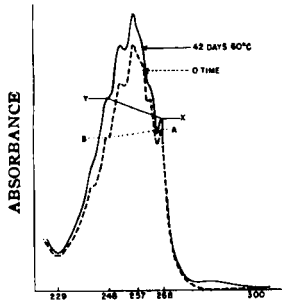


Fig. 2.—Plots showing the influence of butyl rubber extractives on the absorption characteristics of phenylethyl alcohol.

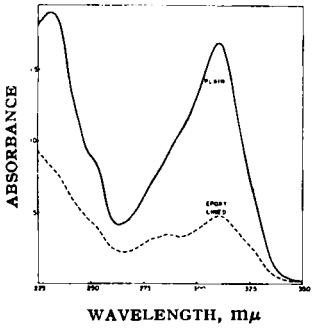


Fig. 3.—Ultraviolet absorption curve of leached extractives. Rubber, natural; solvent, water; storage condition, 6 hours at 115° C., vial inverted.

were measured at 257 mμ, *A* at 268 mμ, and *B* at 248 mμ, and *K* had a value of 0.06. The recalculated data showing the true loss of phenylethyl alcohol are presented in Table VII as the corrected values. These data are now representative of the loss in preservative concentration due to closure effect.

These findings regarding the ineffectiveness of the epoxy lining on rubber stoppers toward preservative sorption from solution were recently substantiated in a report by Garnet (11), who studied the effect of closures on antiseptics in biological products. He found that lacquered rubber stoppers did not solve the problem of loss of thiomersol from vaccine solutions. He reasoned that this lacquer was ineffective because the lacquer membrane was semipermeable and too fragile to withstand the slight distortion brought about by manipulation.

Leaching of Extractives.—The influence of several solvents commonly employed in the preparation of injectable solutions on the leaching of extractives from epoxy lined and unlined stoppers when used on multiple-dose vials was also investigated.

A representative ultraviolet absorption spectrum for the extractives found in the vial solutions containing water, and stoppered with natural rubber closures, is given in Fig. 3. The curves in this figure indicate that considerable absorbance is present and more than one peak in the curve exists. For each solvent the lined closures gave considerable protection against leaching of extractives as evidenced by a two to threefold decrease in absorbance given by the solvents from the vials stoppered with the lined closures compared with the solvents from vials stoppered with unlined closures. This is clearly shown in Table VIII, which summarizes the absorbance values at the peaks in the curves for the extractives found in the solvents from the three rubber stopper compositions tested. It is evident from the data in the table that the protection afforded by the epoxy lining on the neoprene stoppers is considerably less than that found for the natural rubber.

The butyl rubber closures exhibit the least tendency for extractives to be leached into the solvents.

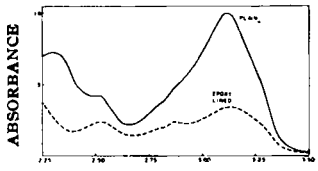


Fig. 4.—Plot showing ultraviolet absorption spectra of extractives leached from the closures after retreatment. Rubber, natural; solvent, water; storage condition, 3 hours at 115° C., vial inverted, second treatment.

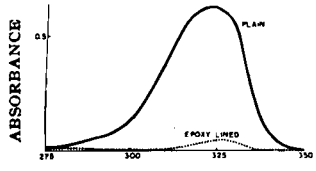


Fig. 5.—Ultraviolet absorption spectra of extractives leached from the rubber stopper after autoclaving for 30 minutes at 115° C., 10 p.s.i. Rubber, natural; solvent, *N,N*-dimethyl acetamide (50%); storage condition, 30 minutes at 115° C. upright.

TABLE VIII.—ABSORBANCE VALUES OF EXTRACTIVES LEACHED BY SEVERAL SOLVENTS IN 10-ml. VIALS FROM EPOXY LINED AND UNLINED RUBBER STOPPERS AFTER AUTOCLAVING THE VIALS IN AN INVERTED POSITION AT 115° C. FOR 6 HOURS

Solvent	Neoprene			Natural			Butyl		
	Wavelength, mμ	Absorbance P	Absorbance L	Wavelength, mμ	Absorbance P	Absorbance L	Wavelength, mμ	Absorbance P	Absorbance L
Water	232	2.01	1.59	310	1.59	0.52	...	...	...
				232	1.38	0.60			
Ethanol, 10%	232	2.30	1.85	312	2.49	0.73	...	...	...
Benzyl alcohol, 2%	...	...	...	312	2.36	0.71	...	...	...
PEG 300, 50%	232	1.81	1.40	325	1.26	0.44	250	0.23	0.26
	256	0.67	0.62	...	...	...	...	...	...
	275	0.55	0.49	...	...	...	...	...	...
N,N-Dimethylacetamide, 50%	275	1.39	1.14	325	3.80	1.52	280	...	0.08
				282	0.99	0.43	...	...	...

TABLE IX.—ABSORBANCE VALUES OF EXTRACTIVES LEACHED BY SEVERAL SOLVENTS IN 10-ml. VIALS FROM EPOXY LINED AND UNLINED RUBBER STOPPERS AFTER AUTOCLAVING THE VIALS IN AN UPRIGHT POSITION AT 115° C. FOR 30 MINUTES

Solvent	Neoprene			Natural			Butyl		
	Wavelength, mμ	Absorbance P	L	Wavelength, mμ	Absorbance P	L	Wavelength, mμ	Absorbance P	L
Water	232	0.60	0.30	...	...	...	...	...	...
Ethanol, 10%	232	0.76	0.51	230	0.09	0.11	...	...	...
Benzyl alcohol, 2%	...	...	...	312	0.07	0.03	...	...	...
PEG 300, 50%	275	0.03	0.03	325	0.16	0.04	...	...	...
N,N-Dimethylacetamide, 50%	...	...	...	325	0.31	0.02	...	...	...

In fact, with four out of the five solvents investigated, essentially no extractives were leached from the rubber. Only with the 50% polyethylene glycol 300 solvent is there significant extractive leached from the closure. With this solvent the same amount of extractive is in the vial solutions stoppered with the lined and unlined closures.

Treatment of the natural rubber closures used in the 6-hour autoclave experiment with water for injection for an additional 3 hours under the same temperature condition resulted in additional extractives coming into the water as shown by the absorption curves in Fig. 4. This illustrates the presence of considerable extractives in the rubber available for leaching by the solutions in the vials.

To determine whether normal autoclaving conditions cause extractives to be leached from the closures, lined and unlined stoppers were used with the vial solutions containing the solvents and autoclaved in an upright position at 115°, 10 p.s.i. for 30 minutes. A representative ultraviolet absorption curve for the extractive found in the vial solutions containing 50% *N,N*-dimethylacetamide and stoppered with natural rubber closures is shown in Fig. 5. The data in Table IX summarize the results obtained with the three closures and several solvents.

The natural rubber stoppers exhibit extractives in four out of five solvents, the neoprene stoppers in three out of five solvents, and the butyl rubber stoppers exhibit no extractives in any of the five solvents. Here, as under the more drastic testing conditions, the epoxy lining affords greater protection against leaching for natural rubber than neoprene rubber.

Although the epoxy lining employed for the three closures was the same, it is interesting that the lining afforded greater protection to the natural than the neoprene rubber stoppers against the leaching of extractives by the solvents used in this study. This may be explained on the basis that after the lacquer is applied to the rubber stoppers they require a curing period at elevated temperatures. During this period, it is possible for ingredients in the rubber formulation to enter the lining and subsequently influence the properties of the lining on the closures. Consequently, although the lacquer applied on the three different rubber stoppers is the same, the lining that results after curing of the lacquer on the rubber may possess different properties, depending on what materials migrated into the lining from the stopper composition.

Identification of Extractives.—In an attempt to identify qualitatively the extractives leached from the three composition closures by the several solvents, the formulation of these closures was studied to determine which ingredients would tend

TABLE X.—ULTRAVIOLET ABSORPTION MAXIMA OF RUBBER CLOSURE INGREDIENTS

Rubber Stopper	Wavelength, mμ				
	Water	10% Ethanol	2% Benzyl Alcohol	50% *N,N*-Dimethyl Acetamide	50% Polyethylene Glycol 300
Neoprene					
1 Imidazoline-type accelerator	232	232	...	...	232
2 Thiazole-type accelerator	...	...	312	320	255
				275	320
					275
3 Diphenylamine antioxidant	...	...	...	285	285
Natural					
1 Thiazole accelerator	312	312	312	325	257
	232	232	...	...	325
	...	...	...	...	230
2 Thiuram accelerator	275	275	275	275	275
3 Substituted butyl phenol antioxidant	...	...	282	282	282
Butyl					
1 Thiazole-type accelerator	312	312	312	325	257
	232	232	...	...	325
	...	...	...	...	230
2 Substituted carbamic acid accelerator	282	282	282	282	277
	258	258	...	...	...
3 Thiazole dicarbamate reaction product	...	...	...	294	...
	...	...	...	282	...
	...	...	...	275	...

to give absorption in the ultraviolet region of the spectrum. Samples of these ingredients were obtained from the rubber stopper manufacturer, and ultraviolet absorption spectra were obtained on these ingedients in the solvents studied. The absorption peaks obtained are summarized in Table X. With this information available, it was possible to identify the ingredient or ingredients being extracted by the solvents in contact with the rubber closures used on the vials.

For the neoprene closures, the material responsible for the absorption at 232 mμ was the primary accelerator (imidazoline type) in the rubber formula. The agent responsible for absorption at 256 and 275 mμ is the secondary accelerator (thiazole type).

In the case of the natural rubber stoppers, the absorption at 230–232, 312, and 325 mμ is due to the primary accelerator (thiazole type). The absorption at 282 mμ is caused by the antioxidant used in the rubber formulation, a substituted butyl phenol.

For the butyl rubber stoppers, the absorbance at 250 mμ is caused by the secondary accelerator (thiazole type). The absorbance at 280 mμ is caused by the primary accelerator, a substituted carbamic acid.

It is important to realize that in addition to the extractives that have been identified, there may be others present in the solvents. These would be materials that do not give an absorption curve in the ultraviolet region of the spectrum.

From the results obtained in this investigation, it is evident that lacquered closures are ineffective in preventing the sorption of materials from solution, are partially effective in preventing extractives from being leached from the rubber by solvents used in the formulation of parenteral solutions. In addition, it appears that the effectiveness of the epoxy lining against leaching of extractives may be dependent upon the rubber composition on which the lining is applied.

Appreciable extractives can be leached from the vial stoppers under normal autoclaving conditions. Subsequent storage of these vials at room temperature for the shelf-life of pharmaceuticals, which nowadays is considered to be approximately 3 years, would no doubt cause further leaching of extractives into solution. Since, for the most part, the materials extracted from the closures by the vial solutions are reactive chemicals, they can cause serious stability, toxicity, and assay problems with the vial contents. Accordingly, before selecting a rubber stopper composition for a vial solution, the closure must be evaluated as to its sorption and extractive characteristics with the solution with which it will be used. This is now of particular importance in light of the proposed regulations on drugs issued by the Food and Drug Administration on February 14, 1963 (12), which state under Section 133.13b that pharmaceutical manufacturers "make adequate provision to determine the stability of products in containers in which they are marketed to insure among other things, that the container is non-reactive and non-absorptive to the drugs." Pharmaceutical and rubber closure manufacturers can no longer disregard the importance of the closure on the stability, toxicity, and assay of the parenteral solution for which it is to be used.

SUMMARY

Epoxy-lined and unlined closures of the same composition were evaluated regarding their relative sorption and leaching of extractives characteristics. Aqueous buffered solutions of the preservatives phenylethyl alcohol and *p*-chloro-β-phenylethyl alcohol filled into multiple-dose vials and stoppered with the lined and unlined closures were used for the sorption studies. In the leaching investigation, several solvents commonly used in parenteral formulations were evaluated with the lined and unlined stoppers. The solvents used were water, 10% ethanol, 2% benzyl alcohol, 50% polyethylene glycol 300, and 50% *N,N*-dimethylacetamide. The results obtained were:

1. The epoxy lining is ineffective as a barrier against closure sorption of the preservatives from solution.
2. The lining for the most part is effective in reducing the amount of extractives being leached from the closures. The degree of effectiveness is dependent upon the closure composition.
3. Of the three rubber compositions evaluated, the butyl rubber exhibited the least tendencies toward sorption of preservative from solution and the leaching of extractives into solution.
4. The extractives leached from the natural, neoprene, and butyl rubber closures by the several solvents were identified and found for the most part to be the accelerators used in the rubber formulations.
5. The extractive leached from the unlined butyl rubber closure interfered with the analysis for the preservatives. The lined closures of the same composition did not exhibit this interference.
6. Extractives were found to be leached from the closures under normal autoclaving conditions used for multiple-dose vial solutions—namely, 115°, 10 p.s.i. for 30 minutes.

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Simple Determination of Some Acid Salts of Organic Bases

By TATJANA BIĆAN-FIŠTER and ANTONIJA ANDREC

The present official methods of analyses for preparations containing acid salts of organic bases, when based on the determination of the isolated base, are time consuming and tedious. An extraction procedure based on the basic hydrolysis of the organic salt in the presence of magnesium oxide was used to convert the salt to the corresponding free base, which was then titrated directly with an acid. The method was applied to tablets containing certain alkaloids or some other organic salts.

THE ASSAY of acid salts of organic bases official in the majority of pharmacopeias is based on the determination of the liberated base which involves prior extraction from the salt carried out by the classical procedure in alkaline media with organic solvents using separators. Although these procedures are satisfactory assay procedures, they have several disadvantages. The number of manipulations involved not only makes the procedure tedious and time consuming, but also allows for more sources of error.

The purpose of this study was to develop a rapid, accurate, and simple method for the determination of acid salts of organic bases which could be applied to the control of tablets containing such salts. In the procedure developed in this study the salts were determined by using a simple extraction procedure based on the basic hydrolysis of the salt in the presence of magnesium oxide, making possible a direct titration of the liberated base with standard acids.

EXPERIMENTAL

Reagents

Chloroform, ethylacetate, ether, glacial acetic acid, and ethanol (96%) reagent grade, were employed. Magnesium oxide was used for chromatography (Merck AG, Darmstadt). U.S.P. Crystal violet indicator solution and methyl red indicator solution and B.P. methyl red-methylene blue indicator solution were employed. Codeine phosphate, papaverine hydrochloride, chlorpromazine hydrochloride, and amphetamine sulfate were commercial samples of U.S.P. quality.

Determination of Salts

Codeine Phosphate.—Accurately weigh approximately 0.3 Gm. of codeine phosphate, transfer into a glass crucible $G_4\sqrt{}$;[1] add 0.5 Gm. magnesium oxide and moisten only slightly with a few drops of water. Mix well with the aid of a glass rod and extract the alkaloid base with four 10-ml. portions of chloroform. Evaporate the chloroform from the filtrate to dryness by heating on a steam bath, dissolve the residue in 10 ml. glacial acetic acid, and titrate the liberated base with 0.1 *N* perchloric acid to a distinct blue color using crystal violet as indicator. Perform a blank determination on the solvents and make any correction necessary.

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[1] √porosity 2r = 5-λ5μ.

TABLE I.—DETERMINATION OF SALTS

Salt	Pharmacopeia Method	Proposed Method
Codeine phosphate	99.1	102.2
		101.7
		101.8
Papaverine hydrochloride	97.3	101.2
		101.0
		101.4
Chlorpromazine hydrochloride	99.0	98.9
		99.2
		98.7
Amphetamine sulfate	98.7	99.8
		99.5
		100.1

TABLE II.—DETERMINATION OF TABLETS

Tablets	Theoretical Content mg./Tablet	Recovery mg./Tablet	Recovery %
Codeine phosphate	300.0	296.0	98.7
		295.0	98.4
		302.0	100.4
Papaverine hydrochloride	40.0	39.7	99.25
		39.6	99.0
		39.2	97.4
Chlorpromazine hydrochloride	250.0	253.0	101.2
		256.0	101.8
Amphetamine sulfate	75.0	73.8	98.8
		73.5	98.0

Papaverine Hydrochloride.—Papaverine hydrochloride was determined in the same way as described for *Codeine Phosphate*.

Chlorpromazine Hydrochloride.—Approximately 0.2 Gm. of chlorpromazine hydrochloride is extracted in the same way as described for *Codeine Phosphate*, with five portions each of 10 ml. ether. Evaporate the ether from the filtrate, dissolve the residue in 2 ml. ethanol, add 20 ml. of 0.1 *N* hydrochloric acid and 15 ml. of water, and titrate the excess of acid with 0.1 *N* sodium hydroxide using the mixed indicator solution methyl red–methylene blue. The titration of the extracted base can be carried out also in nonaqueous media (glacial acetic acid solution) with 0.1 *N* perchloric acid using crystal violet as indicator.

Amphetamine Sulfate.—Approximately 75 mg. of amphetamine sulfate is extracted in the same manner as described for *Codeine Phosphate*, with four portions each of 10 ml. ethylacetate. After adding to the filtrate 40 ml. of glacial acetic acid, the liberated base is titrated with 0.1 *N* perchloric

acid to a distinct blue color, using crystal violet as indicator.

For purposes of comparison, codeine phosphate, chlorpromazine hydrochloride, and amphetamine sulfate were assayed by the methods official in B.P., while papaverine hydrochloride by that official in Ph.Dan. Results of these experiments are recorded in Table I.

Determination of Salts in Tablets

Codeine phosphate, papaverine hydrochloride, chlorpromazine hydrochloride, and amphetamine sulfate in tablets are determined in the same manner as directed in the assay of the salts, the quantity of the powdered tablets being equivalent to the stated amount of the corresponding salt. Results of these determinations are recorded in Table II. Experiments were carried out with standard tablets prepared in this laboratory.

SUMMARY

A simple method for the determination of certain acid salts of organic bases, based on the basic hydrolysis of the salt in the presence of magnesium oxide has been described. The proposed method proved to be a rapid, simple, and accurate one that makes it especially useful for routine work.

Examinations on the possibilities of the application of this procedure to other pharmaceutical preparations will be carried out and reported in a subsequent communication from this laboratory.

Antimicrobial Properties of Aliphatic Thiosemicarbazones

By M. MANOWITZ and G. WALTER

A series of thiosemicarbazones of saturated and α,β-unsaturated aliphatic aldehydes was prepared and tested *in vitro* for activity against various bacteria, yeasts, and molds. Antimicrobial potency was found to depend on chain length and was greatly enhanced by the presence of an unsaturated linkage. Thiosemicarbazones derived from unsaturated aldehydes with chain lengths C-10 to C-12 were the most active against the microorganisms tested.

THE TUBERCULOSTATIC PROPERTIES of thiosemicarbazones have been investigated extensively since the initial publication by Domagk and coworkers (1). A large number of thiosemicarbazones have been prepared and tested for these properties; however, their effect on other microorganisms has received only limited consideration (2–5). Benns, *et al.* (6), determined the antifungal activity of 40 thiosemicarbazones against *Aspergillus niger* and *Chaetomium globosum* and found the most effective compounds were derived from aliphatic aldehydes. Preliminary observations in our laboratories have demonstrated interesting antibacterial properties for certain aliphatic thiosemicarbazones; a further study of the antimicrobial spectrum of a more extensive series of these compounds was indicated. For this purpose, thiosemicarbazones of saturated and unsaturated aliphatic aldehydes were prepared and tested against various bacteria, yeasts, and molds.

EXPERIMENTAL

The thiosemicarbazones of the C-4 through C-12 straight chain saturated aldehydes and of the C-4 through C-13 α,β-unsaturated aldehydes, with the omission of the C-5 unsaturated compound, were included in this investigation. The thiosemicarbazones were prepared by usual methods described in the literature (3).

Antimicrobial Tests.—The antimicrobial properties of the compounds were determined by agar dilution technique employing the following organisms: *Staphylococcus aureus*, ATCC 6538; *S. epidermidis*, ATCC 155; *Bacillus subtilis*, ATCC 9372; *Escherichia coli*, ATCC 11229; *Proteus vulgaris*, ATCC 9920; *Pseudomonas aeruginosa; Bacterium ammoniagenes*, ATCC 6871; *Pityrosporum ovale; Candida albicans*, ATCC 10231; *Trichophyton mentagrophytes*, ATCC 9129; and *Microsporum audouini*, ATCC 11347.

Twofold serial dilutions of the compounds were prepared in alcohol (S.D.30) and 0.2-ml. aliquots of each dilution added to 20-ml. tubes of molten agar. The contents of the tubes were thoroughly mixed and poured into sterile Petri plates. Dextrose tryptone extract agar was employed for the bacteria and Sabouraud's dextrose agar was used for the yeasts and molds.

Bacterial inoculum consisted of 1–100 distilled water dilution of a 24-hour, tryptic soy broth culture grown at 35°. Yeast inoculum was prepared by washing a 3-day-old slant of the organism with 10 ml. of distilled water and diluting the suspended cells 1–100 with distilled water. Mold inoculum consisted of a conidial suspension from the surface growth of a 7-day slant of the organism in 20 ml. of distilled water. Plates were inoculated by placing 1 drop (0.007 ml.) of the inocula on the surface of the hardened agar media with the aid of an Accu-Drop dispenser.[1] Inoculated plates were incubated at 35° for bacterial tests and at 30° for yeasts and molds. Examination of the plates for the presence of growth was made after incubation periods of 48 hours for the bacteria, 4 days for the yeasts, and 14 days for the molds.

RESULTS

Results of the microbiological tests are summarized in Table I. None of the compounds was active against the Gram-negative bacteria (*E. coli, Ps. aeruginosa, Pr. vulgaris*) and therefore these organisms were not included in the table. The data

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[1] Scientific Products, Flushing, Long Island, N. Y.

acid to a distinct blue color, using crystal violet as indicator.

For purposes of comparison, codeine phosphate, chlorpromazine hydrochloride, and amphetamine sulfate were assayed by the methods official in B.P., while papaverine hydrochloride by that official in Ph.Dan. Results of these experiments are recorded in Table I.

Determination of Salts in Tablets

Codeine phosphate, papaverine hydrochloride, chlorpromazine hydrochloride, and amphetamine sulfate in tablets are determined in the same manner as directed in the assay of the salts, the quantity of the powdered tablets being equivalent to the stated amount of the corresponding salt. Results of these determinations are recorded in Table II. Experiments were carried out with standard tablets prepared in this laboratory.

SUMMARY

A simple method for the determination of certain acid salts of organic bases, based on the basic hydrolysis of the salt in the presence of magnesium oxide has been described. The proposed method proved to be a rapid, simple, and accurate one that makes it especially useful for routine work.

Examinations on the possibilities of the application of this procedure to other pharmaceutical preparations will be carried out and reported in a subsequent communication from this laboratory.

Antimicrobial Properties of Aliphatic Thiosemicarbazones

By M. MANOWITZ and G. WALTER

A series of thiosemicarbazones of saturated and α,β-unsaturated aliphatic aldehydes was prepared and tested *in vitro* for activity against various bacteria, yeasts, and molds. Antimicrobial potency was found to depend on chain length and was greatly enhanced by the presence of an unsaturated linkage. Thiosemicarbazones derived from unsaturated aldehydes with chain lengths C-10 to C-12 were the most active against the microorganisms tested.

THE TUBERCULOSTATIC PROPERTIES of thiosemicarbazones have been investigated extensively since the initial publication by Domagk and coworkers (1). A large number of thiosemicarbazones have been prepared and tested for these properties; however, their effect on other microorganisms has received only limited consideration (2–5). Benns, *et al.* (6), determined the antifungal activity of 40 thiosemicarbazones against *Aspergillus niger* and *Chaetomium globosum* and found the most effective compounds were derived from aliphatic aldehydes. Preliminary observations in our laboratories have demonstrated interesting antibacterial properties for certain aliphatic thiosemicarbazones; a further study of the antimicrobial spectrum of a more extensive series of these compounds was indicated. For this purpose, thiosemicarbazones of saturated and unsaturated aliphatic aldehydes were prepared and tested against various bacteria, yeasts, and molds.

EXPERIMENTAL

The thiosemicarbazones of the C-4 through C-12 straight chain saturated aldehydes and of the C-4 through C-13 α,β-unsaturated aldehydes, with the omission of the C-5 unsaturated compound, were included in this investigation. The thiosemicarbazones were prepared by usual methods described in the literature (3).

Antimicrobial Tests.—The antimicrobial properties of the compounds were determined by agar dilution technique employing the following organisms: *Staphylococcus aureus*, ATCC 6538; *S. epidermidis*, ATCC 155; *Bacillus subtilis*, ATCC 9372; *Escherichia coli*, ATCC 11229; *Proteus vulgaris*, ATCC 9920; *Pseudomonas aeruginosa; Bacterium ammoniagenes*, ATCC 6871; *Pityrosporum ovale; Candida albicans*, ATCC 10231; *Trichophyton mentagrophytes*, ATCC 9129; and *Microsporum audouini*, ATCC 11347.

Twofold serial dilutions of the compounds were prepared in alcohol (S.D.30) and 0.2-ml. aliquots of each dilution added to 20-ml. tubes of molten agar. The contents of the tubes were thoroughly mixed and poured into sterile Petri plates. Dextrose tryptone extract agar was employed for the bacteria and Sabouraud's dextrose agar was used for the yeasts and molds.

Bacterial inoculum consisted of 1–100 distilled water dilution of a 24-hour, tryptic soy broth culture grown at 35°. Yeast inoculum was prepared by washing a 3-day-old slant of the organism with 10 ml. of distilled water and diluting the suspended cells 1–100 with distilled water. Mold inoculum consisted of a conidial suspension from the surface growth of a 7-day slant of the organism in 20 ml. of distilled water. Plates were inoculated by placing 1 drop (0.007 ml.) of the inocula on the surface of the hardened agar media with the aid of an Accu-Drop dispenser.[1] Inoculated plates were incubated at 35° for bacterial tests and at 30° for yeasts and molds. Examination of the plates for the presence of growth was made after incubation periods of 48 hours for the bacteria, 4 days for the yeasts, and 14 days for the molds.

RESULTS

Results of the microbiological tests are summarized in Table I. None of the compounds was active against the Gram-negative bacteria (*E. coli, Ps. aeruginosa, Pr. vulgaris*) and therefore these organisms were not included in the table. The data

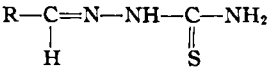
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[1] Scientific Products, Flushing, Long Island, N. Y.

TABLE I.—ANTIMICROBIAL ACTIVITY OF THIOSEMICARBAZONES



Compd. No.	R	Minimum Concn., mcg./ml., Completely Inhibiting Growth of Organism[a]							
		S.a.	*S.e.*	*B.s.*	*B.a.*	*P.o.*	*C.a.*	*T.m.*	*M.a.*
I	$CH_3(CH_2)_2$	x[b]	x	x	x	x	x	50	50
II	$CH_3(CH_2)_3$	x	x	x	x	x	x	100	50
III	$CH_3(CH_2)_4$	x	x	x	x	x	x	100	50
IV	$CH_3(CH_2)_5$	x	x	x	x	x	x	50	50
V	$CH_3(CH_2)_6$	x	x	x	x	x	x	50	50
VI	$CH_3(CH_2)_7$	100	50	100	x	x	x	100	100
VII	$CH_3(CH_2)_8$	12.5	12.5	25	x	x	x	100	50
VIII	$CH_3(CH_2)_9$	12.5	12.5	12.5	12.5	x	x	100	50
IX	$CH_3(CH_2)_{10}$	x	x	x	x	x	x	x	x
X	$CH_3CH{=}CH$	x	x	x	x	x	x	x	x
XI	$CH_3(CH_2)_2CH{=}CH$	50	25	50	100	x	x	50	50
XII	$CH_3(CH_2)_3CH{=}CH$	50	50	100	50	100	x	100	50
XIII	$CH_3(CH_2)_4CH{=}CH$	12.5	6.25	12.5	12.5	50	50	25	12.5
XIV	$CH_3(CH_2)_5CH{=}CH$	3.12	3.12	6.25	6.25	25	25	12.5	3.12
XV	$CH_3(CH_2)_6CH{=}CH$	1.56	.78	1.56	1.56	x	100	3.12	1.56
XVI	$CH_3(CH_2)_7CH{=}CH$	.78	.78	.78	1.56	x	x	3.12	3.12
XVII	$CH_3(CH_2)_8CH{=}CH$	.78	.39	.78	.78	x	x	x	100
XVIII	$CH_3(CH_2)_9CH{=}CH$	x	x	50	50	x	x	x	x

[a] *S.a.* = *S. aureus*; *S.e.* = *S. epidermidis*; *B.s.* = *B. subtilis*; *B.a.* = *B. ammoniagenes*; *P.o.* = *P. ovale*; *C.a.* = *C. albicans* *T.m.* = *T. mentagrophytes*; *M.a.* = *M. audouini*. [b] x denotes growth at 100 mcg./ml.

demonstrated that the thiosemicarbazones of the α,β-unsaturated aldehydes were more potent antimicrobial agents than the thiosemicarbazones of the corresponding saturated aldehydes. Activity against Gram-positive bacteria, in the unsaturated group, increased with increasing chain length from C-4 through C-12, then abruptly decreased at C-13. Compounds XV, XVI, and XVII were the most effective against the bacteria, inhibiting growth at 1 mcg./ml. or less. The unsaturated thiosemicarbazones also demonstrated a corresponding chain length–activity effect against the molds but only up through C-11 (Compound XVI). Activity against yeasts for this group was exerted by Compounds XII, XIII, XIV, and XV.

Most of the saturated thiosemicarbazones possessed slight activity against *T. mentagrophytes* and *M. audouini* (50–100 mcg./ml. range); however, all were inactive against the yeasts and Gram-negative bacteria. Among the saturated compounds, only Compounds VI, VII, and VIII inhibited the growth of the Gram-positive bacteria.

Microbiological tests were also conducted on the individual aldehydes from which the thiosemicarbazones were prepared. Results of these tests demonstrated activity against *T. mentagrophytes* and *M. audouini* at the 50–100 mcg./ml. range for most of the unsaturated aldehydes and for undecanal and dodecanal. None of the aldehydes was effective against bacteria or yeasts at the highest concentration tested (100 mcg./ml.), except 2-dodecenal which inhibited the growth of Gram-positive bacteria at 50 mcg./ml.

DISCUSSION AND SUMMARY

This study has revealed that considerable activity is present among the thiosemicarbazones of aliphatic aldehydes. It is evident that the presence of an α,β-unsaturated linkage in the aldehyde from which the thiosemicarbazone is derived markedly enhances the effectiveness of these compounds. There is an apparent structure-activity pattern displayed by this series of compounds that is a function of the chain length of the molecule. Thiosemicarbazones prepared from aldehydes with chain lengths C-10 to C-12 demonstrated the greatest activity against the bacteria and molds tested.

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Individual Rodent Observation Cages for Pharmacodynamic Studies

By T. LAMAR KERLEY, BARBARA L. CARTER, and JAMES R. BRISCOE

A useful new plastic caging system for mice that offers the advantages of being simple to construct, inexpensive, and easy to clean and use is described. Its greatest advantage is that it provides an effective way to observe large numbers of mice in pharmacodynamic studies.

THE OBSERVATION of large numbers of mice individually has always been a problem for pharmacologists in drug evaluation programs. Steel wire mesh cages with hinged metal tops for such procedures have frequently been used. With standard laboratory cages, it has been difficult not only to observe mice during a procedure, but also to remove them at the conclusion of the work. The cages are usually expensive and difficult to clean.

Often a study, such as the determination of the acute toxicity of a compound, would require the use of as many as 100 or more such cages. Such large numbers make it almost impossible for even a highly skilled observer to note responses of the individual animal. Because mice tend to cling tightly to wire mesh materials and frequently bite into the cage during a convulsive death, removal of the animals at the end of an experiment is, at least, an inconvenient procedure. Moreover, the auditory stimulus from the noisy opening of a metal cage lid frequently induces convulsions prematurely in certain studies. A very satisfactory solution to the problem was found with multicompartmental cage units made of transparent plastic.

MATERIALS

The cages illustrated in Fig. 1 were constructed of $^1/_4$ in. clear acrylic plastic, each compartment measuring 4 × 4 × 4 in. Because of the frequent use of groups of 10 mice, the cages were assembled with 10 compartments per unit, making an overall size of $21^1/_2 \times 10^3/_4 \times 4^1/_4$ in. For observational convenience, the cage units were hung on an upright plywood backboard with conventional hangers available at hardware stores. A single brass spring-return hinge was used for each door with a $^1/_4$-in. air space at each side. The air space also provided for convenient opening of the doors. In our laboratories, doors have been constructed to open either from the top or from the bottom, according to individual preference. The details of construction are illustrated in Fig. 2. It should be noted that the plywood board on which the units are hung forms the back of the cage. This feature simplifies both construction and cleaning. The cleaning difficulties encountered with the individual cages have been reduced to a simple removal of a plastic unit from the hanger which is washed with hot water from a hose. To prevent the formation of rust, brass screws and hinges were used. Prior to assembly, the spring hinges were sprayed with a clear protective plastic coating as a rust-preventive measure; replacement has seldom been necessary. Some possible modifications include the construction of larger units for larger rodents and installation of feeding and watering devices for experiments where longer periods of caging are desired.



Fig. 1.—Typical arrangement of cage units in use.

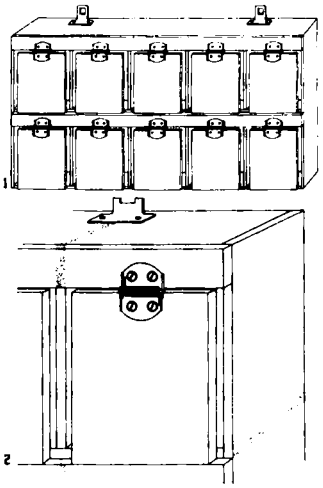


Fig. 2—Line drawing of cages showing construction details.

Among the experiments suitable for this arrangement are anticonvulsant studies using chemoshock techniques, routine toxicologic studies, barbiturate sleeping time determinations, and other experiments requiring temporary storage of mice between drug treatment and another procedure.

Wax pencils have been convenient for marking the occurrence of convulsions or other pertinent events upon the cage door as the event occurs. For example, by sitting in front of a group of these units, one investigator can conveniently observe and mark the occurrence of convulsions in as many as 50 or more mice. Other apparent applications include the intravenous injection of mice accomplished by pulling the tail of the animal through the side space of the door. We have not observed an alteration of drug toxicity due to aggregation of mice as occurs after treatment with *d*-amphetamine, since the mice have no physical contact with one another.

Although the expense of the units could be reduced by using a $^1/_8$-in. thick plastic, we have found the $^1/_4$-in. stock very sturdy and convenient to handle.

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The authors express their gratitude to Mr. George Hollingsworth for the construction of the cage units described in this report and his help in working out the details.

Each unit weighs about 8 lb. and can be constructed in most laboratory shops for a cost of less than $12. for materials. The only similar cages that we have found in use were those reported by Moos, *et al.*(1), where plastic cages for solitary housing of mice for irradiation and longevity experiments were described. A photograph of the present cages, but no description of the units, appeared previously in a report by Burch (2). The caging units have been in use in our laboratory for about 3 years and have become an indispensable laboratory item.

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Circular Thin-Layer Chromatography of Tetracyclines

By GOVIND J. KAPADIA and G. SUBBA RAO

A simple circular thin-layer chromatographic method is described for the separation of hydrochlorides of tetracycline, oxytetracycline, and chlortetracycline employing three sequestering agents—viz., disodium ethylenediaminetetraacetate, tartaric acid, and oxalic acid. Better resolution has been achieved by the use of disodium ethylenediaminetetraacetate in the stationary phase and *n*-butanol saturated with water as the mobile phase.

ALTHOUGH PAPER PARTITION chromatography of tetracyclines has been reported by several workers (1–10), thin-layer chromatography (TLC) of these antibiotics has only been recently investigated. Nicolaus, Coronelli, and Binaghi (11) have studied the chromatography of several tetracyclines with various solvent mixtures by ascending TLC. They were, however, unsuccessful in resolving more than two tetracyclines by a single solvent system on one chromatogram.

The property of tetracyclines of forming chelate complexes with metallic ions suggests that such complexation might occur between these antibiotics and the metallic ions of the adsorbent and/or calcium sulfate added as the binder. In such a case, sequestering agents possibly could effect the separation of these compounds. The use of disodium ethylenediaminetetraacetate (EDTA) and oxalic acid has been promising in the chromatography of certain naturally occurring polyphenolic compounds that showed strong chelating property (12). These sequestering agents might also be employed in the resolution of tetracyclines by TLC.

This report presents the results of preliminary work in which three sequestering agents—*viz.*, EDTA, tartaric acid, and oxalic acid were used—and the separation of hydrochlorides of tetracycline, oxytetracycline, and chlortetracycline was studied. For the development of chromatograms the circular TLC technique of Bryant (13) was modified. The separation of the three antibiotics was achieved by employing all the three sequestering agents, although better separation was accomplished with EDTA.

EXPERIMENTAL

Apparatus.—The Desaga-Brinkmann apparatus for TLC (applicator model "S II") distributed by Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y., was used for the preparation of plates. Glass plates (20 × 20 cm.) with 1/8 in. hole drilled at the center of each were utilized. Pyrex Petri dishes (diameter—14 cm., height—2.5 cm.) were employed as the solvent containers for the development of chromatograms. The adsorbent used was silica gel G (made according to Stahl by Merck, A. G., Darmstadt, Germany, and distributed by Brinkmann).

Preparation of Plates.—*Technique A.*—A slurry of 30 Gm. of silica gel G and 60 ml. of water was made in a dry mortar. This was poured into the applicator and coated over five plates which were then dried at room temperature for 15 minutes. The plates were further activated in an oven at 105–110° for 1 hour and stored in a vacuum desiccator. The adsorbent layer was kept constant at standard 250 μ thickness.

Technique B.—Nine grams of EDTA was dissolved in 60 ml. of water and, using 30 Gm. of silica

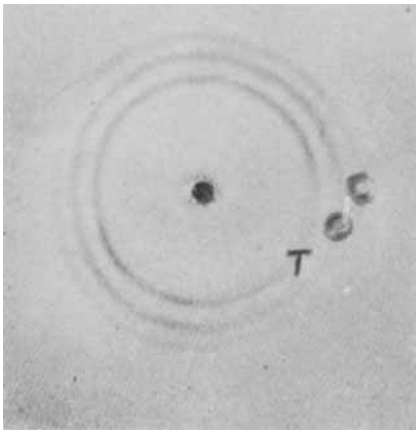


Fig. 1.—Separation of tetracycline (*T*), oxytetracycline (*O*), and chlortetracycline (*C*) hydrochlorides on silica gel G plate admixed with disodium ethylenediaminetetraacetate, using *n*-butanol saturated with water as the solvent.

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The authors are grateful to the Chas. Pfizer Co., Inc., Brooklyn, N. Y., for the samples of tetracycline hydrochloride, oxytetracycline hydrochloride, and oxytetracycline free base. The supply of chlortetracycline hydrochloride by Lederle Laboratories, Pearl River, N. Y., is gratefully acknowledged.

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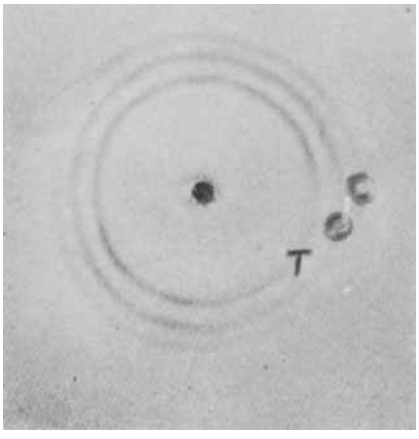


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TABLE I. — R_f VALUES

Antibiotic	Solvent System BW/EDTA	BTW	BOW
Tetracycline HCl	0.23	0.26	0.38
Oxytetracycline HCl[a]	0.27	0.31	0.46
Chlortetracycline HCl	0.30	0.35	0.49

[a] Oxytetracycline base also resolved from the mixture of tetracycline and chlortetracycline hydrochlorides with the same average R_f value as that of its hydrochloride.

gel G, plates were prepared as in *Technique A*. In this case the plates were dried at room temperature for 30 minutes before placing in an oven.

Solvent Systems.—The organic phases of the following mixtures were used as the mobile solvents: *n*-butanol–oxalic acid–water (100 ml.: 5 Gm.: 100 ml.)—BOW, *n*-butanol–tartaric acid–water (100 ml.: 6 Gm.: 100 ml.)—BTW, and *n*-butanol saturated with water—BW.

The plates prepared by *Technique A* were developed by solvent systems BOW and BTW, those by *Technique B* were developed by solvent system BW.

Sample Application.—Any adhering adsorbent in the central hole of the plate was cleared by piercing through with a capillary. A solution of equal quantities of the hydrochlorides of the three tetracyclines in methanol was applied around the circumference of the hole using a lambda pipet so that 20 mcg. of each compound was deposited.

Development Technique.—A paper "wick" (length—$1^1/_8$ in., diameter—$^1/_8$ in.) was made by rolling a piece of Whatman No. 1 filter paper ($2^1/_2 \times$ 1 in.). This was inserted in the hole at the adsorbent side of the plate. The plate was then placed adsorbent side down over a Petri dish containing 50 ml. of developing solvent so that the "wick" dipped in it and the plate hole became the center of the dish. The hole on the clear side of the plate was covered with a vial. The movement of the solvent front was clearly visible; when it reached 6.5 cm. from the center, the plate was removed.

Detection.—The antibiotics were revealed on the chromatograms by spraying the plates with 5% methanolic solution of ferric chloride (4). The reagent gave dark grayish bands with each compound. Before spraying with ferric chloride, three yellowish-green fluorescent bands were visible under ultraviolet light. These bands were located at the same position where colored bands were seen after spraying with the chromogenic reagent. Identity of the bands was achieved by individually chromatographing each antibiotic.

In the chromatograms in which solvent systems BTW and BOW were employed, marked circular portions were observed. These were due to the organic acids used in the solvent mixtures as revealed by spraying 0.5% ethanolic Congo red solution. Blue circular areas of average radii 3.5 cm. and 4.5 cm. were seen with BTW and BOW systems, respectively, when average solvent movement was 6.5 cm. from the center. When sprayed with ferric chloride solution, these areas were a lighter yellow compared to the rest of the plate.

RESULTS

Figure 1 shows the separation of the three tetracyclines using BW solvent system and EDTA in the adsorbent. Table I lists the average R_f values of the compounds obtained with all the three solvent systems. The values were highest with BOW and lowest with BW/EDTA. In these two cases the differences in R_f values of oxytetracycline and chlortetracycline were the same. However, better separation was achieved with BW/EDTA where the bands obtained were sharp. With BOW the bands were broad and some spreading occurred. As a result of this the resolution was not as clear, although each of the two separated distinctly from the tetracycline. The spreading of the bands with BTW was much less, and a satisfactory separation of the antibiotics was observed. Comparison of chromatograms indicated that a better resolution of the compounds was achieved using BW/EDTA.

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Effect of β-Diethylaminoethyl Diphenyl-*n*-propyl Acetate (SKF 525-A) on the Plasma Concentration of C^{14}-Labeled Neuromuscular Blockers

By FLOYD R. DOMER† and W. PINTO-SCOGNAMIGLIO

Although pretreatment with SKF 525-A potentiates the intensity and duration of neuromuscular blockade, it did not alter significantly the plasma radioactivity of dogs given C^{14}-labeled decamethonium (C10) or bis (dimethylaminobutoxy) 1,4-benzene diiodomethylate (1815, I.S.) or rabbits given 1815 I.S.

AMONG THE many biological actions of β-diethylaminoethyl diphenyl-*n*-propyl acetate (SKF 525-A) (1, 2) that of affecting neuromuscular transmission and muscular contraction was first reported by Navis, *et al.* (3). SKF 525-A was shown to potentiate greatly the blockade caused by a number of compounds possessing neuromuscular activity (4). This latter group of workers proposed the theory that the SKF 525-A caused its potentiation by displacing the neuromuscular blocking agent from nonspecific receptors to the neuromuscular junction. Subsequent work *in vitro* with a nitrocellulose membrane indicated that SKF 525-A indeed is quite potent in its ability to displace a neuromuscular blocker (5).

The present report deals with another attempt to elucidate the mechanism of action of SKF 525-A in potentiating neuromuscular blockade. The approach was that of using a C^{14}-labeled neuromuscular blocking agent and measuring the radioactivity in the blood of dogs and rabbits.

C^{14}-Labeled decamethonium (C10), 0.2 mg./Kg., or bis(dimethylaminobutoxy) 1,4-benzene diiodomethylate (1815, I.S.), 0.2 mg./Kg., were administered to dogs anesthetized with chloralose as previously described (4). Blood samples were taken from the femoral vein at 1, 2, 4, 7, 10, 15, and 30 minutes after the $C^{14}C10$ administration. At 45 minutes a second dose of the $C^{14}C10$ was given. Plasma samples were prepared and counted in a refrigerated liquid scintillation counter as previously described (6). A 10 mg./Kg. dose of SKF 525-A was given intravenously 10 minutes before the first dose of radioactive C10 or 1815, I.S. in the test experiments. Table I gives the results obtained in a pair of dog experiments with the SKF 525-A pretreatment. The circulating blood level of the neuromuscular blocking agent was higher than in the control animal during the first 4 minutes. Subsequently, it decreased more rapidly than in the control. Similar results were obtained in dogs given C^{14}-labeled 1815, I.S.

Rabbits were anesthetized with urethane (1.5 Gm./Kg. s.c.), given artificial respiration throughout the experiment, and C^{14} 1815, I.S. (0.25 mg./Kg.) was given intravenously. Blood samples were obtained from the heart at 1, 5, and 10 minutes. At 20 minutes, a second dose of the C^{14} 1815, I.S. was given and a second series of blood samples taken at 1, 5, and 10 minutes. The test animals were given SKF 525-A (10 mg./Kg. i.v.) 10 minutes prior to the first administration of C^{14} 1815, I.S. Table II gives the results obtained in five rabbit experiments. There was no significant difference in the circulating blood level of radioactive neuromuscular blocker between control animals and those pretreated with SKF 525-A ($p > 0.2$). However, the treated animals did have slower rates of decrease in blood radioactivity.

The fact that there was no elevation in the circulating blood level of C^{14}-labeled neuromuscular blocking agents in the rabbit or the dog would at first appear to be contrary to the hypothesis of displacement from nonspecific receptors to the neuromuscular junction. However, this need not necessarily be the case. Waser (7) using C^{14}-labeled calabash curarine found that pretreatment with SKF 525-A doubled the amount of radioactivity found in the end-plates of mice diaphragms. This indicates directly that there is an increased interaction between the labeled compound and the endplate in the pretreated animals. Similarly, in our experiments, the SKF 525-A pretreatment could have increased the affinity of the receptors for the labeled compound without affecting the total number of receptors involved. This would result in a decrease in the amount of circulating radioactive compound. Another possibility could be that the neuromuscular receptors represent such a small percentage of all the body receptors that the change caused by the SKF 525-A would be too small to be detected by our procedure. Finally, the theory advanced by Paton (8) that the *rate* of attachment

TABLE I.—PLASMA LEVELS OF C^{14}-LABELED DECAMETHONIUM IN DOGS

Injection	Time, min.	—$C^{14}C10$, mcg./ml.— Control[a]	Pretreated with SKF 525-A[a]
SKF 525-A, 10 mg./Kg. i.v.	−10	...	...
$C^{14}C10$, 0.2 mg./Kg. i.v.	0	...	...
	1	3.05	3.53
	2	1.61	2.71
	4	1.28	1.59
	7	1.93	1.39
	10	1.78	1.08
	15	1.63	0.76
	30	1.37	0.44
$C^{14}C10$, 0.2 mg./Kg. i.v.	45	...	...
	46	2.95	3.85
	47	2.39	3.08
	49	2.02	2.06
	52	1.17	1.74
	55	1.24	1.32
	60	1.32	1.06
	75	1.18	0.67

[a] Each column represents the results obtained in a single dog experiment.

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† Present address: Department of Pharmacology, Tulane University, New Orleans, La.

TABLE II.—PLASMA LEVELS OF C^{14}-LABELED 1815, I.S. IN RABBITS

Injection	Time, min.	C^{14} 1815, I.S., mcg./ml. Control Mean ± S.E.[a]	C^{14} 1815, I.S., mcg./ml. Pretreated with SKF 525-A Mean ± S.E.[a]
SKF 525-A, 10 mg./Kg. i.v.	−10	...	...
	0	...	...
C^{14} 1815, I.S. 0.25 mg./Kg. i.v.			
	1	3.22 ± 0.73	2.71 ± 0.70
	5	1.48 ± 0.29	1.52 ± 0.66
	10	0.94 ± 0.34	1.26 ± 0.45
	20	...	...
C^{14} 1815, I.S. 0.25 mg./Kg. i.v.			
	21	3.44 ± 1.57	4.40 ± 1.41
	25	2.04 ± 0.99	3.01 ± 1.58
	30	1.78 ± 1.01	2.76 ± 1.80

[a] Five rabbits used in each case.

to, and detachment from, receptors is important for stimulation would also be compatible with the present findings. This would mean that the SKF 525-A is increasing the rate at which the neuromuscular blocker-receptor combination is being made and broken without having influence on the total amount of drug being present.

Evidence that SKF 525-A itself has an effect upon neuromuscular transmission and receptor excitability have been found by a number of authors (3, 9, 10). This makes it quite difficult to separate clearly the action(s) of SKF 525-A involved in the observed potentiation of neuromuscular blocking agents. Further attempts will be necessary to clarify this problem.

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Synthesis of N-Substituted and N,N′-Disubstituted Benzyl Derivatives of 5,5-Disubstituted Barbiturates

By EDWARD J. ROWE and MELVIN H. WEINSWIG

N-Substituted and N,N′-disubstituted benzyl derivatives of barbital, phenobarbital, and amobarbital have been synthesized by the use of a strongly basic anion-exchange resin. The barbiturate is first absorbed on the resin and the resin then agitated mechanically with an ethanol solution of benzyl chloride. The reaction proceeds at room temperature. The yields in all cases ranged from 50 to 80 per cent.

IN A PREVIOUS REPORT (1) a method was described for the synthesis of benzyl ethers of a variety of phenols by the use of a strongly basic anion-exchange resin. The method involved the reaction of the phenolate form of the resin with benzyl chloride. This report presents an analogous method for the synthesis of N-benzyl and N,N′-dibenzyl derivatives of three 5,5-disubstituted barbituric acids (phenobarbital, barbital, and amobarbital). The synthesis of these derivatives involves the reaction of the barbiturate form of a strongly basic anion-exchange resin with benzyl chloride. The resin employed is based on polystyrene and contains quaternary ammonium groups. The reaction proceeds at room temperature. A mixture of N-benzyl and N,N′-dibenzyl derivatives is obtained from the reaction. Separation of the derivatives is based upon the solubility of the N-benzyl derivative in dilute alkali. Table I summarizes the benzyl derivatives of phenobarbital (5-ethyl-5-phenyl barbituric acid), barbital (5,5-diethyl barbituric acid), and amobarbital (5-ethyl-5-isoamyl barbituric acid) prepared by the use of a strongly basic anion-exchange resin. The yields are based upon the amount of the barbiturate converted to the N-benzyl and N,N′-dibenzyl derivatives. The N,N′-dibenzyl derivative is the main product when an equivalency of the resin in the OH form is used with the barbiturate form and the quantity of the benzyl chloride is doubled.

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EXPERIMENTAL

Resin Preparation.—Dowex 1X4[1] supplied commercially in the chloride form (20–50 mesh) was converted to its hydroxyl form by the usual column

[1] Manufactured by Dow Chemicel Co., Midland, Mich., and purchased from J. T. Baker Co., Phillipsburg, N. J.

TABLE II.—PLASMA LEVELS OF C^{14}-LABELED 1815, I.S. IN RABBITS

Injection	Time, min.	C^{14} 1815, I.S., mcg./ml. Control Mean ± S.E.[a]	C^{14} 1815, I.S., mcg./ml. Pretreated with SKF 525-A Mean ± S.E.[a]
SKF 525-A, 10 mg./Kg. i.v.	−10	...	...
	0	...	...
C^{14} 1815, I.S. 0.25 mg./Kg. i.v.			
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TABLE I.—BENZYL DERIVATIVES OF 5,5-DISUBSTITUTED BARBITURIC ACIDS BY THE USE OF A STRONGLY BASIC ANION-EXCHANGE RESIN

Barbiturate	Yield, % 50 meq. Dowex 1×4 (barbiturate form) 0.075 mole benzyl chloride	Yield, % 50 meq. Dowex 1×4 (barbiturate form) 50 meq. Dowex 1×4 (OH form) 0.15 mole benzyl chloride	M.p., °C.[a]	Anal.[b] Calcd.	Found
Phenobarbital, N-benzyl	47.6	19.6	112.5[c]	C 70.81	70.65
				H 5.59	6.05
				N 8.70	8.90
Phenobarbital, N,N′-dibenzyl	31.0	48.5	90[d]	C 75.73	75.65
				H 5.83	6.10
				N 6.80	6.89
Barbital, N-benzyl	32.3	6.57	127[e]	C 65.70	65.26
				H 6.57	6.59
				N 10.22	10.35
Barbital, N,N′-dibenzyl	37.9	74.7	124[f]	C 72.53	72.00
				H 6.97	7.00
				N 7.69	7.64
Amobarbital, N-benzyl	19.62	9.5	90[g]	C 68.35	67.88
				H 7.60	7.76
				N 8.86	8.79
Amobarbital, N,N′-dibenzyl	33.5	53.3	53[h]	C 73.84	74.32
				H 7.38	7.56
				N 6.90	7.04

[a] All melting points on calibrated Fisher-Johns apparatus. [b] Carried out by Weiler and Strauss, Oxford, England. [c] Literature (3) m. p. 113°. [d] Not reported in literature. [e] Literature (3, 4) m.p. 127°. [f] Not reported in literature. [g] Literature (5) m. p. 89–90°. [h] Literature (6) oil.

technique with 5% aqueous sodium hydroxide (four to five times the volume of resin). The resin was rinsed with distilled water until the washings were neutral and then with absolute methanol. The residual methanol was removed by passing a current of carbon dioxide-free air through the column of resin. The quantity of resin employed for each run of benzyl derivatives was based upon the absorption capacity of the resin determined by the method of Kunin and Myers (2).

Absorption of the Barbiturate on the Resin.—A general procedure was followed. The barbiturate as the sodium salt (0.05 mole) was dissolved in 300 ml. distilled water and passed through the resin in a column 25 mm. in diameter. The resin column was first rinsed with distilled water until the washings were neutral to phenolphthalein T.S., then with two 100-ml. portions of ethanol.

Synthesis of Benzyl Barbiturates.—A general procedure was followed for the synthesis of the benzyl barbiturates. The preparation of N-benzyl and N,N′-dibenzyl phenobarbital illustrates the procedure used.

An 18.9-Gm. (0.15 mole) quantity of benzyl chloride dissolved in 300 ml. ethanol was added to the column containing the Dowex 1X4 resin (43 Gm.), upon which 11.6 Gm. (0.05 mole) of phenobarbital U.S.P. had been absorbed. The mixture was then agitated for 48 hours by rotating the column horizontally with an electric motor at 75 r.p.m. At the end of this period, the column was drained and the resin washed with three 75-ml. portions of ethanol. Evaporation of the eluate and washings left a yellowish syrupy residue containing a mixture of N-benzyl and N,N′-dibenzyl phenobarbital.

N,N′-Dibenzyl Phenobarbital.—Separation of N,N′-dibenzyl phenobarbital was accomplished by treating the yellowish syrupy residue with 1 *N* sodium hydroxide solution and extracting the resulting alkaline-aqueous mixture with several portions of ether. Evaporation of the ether extractives, first washed free of alkali, yielded N,N′-dibenzyl phenobarbital which when crystallized from 95% ethanol melted at 90°. The yield was 6.41 Gm. (31%).

Anal.—Calcd. for $C_{26}H_{24}N_2O_3$: C, 75.73; H, 5.88; N, 6.80. Found: C, 75.65; H, 6.10; N, 6.89.

N-Benzyl Phenobarbital.—The alkaline-aqueous portion from which the N,N′-dibenzyl phenobarbital was removed was acidified with diluted hydrochloric acid and extracted with several portions of ether. The combined ether extractives were washed free of acid and evaporated to dryness. A crystalline residue was obtained which when recrystallized from 70% ethanol melted at 112.5°; literature (3) m.p. 113°. The yield was 7.66 Gm. (47.6%).

Anal.—Calcd. for $C_{19}H_{18}N_2O_3$: C, 70.81; H, 5.59; N, 8.70. Found: C, 70.65; H, 6.05; N, 8.90.

SUMMARY

A method is presented for the synthesis of N-benzyl and N,N′-dibenzyl derivatives of three 5,5-disubstituted barbituric acids by the use of a strongly basic anion-exchange resin. The barbiturate form of the resin is treated with an ethanol solution of benzyl chloride. The reaction proceeds at room temperature.

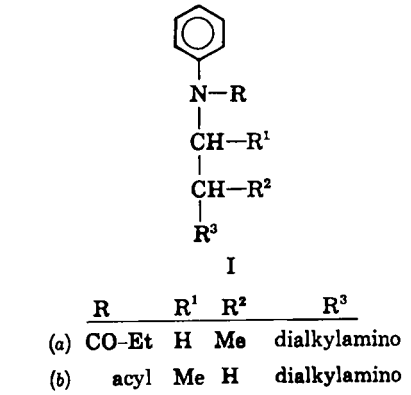
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Resolution and Absolute Configuration of (+)-α-Anilinopropionic Acid

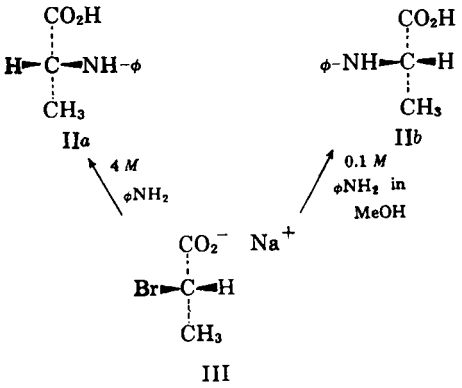
Sir:

We have previously reported (1, 2) the absolute configuration of certain basic anilide analgesics of type I*a* (3, 4). As a continuation of our interest in this area, we wished to determine further the configuration of various analgesics represented by formula I*b* (3–6). Since these compounds (I*b*) contain an anilino moiety attached to an asymmetric carbon atom, a configurational correlation could be accomplished by relating such compounds to a configurationally known α-anilino structure. There are, however, no structures possessing features of I*b* whose absolute stereochemistry is known. A compound which conceivably can be transformed into structures of type I*b* is α-anilinopropionic acid (7) (II). We wish to describe its optical resolution and configurational assignment.



I

	R	R¹	R²	R³
(*a*)	CO–Et	H	Me	dialkylamino
(*b*)	acyl	Me	H	dialkylamino



III

The resolution of II*a* was accomplished by crystallization of its quinine salt from acetone-methanol. Large triclinic crystals, m.p. 199–201°, $[\alpha]_D^{22°}$ − 126° (*c* 2% in water), were formed after the solution stood for 24 hours at 25°. Two subsequent recrystallizations produced crystals which showed no change in rotation or melting point.

Anal.—Calcd. for $C_{29}H_{35}N_3O_4$: C, 71.21; H, 7.21; N, 8.58. Found: C, 71.11; H, 7.33; N, 8.90.

Treatment of the quinine salt with aqueous 1 *N* sodium hydroxide followed by extraction with chloroform and acidification of the aqueous solution to pH 4 afforded the resolved (+)-amino acid (II*a*), m.p. 149–150°, $[\alpha]_D^{23°}$ +71° (*c* 2% in ethanol). The solid state infrared spectrum of II*a* is very similar to racemic II (7).

Our configurational assignment of II*a* is based upon the conclusions of Cowdrey, Hughes, and Ingold (8), who have rigorously established the mechanism and steric course of displacement reactions on optically active sodium α-bromopropionate. L-(−)-α-Bromopropionic acid was prepared according to the procedure of Greenstein (9) and converted to the sodium salt by titration with methanolic sodium hydroxide or sodium methoxide. Removal of solvent resulted in a quantitative yield of the sodium salt (III), $[\alpha]_D^{25°}$ +6.0° (*c* 5% in water). When III was heated in pure aniline, partially racemic II*a*, $[\alpha]_D^{22°}$ +28.5° (*c* 4% in ethanol), was produced. Carrying out the reaction of 0.1 *M* III in 4 *M* methanolic aniline likewise afforded II*a*, $[\alpha]_D^{23°}$ +7.0° (*c* 4% in ethanol). When the concentration of aniline was reduced to 0.1 *M*, the enantiomeric product (II*b*), $[\alpha]_D^{23°}$ −9.0° (*c* 4% in ethanol), was formed.

The aforementioned results are consistent with the established stereochemical course of displacement reactions of III (8). In both pure or 4 *M* aniline the net effect is inversion due to S_N2 attack by aniline. However, in 0.1 *M* aniline there is net retention of configuration due to neighboring carboxylate anion participation (8). Since III is known to have the L-configuration (10), an S_N2 displacement by aniline should afford a product in the D-series. Thus, (+)-α-anilinopropionic acid is represented by projection formula II*a* and possesses the D-configuration.

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Books

REVIEWS

A Bibliography of the Tabletting of Medicinal Substances. Compiled by A. J. EVANS and D. TRAIN. The Pharmaceutical Press, 17 Bloomsbury Square, W.C. 1, England, 1963. 159 pp. 14 × 18.5 cm.

A comprehensive bibliography of publications on medicinal tabletting is presented classified into five main groups: general, tablets, tabletting practice (with subdivisions for granulation, compression, coating, standardization and variation, packaging and storage), materials, and fundamentals. In all, it contains about 900 references from the 1945–1961 period as well as references to fundamental papers published since 1935. The authors explain that they hope to supplement the volume with additional references as well as publications appearing since 1961. Author and subject indexes are provided.

Human Aging. Edited by J. E. BIRREN, R. N. BUTLER, S. W. GREEHOUSE, L. SILOLOFF, and M. R. YARROW. Public Health Service, National Institutes of Health, Bethesda, Md. Available from the Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. 1963. xi + 328 pp. 17 × 25 cm. Price $3.

The efforts of 22 investigators from the National Institutes of Mental Health toward elucidating the biological and behavioral aspects of the human aging process are presented. The entire volume deals with the study of 47 aged males, and the authors note that the study should be considered a pilot rather than a definitive study. Some 15 phases of the study are described in detail.

The Clinical Chemistry of Monoamines. Edited by H. VARLEY and A. H. GOWENLOCK. Elsevier Publishing Company, Inc., 52 Vanderbilt Ave., New York 17, N. Y., 1963. xvi + 242 pp. 16.5 × 24 cm. Price $11.

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media, diagnostic materials and methods, and special fungi stains. A brief glossary is also included.

Physical and Technical Pharmacy. Edited by H. M. BURLAGE, C. O. LEE, and L. W. RISING. The Blakiston Div., McGraw-Hill Book Co., Inc., 330 West 42nd St., New York 36, N. Y., 1963. ix + 740 pp. 15 × 23 cm. Price $16.

Organized to meet the need for a student text to correlate principles of mathematics, physics, and chemistry with pharmaceutical procedures, methods, and technology, this volume presents materials on mathematics, and pharmaceutical calculations, the physicochemical sciences as they apply to pharmaceutical technology, stability of drugs and pharmaceutical systems with particular reference to storage conditions, and adjuncts of importance in the formulation of satisfactory products. Ten pharmaceutical scientist contributors to the volume have assisted the editors in presenting a volume which will be a significant contribution to pharmaceutical education.

Hallucinogenic Drugs and Their Psychotherapeutic Use. Edited by R. CROCKET, R. A. SANDISON and A. WALK. Charles C Thomas, Springfield, Ill., 1963. xiii + 191 pp. 15.5 × 25 cm. Price $7.50.

The proceedings of the quarterly meeting of the Royal Medico-Psychological Association held in February 1961 are presented. The papers are grouped under six topics: the historical and psychopharmacological background, hallucinogenic agents and their general application, techniques and methodology, the use of hallucinogens in specific conditions, clinical observations and phenomenological interpretation, and the moral, religious, and social significance of experience under hallucinogenic drugs. The purpose of the conference was to bring investigators from fields other than the clinical and psychotherapeutic areas to discuss the psychic changes accompanying the administration of the hallucinogens; the editors have created a stimulating and valuable volume.

An Introduction to Clay Colloid Chemistry. By H. VAN OLPHEN. Interscience Publishers, Div. of John Wiley & Sons, 605 Third Ave., New York 16, N. Y., 1963. xvi + 301 pp. 15.5 × 23 cm. Price $10.

Modern concepts in colloid and surface chemistry are discussed as they apply to clay systems. The book stresses the effect of changes in fluid composition on the forces acting between suspended clay particles, and the consequences of such changes on the bulk physical and mechanical properties of the suspensions which are important in clay technology. The treatment in the text is elementary which will limit its usefulness to workers active in clay technology but will provide a workable background knowledge for persons less knowledgeable in the field.

Man and His Future. Edited by G. WOLSTENHOLME. Little, Brown and Co., Boston, Mass., 1963. vi + 410 pp. 13 × 20.5 cm. Price $6.

The biological, environmental, and social aspects of man and his future were treated in a symposium which included outstanding scientists such as Sir Julian Huxley, J. B. S. Haldane, and Carleton Coon. The controversial, provocative, and uninhibited deliberations reported present a review of current thinking among research workers.

The Pathogenesis of Leprosy. Edited by G. E. W. WOLSTENHOLME and M. O'CONNOR. Little, Brown and Co., Boston, Mass., 1963. ix + 101 pp. 12 × 18.5 cm. Price $2.95.

Another booklet in the Ciba Foundation Study Group series, this volume serves to focus attention on the progress and problems of research on leprosy. Papers presented were devoted to experimental observations related to the histopathology of leprosy, cytopathology of the Virchow cell of human leprosy, applicability of experimental murine leprosy to the study of human leprosy, experimental studies on human leprosy, and leprosy bacilli in mouse foot-pads.

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Review Article

Naturally Occurring Coumarins and Related Physiological Activities

By TAITO O. SOINE

SPÄTH (1), DEAN (2, 3), and Reppel (4) have written comprehensive reviews pertaining to the chemistry of the naturally occurring coumarins, the latest being that of Dean (3) in 1963. Mention should be made of the compilation by Karrer (5) in 1958 wherein he lists the coumarins that had been isolated from natural sources up to the date of publication, together with references to their isolation, structure elucidation, and synthesis. Most of the authors have commented briefly on the physiological and pharmacological activities associated with this group of compounds as well. The survey of von Werder (6) in 1936 concerning the coumarins as therapeutic agents should be mentioned also in this respect. Further, Bose (7) has summarized most of the biochemical properties of the natural coumarins. Nevertheless, a perusal of the literature indicates that no recent review of the physiological activities of coumarins has been forthcoming; therefore, the present writing attempts to bring together some of the interesting work in this connection.

Coumarins were discovered originally in plants; the name of the group derives from "coumarona," the common name of *Coumarouna odorata*, from which coumarin itself was isolated first by Vogel in 1820. For many years naturally occurring coumarins were the only ones known, and it was not until the synthetic methods of Perkin and von Pechmann in the latter part of the 19th century combined to produce virtually any synthetic coumarin desired. In the words of Späth, the synthetic output of coumarin derivatives of almost every imaginable type was limited only by "die Phantasie und die experimentellen Moglichkeiten der Chemiker." However, it is not the purpose of the present writing to cover the vast literature pertaining to synthetic coumarins, except as it impinges on the basic premise of the review—namely, the physiological activity of naturally occurring coumarins. In this sense, those synthetic derivatives that bear upon the definition of the structure-activity profile of the natural prototype will be considered. It is obvious, as in many other fields, that the natural prototype may not be the best representative exhibiting a given physiologic activity. Nonetheless, the natural product serves a useful purpose in directing further synthetic effort and, on occasion, withstands the competition of its synthetic congeners (*e.g.*, coumestrol and psoralen). It is quite safe to say, however, that all medicinally useful coumarins have had their origins in natural products.

Natural Occurrence and Biosynthesis.—Coumarins occur in all plant parts from roots to flowers and fruits and are found widely spread in various plant families. Späth (1) and Reppel (4) have listed the plant sources and the parts of plants associated with coumarins. In general, they

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The author thanks Mr. P. K. Gupta, graduate student in pharmaceutical chemistry, for assistance in surveying the literature.

are found especially in the grasses (*Poae*), orchids (*Orchidaceae*), legumes (*Leguminosae*), umbellifers (*Umbelliferae*), *Rutaceae*, *Labiatae*, and other families of lesser importance. They are rarely, if ever, found in the conifers, cacti, lilies, or primrose. More recently, a few complex coumarins have been found as metabolic products of bacteria and fungi, the most recent being the aflatoxins which are the result of *Aspergillus flavus* infection of oil-seed cake derived from peanuts (8). Only a few coumarins have been obtained from animal sources, the outstanding ones being the two yellow pigments isolated by Lederer (9) from castoreum. The simple hydroxy and methoxy compounds, as well as coumarin itself, occur widely in many different families, but as the complexity of the compounds increases they seem to be more and more restricted with respect to familial occurrence. Thus, coumarin, umbelliferone, scopoletin, and esculetin occur extensively in many families; but the more complex psoralen, nodakenin, xanthyletin, and calophyllolide occur in only one or two families. However, in the latter case, the isolations have not been confined always to a single genus or species.

A complete consideration of the biosynthesis of

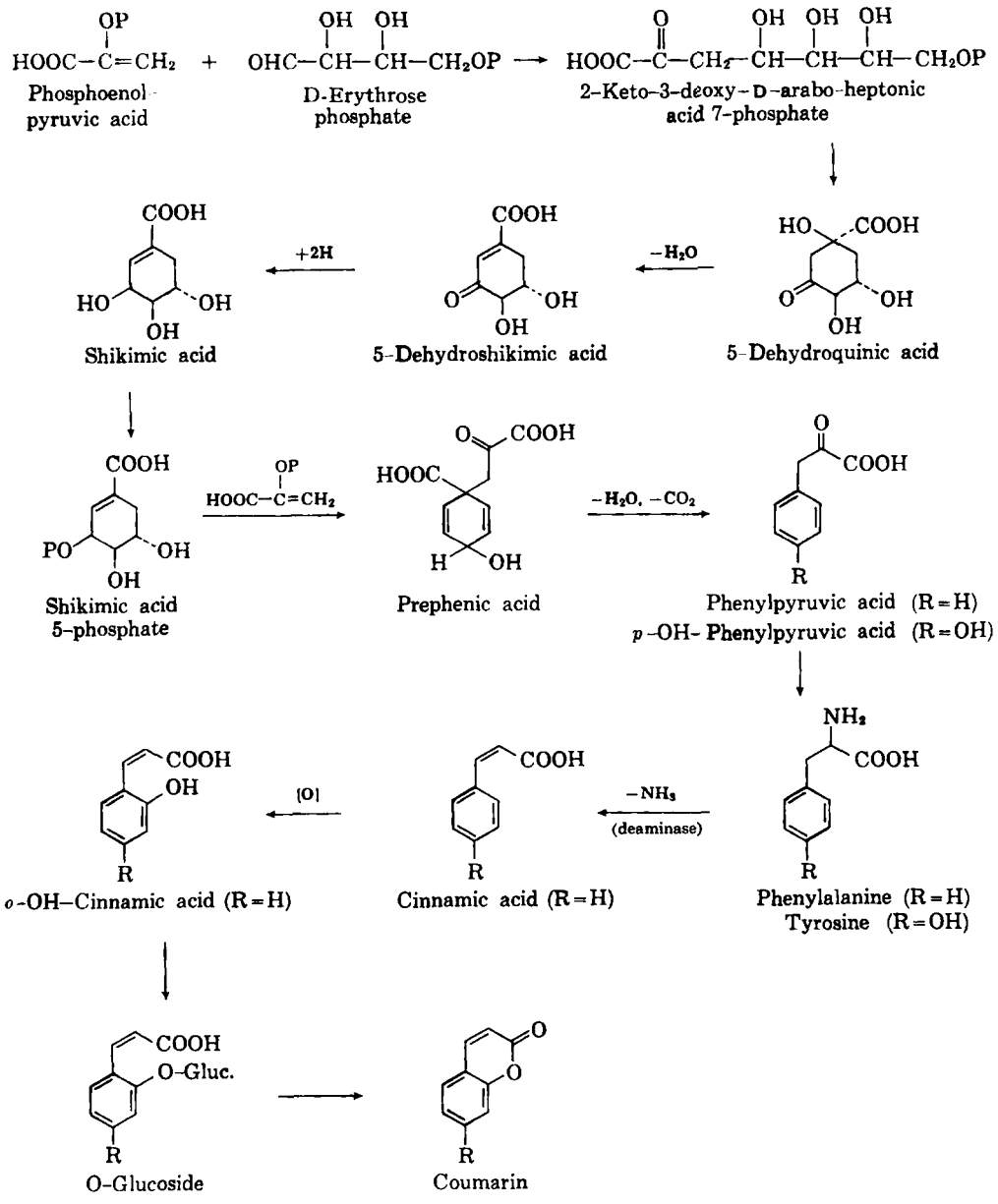


Fig. 1.—Shikimic acid pathway to coumarins.
Key: P = phosphate, R = H or OH as indicated, and Gluc. = glucosyl.

coumarins is somewhat beyond the scope of this review, but it may be pointed out that this field of interest is still in its infancy and much can be expected in the future. Being phenylpropanoid compounds, it has been of considerable interest to researchers to determine which of the two known (10) major pathways is involved in coumarin biosynthesis. These are the Birch-Donovan acetate pathway and the shikimic acid pathway. The acetate route involves head-to-tail condensation of acetate units to build up hydroxylated aromatic rings and has been shown *not* to be the major pathway to coumarins, although coumestrol appears to be an exception, being obtained by way of the isoflavone route (11). On the other hand, the shikimic acid pathway (Fig. 1) has been demonstrated as definitely involved. The initial step is a condensation of phosphoenolpyruvic acid with D-erythrose phosphate to form 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate which cyclizes to 5-dehydroquinic acid. This, in turn, proceeds to 5-dehydroshikimic acid and then to shikimic acid which, by way of its 5-phosphate, condenses with another mole of phosphoenolpyruvic acid to form prephenic acid. The latter aromatizes to yield phenylpyruvic acid (or its *p*-hydroxy derivative), then proceeds to phenylalanine (or tyrosine). Enzymatic deamination of phenylalanine, for example, provides cinnamic acid which has been shown to undergo *o*-hydroxylation followed by glucoside formation. The glucoside, in some manner, is finally converted to coumarin. The above sketch of the biosynthetic pathway to coumarins is admittedly brief; the reader is directed to the recent papers by Grisebach and Ollis (12) and Brown (13) for a more complete discussion together with key references. Brown points out that there are actually only a few of the simple coumarins about which much is known, largely through C^{14} tagging experiments. The growing activity in the field, however, is well illustrated by the work of Chambers, *et al.* (14), and Bunton, *et al.* (15), on the biosynthesis of novobiocin, and the work of Grisebach, *et al.* (16), on the biosynthesis of coumestrol.

Isolation Methods.—Ordinarily, the plant material is milled to a suitable state of subdivision and extracted in conventional types of apparatus. That the selection of the proper plant parts may be of great importance is emphasized by the comparative studies on isolation of visnadin by Smith, *et al.* (17), and Abu-Shady (18). In the former isolation, the total extract of the seeds of *Ammi visnaga* L. was worked up by a lengthy and tedious chromatographic procedure to obtain finally visnadin, together with its congeners, samidin, and dihydrosamidin. On the other hand, Abu-Shady utilized only the seed-free umbels and, by a relatively straightforward extraction and crystallization, obtained visnadin in good yield and in an excellent state of purity.

For the most part, isolation procedures depend upon successive extraction with solvents of increasing polarity. Thus, petroleum ether is frequently used as the initial extraction menstruum and has the advantage that most of the oxygenated coumarinic materials are not particularly soluble in it, although it must be pointed out that the oils extracted together with the coumarins tend to exert a solubilizing effect. A quick wash of the plant material with ice-cold petroleum ether (19) often removes much of this oil without undue sacrifice of coumarin material. As a result, coumarins quite frequently crystallize directly from the concentrated extract either during Soxhlet extraction or on standing and further concentration and cooling of the extract. Advantage, however, may be taken of the alkali hydroxide solubility and acid insolubility of most coumarins to separate them from other alkali-insoluble constituents in the extract. Plant acids are separated easily from coumarins by suitable use of sodium bicarbonate or sodium carbonate solutions which solubilize the acids only, the coumarins being soluble only in alkali hydroxides with or without the use of added alcohol. Petroleum ether may be omitted (and often is) and direct ether extraction may be employed. In either case, the above purification procedure may be used, although one must be cognizant of the possibility of facile hydrolysis of alkali-sensitive groups. This is particularly true of compounds such as samidin and its congeners (17) and also of pteryxin and suksdorfin (20). In these cases, a rapid expulsion of the 4′-acyloxy group takes place in the presence of alkali. Following extraction with petroleum ether and/or ether, the use of methanol or ethanol as the menstruum often results in the fortuitous crystallization of coumarinic glycosides. However, if no crystallization occurs, the glycosidic materials may be obtained by treatment of the extract in the usual way, such as the lead method, etc. In the event that the glycoside *per se* is not desired or is not obtainable, it is customary to cleave the coumarinic glycosides hydrolytically with acid or enzyme, then to recover the aglycones in the normal fashion already described. In any event, an effort should be made to identify the coumarins present in the extracts *before* chemical treatment so that comparisons can be made following such treatment to detect

possible hydrolytic cleavages. This is conveniently done with paper or thin-layer chromatographic techniques, taking advantage of the fluorescence of most coumarins under ultraviolet light. Isolation methods employing sublimation techniques are of limited usefulness due to the real possibility of structural modifications resulting in artifacts as a consequence of thermal lability. Nevertheless, the method is employed in the case of some of the simpler coumarins if care is taken to determine that the isolated products were originally present in the plant material.

Purification of crude coumarin fractions may be carried out by fractional crystallization techniques or by separations employing column chromatography, preparative thin-layer chromatographic techniques (21), or any of the other commonly used preparative chromatographic procedures. Examples of the power of the chromatographic methods in effecting separations are the isolations of Stanley, *et al.* (22), on lemon oil constituents and of Svendsen (23) in identifying the components of Norwegian umbellifers. A very helpful device in this respect has been the chromatostrip (24) for following the elution pattern from a column. The chromatostrip technique enables quick determinations of purity of fractions and is not unlike thin-layer chromatography, albeit less refined. Chromatography is a powerful means for demonstrating the homogeneity of unknown and known coumarins and frequently serves for identification purposes.

Chemical and Physical Properties.—Except

TABLE I.—GENERAL CHEMICAL REACTIONS OF COUMARIN NUCLEUS[a]

Reagent or Reaction Type	Result
Acids	No reaction. Solution in concentrated sulfuric acid often gives visible blue fluorescence
Ammonia	No reaction even under pressure and high temperatures
Hot dilute aqueous NaOH for short period of time	Formation of water-soluble yellow sodium coumarinate which regenerates original coumarin with acid
Hot dilute aqueous NaOH for prolonged heating period (or use of yellow HgO catalysis for shorter period of time)	Isomerization of initially formed sodium coumarinate (*cis*) to sodium coumarate (*trans*) which gives free coumaric acid instead of lactone on acidification
Alkali fusion or hot concentrated alkali	Usually provides benzenoid ring system as a phenol
Potassium carbonate and methyl iodide (or sulfate)	Methylates any phenolic hydroxyls but does not open coumarin ring
Sodium hydroxide and methyl iodide (or sulfate)	Forms *o*-methoxycinnamic acids
Reduction	
(*a*) H_2 with Pd/C	Smooth hydrogenation of 3:4 bond
(*b*) H_2 with Raney Ni (40–100°)	Hydrogenation of 3:4 bond
(*c*) H_2 with Raney Ni (200–250°)	Hydrogenation of entire ring system to give octahydrocoumarin and hexahydrochroman
(*d*) H_2 with Cu chromite	Reduction to 3-(*o*-hydroxyphenyl)-propyl alcohol
(*e*) Na and alcohol	Reduction to 3-(*o*-hydroxyphenyl)-propyl alcohol
(*f*) Na-Hg in dilute solutions	Reduces 3:4 bond to give sodium melilotate
(*g*) Na-Hg in concentrated solutions	Bimolecular reduction to give acid which provides dihydrodicoumarin on distillation
(*h*) Zinc and alkali (or AcOH)	Very similar to (*f*) and (*g*) above
(*i*) $LiAlH_4$	Yields *o*-hydroxycinnamyl alcohol and 3-(*o*-hydroxyphenyl)-propyl alcohol
Oxidation	
(*a*) Nitric acid or permanganates	Gives succinic acid if coumarin is first reduced at 3:4 position but none if not reduced (method for identifying coumarin system)
(*b*) Ozone	Order of attack is: double bonds in side chains, furan ring double bond, and finally 3:4 bond
(*c*) CrO_3	Resistant with respect to coumarin ring
(*d*) Permanganates	Usually results in a phenolic carboxylic acid
(*e*) Per acids	Resistant but forms epoxides of side chain double bonds
(*f*) Alkaline persulfates	Form 6-hydroxy coumarins
(*g*) Hydrogen peroxide (alkaline)	Especially useful with furanocoumarins, leaving furan-2:3-dicarboxylic acid
Mercuration	
(*a*) Mercuric chloride	Addition to 3:4 bond
(*b*) Mercuric acetate	Formation of 3,6,8-triacetoxymercuri-4-methoxydihydrocoumarin. This, on Br_2 treatment, gives 3,6,8-tribromocoumarin
Diazonium salts (with K_2CO_3)	Couple in 6 position
Grignard reagent	Adds first to carbonyl which may give an unsaturated ketone or, with excess reagent, can add a second mole to eventually give 2,2-dialkyl-1,2-benzopyrans or Δ^3-chromenes

for coumarin and dicumarol, all other naturally occurring coumarins bear an oxygen substituent at the 7 position (Table III) so that, in reality, one could say that umbelliferone (7-hydroxycoumarin) is the parent compound rather than coumarin. However, all coumarins are characterized by the benz-α-pyrone system which makes identification fairly straightforward in most cases. This lactonic character enables coumarins to be water-solubilized by alkali hydroxides with the usual appearance of yellow in the solution. The yellow coloration is particularly valuable for diagnostic purposes if alcohol is needed to bring the coumarin into solution sufficiently to enable alkali to react with it. Acidification (even with CO_2) of aqueous solutions results in prompt relactonization and recovery of the original coumarin barring hydrolytic cleavages. The speed of relactonization is such that it is virtually impossible in most cases to obtain the intermediate *o*-hydroxycinnamic acid unless a hydrogen bonding group such as nitro or carbonyl is present in the 8 position to slow down the process by bonding with the phenolic hydroxyl group.[a] Many of the other chemical properties of coumarins are given in Table I which summarizes most of those reactions common to this nucleus.

An interesting property of coumarins is their ability to fluoresce under ultraviolet light. Fluorescence of coumarins is not necessarily a unique feature of this group compared to other plant constituents but is, nevertheless, a useful diagnostic feature when used properly. The studies of Goodwin and Kavanagh (25, 26) on fluorescence as a function of pH are interesting in this respect as are those of Feigl, *et al.* (27), on

TABLE I.—(continued)

Reagent or Reaction Type	Result
Hydroxylamine	Adds 3 moles of NH_2OH in absence of alkali or 2 moles in presence of alkali to give a hydroxamic acid. Added $FeCl_3$ in latter case gives deep violet color (test for coumarins)
Diazomethane	Usually methylates only phenolic hydroxyls but can also form a pyrazoline derivative across 3:4 bond
Sodium bisulfite	Bisulfite addition compound at 4 position
Halogenation	
(*a*) Cl_2 or Br_2	Adds to 3:4 bond and then alkali will remove HX to give a 3-halogenocoumarin. Alkali converts the latter to coumarilic acids
(*b*) *N*-Bromosuccinimide	Forms 3-bromocoumarin directly
Nitration	Principally at 6 position; small amount at 8 position
Light	Causes photodimerization in some cases
Phosphorus pentasulfide	Forms thiocoumarins that are readily converted to the oxime and phenylhydrazone of the coumarin; otherwise unobtainable from coumarin directly
Diels-Alder conditions	Adds butadienes across 3:4 bond but addition is better to *o*-methoxycinnamic acids
Michael reaction conditions	Adds cyanoacetamide, malonic ester, and ethylphenylacetate
Special reactions of 7-hydroxycoumarin (umbelliferone)	
(*a*) Duff reaction	Forms 8-formyl-7-hydroxycoumarin
(*b*) Claisen migration on 7-allyloxycoumarin	Forms 8-allyl-7-hydroxycoumarin
(*c*) Fries rearrangement of esters of 7-hydroxycoumarin	Forms 8-acyl-7-hydroxycoumarin which on alkali treatment yields 2-acylresorcinols (Nidhon synthesis)
Special reactions of 4-hydroxycoumarin (benzotetronic acid)	
(*a*) Bicarbonates	Confer water solubility
(*b*) Aniline	Forms 4-anilino derivative
(*c*) PX_5	Forms 4-halogeno derivative
(*d*) Diazonium salts	Couple at 3 position to give 3-phenylhydrazone
(*e*) Aldehydes	Addition due to active methylene at 3 position
(*f*) Hydrolysis with aqueous alkali	Mild conditions give *o*-hydroxyacetophenone; more rigorous conditions give salicylic acid
(*g*) $\alpha\beta$-Unsaturated ketones	Adds in Michael manner
(*h*) Diazomethane	Mainly methylation of 4-hydroxyl but small amount of 2-methoxychromone also forms due to tautomeric 2-hydroxychromone
(*i*) Ferric chloride	No color in spite of enolic character due to unfavorable steric disposition for complex formation
(*j*) Hydrogenation	Difficult but proceeds to 4-hydroxydihydrocoumarin which dehydrates readily to reform the coumarin

[a] For a comprehensive survey of coumarin chemistry see Wawzonek, S., "Heterocyclic Compounds," Vol. 2, edited by Elderfield, R. C., John Wiley and Sons, Inc., New York, N. Y., 1951, p. 173.

the use of fluorescence *via* photocatalysis as a sensitive and specific test for coumarin. Ichimura (28) has published the spectra of numerous coumarins, and Wheelock (29) has studied the relationship of structure to fluorescent properties in an extensive series of coumarins. Crosby and Berthold (30) describe the paper chromatography of several coumarins and the usefulness of fluorescence spectra for characterizing the eluted coumarins. Most coumarins fluoresce blue under ultraviolet light and many of the 7-oxygenated ones even fluoresce in visible light, especially if dissolved in concentrated sulfuric acid. In alkaline solution the fluorescence usually turns green and may even disappear, particularly when the hydroxyl at the 7 position is free. A greenish fluorescence is noted with many of the furanocoumarins in a neutral state. Fluorescence is not particularly enlightening in determining structural features but is very helpful in locating and recovering coumarin spots on a chromatogram without use of a chemical spray.

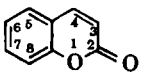
Because coumarins have well defined absorption bands in the ultraviolet region, they have been studied extensively in the hope that these spectra might have structural significance. The ultraviolet absorption pattern of the coumarin nucleus can be attributed largely to the benz-α-pyrone structure and Nakabayashi, *et al.* (31), assume that the two absorption bands of coumarin at 270 mμ and 312 mμ are those of benzene at *ca.* 200 mμ and 240–260 mμ that had shifted to these regions. The α-pyrone moiety absorbs at about 300 mμ (log ϵ, *ca.* 4), and variations from this are attributed to the pronounced effect of substituents and their location. Ganguly and Bagchi (32) considered the spectra of coumarins and chromones with a view toward finding differences that would eliminate confusion between these two superficially similar groups. They noted that chromones have strong absorption at 240–250 mμ with a log ϵ over 3.8, whereas the coumarins usually have a deep minimum at this wavelength. With respect to nonhydroxylated coumarins, they found that methyl substitution had little effect on the positions of the parent absorption bands. On the other hand, Sen and Bagchi (33) found that introduction of a hydroxyl group into the nucleus caused a general bathochromic shift of the principal absorption bands. In assessing the differences between hydroxychromones and hydroxycoumarins they found that the former show a considerable amount of absorption around 250 mμ with the log ϵ invariably over 4.1, whereas the log ϵ in the case of the hydroxycoumarins was never over 3.75. Furthermore, the minima of the hydroxycoumarins were between 255–262 mμ, but those of the hydroxychromones were over 280 mμ. There have been many studies (22, 34–42) on the ultraviolet spectral characteristics of the coumarins and, because of the variations shown by the naturally occurring variety, it is desirable to consult the original papers cited for specific details. One point that is worth mentioning is the bathochromic shift that usually ensues together with an increase in the extinction coefficient when the spectrum of a given coumarin is taken in an alkaline medium. For example, the maximum of umbelliferone at 325 mμ (log ϵ, 4.15) shifts to 372 mμ (log ϵ, 4.23) in alkaline solution.

Infrared analysis has found its place in the structural characterization of coumarins also. The particular value of such analysis, aside from detecting groups that are not fundamentally related to the coumarin nucleus, lies in its ability to assign a lactone function to the coumarin. Characteristic bands for the α-pyrone moiety are found, as a rule, in the region of 1715–1745 cm.$^{-1}$ together with the conjugated aromatic double bond at about 1625–1640 cm.$^{-1}$ Aromatic absorption bands are found in the usual regions.

High resolution nuclear magnetic resonance (NMR) spectra also have been of use in the structural definition of coumarins. In some cases, the structural argument has been based almost solely on the NMR spectrum (43, 44). In other cases, this analytical procedure has been used to assign a structure where it would have been difficult or impossible to do it chemically (45, 46). Dharmatti, *et al.* (47), have examined the high-resolution NMR spectra of coumarin and a number of its derivatives and have noted that the calculated and observed chemical shifts and coupling constants for the four protons in the benzene ring of coumarin agree satisfactorily. The chemical shifts for the protons in the 3 and 4 positions of coumarin are almost the same as those observed for the ethylenic protons in *o*-coumaric acid; thus, they do not substantiate the earlier view of a naphthalene-type resonance in coumarins (48). The coupling constant ($J_{3,4}=9.8$) confirms that the protons at the 3 and 4 positions are *cis* to each other as expected. They point out that the NMR spectra provide a convenient method for distinguishing between 3 and 4 substituted coumarins on the basis of the observed chemical shifts.

The use of mass spectrometry in coumarin

TABLE II.—NATURALLY OCCURRING COUMARINS



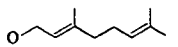
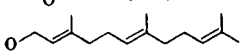
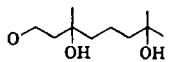
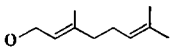
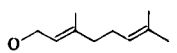
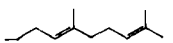
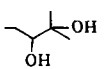
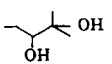
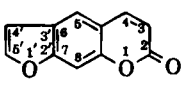
No.	Name (Synonyms)	M.p., °C.	Position of Substituent 4	5	6	7	8	$[\alpha]_D$ (solv., °C.)	Refs.
					A. Simple Coumarins				
1.	Coumarin	67–68	H	H	H	H	H	...	(5)
2.	Umbelliferone (skimmetin, dichrin A)	223–224	H	H	H	OH	H	...	(163–167)
3.	Skimmin	219–221	H	H	H	O-Glucoside	H	−79.8 (pyr., 18)	(168, 169)
4.	Neohydrangin	204	H	H	H	O-Diglucoside	H	−130.9 (H_2O, 21)	(170)
5.	Herniarin (ayapanin)	114	H	H	H	OCH_3	H	...	(165, 171, 172)
6.	Umbelliprenin	61–63	H	H	H		H	...	(173)
7.	Auraptene	68	H	H	H		H	...	(174, 175)
8.	Marmin	125	H	H	H		H	...	(176, 177)
9.	Esculetin (cichorigenin, crategin, aesculetin)	268–272 dec.	H	H	OH	OH	H	...	(164, 178–182)
10.	Esculin (aesculin)	205	H	H	O-Glucoside	OH	H	−146 (CH_3OH, 15)	(178, 179, 183, 184)
11.	Cichoriin	213–215	H	H	OH	O-Glucoside	H	−104.5 (dioxan)	(185, 186)
12.	Scopoletin	205–205	H	H	OCH_3	OH	H	...	(187–190)
13.	Scopolin	217–219	H	H	OCH_3	O-Glucoside	H	...	(187, 191, 192)
14.	Angelical	256–257	H	H	CHO	OCH_3	H	...	(193)
15.	Glabralactone	129–130	H	OCH_3	H	OCH_3		...	(194, 195)
16.	Fabiatrin	236–238	H	H	OCH_3	O-Primveroside	H	−140 (H_2O, 20)	(196)
17.	Ayapin	231–232	H	H	O—CH_2—O		H	...	(197, 198)
18.	Esculetin dimethyl ether (scoparone, scoparin)	144–146	H	H	OCH_3	OCH_3	H	...	(180, 199)
19.	Daphnetin	255–256 dec.	H	H	H	OH	OH	...	(200–202)
20.	Daphnin	223–224 dec.	H	H	H	O-Glucoside	OH	−123.9 (CH_3OH, 22)	(202–206)
21.	Daphnetin glucoside	197–198	H	H	H	OH	O-Glucoside	+4.57 (CH_3OH, 23)	(207)
22.	Citropten (limettin)	146–147.5	H	OCH_3	H	OCH_3	H	...	(208–211)
23.	5-Geranoxy-7-methoxy coumarin	86–87	H		H	OCH_3	H	...	(212)
24.	Collinin	67–68	H	H	H		OCH_3	...	(213)

TABLE II.—(*continued*)

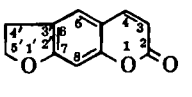
No.	Name (Synonyms)	M.p., °C.	Position of Substituent 4	5	6	7	8	[α]D (solv., °C.)	Refs.
25.	Fraxinol	171–173	H	OCH_3	OH	OCH_3	H	...	(214)
26.	Fraxetin	227–228	H	H	OCH_3	OH	OH	...	(215–218)
27.	Fraxin (paviin)	205	H	H	OCH_3	OH	O-Glucoside	...	(215, 219, 220)
28.	Fraxidin	196–197	H	H	OCH_3	OCH_3	OH	...	(217, 220, 221)
29.	Isofraxidin (calycanthegenol)	148–149	H	H	OCH_3	OH	OCH_3	...	(217, 221)
30.	Calycanthoside (isofraxidin glucoside)	219–220	H	H	OCH_3	O-Glucoside	OCH_3	−42° (CH_3OH)	(222)
31.	6,7,8-Trimethoxy coumarin	104	H	H	OCH_3	OCH_3	OCH_3	...	(216, 223)
32.	Osthenol	124–125	H	H	H	OH		...	(224)
33.	Vellein	187.5–189	H	H	H	O-Glucoside		...	(225)
34.	Osthol	83–85	H	H	H	OCH_3		...	(226–228)
35.	Merangin (auraptene)[a]	98	H	H	H	OCH_3		−33.4 (C_2H_5OH, 20)	(5, 229, 332, 333)
36.	7-Demethylsuberosin	133.5–134	H	H		OH	H	...	(223)
37.	Suberosin	87–88	H	H		OCH_3	H	...	(230)
38.	Ostruthin	117–119	H	H		OH	H	...	(231–234)
39.	Geijerin	121	H	H		OCH_3	H	...	(235, 236)
40.	Toddaculine	95	H	OCH_3		OCH_3	H	...	(237)
41.	Angelicone	130	H	OCH_3		OCH_3	H	...	(238)
42.	Toddalolactone	131–132.5	H	OCH_3		OCH_3	H	+55.9 ($CHCl_3$, 30)	(239, 240)
43.	Aculeatin hydrate[b]	131–132.5	H	OCH_3		OCH_3	H	+50.9 ($CHCl_3$, 26)	(241)
44.	Aculeatin	113	H	OCH_3		OCH_3	H	−16.8 (EtOAc, 24)	(241)
45.	Brayleyanin	95	H	H	OCH_3			...	(242)

46.	Dalbergin	209–210	C_6H_5	H	OH	OCH_3	H	...	(243–245)
47.	Mammein	128.5–129	*n*-C_3H_7	OH		OH		...	(246–248)
48.	4-Phenyl-5,7-dihydroxy-6-isovaleryl-8-isopentenyl coumarin	98–109	C_6H_5	OH		OH		...	(249–250)

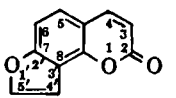
B. Furanocoumarins



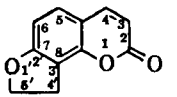
Psoralen Type



Dihydropsoralen Type



Angelicin Type



Dihydroangelicin Type

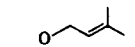
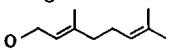
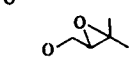
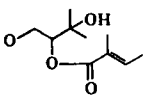
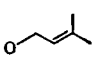
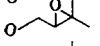
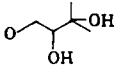
			Psoralen Type					
			Position of Substituent					
			5	4′	5′	8		
49.	Psoralen	161–163	H	H	H	H	...	(78, 251)
50.	Bergaptol	278–282	OH	H	H	H	...	(212, 252, 253)
51.	Bergapten (heraclin, majudin)	188–191 (sub.)	OCH_3	H	H	H	...	(254–257)
52.	Isoimperatorin	109		H	H	H	...	(258, 259)
53.	Bergamottin	59–61		H	H	H	...	(234, 260)
54.	Oxypeucedanin	141–143		H	H	H	Slightly levorotatory	(232, 261, 262)
55.	Ostruthol	136–137		H	H	H	−18.3 (pyrid., 15)	(263)
56.	Xanthotoxol	249–251	H	H	H	OH	...	(264–266)
57.	Xanthotoxin	145–146	H	H	H	OCH_3	...	(267–269)
58.	Prangenin	97	H	H	H	OC_4H_9(*n*)	...	(270, 271)
59.	Imperatorin(marmelosin, ammidin)	102–105	H	H	H		...	(264, 266, 272)
60.	5-Methoxy-8-hydroxy-psoralen	210–212	OCH_3	H	H	OH	...	(273)
61.	Isopimpinellin	148–151 dec.	OCH_3	H	H	OCH_3	...	(274, 275)

TABLE II.–(*continued*)

No.	Name (Synonyms)	M.p., °C.	Position of Substituent 5	4′	5′		[α]D (solv., °C.)	Refs.
62.	Phellopterin	102	OCH_3	H	H		...	(276, 277)
63.	Byakangelicol	106	OCH_3	H	H		+34.77 (pyrid., 25)	(276–278)
64.	Ferulin[d]	87	OCH_3	H	H		+27.31 (23) (pyrid., 23)	(279)
65.	Byakangelicin	125–126	OCH_3	H	H		+24.62 (pyrid., 25)	(276, 280)
66.	Psoralidin[e]	315 dec.	OH	H	H		...	(281, 282)
67.	Peucedanin (oreoselone methyl ether)	109	H	OCH_3			...	(283, 285)

DIHYDROPSORALEN TYPE

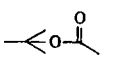
68.	Nodakenetin	185–192	H	H		H	−25.4 ($CHCl_3$, 24)	(286, 287, 368)
69.	Marmesin[f]	189.5	H	H		H	+26.8 ($CHCl_3$, 34)	(288, 289, 368)
70.	Nodakenin	215–219	H	H		H	+56.6 (H_2O, 30)	(286, 287)
71.	Marmesinin	259–260 dec.	H	H		H	−60 (50% C_2H_5OH, 25)	(290, 291)

ANGELICIN TYPE

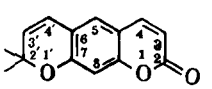
			Position of Substituent 5	6	5′	4′		
72.	Angelicin (isopsoralene)	138–140	H	H	H	H	...	(292, 293)
73.	Isobergapten	218–222	OCH_3	H	H	H	...	(253, 294)
74.	Sphondin	192–193	H	OCH_3	H	H	...	(295, 296)
75.	Sphondylin	161–163	A monomethoxyfuranocoumarin of uncertain structure, isomeric with No. 73 and 74				...	(295)
76.	Pimpinellin	117–119	OCH_3	OCH_3	H	H	...	(275 297)

DIHYDROANGELICIN TYPE

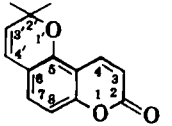
77.	Columbianetin	164.5–165	H	H		H	+20 (diox., 27)	(46)
78.	Columbianin	275–276(with $2H_2O$)	H	H		H	+118 (H_2O, 23)	(46)
79.	Columbianadin	121–122	H	H		H	+26.5 (diox., 27)	(46)
80.	Discophoridin	134–135	H	H		H	+20.4 (C_2H_5OH, 20)	(43)
81.	Athamantin	58–60	H	H			+96 (CH_3OH, 22)	(39, 298–300)

82.	Edultin	138-140	H	H			+41.13 (pyrid., 10.5)	(301)

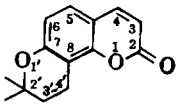
C. Pyranocoumarins (Chromeno-α-pyrones)



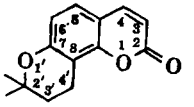
Xanthyletin Type



Alloxanthyletin Type



Seselin Type

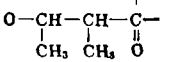


Dihydroseselin Type

XANTHYLETIN TYPE

			Position of Substituents 4	5	8		
83.	Xanthyletin	128–131	H	H	H	...	(302–304)
84.	Xanthoxyletin	132	H	OCH_3	H	...	(305, 306)
85.	Luvangetin	108–109	H	H	OCH_3	...	(307–309)

ALLOXANTHYLETIN TYPE

			Position of Substituents 4	7	8		
86.	Alloxanthoxyletin	115.5	H	OCH_3	H	...	(310)
87.	Calophyllolide	158	$-C_6H_5$	OCH_3		...	(311, 312)
88.	Inophyllolide	188	$-C_6H_5$			...	(312)

SESELIN TYPE

			Position of Substituents 5	6	3′	4′		
89.	Seselin	119–120	H	H	H	H	...	(313–315)
90.	Braylin	150	H	OCH_3	H	H	...	(242)

DIHYDROSESELIN TYPE

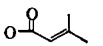
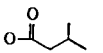
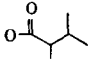
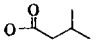
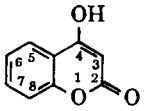
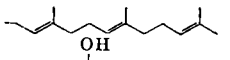
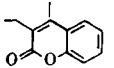
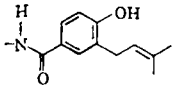
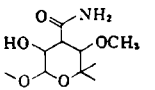
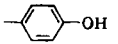
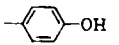
91.	Samidin	138–139	H	H		$OOC-CH_3$	+100 (dioxan)	(17, 316)
92.	Dihydrosamidin	111–113	H	H		$OOC-CH_3$	+63 (dioxan)	(17)

TABLE II.—(*continued*)

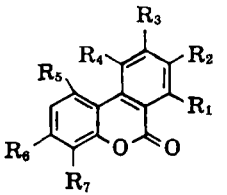
No.	Name (Synonyms)	M.p., °C.	Position of Substituent 5	6	3′	4′	[α] (solv., ° C.)	Refs.
93.	Visnadin (provismine)	85–88	H	H		OOC–CH₃	+38 (dioxan)	(17, 18, 316)
94.	Suksdorfin	140.5–141	H	H	OOC–CH₃		+4 (C_2H_5OH, 24)	(20, 317)
95.	Pteryxin	81.5–82.5	H	H	OOC–CH₃		+10 (C_2H_5OH, 22)	(20, 318)

D. 4-Hydroxycoumarins



No.	Name (Synonyms)	M.p., °C.	Position of Substituents 3	5	7	8	[α] (solv., ° C.)	Refs.
96.	Ammoresinol	107–108		H	OH	H	...	(319–322)
97.	Dicumaro	288–289		H	H	H	...	(49, 323)
98.	Novobiocin (albamycin, cathomycin, cardelmycin, spheromycin, etc.)	...		H		CH_3	...	(324–329)
99.	Isoshekkangenin[g]	228		OHC_3	OH	H	...	(330, 331)
100.	Isoshekkanin[g]	253		OCH_3	O–Glucoside	H	...	(330)

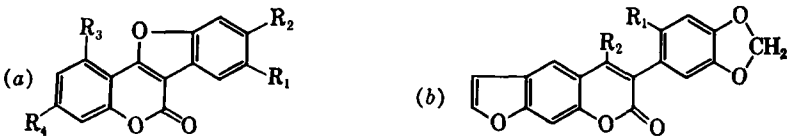
E. 3,4-Benzocoumarins



No.	Name (Synonyms)	M.p., °C.	Position of Substituents R_1	R_2	R_3	R_4	R_5	R_6	R_7	[α] (solv., ° C.)	Refs.
101.	2′:3″-Dihydroxydibenz-α-pyrone	360 dec.	H	OH	H	H	H	OH	H	...	(9)

102.	Alternariol	350 dec.	OH	H	OH	H	CH_3	OH	H	...	(334)
103.	Alternariol methyl ether	267	OH	H	OH (or OCH_3)	H	CH_3	OCH_3 (or OH)	H	...	(334)
104.	3:5:3′:5′-Dihydroxydiphenic acid dilactone	360	H	OH	H	O——OC		OH	H	...	(9)
105.	3:5:3′:5′-Dihydroxy-4:4′-dimethoxydiphenic acid dilactone	337–338	H	OH	OCH_3	O——OC		OH	OCH_3	...	(9, 335)
106.	Ellagic acid	360	H	OH	OH	O——OC		OH	OH	...	(9, 336, 337)

F. 3 Phenylcoumarins (and derivatives)



(*a*)

		Position of Substituents					
		R_1	R_2	R_3	R_4		
107.	Coumestrol	H	OH	H	OH	...	(68–70)
108.	Wedelolactone	OH	OH	OH	OCH_3	...	(338, 339)

(*b*)

		Position of Substituents				
		R_1	R_2			
109.	Pachyrrhizin	OCH_3	H		...	(340)
110.	Erosnin	O			...	(341, 342)

G. Miscellaneous Coumarins

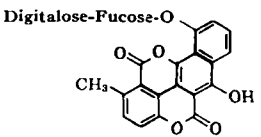
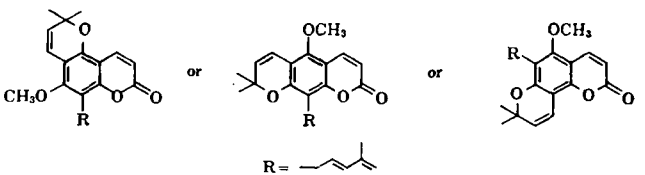
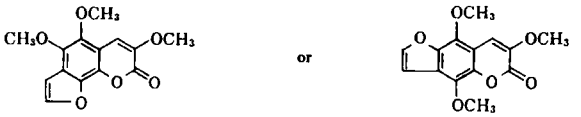
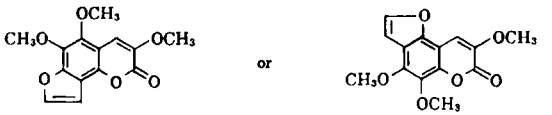
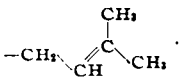
			Structure(s)		
111.	Chartreusin (antibiotic X-465A)	184–186		+132 (pyr., 25) −33 (gl. acetic acid, 25)	(343–347)
112.	Avicennin	141–142		...	(45, 348–350)

TABLE II.—(*continued*)

No.	Name (Synonyms)	M.p., °C.	Structures	[α]D (solv., °C.)	Refs.
113.	Halfordin	136–137		...	(351)
114.	Isohalfordin	151–152		...	(351)
115.	Aflatoxin B (I) Aflatoxin G (II)	268–269 244–246	 I II	... ...	(8, 44)

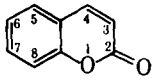
[a] See also entry No. 7. Priority of the name rests with No. 7. [b] Aculeatin hydrate is identical to No. 42 or is an isomer. [c]  is to be interpreted as  . [d] Formula claimed is that of No. 63; matter has not been cleared up yet. Very doubtful that this formula is correct (see *Reference 282*). [f] No. 69 is the optical isomer of No. 68. [g] The validity of this structure is doubtful.

structural studies has not been exploited to any degree yet. However, the technique has been used to determine the exact molecular weights of compounds in scarce supply (44). Molecular weights may be obtained also by X-ray determination of the unit-cell dimensions for a given compound which, together with the density of the crystals, enables a precise calculation of this value that can be very essential to structural elucidation. The latter technique has been helpful in the determination of the structure of novobiocin, a coumarin-type antibiotic.

Classification.—There are several structural categories into which the coumarins could be divided based on the desires and needs of the classifier. The classification employed in Table II suffices for the purposes of this review. It structurally defines the known coumarins and gives some of the pertinent physical constants and references to the original literature. The various complex coumarins in the first category, *Simple Coumarins*, may indicate something of an overstatement insofar as the connotation of simplicity is concerned, when one considers the relative complexity of some of the entries. Nevertheless, for ease of classification, this group was handled best in this manner. The other classes, *i.e.*, *furanocoumarins*, *pyranocoumarins*, 4-*hydroxycoumarins*, 3,4-*benzocoumarins*, and 3-*phenylcoumarins* have relatively straightforward structural relationships. The various structural features to be found in natural coumarins are recorded in Table III and illustrate the variety of substituents and their positions to be found in this group.

Physiological Activity.—Although the naturally occurring coumarins are a large and important group of plant products, it is doubtful

TABLE III.—GENERAL STRUCTURAL FEATURES OF NATURAL COUMARINS



Position	Substituent	Comment
3	—AR—	Aromatic ring fused across 3:4 bond
	—AR	Phenyl ring, usually phenolic. May or may not be bridged at an *o*-position to the 4 position by an ether linkage)
	—R	Farnesyl
	—OR	Methoxyl (only in halfordin and isohalfordin)
	—CO—$(CH_2)_2$—	Bridged to 4 position
	—COO$(CH_2)_2$—	Bridged to 4 position
	—CH_2-coum.	Bis compound (see dicumarol)
	—NHCO—R	See novobiocin
4	—OH	High degree of enolic character
	—R	Only *n*-propyl (see mammein)
	—AR	Phenyl
	—AR—	Fused across 3:4 position
5	—OH	Typically phenolic
	—OR	(*a*) Methoxyl, isopentenyloxy (and its epoxide and hydrated epoxide), and geranoxy (*b*) Part of pyrano ring (and possibly furano) attached to 6 position
	—R	Methyl (only in alternariol and its methyl ether)
	—COO—	Lactone bridge to benzene ring fused at 3:4 position (see ellagic acid and congeners)
6	—OH	Typically phenolic
	—OGl	Glucoside
	—OR	Methoxyl, and methylenedioxy (to 7 position)
	—R	(*a*) Isopentenyl (and its epoxide and hydrated epoxide) and geranyl (*b*) Point of attachment of pyrano or furano ring system originating at 5 or 7 position
	—CO—R	Isopentenoyl and isopentanoyl (isovaleroyl)
7	—H	Found only in coumarin and dicumarol
	—OH	Typically phenolic. Present in all natural coumarins as free OH or one of the combined forms below
	—OGl	Glucoside (or, in one case, a primveroside)
	—OR	(*a*) Methoxyl, methylenedioxy (to 6 position), isopentenyloxy, geranoxy (and its dihydroxy derivative), and farnesyloxy (*b*) Part of ring system attached as furano or pyrano type to 6 or 8 position
8	—OH	Typically phenolic
	—OGl	Glucoside
	—OR	Methoxyl, *n*-butoxyl, isopentenyloxy (and its epoxide and hydrated epoxide)
	—R	Isopentenyl (and its epoxide and hydrated epoxide)
	—CO—R	Isopentanoyl (isovaleroyl)

that their full range of physiological actions has been appreciated by most investigators. There has been, however, a continuing effort and a buildup of literature in this area with an occasional sporadic flurry of publication concerned with certain coumarins that have caught the wider interest of scientists. Bose (7), as previously mentioned, has considered the biochemical properties of the natural coumarins in an effort to show that these products are not mere metabolic products of the living cell, but that they possess varied and often remarkable physiological actions. Thereby, he stimulated an interest in coumarins for others. In surveying the literature, the present author has succumbed to the temptation to depart from the announced intention of covering only naturally occurring coumarins and has, to a limited extent, covered some activities of synthetic coumarins that seemed of interest. Table IV summarizes the various physiological activities that are known to exist in the coumarin compounds with particular emphasis on activity associated with the natural products. A selected group of physiological activities will be discussed in more or less detail; the remainder, which represents only one or two investigations at best, will be taken care of by simply referring to the work.

ANTICOAGULANT ACTIVITY

Recognition of the coumarin moiety as an anticoagulant species dates back to the report of Link, *et al.* (49), concerning studies on the toxic agent of spoiled sweet clover hay. The often fatal toxic material causing serious hemorrhagic conditions in cattle was found to be 3,3′-methylenebis-(4-hydroxycoumarin) (*Dicumarol*, Table V). Following this discovery, the problem of structure-activity relationships was examined by numerous investigators, prominent among which are Link (50), Mentzer, *et al.* (51), Chmielewska and Cieslak (52), and Arora and Mathur (53). Link came to the conclusion that the minimum requirements for hypoprothrombinemic activity were an intact 4-hydroxycoumarin with a substituent at position 3, a keto group on the 3-substituent in a 1:5 position to the 4-hydroxyl and, for maximal activity, a bis arrangement was necessary. Mentzer and his co-workers also felt that this arrangement was needed, although subsequent work has shown that this is a nonessential feature. These early studies, of course, led to synthetic efforts in other laboratories, and today there is a substantial literature connected with compounds of this nature. The most important of these synthetic discoveries are listed in Table V. Most of the synthetic work has been directed toward correcting the shortcomings of the naturally occurring prototype, dicumarol, although it still enjoys extensive usage. The principal faults were a slow onset of action and too prolonged an action which was difficult to terminate—although the action could be stopped with vitamin K (or menadione), provided the dicumarol level was not too high (54). It may be observed, however, that the coumarins as a group are singularly free of toxic side reactions and may be given for years without development of untoward effects except hemorrhage from overdosage. It is worth noting that the activity of 3-(α-acetonylbenzyl)-4-hydroxycoumarin (warfarin), for example, was so great that it was employed as an effective hemorrhagic rodenticide for years before its acceptance into human

TABLE IV.—SOME PHARMACOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF COUMARINS

Activity	Ref.
1. Inhibition of germination and root growth ("blastocholine effect"), *i.e.*, plant growth regulation	(7)[a]
2. Cytogenetic and cytological properties	(7, 352–354)
3. Curare-like action	(7)
4. Spasmolytic action	(7, 20, 317, 318, 355)
5. Narcotic effect	(7)
6. Sedative and hypnotic effects	(7) text[b]
7. Central nervous system paralysis	(7)
8. Central nervous system stimulation	(7, 356)
9. Respiratory stimulation	(357)
10. Uricosuric action	(7)
11. Vitamin-P-like action on capillary fragility	(7)
12. Anticoagulant action	(7) text
13. Hemostatic effect	(7)
14. Bacteriostatic, tuberculostatic, and antifungal action	(7) text
15. Anthelmintic action	(7) text
16. Piscicidal action	(7, 358–360)
17. Dermal photosensitization	(7) text
18. Antileucodermic action	(7) text
19. Bacterial photosensitization	text
20. Antiveratrinic action	(361)
21. Anti-retinal pigment degeneration effect	(367)
22. Analgesic action	text
23. Hypercholesteremic effect	(362)
24. Hypocholesteremic effect	(363)
25. Antidigitoxin activity	(364)
26. Hypothermal action	text
27. Estrogenic action	text
28. Molluscacidal action	text
29. Vasodilator action	text
30. Human chorionic gonadotrophin inhibitory action	(365)
31. Choleretic action	(366)

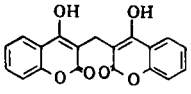
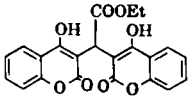
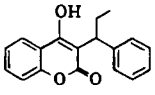
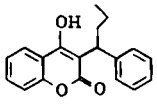
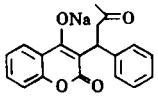
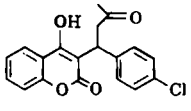
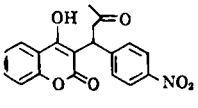
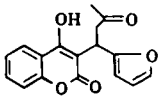
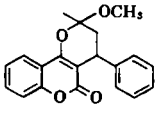
[a] *Reference* 7 gives a relatively recent survey of biochemical properties associated with coumarins and, in this table, reference to it suggests that the reader consult the original article for leading references. [b] "Text" indicates that an adequate discussion together with pertinent references is in this review.

therapy (*i.e.*, as coumadin). An interesting note in connection with racemic warfarin is that recently it has been resolved, its absolute configuration determined, and that levorotatory (S)-warfarin has been shown to be seven times as active as (R)-warfarin (55).

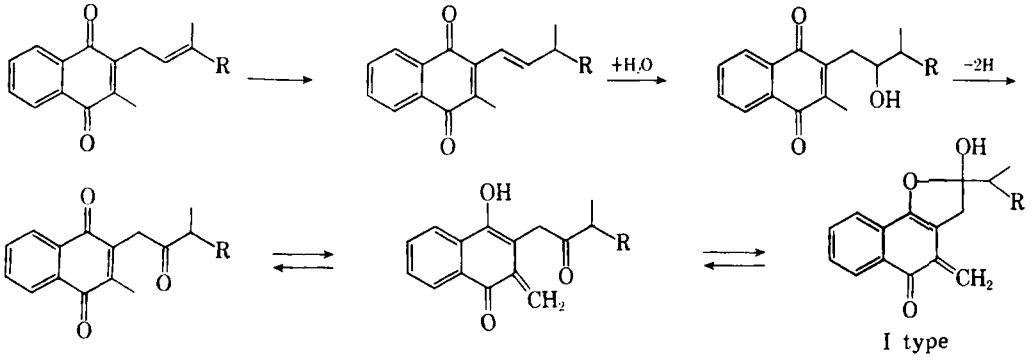
The activity associated with the coumarin-type molecule is currently (56) considered to be a vitamin K antagonism in which the anticoagulant competes with vitamin K in its blood clotting role. Thus, the clotting time is increased *in vivo* (but not *in vitro*) by a decrease in the prothrombin concentration of the blood presumably by interfering with its production in the liver. Prothrombin production is believed to arise from a combination of vitamin K (a compound having a 2-methyl-1:4-naphthoquinone nucleus) with an apoenzyme (AE) to form the active enzyme (AEK). The K vitamin acts as the prosthetic group of the enzyme. The coumarins (B) can compete successfully with the K vitamin for the apoenzyme by virtue of structural similarity and thus exert antivitamin K activity. The equation, $AEK + B \rightleftarrows AEB + K$, expresses this relationship as a reversible system and, thus, vitamin K can reverse the coumarin activity if present in sufficient concentration. Reduction of prothrombin production, of course, ensues if the form AEB predominates.

In keeping with this concept, Chmielewska and Cieslak (52) have hypothesized that the

TABLE V.—SOME USEFUL ANTICOAGULANT COUMARINS

Structure	Generic Name	Proprietary Name(s)
	Bishydroxycoumarin	Dicumarol
	Ethyl biscoumacetate	Tromexan ethyl acetate, pelentan
	...	Marcumar, marcoumar
	...	Liquamar
	Warfarin (sodium)	Coumadin (sodium)
	Coumachlor	Tomorin
	Acenocoumarol	Sintrom
	...	Fumarin
	Cyclocoumarol	Cumopyran

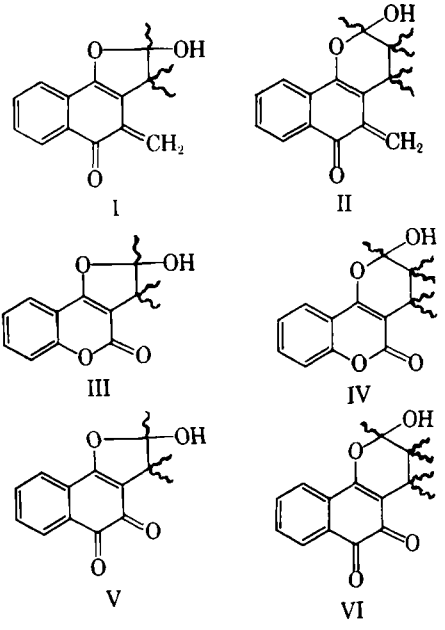
structure necessary for vitamin K activity is expressed by formulas I and II with formula I type arising from biological oxidation of the phytyl or difarnesyl chain of vitamins K as

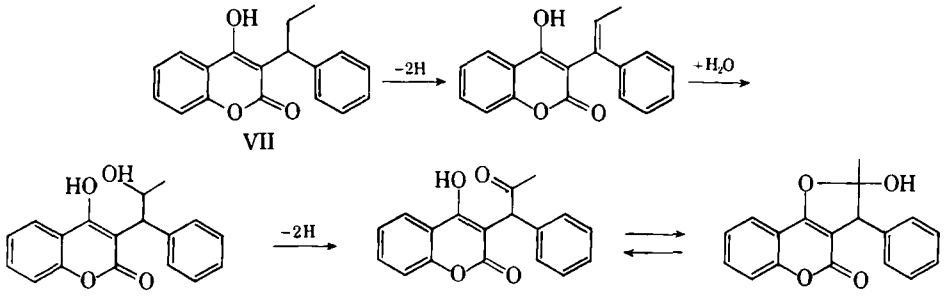


The problem associated with adapting dicumarol, in the face of the accepted bis-formula, was ingeniously solved by showing that dicumarol actually existed in a tautomeric keto-enol form

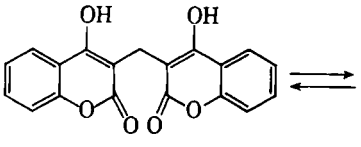
They further postulated that the *o*-quinoid group, O=C—C=CH_2, acts as the active center, whereas the hemiketal group is used to attach the active molecule to the protein part of the enzyme. In keeping with this hypothesis, they suggest that coumarin type antivitamin K compounds (III, IV) as well as V and VI (3-substituted derivatives of 2-hydroxy-1:4-naphthoquinone) which are known hemorrhagic agents lack the active center but contain the hemiketal linkage and, being similar in shape, successfully compete with vitamins K for the apoenzyme. Two active coumarins, however, failed to have the structural requisites to fit into their hypothesis—namely, 3-(1′-phenyl-*n*-propyl)-4-hydroxycoumarin (VII) and dicumarol itself. The known activity of VII was suggested by the authors as accounted for by a probable biological oxidation at the 2′-position, a postulation that was supported by their preparation of model compounds incapable of oxidation at the 2′-position, all of which were inactive. The suggested sequence of events in the proposed biological oxidation is

(VIII) which would, of course, leave the way open for formation of a hemiketal structure

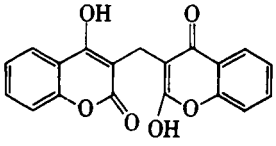




related to formula IV. In conformity to this,



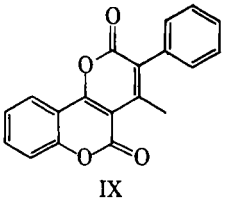
Dicumarol



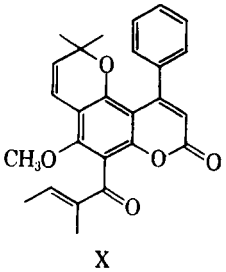
VIII

one can cite Link's cumopyran (see Table V) as an active agent.

Although the findings of Chmielewska seem rather conclusive, Arora and Mathur (53) have studied a series of coumarins recently with a view toward further amplification of the structure-activity relationships. In general, their findings support the Chmielewska-Cieslak hypothesis insofar as the 4-hydroxycoumarins are concerned. They found, however, that introduction of a methoxyl group into the 4-hydroxycoumarin molecules (including dicumarol) potentiated activity but that additional loading of the molecule with methoxyl groups could inhibit or augment activity, depending on the location of the groups. In particular, they felt that a methoxyl at position 8 had a special potentiating effect. A single compound (IX) in their series, at least formally related to formula IV, showed considerable activity, although they felt their observation to be on such a limited basis that it had little significance. Calophyllolide (X), however, with an activity superior



IX



X

to that of Dicumarol is unexplainable on the basis of the Chmielewska-Cieslak hypothesis. This naturally occurring coumarin apparently has excellent anticoagulant activity (53) but is not a 4-hydroxycoumarin, nor does it have a substituent in the 3-position. Arora and Mathur suggest that the inhibitory effect of the 4-phenyl group (noted in their studies on 4-phenylcoumarins) is probably counteracted by several potentiating factors which presumably are the 7-methoxyl, the 8-acyl group, and the additional dimethylpyrano group bridging positions 5 and 6. In conclusion, the latter authors state that anticoagulant activity is "governed not by individual structural features but by a combination of several, none of which can be defined precisely at the present time." Among the general structural features that could be important, however, they cited molecular shape, a six-membered heterocyclic ring (other than pyrone) such as a cyclic acetal, a substituent at position 8, and etherification of free phenolic groups. The last point was felt to be important by these authors, inasmuch as their findings indicated that ionization was possibly correlated with vitamin K activity.

ESTROGENIC ACTIVITY

A discussion of estrogenic activity in coumarin type compounds may be backgrounded advantageously by a brief consideration of the closely related isoflavones which, historically, were the first of the plant phenolics to show such activity. Indeed, the isoflavones may well be the source of coumarin estrogens (*e.g.*, coumestrol) in the plant as will become evident in the following discussion and from comments made in the consideration of biosynthesis in this paper. Estrus promoting substances in plants, however, have been known since 1926, shortly after the development of the Allen-Doisy test and are well reviewed by Bradbury and White (57) as well as Cheng, *et al.* (58). Continuing interest in naturally occurring estrogens has resulted recently in the isolation and characterization (59, 60) of miroestrol, virtually as active as diethylstilbestrol, from *Pueraria mirifica.*

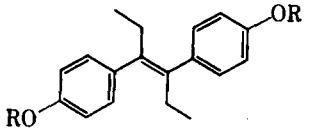
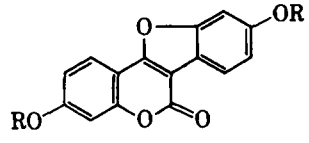
Recognition of the role of isoflavones as estrogens probably stems from the observations of Bennetts, *et al.* (61), in 1946 in connection with a serious sheep breeding problem encountered in western Australia. The problem of infertility was found to be caused by grazing on pastures of subterranean clover (*Trifolium subterraneum* L.). This finding was confirmed by Curnow, *et al.* (62), and was followed by the actual isolation of genistein (IVe),[1] an isoflavone, from this plant by Bradbury and White (63) and the suggestion by Biggers and Curnow (64) that this compound represented the principal estrogenic substance in the plant. Further studies have resulted in the demonstration of estrogenic activity in numerous other forage plants and the isolation

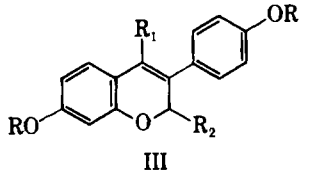
[1] Numbers such as IVe represent the structure to be found under that number in Table VI.

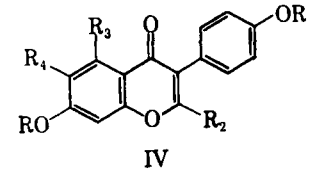
and synthesis of other interesting isoflavones such as formononetin (IVc), biochanin-A (IVf), and daidzein (IVd). The activities are well summarized by Cheng, *et al.* (58), and Micheli, *et al.* (65) (see Table VI).

The probable structural relationship of the isoflavones to diethylstilbestrol (Ia) did not escape the attention of workers in this field. Indeed, even a relationship with estradiol was visualized by some. The structure of coumestrol (IIa) may also be compared in this context. It was suggested by Biggers that genistein was probably a proestrogen, being metabolized in the animal body to the true estrogen. Thus, a number of compounds were prepared to explore the structure-activity relationships and to make compounds more closely resembling diethylstilbestrol. These studies are well summarized by Bradbury and White (57) and need not be discussed in detail here. Briefly, these studies led to the conclusion that, at best, the isoflavones were weakly estrogenic but that certain structural features seemed to promote activity in some measure. Among these features was the presence of a 5-hydroxy group that was suggested as being involved in hydrogen bonding with the carbonyl at position 4, presumably bringing about a greater contribution of the tautomeric enolic structure as against the ketonic structure. Coplanarity of the molecule as a whole was emphasized by Bradbury and White (66) in 1953 as being important to estrogenic activity. These workers also observed that reduction of the 2:3 double bond to form isoflavanones reduced activity. In contrast, it was noted (67) that isoflav-3-ens (III), as a group, were much more active than the isoflavones, possibly because of the much greater similarity to the diethylstilbestrol molecule. In this connection, it was noted that a 2- or 4-alkyl (or better, aryl) substituent conferred unusual activity on the isoflav-3-ens, whereas a methoxyl group at position 5 dropped activity. The isoflav-3-ens undoubtedly function as diethylstilbestrol does because structural modifications in both groups seem to affect activity similarly.

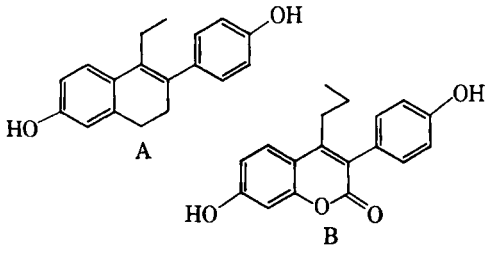
TABLE VI.—COMPARATIVE ESTROGENIC POTENCIES[a]

Compd. and Structure, Type	No.	Substituents	Dose Level, mg.[b]
I. Diethylstilbestrol derivatives		R	
 I	(*a*)	H—	0.00008
	(*b*)	CH_3CO —	0.00010
	(*c*)	$CH_3(CH_2)_{14}CO$—	0.00021
	(*d*)	CH_3—	0.00045
II. Coumestrol derivatives		R	
 II	(*a*)	H—	0.25
	(*b*)	CH_3CO—	0.33

III. Related isoflav-3-ens		R	R_1	R_2	
 III	(*a*)	CH_3CO—	C_2H_5—	CH_3—	0.00014
	(*b*)	CH_3—	C_2H_5—	CH_3—	0.00038
	(*c*)	CH_3CO—	H—	CH_3—	0.031
	(*d*)	CH_3—	H—	CH_3—	0.051
	(*e*)	CH_3—	C_6H_5—	H—	0.005
	(*f*)	CH_3CO—	C_2H_5—	H—	0.012
	(*g*)	CH_3—	C_2H_5—	H—	0.060
	(*h*)	CH_3—	CH_3 —	H—	0.208

IV. Related isoflavones		R	R_1	R_2	R_3	R_4	
 IV	(*a*)	H—	H—	CH_3—	H—	H—	21
	(*b*)	CH_3CO—	CH_3CO—	CH_3—	H—	H—	...
	(*c*)	H—	CH_3—	H—	H—	H—	31
	(*d*)	H—	H—	H —	H—	H—	11
	(*e*)	H—	H—	H—	HO—	H—	8
	(*f*)	H—	CH_3—	H—	HO—	H—	18
	(*g*)	H—	CH_3—	H—	H—	CH_3O—	(15)[c]

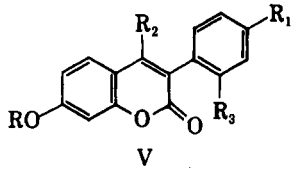
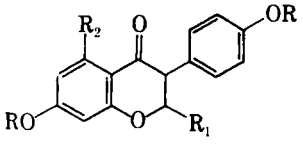
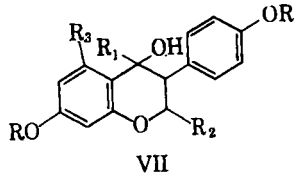
The matter of estrogenic activity of plant phenolics stood at this point until 1957 when Bickoff, *et al.* (68, 69), working with ladino clover (*Trifolium repens*), isolated an estrogenic coumarin which they named coumestrol (IIa). They later synthesized it as well (70). Further study showed that the same compound was the predominant one in ladino clover, strawberry clover (*Trifolium fragiferum*), and alfalfa (*Medicago sativa*). Comparison of estrogenic potencies showed that coumestrol was approximately 30 times as potent as genistein, although still far short of diethylstilbestrol. Interestingly enough, prior to the isolation of coumestrol, Mentzer, *et al.* (71, 72), as early as 1946, had anticipated the estrogenic activity of coumarins on purely theoretical grounds. These workers (72) based their researches on several diverse comparisons of structurally analogous compounds exhibiting similar activities and proposed that estrogenic activity should be exhibited by coumarins structurally related to diethylstilbestrol, but more particularly by preparing compounds directly related to 1-ethyl-2-*p*-hydroxyphenyl-3,4-dihydro-6-hydroxynaphthalene (A) which had been prepared by Dodds, *et al.* (73). Compound A was estrogenic in a dose of 100 mcg. and, essentially, Mentzer's modification was the replacement of $—CH_2CH_2—$ in A with the —COO— group. The most active compound in their series proved to be 3-(*p*-hydroxyphenyl)-4-*n*-propyl-7-hydroxycoumarin (B). Bickoff, *et al.* (68), compared this compound



with coumestrol and found the same order of activity (compare also IIa and Vj in Table VI).

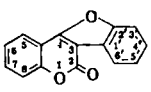
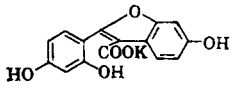
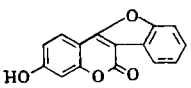
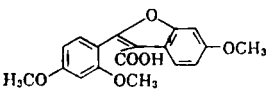
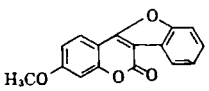
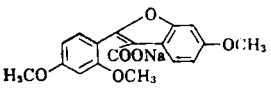
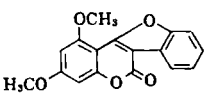
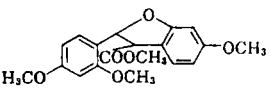
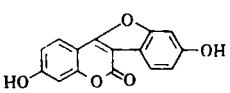
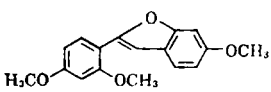
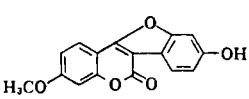
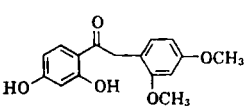
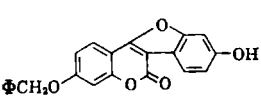
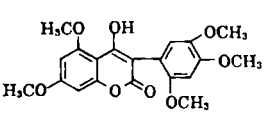
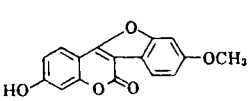
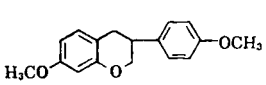
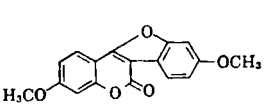
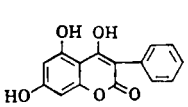
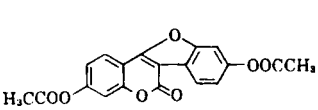
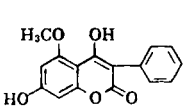
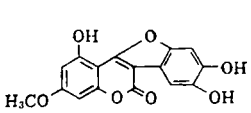
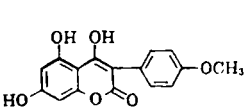
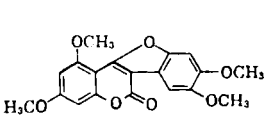
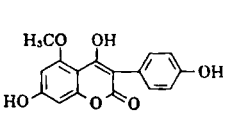
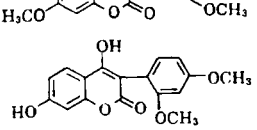
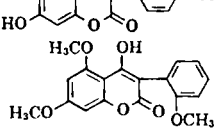
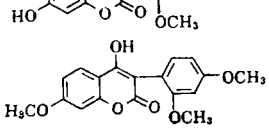
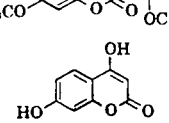
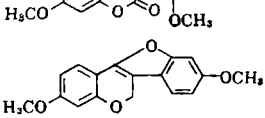
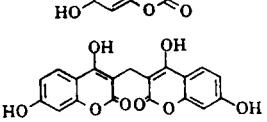
In a further study of structure-activity relationships, Bickoff, *et al.* (74), compared cou-

TABLE VI.—(continued)

Compd. and Structure, Type	No.	Substituents				Dose Level, mg.[b]
		R	R_1	R_2	R_3	
V. Related coumarins						
 V	(*a*)	H—	HO—	H—	H—	37
	(*b*)	CH_3CO—	CH_3COO—	H—	H—	40
	(*c*)	CH_3—	CH_3O—	H—	H—	(50)
	(*d*)	CH_3CO—	CH_3COO—	CH_3COO—	H—	(50)
	(*e*)	H—	HO—	HO—	H—	(40)
	(*f*)	H—	CH_3O—	HO—	H—	(50)
	(*g*)	H—	CH_3O—	HO—	CH_3O—	(25)
	(*h*)	H—	CH_3O—	*n*-C_3H_7—	H—	0.031
	(*i*)	CH_3—	CH_3O—	*n*-C_3H_7—	H—	0.032
	(*j*)	H—	HO—	*n*-C_3H_7—	H—	0.10
	(*k*)	H—	HO—	C_2H_5—	H—	(0.10)[d]
	(*l*)	H—	H—	C_2H_5—	H—	0.
	(*m*)	H—	CH_3O—	CH_3—	H—	0.2
VI. Related isoflavanones		R	R_1	R_2		
 VI	(*a*)	CH_3CO—	H—	H—		25
	(*b*)	H—	H—	H—		..
	(*c*)	CH_3CO—	CH_3—	H—		..
	(*d*)	H—	CH_3—	H—		..
	(*e*)	CH_3CO—	H—	CH_3COO—		18
VII. Related isoflavan-4-ols		R	R_1	R_2	R_3	
 VII	(*a*)	CH_3—	C_6H_5—	H—	H—	0.0035
	(*b*)	CH_3CO—	H—	CH_3—	H—	0.320

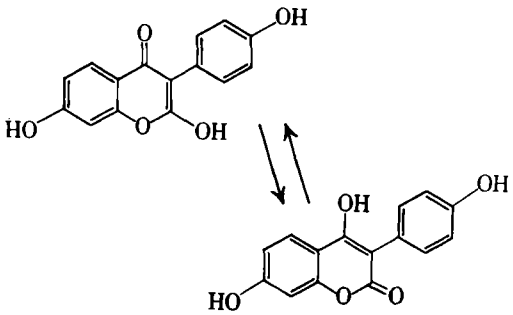
[a] This table is adapted from the tabular material given by Micheli, R. A., Booth, A. N., Livingston, A. L., and Bickoff, E. M., *J. Med. Pharm. Chem.*, **5**, 321(1962). [b] Represents the quantity of material fed to produce a 25-mg. uterine response compared to a uterine weight of 10 mg. for untreated animals. [c] Dose level in parentheses means that the compound was inactive at the indicated level. [d] Only slightly active at this level; higher dosage levels not tested because of lack of material.

TABLE VII.—RELATIVE ESTROGENIC ACTIVITIES OF COUMESTROL AND RELATED COMPOUNDS

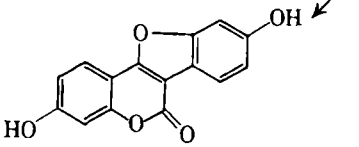
Compd. No.	Structural Formula	Relative Activity	Compd. No.	Structural Formula	Relative Activity
1		0	16		754
2		152	17		20
3		40	18		15
4		0	19		26
5		1000	20		3
6		540	21		16
7		189	22		0
8		150	23		0
9		359	24		0
10		850	25		0
11		1	26		3
12		0	27		0
13		22	28		0
14		0	29		0
15		0	30		0

mestrol with numerous structurally related compounds and obtained the results in Table VII. A number of interesting comparisons were made from a study of the results, although in no case was greater activity obtained than that of coumestrol itself. From the tabulated results it was observed that the phenolic hydroxyl groups in positions 4′ and 7 were quite important, inasmuch as their absence (compound 1, Table VII) led to inactivity. Some return of activity was noted with the presence of the hydroxyl at position 7 (compound 2, Table VII). Etherification of the phenolic groups (compounds 3 and 9, Table VII) results in reduced activity, a finding that is in conformity with almost all phenolic estrogens, except for the anomalous case of the 4-alkylcoumarins (65) where the reverse is true. It may be observed that etherification of the 4′-hydroxyl (compound 8, Table VII) reduces activity more than similar treatment of the 7-hydroxyl (compound 6, Table VII). Other nuclear substitution (*i.e.*, OH or OCH_3) as in the 3′ and 5 positions (compounds 11–14, Table VII) almost eliminates activity. Acetylation of the hydroxyl groups of coumestrol (compound 10, Table VII) results in very little loss in activity, possibly due to facile hydrolysis of these groups *in vivo* to regenerate coumestrol. The ether link bridging the 4 and 2′ positions appears to contribute substantially to activity since the lack of the ether bridge (compound 23, Table VII) produces a virtually inactive compound. Compounds 13 and 14 exhibit similar activity losses by opening of the 4:2′ ether bridge, although in this case the compounds bear a hydroxyl in position 4. Lack of activity in 4-hydroxycoumarins is general and is attributed to destabilization of the 3:4 double bond and, therefore, of the stilbene system which is now free to exist in tautomeric keto-enol forms. It has been suggested that the greater activity of coumestrol when compared with the isoflavones may be due to the principally ketonic character of the isoflavone at position 4. Reduction of the double bond at the 3:4 position (compounds 15, 23, Table VII) brings about loss of activity even though the ether linkage is still present in compound 15 (the C=O at position 2, however, is replaced by $—CH_2—$). Among the interesting observations made was that opening of the lactone ring to form the potassium salt of the resulting *o*-hydroxycinnamic acid (compound 16, Table VII) did not decrease activity, whereas formation of the corresponding *o*-methoxycinnamic acid, its methyl ester, and its salt (compounds 17–19, Table VII) virtually abolished activity. This difference in activity is undoubtedly because the potassium salt can readily revert back to coumestrol in the acid gastric environment, whereas the *o*-methoxy compound is incapable of doing so.

It has been suggested by Whalley (75) in a review of estrogens by Biggers that coumestrol derives its estrogenic activity by virtue of its stilbene-like structure which is analogous to that of diethylstilbestrol and that, furthermore, it bears a close structural relationship to diethylstilbestrol and estradiol. Structural alterations that disturb this stilbene-like structure would, therefore, reduce the similarity as has been suggested in the case of the 4-hydroxycoumarins with consequent lessening of estrogenic potency. Bate-Smith, quoted by Biggers (75), also suggests the possibility that coumestrol may be related to the isoflavones by postulating the conversion of an isoflavanol to coumestrol as in



Isoflavanol corresponding to Daidzein



Coumestrol

That the above speculation had a solid foundation is borne out by the findings of Grisebach and Barz (16).

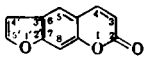
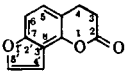
In conclusion, the reader's attention should be directed to the recent comprehensive study by Micheli, *et al.* (65), in which the authors examine the question of estrogenic structure-activity relationships of coumestrol, plant phenolics, and synthetic estrogens on a comprehensive and comparable basis. The findings of this group are summarized in Table VI adapted from their paper. Although their findings are ex-

tremely interesting, the full consideration of the paper is not within the scope of this review; the interested reader is urged to consult the original paper for details.

DERMAL PHOTOSENSITIZING ACTIVTY

The role of certain plant juices and extractives as dermal photosensitizing agents has been known for many years. Among the plants

TABLE VIII.—QUALITATIVE TESTS ON HUMAN SKIN IRRADIATED WITH LONG-WAVE ULTRAVIOLET LIGHT (3655 Å.)[a]

No.	Name		Activity[b]
		NATURAL FURANOCOUMARINS	
1.	Psoralen		++++
2.	Xanthotoxin	(8-Methoxypsoralen)	+++
3.	Bergapten	(5-Methoxypsoralen)	+++
4.	Angelicin		+
5.	Isobergapten	(5-Methoxyangelicin)	+
6.	Oxypeucedanin	[5-(β,γ-Oxido-isoamyloxy)-psoralen]	Sunlight only
7.	Xanthotoxol	(8-Hydroxypsoralen)	−
8.	Imperatorin	(8-Isoamyleneoxypsoralen)	−
9.	Bergaptol	(5-Hydroxypsoralen)	−
10.	Isopimpinellin	(5,8-Dimethoxypsoralen)	−
11.	Ostruthol	[5(β,γ-Dihydroxy-isoamyloxy)-psoralen angelic acid monoester]	−
		SYNTHETIC FURANOCOUMARINS	
	Related to Psoralen		
12.	4′,5′-Dihydropsoralen		−
13.	3,4-Dihydropsoralen		−
14.	4′-Methylpsoralen		+++
15.	4,4′-Dimethylpsoralen		+++
16.	4′-Phenyl-4-methylpsoralen		−
17.	Dimer of psoralen		−
18.	Thiopsoralen		−
	Related to Xanthotoxin		
19.	3,4-Dihydroxanthotoxin		−
20.	3-Methylxanthotoxin		+
21.	4-Methylxanthotoxin		+++
22.	4′-Methylxanthotoxin		++
23.	4′,3-Dimethylxanthotoxin		+
24.	4′,4-Dimethylxanthotoxin		+
25.	5′,4-Dimethylxanthotoxin		+++
26.	5′-Phenyl-4-methylxanthotoxin		−
27.	5-Chloro-xanthotoxin		+
28.	5-Nitro-xanthotoxin		−
29.	5-Amino-xanthotoxin		−
30.	5-Acetylamino-xanthotoxin		−
31.	8-Benzyloxypsoralen		+
32.	Thioxanthotoxin		−
	Related to Bergapten		
33.	4′,5′-Dihydrobergapten		−
34.	4-Methylbergapten		+
35.	4-Methyl-4′,5′-dihydrobergapten		−
36.	Allobergapten[c]		+
37.	4′,5′-Dihydroallobergapten		−
38.	4′,5′,3,4-Tetrahydroallobergapten		−
39.	4-Methylallobergapten		+
40.	4-Methyl-4′,5′-dihydroallobergapten		−
41.	5-Ethoxypsoralen		++
42.	5-Isopropoxypsoralen		+
43.	5-*n*-Propoxypsoralen		− (Sunlight +)
44.	5-*n*-Butoxypsoralen		− (Sunlight +)
45.	5-Isoamyloxypsoralen		− (Sunlight +)
46.	Psoralen-5-oxyacetic acid ethyl ester		−
47.	5-Benzyloxypsoralen		−
48.	8-Nitrobergapten		−
49.	8-Aminobergapten		−
50.	8-Acetylaminobergapten		−
51.	Bergapten-8-carboxylic acid methyl ester		+
52.	4′,5′-Dihydrobergapten-8-carboxylic acid methyl ester		−
53.	Bergapten-8-carboxylic acid		−
54.	4′,5′-Dihydrobergapten-8-carboxylic acid		−
55.	Dimer of bergapten		−
	Related to Angelicin		
56.	4-Methylangelicin		+
57.	Dimer of angelicin		−
	Related to Isopimpinellin		
58.	5,8-Dihydroxypsoralen		−
59.	Psoralenequinone		−

[a] Adapted from Musajo, L., and Rodighiero, G., *Experientia*, **18**, 153(1962). [b] ++++, Maximum activity; −, inactivity.
[c] Allobergapten has the structure

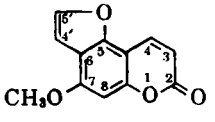


TABLE IX.—QUANTITATIVE TEST ON HUMAN SKIN[a,b]

Compd.	Minimum Length of Irradiation Needed for Outcome of Erythema, Min.	Relative Activity
Psoralen	6	100
4′-Methylpsoralen	10	60
Xanthotoxin	16	37.5
4,5′-Dimethylxanthotoxin	18	33.3
4,4′-Dimethylpsoralen	20	30
4-Methylxanthotoxin	20	30
Bergapten	22	27.5
5-Ethoxypsoralen	25	24
4′-Methylxanthotoxin	25	24
5-Isopropoxypsoralen	35	17.1
4-Methylbergapten	40	15
5-Chloroxanthotoxin	40	15
Angelicin	50	12
4′,4-Dimethylxanthotoxin	50	12
Allobergapten	50	12
4-Methylallobergapten	50	12
Bergapten-8-carboxylic acid methyl ester	50	12
3,4′-Dimethylxanthotoxin	55	11
3-Methylxanthotoxin	60	10
Isobergapten	60	10
4-Methylangelicin	60	10
4-Benzyloxypsoralen	60	10

[a] Adapted from Musajo, L., and Rodighiero, G., *Experientia*, **18**, 153(1962). [b] The substance is applied at a concentration of 5 mcg. per cm. and irradiated with a Philips HPW 125 lamp (3655 Å.) at 15 cm. from the skin.

involved are some of the common ones such as parsley, celery, figs, and parsnip (76, 77). Juices of various parts of these plants, after contact with the skin and exposure to sunlight, cause areas of erythema, hyperpigmentation and, occasionally, vesiculation on the skin. In retrospect, it now appears that plants causing these effects all contain furanocoumarins as the active agents. Possibly one of the first observations implicating these compounds with photosensitizing activity was that of Jois, *et al.* (78), in 1933 in connection with the Ayurvedic treatment of leucoderma with seeds of *Psoralea corylifolia*. These workers actually isolated psoralene and characterized it as a furanocoumarin, but the clinicians apparently were unable to grasp the significance of the finding, and it remained for others to bring it to light years later. Kuske (79), in 1938, through a consideration of plant extracts and pure furanocoumarins, ascertained that the latter were the responsible agents in the dermal response. Following this, in 1947, Fahmy and Abu-Shady (80) isolated three crystalline principles from the seeds of the empirically used antileucodermic vegetable drug *Ammi majus* L., *viz.*, ammoidin, ammidin, and majudin which were soon shown (81) to be the well known xanthotoxin, imperatorin, and bergapten, respectively. The story of their investigation, proceeding from an unidentified crude drug to the isolation of active crystalline principles, is well told by the Egyptian dermatologist, El Mofty (82). The method finally developed for treatment of vitiliginous areas, utilizing the dermal photosensitizing properties of the furanocoumarins, was either to paint the areas externally with a solution or to administer the drug orally and, in either case, to expose the patient to sunlight or ultraviolet irradiation. In responsive patients, repigmentation of the vitiliginous areas follows either with or without previous vesiculation.

The discovery of this unique activity of the furanocoumarins coupled with their utility in the treatment of leucoderma stimulated much activity among researchers seeking to evaluate other naturally occurring furanocoumarins as well as related synthetic compounds for the structure-activity profile of this interesting chemical nucleus. It is not possible to cover all of the modifications that have been attempted, but certain obvious structural requirements have been defined. Especially active in this area have been Musajo and co-workers in Italy (83–85) and the American group at the University

TABLE X.—FURANOCOUMARINS TESTED ON GUINEA PIG SKIN[a,b,c]

Compd.	Activity[d]
4-Methylpsoralen	++++
5′-Methylpsoralen	++++
5′,8-Dimethylpsoralen	++++
4,4′-Dimethylpsoralen	+
4,5′,8-Trimethylpsoralen	++++
3,4,5′,8-Tetramethylpsoralen	+
8-*n*-Propyl-4,5′-dimethylpsoralen	++
3-*n*-Butyl-4,5′,8-trimethylpsoralen	+
8-Acetyl-4,5′-dimethylpsoralen	++
8-Bromo-4,5′-dimethylpsoralen	++
8-Acetamido-4,5′-dimethylpsoralen	+
8-Acetyl-4,5′-dimethylpsoralen semicarbazone	+
3,4-Benzo-5′,8-dimethylpsoralen	+
3,4-Cyclohexeno-5,8-dimethylpsoralen	+
Anhydromarmesin	+
Marmesin	+
5′-Methylangelicin	+
3-Bromo-4′,5′-dihydroxanthotoxin	±
8-Amino-4,5′-dimethylpsoralen	−
4′,5′-Dihydroxanthotoxin	−
4′,5′-Dihydro-4-methylpsoralen	−
3,5-Dibromo-4′,5′-dihydro-8-methoxypsoralen	−
5-Bromo-8-methoxypsoralen	−
4′,5′-Dimethylangelicin	−

[a] Adapted from Musajo, L., and Rodighiero, G., *Experientia*, **18**, 153(1962). [b] Based on the work of Pathak, M. A., Fellman, J. H., and Kaufman, K. D., *J. Invest. Dermatol.*, **35**, 165(1960). [c] The administered amounts varied up to 1000 mcg. per cm.²; a 250-w. lamp, emitting U.V. radiations with $\lambda > 3200$ Å. was used and irradiation time was 45 minutes; distance 12–15 cm. [d] ++++, Maximum activity; −, inactivity.

of Oregon, among whom the active contributors have been Pathak, Fowlks, Fitzpatrick, etc. (86, 87). Musajo and Rodighiero (76) have reviewed the field recently, although the interested reader will wish to consult the proceedings of the 1959 symposium on psoralens and radiant energy as well (88). Members of the American group have been interested especially in the more fundamental aspects of photodynamic action, such as the biochemistry of melanin formation (89, 90), the mechanism of the photodynamic effect (91), the inhibition of enzyme systems (92), and the sensitization of bacteria (93, 94) by the furanocoumarins. The Italian group also has been active recently in trying to determine the mechanism of activity of these compounds (95).

Musajo and Rodighiero (76) have tabulated the relative qualitative (Table VIII) and quantitative (Table IX) activities of the naturally occurring coumarins and the synthetic congeners related to the natural prototypes. Table X summarizes the structure-activity relationships developed by Pathak, *et al.* (87). A consideration of the data offered in these tables indicates that maximum photosensitizing activity lies in the parent compound, psoralene, and that the various structurally related compounds have more or less reduced activity, depending on the ring system and nature of substituents. Significant activity requires a linear unreduced furanocoumarin ring system. Free phenolic groups inactivate the molecule, but the methyl ethers of the two possible phenols are both active. However, the dimethyl ether of the diphenol is inactive. Etherifying groups larger than methyl result in progressive reduction of activity as the size of the group increases. Nuclear substitution with methyl groups can cause significant retention of activity or loss depending on the position of the group. Thus, a methyl at the 4, 4′, 5′, or 8 positions may or may not inhibit activity, but a methyl at the 3 position invariably does so. Little success has been noted with the introduction of nitro, amino, or acetylamino groups. A unique ring modification in the form of the oxazolocoumarins (96) also leads to inactivity.

Fowlks (91) has analyzed the available evidence in an effort to determine the mechanism of the photodynamic effect of these compounds as well as of the multitude of structurally different but similarly active compounds. He feels that the primary event of photosensitization is the absorption of light by the photosensitizer molecule or by a complex of the photosensitizer with protein or nucleic acid. Following this, the events can become rather complicated, but it appears probable that an activated photosensitizer molecule can cause one of three results: (*a*) chemical combination of photosensitizer with a sensitive cell constituent, (*b*) chemical reaction, *i.e.*, oxidation of a sensitive cell constituent or, (*c*) indirect excitation of cell constituents which, through chemical changes, cause inactivities of vital cellular activities. He further suggests that there may well be a balance between damage suffered by the cell and its ability to repair the damage. Nonrepairable damage could lead to immediate or delayed death or to mutation. Indeed, mutation (97) is evident upon application of furanocoumarins to onion root tips, and 5-methoxypsoralen and psoralen are said to be almost as effective mutagenic agents as the most effective agent known, tryptaflavin. In connection with the observations of Fowlks, Pathak and Fellman (98) have noted that all biologically active furanocoumarins inducing photosensitization possess absorption and fluorescensce peaks of 320–360 and 420–460 mμ, respectively. Others, outside this range, are inactive. It also has been noted that the long wavelength ultraviolet lamp (3200 Å.) is needed to potentiate the response of the skin (erythema and sun-tanning) to the coumarins. It is thought that the skin response is associated in large measure with the capture of radiant energy of the proper wavelength (*i.e.*, 320–340 mμ) and that any changes in molecular shape, substitution pattern, etc., which disturb this specificity lead inevitably to decreased activity. Thus, the absorption of light of specific wavelength and the emergence of light of another wavelength in intimate contact with a sensitive cellular component is thought to be crucial to successful photosensitizing activity. A further observation may be made in that furanocoumarins have been quite definitely shown not to act by a photo-oxidative mechanism although other photosensitizing molecules such as the hematoporphyrins are known to act by way of this mechanism. Rodighiero and Capellina (99) have shown that, although furanocoumarins are dimerized under the influence of irradiation, the dimers are biologically inactive. The possibility that free radicals may be generated from excited furanocoumarin molecules under ultraviolet irradiation has been explored by Pathak, *et al.* (100), but, granting the biological changes that could occur in such an irradiated system, this probably does not adequately explain the marked activity of these compounds (76). Very recently, Musajo, *et al.* (95), have observed some interesting reactions of furanocoumarins with flavinmononucleotide (FMN). It appears that FMN will undergo reaction with furanocoumarins, but only with those that are photodynamically active and that

the reaction products appear to have been modified in the furan ring principally. Furthermore, they have demonstrated (albeit, in rather large doses) an antagonistic action of FMN toward the erythemal response expected from the psoralen-type molecule.

In spite of these findings, the precise mechanism whereby furanocoumarins function in the treatment of leucoderma is unknown. The biochemical events leading to the formation of the skin pigment, melanin, have been clarified, but no clearcut implication of the coumarins with it has been demonstrated. The formation of melanin is believed (89, 90, 101) to occur as depicted in Fig. 2. The coumarins apparently do not influence the tyrosine-tyrosinase reaction even under irradiation (76, 102, 103). A recent paper by Judis (104) indicates that xanthotoxin may cause the conversion of dihydroxyphenylalanine (DOPA) to melanin under the influence of ultraviolet light. Sen (101) finds himself tempted to look for another alternate additional pathway for melanin formation by using Robinson's scheme (105) for the formation of hydroxyindoles. Thus, it appears that the mechanism is uncertain and will have to await further clarification.

ANTIBACTERIAL ACTIVITY

Although coumarin itself has low antibacterial activity, other coumarins have been observed to have such activity in varying degrees. In particular, Dicumarol (106–108) has shown excellent activity against certain bacteria, *e.g.*, *Bacillus anthracis*, *Staphylococcus aureus*, *S. albus*, *Streptococcus pyogenes*, and *Pasteurella avicida*, although it has no growth suppressant action against some microorganisms. Cavallito (109) suggests that the predominantly Gram-positive activity of relatively lipophilic dicumarol is accounted for by the association of hydrophilic properties with Gram-negative activity and lipophilic character with Gram-positive activity. Ukita and co-workers (110) observed remarkable antibacterial activity against *Straphylococcus aureus* and *Mycobacterium tuberculosis* (avian type) by another (synthetic) coumarin, namely 3-acetyl-4-hydroxycoumarin. They point out that this compound is related to a number of other potent antibacterial compounds containing a tricarbonyl-methane unit, $[CH(-\overset{|}{C}=O)_3]$, as a common structural feature. Among the interesting noncoumarinic natural compounds containing this structural feature was the antibacterial lichen pigment, usnic acid. These workers also examined the effect of increasing the chain length of the 3-acyl group and found maximum activity in 3-*n*-decanoyl-4-hydroxycoumarin. Similar findings were reported by Toda, *et al.* (111). More recently, Ukita's group (112, 113) has studied the mode of action of the 3-

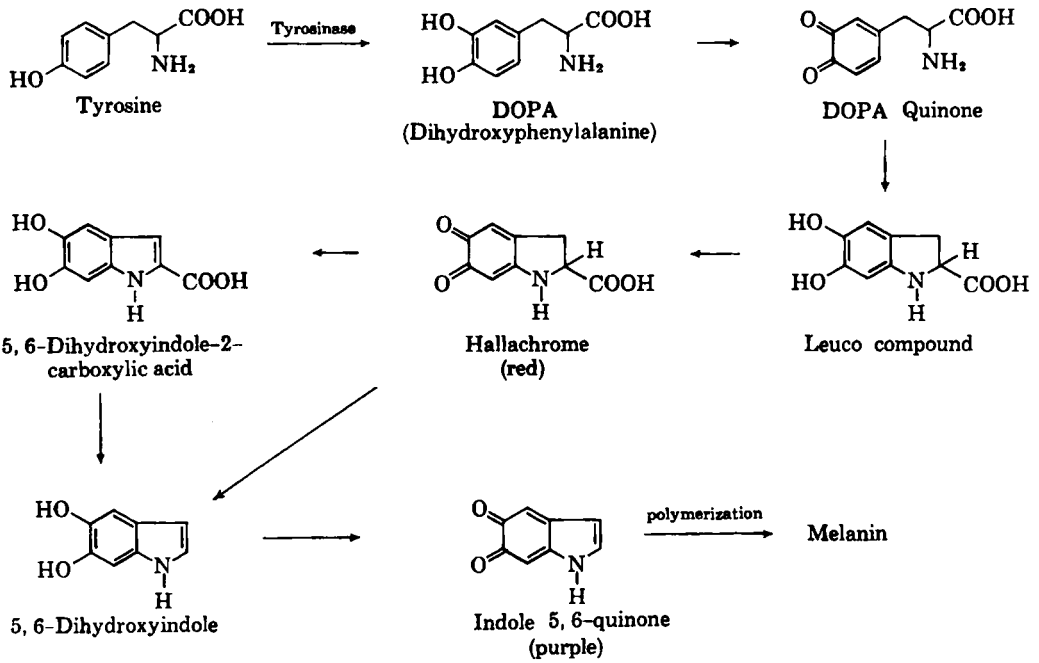


Fig. 2.—Enzymatic oxidation of tyrosine to melanin.

acetyl compound and concluded that it acts by an uncoupling effect on oxidative phosphorylation. It is interesting to note that Dicumarol is, likewise, a potent uncoupling agent (114). Indeed, Martius and Nitz-Litzow (115) were prompted to suggest that there was a direct relationship between the anticoagulant activity of Dicumarol and its congeners and the uncoupling effect, a hypothesis that since has become untenable.

Possibly the most important coumarin-type antibacterial agent is the antibiotic, novobiocin, isolated as a fungal metabolite of *Streptomyces niveus*. This unusual glycosidic coumarin has the distinction of being the first 4-hydroxycoumarin isolated as a fungal metabolite and is also the first to contain nitrogen as a part of the molecule. Its excellent antibacterial spectrum, chiefly against Gram-positive organisms and *Proteus vulgaris*, has led to its wide acceptance in medicine. It has also been found that it has a good action against *Leuconostoc mesenteroides*, the organism responsible for plugging of pipes in the sugar industry (116). Conversion to dihydronovobiocin resulted in a compound still retaining a wide spectrum of activity (116, 117). The aglycone derived from dihydronovobiocin by way of acid hydrolysis is also said to have activity (116), but the isoaglycone obtained by similar treatment of novobiocin is inactive. Recent patents (118, 119) have claimed good antibacterial activity for 3-amino-4,7-dihydroxy-8-methylcoumarin and the amide derivatives derived from novobiocin as well as for the esters of novobiocin which are claimed to have increased oil solubility and, thus, better properties for incorporation into oily ointment bases. The Japanese group of Okumura, *et al.* (120–122), has studied the compounds related to novobiocin in an effort to determine the structural features that contribute to its activity. Their first study described the synthesis of a number of 3-acylamino-4-hydroxycoumarin derivatives, several of which showed good antibacterial activity. One of the studies (121) undertaken was on 3-alkenyl-substituted 4-hydroxybenzoic acids related to the corresponding structural unit in novobiocin. The ethyl esters of some of these acids (*i.e.*, 3-(3-methyl-2-butenyl)-4-hydroxybenzoic acid and 3-allyl-4-hydroxybenzoic acid) exhibited antibacterial activity remindful of that shown by the well known *p*-hydroxybenzoates. In the final summation of their structure-activity studies (122) they pointed out that the presence of 3-(*O*-carbamoyl)-novobioside bonded at the 7 position of 3-(3-alkenyl-4-hydroxybenzamido)-4-hydroxycoumarin played an important role in the activity of novobiocin. Rodighiero, *et al.* (123), also have examined derivatives of 3-aminocoumarin for antibacterial activity and have found their best compound to be 3-amino-7,8-dihydroxycoumarin which was particularly effective against *Streptococcus pyogenes* and moderately effective against a variety of other bacteria.

Another coumarinic antibiotic that has been of interest is chartreusin, isolated as a metabolite from *Streptomyces chartreusis* (343). It is pre-

TABLE XI.—EFFECT ON FLOW RATE THROUGH ISOLATED RABBIT HEART OF VARIOUS PYRANOCOUMARINS

Compd.	Concn.	Rate of Flow—Control ml./2 min.	Rate of Flow—Sample, ml./2 min.	Increase, %	Relative Potency
Khellin	1:60,000	26.5	37	40	1
Visnagan	1:150,000	30.7	43	41	2.5
Khellin	1:30,000	28.0	39.7	41.8	1
Visnadin	1:60,000	21.0	49.1	131.6	...
Visnadin	1:300,000	28.5	39.7	39.2	10
Khellin	1:30,000	26.5	37.0	39.6	1
Visnadin mother liquors	1:240,000	23.5	30.8	31.2	Approx. 8
Khellin	1:15,000	24.7	45.6	84.6	1
Dihydrosamidin	1:75,000	24.7	45.0	82.2	5
Khellin	1:15,000	29.2	47.7	63.2	1
Papaverine·HCl	1:75,000	27.2	37.2	36.7	2.5
Papaverine·HCl[a]	1:205,000	23	41	78	...
Pteryxin	1:225,000	22	45	105	...
Papaverine·HCl	1:140,000	15	28	87	...
Pteryxin	1:185,000	16	37	131	...
Papaverine·HCl	1:140,000	12	28	133	...
Pteryxin	1:140,000	12	28	133	...
Papaverine·HCl	1:89,000	17	22	30	...
Pteryxin	1:176,000	23	44	91	...
Papaverine·HCl	1:112,000	14	28	100	...
Pteryxin	1:120,000	16	30	88	...

[a] This value and those following are taken from the Ph.D. dissertation of Robert E. Willette entitled "Structural Studies of Certain Pharmacologically Active Coumarins Isolated from Umbelliferae," University of Minnesota, Minneapolis, 1960, p. 56. The data were supplied by Hazleton Laboratories, Falls Church, Va. The same laboratory supplied the data in the first part of the table, these data having been reported in *J. Am. Chem Soc.*, **79**, 3538(1957).

dominantly active against Gram-positive organisms and mycobacteria (344) and also acts against *Micrococcus pyogenes* v. *aureus* phage. It exhibits possible cumulative toxicity and has not been exploited commercially. Work on the structure (345, 346) finally resulted in the elucidation of its structure by Simonitsch, *et al.* (347).

Tuberculostatic activity by coumarins has been noted in a few cases. Bersch and Döpp (124) examined this activity as related to *p*-aminosalicylic acid (PAS) and found that *trans*-*p*-aminocinnamic acid was one-fourth as active as PAS and that the analogous 7-amino-4-methylcoumarin was one-half as active as PAS. Rodighiero, *et al.* (125), working with furanocoumarins, found that only psoralen and xanthotoxin (coumarin less so) were able to inhibit growth of the tubercle bacillus significantly.

Antifungal activity has been noted, in passing, by many authors. One of the more recent studies is that of Chakraborty, *et al.* (126), who found that of 17 natural coumarins tested, the psoralenes, including psoralen and imperatorin, were the most effective antifungal agents tested.

VASODILATOR ACTIVITY

Although the coumarins are well regarded in the managment of symptomatic coronary arteriosclerosis, there is a growing opinion that holds the mechanism of activity to be vasodilatory rather than anticoagulant (127–131). One of the most recent studies in support of this hypothesis is that of Blake, *et al.* (131), who have shown, by a new plethysmographic technique, that the coronary vasodilator activity of dicumarol and warfarin on swine coronary arteries is comparable to the activity of aminophylline and nitroglycerin. The authors suggest that the vasodilator activity is brought about by a direct effect of the unchanged compound on the wall of the artery rather than by some indirect means (*e.g.*, by a metabolite or *via* the central nervous system). It was determined further that the concentrations of bishydroxycoumarin achieved in their experiments were approximately of the same order as those obtained in conventional anticoagulant therapy.

Other coumarins have been investigated also as coronary vasodilators following the original studies on *Ammi visnaga* L. which resulted in the marketing of khellin, a furanochromone, as a coronary vasodilator. The fact that certain impure preparations of khellin showed a better vasodilatory activity than the pure chromone in controlling angina of effort stimulated a search for the obviously more potent contaminant in the noncrystallized "visnagan" fraction of Samaan (132). This contaminant, originally described as "visnagan," was isolated in a crystalline state as the coumarin, visnadin, in 1952 by Smith, *et al.* (133). More recently, by carrying out more refined isolations (134) they obtained and structurally defined three strongly vasodilatory coumarins, *viz.*, visnadin, samidin, and dihydrosamidin. By studying the effect of these principles on the rate of flow through isolated rabbit heart, they obtained the results shown in Table XI. Willette and Soine, for the closely related coumarins pteryxin and suksdorfin (135), have shown a somewhat more potent action (see Table XI). Visnadin has been employed abroad for the treatment of angina pectoris (136, 137) with some success, although there is no U. S. product of comparable nature.

MOLLUSCACIDAL ACTIVITY

An interesting study by the Egyptian researchers, Schönberg and Latif (138), has demonstrated the possibility of snail control, at least in countries where furanocoumarin-containing plants can be grown with little expense. They have shown that bergapten and isopimpinellin (xanthotoxin less effectively) exert a molluscacidal activity comparable to some of the most powerful synthetic organic agents known, *e.g.*, dinitro-*o*-dicyclohexylphenol and sodium pentachlorophenate (Dow G) The objective, of course, is to control the snail *Biomphalaria boissi* which is known to be the intermediate aquatic host for *Schistosoma mansoni*, the blood fluke responsible for human schistosomiasis (*i.e.*, bilharziasis).

Utilizing snails obtained from their natural habitat as test objects under controlled conditions, they were able to show pronounced molluscacidal activity. Their preliminary observations were confirmed and extended by Evans and Zachary of the U. S. Naval Medical Research Unit No. 3, Cairo, and are summarized in Table XII. Xanthotoxin has not been included in the table because it showed no activity at 5 p.p.m. and only a 25% kill at 10 p.p.m. At 50 p.p.m.,

TABLE XII.—COMPARATIVE MOLLUSCACIDAL ACTIVITIES

Compd.	5 p.p.m.	2 p.p.m.
Bergapten	32/32 (100%)[a]	22/32 (69%)
Isopimpinellin	22/32 (69%)	3/32 (9%)
Dinitro-*o*-cyclohexylphenol	13/16 (81%)	14/32 (44%)
Sodium pentachlorophenate	10/16 (63%)	2/32 (6%)

[a] Percentage kill.

however, it gave a 100% kill. In the table the parentheses include the percentage kill. Schönberg and Latif have suggested that it might be possible to control snails economically by growing plants (*e.g.*, *Ammi majus*) containing the furanocoumarins, harvesting them, and simply throwing them into the channels infected with snails. As far as the author is aware, the suggestion has not been implemented.

ANTHELMINTIC ACTIVITY

It has long been known that certain naturally occurring lactones, *e.g.*, γ-butyrolactone, santonin, etc., possess anthelmintic properties. That such activity also was present in the coumarin lactones was demonstrated by Baldwin (139). Since then most of the interest in coumarins as anthelmintics has been among the Japanese. Ito, *et al.* (140, 141), found only weak activity in coumarin, 2-thiocoumarin, and 4-methylcoumarin, but Nakabayashi, *et al.* (142), working with *Ascaris suilla*, showed that methyl, hydroxy, and methoxyl derivatives of coumarin possessed significant activity and that the 7- and 3-methylcoumarins were particularly effective, although the presence of both 7-methyl and 3-alkyl substituents simultaneously depressed activity (143). Longer alkyl chains than methyl depressed activity likewise. Comparison of 3:4-dihydrocoumarin and its methyl derivatives with the corresponding unreduced compounds (144) indicated that there was no parallelism of action between the two groups and that the dihydro derivatives were uniformly less effective than the parent compounds. Ito and Kitagawa (145) found the same to be true when they compared the efficacies of coumarin, 2-thiocoumarin, and 4-methylcoumarin and their 3:4-dihydro derivatives. They noted that compounds melting between 70 and 100° all seemed to have good efficacy, and that those melting over 100° were uniformly inactive. These workers also studied the mode of action of these compounds against *Ascaris suilla* (146). Ishifuku, *et al.* (147), studied a number of umbelliferone and esculetin derivatives and found that the isopropyl ether of the former provided the greatest activity and that esculetin derivatives were, at best, only weakly active. They noted, too, that there was correlation between a low melting point and anthelmintic activity. Nakabayashi (148) showed that, in general, the several methylthiocoumarins were relatively less effective than the corresponding coumarins. He also examined the α- and β-naphthocoumarins and the 1- and 4-methyl as well as the tetrahydro-, hexahydro-, and dodecahydro derivatives (149). The most potent compound tested was 4-methyl-hexahydro-α-naphthocoumarin.

SEDATIVE AND HYPNOTIC ACTIVITY

Kreitmair (150) showed that coumarin possessed a definite hypnotic and sedative action on mice on oral administration but discounted any possible therapeutic application because of the high toxicity that made the margin of safety too small. The toxicity of coumarin has also been studied by Hazleton, *et al.* (151), who were unable to show really harmful effects but pointed out that longer term studies should be carried out. Ito, *et al.* (152), examined several coumarins for analgesic and hypnotic effects. They concluded that the intact 3:4 double bond was necessary for activity and noted that the potency was in the order of coumarin nucleus with α-pyrone ring *o*-condensed with naphthalene, the same condensed with benzene, and the benzene ring condensed to the 6 position. A surprisingly powerful effect was noted with ethyl coumarin-3-carboxylate alone among the ethyl coumarin carboxylates. They noted no great differences between α- and γ-pyrone ring systems. Ito, *et al.* (153), in 1953 again studied several different coumarins and found activity in coumarin-3-carboxylic acid diethylamide. Introduction of $-CH_2-$ between the coumarin ring and $-CON(C_2H_5)_2$ caused loss of activity as did conversion to the isomeric isocoumarin compound. They confirmed their earlier finding in this group of compounds that reduction of the 3:4 double bond was deleterious to activity. Kitagawa (154) observed that an alkyl group in the 3 position gave a hypnotic effect if the number of carbons was odd but imparted toxicity if even (peak with ethyl). The effect of the methyl group, in decreasing order of activity, was 8-, 3-, and 4-methyl. However, a significant finding was that the difference between the hypnotic and lethal dose was slight except with the 4- and 8-methyl compounds. Kitagawa (155) was able also to relate the hypnotic effect of coumarin derivatives to the suppressive action on the cytochrome level in conformity with the general proposal of Michaelis and Quastel (156) relating to hypnotics and anesthetics.

ANALGESIC ACTIVITY

In 1957, Stern, *et al.*, published a paper relating to the analgesic and antipyretic effects of vitamin K and dicumarol with special reference to 4-hydroxycoumarin (157) They made the startling statement that they were able to obtain

analgesic effects from 4-hydroxycoumarin with a minimum dose of 0.025 mcg./Kg. This proposal was examined by Adami (158) and Adami, *et al.* (159), in which they administered 4-hydroxycoumarin to albino rats in a well controlled study in doses of 0.010 to 250 mcg./Kg. orally, intraperitoneally, and subcutaneously. They found no analgesic activity of significance.

HYPOTHERMAL ACTIVITY

Kitagawa, *et al.* (160–162), examined a large group of coumarins for their ability to reduce body temperature. They concluded that most coumarins possess an effect of this kind. Among the many relationships noted they observed that methyl or propyl in the 3 position had a particularly strong action. Reduction of the 3:4 double bond reduced activity, but the introduction of a 2-thio group had little effect. A methyl group attached to the α-pyrone ring was preferable to having it attached to the benzene moiety of coumarin. With respect to 2-thiocoumarin they observed that there was a complete parallelism between the amount distributed in the brain and the hypothermal action.

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Research Articles

Quinazolines and 1,4-Benzodiazepines XVII

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By R. IAN FRYER, R. A. SCHMIDT, and L. H. STERNBACH

The synthesis of a series of 5-pyridyl analogs of the psychopharmacologically active class of compounds, 1,4-benzodiazepines, is described. These compounds are being screened for psychosedative, muscle relaxant, and anticonvulsant activity.

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The 2-aminobenzoylpyridines III*a,b* (H)[3] used as starting materials for these syntheses are described by Schofield and co-workers (4, 5). However, as recent work by Raynolds and Levine (6) makes 4-phenacyl pyridine readily available, we were able to prepare 4-(2-aminobenzoyl)pyridine III*b* (H) by the much easier route used by Schofield and Ockenden for the synthesis of 2-(2-aminobenzoyl)pyridine III*a* (H).[4] These workers utilized the oxidative fission of the 2,3-double bond of the appropriate indole I*a* (H), followed by hydrolysis of the 2-(2-benzamidobenzoyl)-pyridine II*a* (H) thus obtained.

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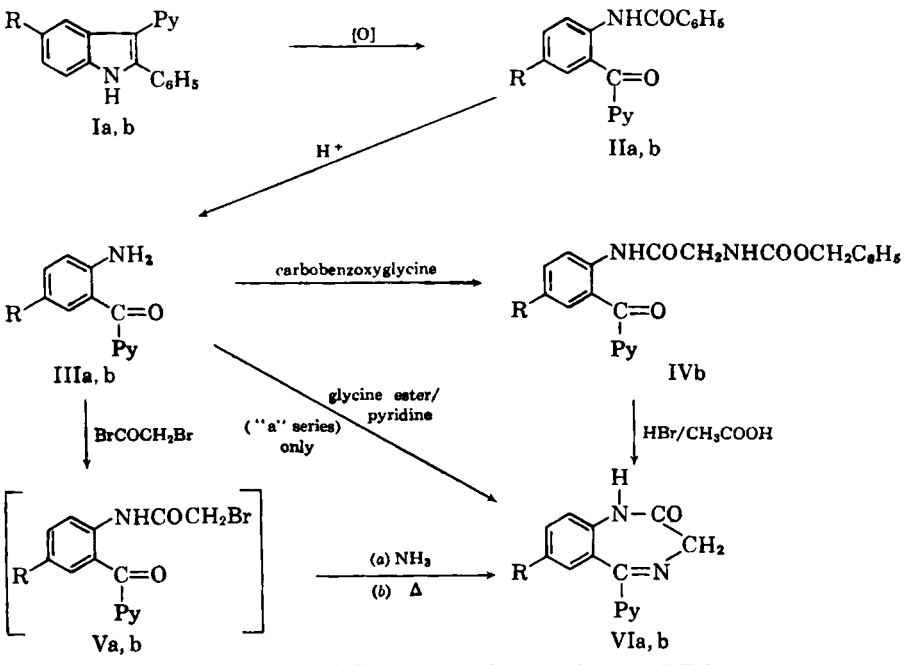
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a series, Py = 2-C_5H_4N *b* series, Py = 4-C_5H_4N

Scheme I

Attempts to convert III*a* and *b* into benzodiazepinones by condensation with glycine ester hydrochloride showed a significant difference in their reactivity. III*a* (H) reacted readily and gave the desired product VI*a* (H), whereas attempts to form VI*b* (H) by this method were not successful. The second method (III→V→VI) used for the synthesis of these benzodiazepinones gave the desired result for both isomers. The first intermediates, the two bromoacetamido derivatives V*a,b* (H), were prepared in an acid medium to avoid quaternization of the pyridine nitrogen. Two methods were generally employed. The first consisted of treating the dihydrobromide of the aminobenzoyl pyridine in *N,N*-dimethylformamide with 1 mole of bromoacetyl bromide and slowly titrating the mixture with two equivalents of pyridine. The second procedure, which was preferred, consisted of treating III*a,b* (H) in acetic acid with bromoacetyl bromide. Since attempts to obtain the pure bromoacetamido derivatives, V*a,b* (H), as crystalline compounds were unsuccessful, the crude bromoacetyl compounds were converted directly to VI*a,b* (H) by reaction with ammonia. The intermediate aminoacetamidobenzoylpyridines were not isolated. An alternative method for the synthesis of VI*b* (H) proceeded *via* the carbobenzoxyglycyl derivative IV*b*. This compound was prepared using essentially the method that Sheehan and Hess devised for the synthesis of peptides (7, 8). Cleavage of the carbobenzoxy group with hydrogen bromide in glacial acetic acid (9) gave VI*b* (H) directly without isolation of the intermediate, 4-(2-aminoacetamidobenzoyl)pyridine.

The 5-bromo-2-phenyl-3-(2-pyridyl)indole I*a* (Br) and the 1-acetyl derivative of 5-bromo-2-phenyl-3-(4-pyridyl)indole I*b* (Br), prepared by Fischer syntheses, gave the (2-benzamido-5-bromobenzoyl)pyridines II*a,b* (Br) after oxidation. The acetyl derivative of I*b* (Br) was utilized since we were unable to oxidize the parent indole I*b* (Br) successfully in this instance.

Hydrolysis of these compounds afforded the two 2-amino-5-bromobenzoylpyridines, III*a,b* (Br). A simpler method for the preparation of these 5-bromo compounds was the direct bromination of the 2-aminobenzoylpyridines, III*a,b* (H), with bromine in acetic acid.[5] This yielded products which were in every respect identical with the one synthesized by the above unequivocal method. Chlorination of III*a* (H) with chlorine in acetic acid gave a chloro derivative which, by analogy, would be III*a* (Cl).

A dibromo compound was isolated as a by-product from the bromination of III*b* (H). By analogy with similar bromination reactions of 2-aminoacetophenone (10), it is believed to be 4-(2-amino-3,5-dibromobenzoyl)pyridine; but this structure was not established conclusively.

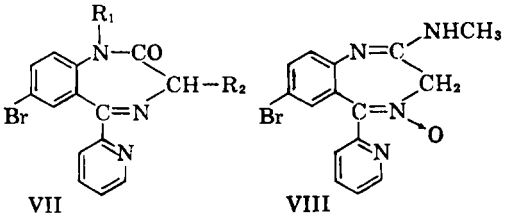
The three halogenated aminobenzoyl pyridines,

[5] Bromination of the acetyl or benzoyl derivatives of III*a,b* (H) proceeded very slowly or not at all, and the yields of the bromo compounds were extremely small when isolated.

IIIa,b (Br), and IIIa (Cl), were converted to the corresponding 7-halo-1,3-dihydro-5-pyridyl-2*H*-1,4-benzodiazepin-2-ones, VIa,b (Br) and VIa (Cl)—as described above—*via* the bromoacetyl derivatives Va,b (Br) and Va (Cl), respectively.

Nitration of the two 1,3-dihydro-5-pyridyl-2*H*-1,4-benzodiazepin-2-ones, VIa,b (H), with potassium nitrate in cold concentrated sulfuric acid gave the two corresponding 1,3-dihydro-7-nitro-5-pyridyl-2*H*-1,4-benzodiazepin-2-ones, VIa,b (NO_2). The nitro group was assumed to be in position 7, based on analogous reactions carried out on 1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-ones described in an earlier paper (11).

Using known procedures (12), IIIa (Br) was converted to the three 5-(2-pyridyl)-1,4-benzodiazepine derivatives VII (R_1 = CH_3, R_2 = H), VII (R_1 = H, R_2 = CH_3), and VIII.



VII VIII

PHARMACOLOGICAL RESULTS

Preliminary screening data indicate that a number of these compounds are nontoxic centrally acting agents with anticonvulsant and psychosedative properties. As other results become available, it is anticipated that they will be reported as an integral part of an overall study of the 1,4-benzodiazepine class of compounds.

EXPERIMENTAL[6]

All melting points are corrected. The infrared absorption spectra of starting materials and reaction products were compared wherever necessary in order to establish structural changes. The infrared spectra were determined in 0.3 to 0.4% chloroform solutions using a Perkin-Elmer model 21 spectrophotometer.

1,3-Dihydro-5-(2-pyridyl)-250-1,4-benzodiazepin-2-one, VIa (H).—*A.* Bromoacetyl bromide (9 ml., 0.1 mole) was added dropwise (15 minutes) to a stirred solution of 2-(2-aminobenzoyl)pyridine IIIa (H) (4), (19.8 Gm., 0.1 mole), in glacial acetic acid (1 L.). The mixture was stirred at room temperature for 2 hours, then concentrated to a gum under reduced pressure. Liquid ammonia (approximately 500 ml.) was carefully added to the residue and allowed to evaporate slowly. The crude product was stirred for 2 hours with 500 ml. of water and filtered. Recrystallization of the precipitate from acetone gave 16.4 Gm. (69%) of VIa (H) as white prisms, m.p. 232–234° dec.

Anal.—Calcd. for $C_{14}H_{11}N_3O$: C, 70.87; H, 4.67. Found: C, 71.18; H, 4.98.

B.—A mixture of 2-(2-aminobenzoyl)pyridine (2.0 Gm., 0.01 mole), glycine ethyl ester hydrochloride (4.2 Gm., 0.03 mole), and pyridine (100 ml.) was refluxed for 8 hours. The solvent was removed under reduced pressure, and the residue was partitioned between water (50 ml.) and benzene (50 ml.). The aqueous layer was extracted with benzene (2 × 50 ml.). The organic layers were combined, washed with water (2 × 25 ml.), and concentrated to 25 ml. The addition of 25 ml. of petroleum ether (b.p. 30–60°) gave 0.5 Gm. (21%) of crystalline material on standing, m.p. 231–232°.

2-(2-Amino-5-bromobenzoyl)pyridine, IIIa (Br).—*A.*—*From 2-Phenacylpyridine.*—2-Phenacylpyridine *p*-bromophenylhydrazone was cyclized with concentrated hydrochloric acid to give 5-bromo-2-phenyl-3-(2-pyridyl)indole Ia (Br) (colorless prisms from ethanol, m.p. 175–177°). This compound was not purified further but oxidized with chromium trioxide (4) to give 2-(2-benzamido-5-bromobenzoyl) pyridine IIa (Br), m.p. 138–140° dec., (yellow prisms from ethanol).

Anal.—Calcd. for $C_{19}H_{13}BrN_2O_2$: C, 59.86; H, 3.44. Found: C, 59.88; H, 3.65.

Hydrolysis of IIa (Br) with concentrated hydrochloric acid gave, after recrystallization from ligroin (b.p. 90–120°), IIIa (Br) as yellow needles, m.p. 96–98°.

Anal.—Calcd. for $C_{12}H_9BrN_2O$: C, 52.01; H, 3.27. Found: C, 52.03; H, 3.57.

B.—*From 2-(2-Aminobenzoyl)pyridine.*—A solution of bromine (35 ml., 0.70 mole) in glacial acetic acid (600 ml.) was added dropwise to a stirred solution of 2-(2-aminobenzoyl)pyridine IIIa (H) (131 Gm., 0.66 mole) kept at 15–17° with an ice bath. The reaction mixture was allowed to warm to room temperature and stirred for 20 hours. The red crystalline hydrobromide was separated by filtration and washed with cold glacial acetic acid. The base was liberated by decomposition of the salt with water (2.5 L.). The product was separated by filtration, dried, and recrystallized from ligroin (b.p. 90–120°). By making the filtrate basic with sodium hydroxide solution, additional product was obtained which was filtered, dried, recrystallized, and added to the first crop. The product (154.5 Gm., 84%) was obtained as bright yellow needles, melting point and mixed melting point with the product obtained by *Method A*, 96–98°.

2-(2-Amino-5-chlorobenzoyl)pyridine, IIIa (Cl).—A solution of chlorine (1.36 Gm., 0.019 mole) in glacial acetic acid (50 ml.) was added over a 15-minute period to a cold, stirred solution of IIIa (H) (3.57 Gm., 0.018 mole) in glacial acetic acid (25 ml.). The mixture was stirred for 15 minutes and allowed to stand overnight at room temperature. The solvent was removed under reduced pressure, and the residue was digested with hot water (50 ml.) and allowed to cool. After 3 days of standing, the solution deposited 1.0 Gm. of yellow crystalline solid which was filtered and recrystallized from ligroin (b.p. 90–120°) to give 0.84 Gm. (20%) of product as yellow prisms, m.p. 99–101°.

Anal.—Calcd. for $C_{12}H_9ClN_2O$: C, 61.94; H, 3.90. Found: C, 62.09; H, 4.04.

7-Bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one, VIa (Br).—A mixture of IIIa (Br) (1.0 Gm., 3.6 mmole), glycine ethyl ester hydrochloride (0.75 Gm., 5.4 mmoles) and pyridine (100 ml.) was refluxed and worked up as described for

[6] The *Experimental* was performed in part with Mr. R. Czajkowski.

the *preparation of VIa (H) Method B* to give, after recrystallization from acetone, 235 mg. (20%) of VIa (Br) as colorless prisms, m.p. 237 to 238.5°.

Anal.—Calcd. for $C_{14}H_{10}BrN_3O$: C, 53.18; H, 3.19. Found: C, 53.36; H, 3.38.

7 - Chloro - 1,3 - dihydro - 5 - (2 - pyridyl) - 2H - 1,4-benzodiazepin-2-one, VIa (Cl).—Using the procedure described for the *preparation of VIa (H) Method A*, 4.66 Gm. of IIIa (Cl) gave, after recrystallization from ethanol, 3.1 Gm. (57%) of VIa (Cl) as yellow prisms, m.p. 225–226° dec.

Anal.—Calcd. for $C_{14}H_{10}ClN_3O$: C, 61.88; H, 3.71. Found: C, 61.92; H, 3.91.

1,3 - Dihydro - 7 - nitro - 5 - (2 - pyridyl) - 2H - 1,4-benzodiazepin-2-one, VIa (NO_2).—A solution of potassium nitrate (5.04 Gm., 0.05 mole) in concentrated sulfuric acid (30 ml.) was added dropwise at 5° to a solution of VIa (H) (11.2 Gm., 0.047 mole) in concentrated sulfuric acid (100 ml.). The reaction mixture was stirred at 5° for 2 hours, then poured onto ice in a vessel cooled by an ice-salt bath. The solution was kept at 0° and made alkaline (pH 8) by the addition of ammonium hydroxide (450 ml.). The product was filtered and recrystallized from acetone to give 6 Gm. (45%) of VIa (NO_2) as colorless prisms, m.p. 254–255° dec.

Anal.—Calcd. for $C_{14}H_{10}N_4O_3$: C, 59.57; H, 3.57; N, 19.85. Found: C, 60.00; H, 4.06; N, 20.04.

7 - Bromo - 1,3 - dihydro - 1 - methyl - 5 - (2 - pyridyl)-2H-1,4-benzodiazepin-2-one, VII (R_1 = CH_3, R_2 = H).—Alkylation of VIa (Br) (7.5 Gm., 0.024 mole) with methyl iodide by a previously described procedure (3) gave 5.51 Gm. (70%) of VII (R_1 = CH_3, R_2 = H) as pale yellow crystals, m.p. 136–137°.

Anal.—Calcd. for $C_{16}H_{12}BrN_3O$: C, 54.56; H, 3.66. Found: C, 54.71; H, 3.73.

7 - Bromo - 1,3 - dihydro - 3 - methyl - 5 - (2 - pyridyl)-2H-1,4-benzodiazepin-2-one, VII (R_1 = H, R_2 = CH_3).—By substituting α-bromopropionyl bromide for bromoacetyl bromide and 2-(2-amino-5-bromobenzoyl)pyridine for 2-(2-aminobenzoyl)pyridine in the procedure described for the *preparation of VIa (H) Method A*, a 30% yield of VII (R_1 = H, R_2 = CH_3) was obtained as cream-colored crystals from acetone, m.p. 228–229°.

Anal.—Calcd. for $C_{16}H_{12}BrN_3O$: C, 54.66; H, 3.66. Found: C, 54.51; H, 3.39.

7 - Bromo - 2 - methylamino - 5 - (2 - pyridyl) - 3H-1,4-benzodiazepine 4-oxide, VIII.—2-(2-Amino-5-bromobenzoyl)pyridine was converted to the oxime(12), m.p. 163–166°.

Anal.—Calcd, for $C_{12}H_{10}BrN_3O$: C, 49.33; H, 3.45. Found: C, 49.41; H, 3.33.

Then, by treating with chloroacetyl chloride, it was converted to 2-chloromethyl-4-(2-pyridyl)-6-bromoquinazoline 3-oxide (12), m.p. 206° dec.

Anal.—Calcd. for $C_{14}H_9BrClN_3O$: C, 47.96; H, 2.59. Found: C, 48.07; H, 2.65.

Reaction of the quinazoline derivative with methylamine in methanol (12) resulted in ring enlargement and formation of the 5-pyridyl-1,4-benzodiazepine 4-oxide, which was isolated in the usual manner (12). It formed cream-colored prisms after recrystallization from acetone, m.p. 231–233° dec.

Anal.—Calcd. for $C_{15}H_{12}BrN_4O$: C, 52.19; H, 3.79. Found: C, 52.46; H, 4.04.

4-(2-Aminobenzoyl)pyridine, IIIb (H).—Using the methods described by Ockenden and Schofield (4), 4-phenacylpyridine (6) was converted *via* the phenylhydrazone into 2-phenyl-3-(4-pyridyl)indole *Ib* (H), m.p. 257–258°, colorless prisms from acetic acid.

Anal.—Calcd. for $C_{19}H_{14}N_2$: C, 84.42; H, 5.22. Found: C, 84.57; H, 5.57.

The indole was oxidized with chromium trioxide to give 4-(2-benzamidobenzoyl)pyridine II*b* (H), m.p. 129–130°, (colorless prisms from ethanol).

Anal.—Calcd. for $C_{19}H_{14}N_2O_2$: C, 75.48; H, 4.67. Found: C, 75.61; H, 4.51.

This was hydrolyzed with concentrated hydrochloric acid to give III*b* (H). This crystallized from methanol as yellow needles melting at 159.5 to 160°.[7]

Anal.—Calcd. for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09. Found: C, 72.56; H, 4.66.

[2 - (4 - Pyridylcarbonyl)phenyl]carbamoylmethylcarbamic Acid Benzyl Ester, IVb (H).—A mixture of 4-(2-aminobenzoyl)pyridine (5.0 Gm., 0.025 mole) and carbobenzoxy glycine (5.3 Gm., 0.025 mole) in methylene chloride (200 ml.) was cooled to 19°. *N,N'*-Dicyclohexylcarbodiimide (5.2 Gm., 0.025 mole) was added, and the solution stirred for 2 hours. The mixture was refrigerated (+5°) for 3 days; *N,N'*-dicyclohexyl urea was removed by filtration and discarded. The methylene chloride solution was concentrated *in vacuo* at room temperature. The residue was dissolved in benzene and was chromatographed through a column of florisil (90 Gm.) after filtration. The benzene fractions were discarded, and the eluant changed to ether. The ether fractions gave 3.5 Gm. (35.7%) of white needles which were recrystallized from a benzene–petroleum ether mixture, m.p. 130 to 130.5°.

Anal.—Calcd. for $C_{22}H_{19}N_3O_4$: C, 67.85; H, 4.92. Found: C, 68.03; H, 4.99.

1,3 - Dihydro - 5 - (4 - pyridyl) - 2H - 1,4-benzodiazepin-2-one, VIb (H).—*A.—From the Carbobenzoxyglycine Derivative, IVb (H).*—A solution of hydrogen bromide in glacial acetic acid (33% w/w), (20 ml.) was added dropwise at room temperature to a stirred solution of the carbobenzoxy compound IV*b* (H) (3.5 Gm., 9.0 mmole) in glacial acetic acid (10 ml.). The mixture was stirred for an additional 4 hours and diluted with 1 L. of diethyl ether. The precipitated hydrobromide (2.8 Gm., m.p. 254–255°) was separated by filtration and dissolved in water (100 ml.). The solution was made alkaline (pH 8) with concentrated ammonium hydroxide; the resulting precipitate was separated by filtration and recrystallized from benzene to give 1.6 Gm. (75%) of white needles, m.p. 206–207°.

Anal.—Calcd. for $C_{14}H_{11}N_3O$: C, 70.87; H, 4.67. Found: C, 71.00; H, 4.82.

B.—From 4-(2-Aminobenzoyl)pyridine, IIIb (H).—1. A solution of 4-(2-aminobenzoyl)pyridine III*b* (H) (5.0 Gm., 0.025 mole) in *N,N*-dimethylformamide (20 ml.) was saturated with hydrogen bromide. Excess hydrogen bromide was removed by warming under reduced pressure. Bromoacetyl bromide (5.1 Gm., 0.024 mole, 2.2 ml.) was added dropwise to the resulting mixture with stirring. A solution of pyridine (4.0 Gm., 0.05 mole, 4.0 ml.) in *N,N*-dimethylformamide (5 ml.) was added over a 5-minute period. The mixture was stirred for a few hours; approximately 300 ml. of liquid ammonia was carefully added. The ammonia was allowed to

[7] Schofield (5) reports m.p. 160–161°

evaporate overnight, and the reaction mixture was taken up in benzene (100 ml.). Ammonium bromide was removed by filtration. The benzene solution was washed with water (3 × 100 ml.) and concentrated under reduced pressure. Recrystallization of the residue from an acetone–hexane mixture gave 2.5 Gm. (41%) of white needles, m.p. 206–207°.

2. A solution of 4-(2-aminobenzoyl)pyridine III*b* (H), (16 Gm., 0.08 mole) in glacial acetic acid, was treated with bromoacetyl bromide and then with ammonia as described for the *preparation of VIa (H), Method A* to give 7.0 Gm. (36.5%) of crude VI*b* (H), melting at 203–205°.

4-(2-Amino-5-bromobenzoyl)pyridine IIIb (Br) and 4-(2-Amino-3,5-dibromobenzoyl)-pyridine.—*A. —From 4-Phenacylpyridine.*—The crude *p*-bromophenylhydrazone of 4-phenacylpyridine was cyclized in hydrochloric acid to give 5-bromo-2-phenyl-3(4-pyridyl)indole, I*b* (Br), m.p. 312–313° (yellow prisms from acetic acid).

Anal.—Calcd. for $C_{19}H_{13}BrN_2$: C, 65.34; H, 3.75. Found: C, 65.04; H, 3.96.

Acetylation of the indole was accomplished by treating the compound under reflux in a mixture of acetic anhydride and potassium acetate for 3 hours. The reaction mixture was cooled, poured into water. and the product extracted into ether. Removal of the solvent and recrystallization of the residue from acetone gave 1-acetyl-5-bromo-2-phenyl-3(4-pyridyl) indole as white prisms, m.p. 172–173°.

Anal.—Calcd. for $C_{21}H_{15}BrN_2O$: C, 64.46; H, 3.86. Found: C, 64.16; H, 3.84.

This product was then oxidized with trifluoroperacetic acid (13, 14). The crude product was hydrolyzed with 50% (v/v) sulfuric acid to give III*b* (Br). After crystallization from chloroform, yellow prisms were obtained melting at 213–214°.

Anal.—Calcd. for $C_{12}H_9BrN_2O$: C, 52.01; H, 3.27. Found: C, 51.95; H, 3.13.

B.—From 4-(2-Aminobenzoyl)pyridine.—A solution of bromine (25 Gm., 0.16 mole, 8 ml.) in glacial acetic acid (5 ml.) was added dropwise with stirring to a solution of 4-(2-aminobenzoyl)pyridine, III*b* (H), (30 Gm., 0.15 mole) in glacial acetic acid (200 ml.) cooled in an ice bath. The mixture was stirred for 10 minutes, diluted with water (300 ml.), and made alkaline with solid sodium hydroxide. The crystalline residue was separated by filtration, washed with water, then dissolved in benzene. Residual water was removed by azeotropic distillation. The benzene solution was concentrated to a small volume and chromatographed on grade I neutral alumina (300Gm.).

Elution of the column with chloroform gave the dibromo compound (8.3 Gm.), m.p. 139–140°, which gave yellow needles on recrystallization from ether, m.p. 141–142°.

Anal.—Calcd. for $C_{12}H_8Br_2N_2O$: C, 40.37; H, 2.54. Found: C, 40.13; H, 2.38.

Eluting with 50% benzene/ether (v/v) gave 15.8 Gm. of the monobromo derivative III*b* (Br). Further elution of the column with methanol gave 4 Gm. of starting material. The dibromo ketone could also be prepared by further bromination of III*b* (Br) under the conditions described above.

7 - Bromo - 1,3 - dihydro - 5 - (4 - pyridyl) - 2H - 1,4-benzodiazepin-2-one, VIb (Br).—The 1,4-benzodiazepinone was prepared from 13 Gm. (0.36 mole) of III*b* (Br) as described for *IIIb(H), Procedure B.1.* The crude product (8.8 Gm.) was purified by crystallization and yielded 3 Gm. of starting material and 4.5 Gm. (39%) of the desired product. Recrystallization from acetone gave white needles, m.p. 228 to 228.5°.

Anal.—Calcd. for $C_{14}H_{10}BrN_3O$: C, 53.18; H, 3.19. Found: C, 53.09; H, 3.38.

1,3 - Dihydro - 7 - nitro - 5 - (4 - pyridyl) - 2H - 1,4-benzodiazepin-2-one, VIb (NO_2).—A solution of 5-(4-pyridyl)-1,4-benzodiazepinone, VI*b* (H), (6.0 Gm., 0.025 mole) in concentrated sulfuric acid (30 ml.) was nitrated as described under the *preparation of VIa (NO_2).* The reaction mixture was worked up as described above and gave 3.0 Gm. (42.5%) of pale yellow needles after recrystallization from acetone, m.p. 242 to 242.5°.

Anal.—Calcd. for $C_{14}H_{10}N_4O_3$: C, 59.57; H, 3.57. Found: C, 59.71; H, 3.26.

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Whole Body Radioactivity Measurement and Significance

By G. E. SCHIFFERDECKER, W. V. KESSLER, and J. E. CHRISTIAN

The total body potassium in humans was determined by measuring the K^{40} radioactivity in a whole body liquid scintillation counter. These values were used to study the lean body mass and the fat content as a function of age and physical fitness. The effect of an anorectic-tranquilizer drug combination and food substitute therapy on the body composition of obese humans undergoing weight loss was also investigated. The techniques reported for the *in vivo*, nondestructive detection and measurement of low levels of γ-emitting radionuclides in large animals and man has opened up entirely new opportunities for research of significance to the pharmaceutical sciences, which heretofore has not been possible.

THE FIRST WHOLE body measurements of the radioactivity in humans were made with high pressure ionization chambers. These were constructed independently by Sievert (1) in Stockholm, Sweden, and by Burch and Spiers (2) in Leeds, England. These detectors were developed because of a need for instruments capable of measuring small quantities of radioactive materials which were present in the body as a result of contamination. A neutrino detector, a large volume liquid scintillation counter designed and used for the neutrino program of Cowan and Reines (3), was adapted to whole body counting after a few modifications.

The first liquid scintillation counter designed specifically for whole body counting of humans was reported by Anderson, *et al.* (4), at the Los Alamos scientific laboratory. A 2π large volume liquid scintillation counter located at Purdue University was used in the studies reported here. The design and basic operating characteristics of this counter have been previously described (5).

The recent development of whole body liquid scintillation counters for the *in vivo* nondestructive detection and measurement of low levels of γ-emitting radionuclides in large animals and man has opened up entirely new opportunities for research of significance to the pharmaceutical sciences. The *in vivo* estimation of total body potassium by counting naturally occurring K^{40} has led to techniques for the determination of body composition in terms of lean body mass and total body fat (6–8) and the study of the effect of many factors, including drugs, on body composition. In addition to the determination of the natural γ-emitting radionuclides, the same techniques may be used to determine minute amounts of radioisotopes introduced into the living system. The results reported in this paper illustrate some of the potential applications.

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EXPERIMENTAL

Operational Characteristics of the 2π Liquid Scintillation Counter. -A description of the detector

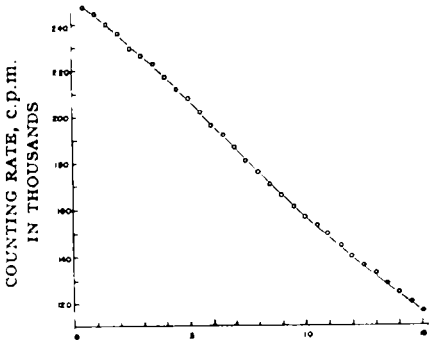


Fig. 1.—Effect of vertical placement of a Cs 137 source.

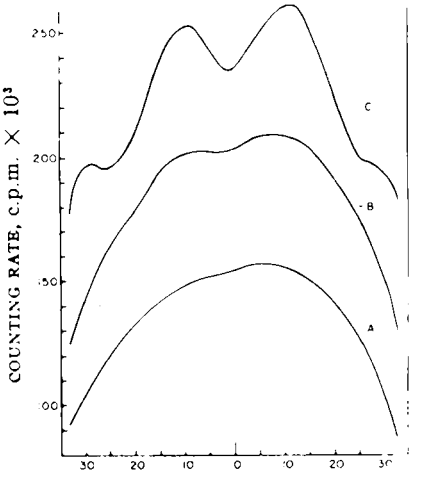


Fig. 2.—Effect of horizontal placement of a Cs-137 source. Key: A, source 10 in. above conveyor; B, source 5 in. above conveyor; C, source on conveyor.

TABLE I.—POTASSIUM, LBM, AND FAT VALUES OF HUMANS OF NEARLY CONSTANT WEIGHT OVER 12 WEEKS

Subject[a]	Age, Yr.	Wt., Kg.	Potassium,[b] Gm.	LBM,[b] Kg.	Fat,[c] Kg.
A	45	71.7	136.5 ± 3.5	51.0 ± 1.4	20.7
B	30	68.1	144.5 ± 3.5	54.0 ± 1.4	14.1
C	37	65.8	141.9 ± 3.4	53.1 ± 1.3	12.7
D	24	61.3	130.9 ± 2.9	48.9 ± 1.1	12.4
E	26	73.5	163.1 ± 3.0	61.1 ± 1.1	12.4
F	27	74.9	179.4 ± 3.0	67.1 ± 1.1	7.8
G	24	65.4	92.2 ± 2.8	34.4 ± 1.0	31.0
H	27	55.4	124.0 ± 3.2	46.2 ± 1.1	9.2

[a] All subjects were males except subject G. [b] Average of ten to 12 individual values taken at approximately weekly intervals. The observed standard deviations are indicated. [c] The fat content was calculated by difference between the total body weight and the average LBM.

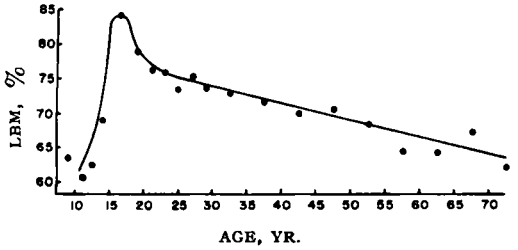


Fig. 3.—Average per cent lean body mass values of male humans as a function of chronologic age.

tank, electronics, steel shield, sample conveyor system, stability, and reproducibility of the counter has been previously reported (5). The high voltage supply for the photomultiplier tubes was operated at 1580 v. The high voltage supplied to each tube was adjusted with a variable series resistance so that all tubes had the same gain. The base discriminator and window settings for the K^{40} channel were 16.0 and 10.0, respectively; those for the Cs^{137} channel were 4.0 and 5.0, respectively. The base discriminator range of 100 pulse height units corresponded to about 85 v. for both the K^{40} and Cs^{137} analyzers. The maximum window width of 10 pulse height units was 20% of the base discriminator range. This corresponded to a maximum window width of about 17 v. The counting efficiency for a Cs^{137} point source[1] was 30.5% and for a K^{40} source (3.32 Kg. potassium chloride) was 18.6% when these sources were positioned in the center of the sample well.

The effect of horizontal and vertical positioning on the counting rate of a Cs^{137} source in the sample well was studied. The source was positioned in a plastic tube supported on wooden holders. The vertical displacement of the source was made at the center of the sample well with 1-minute measurements of the counting rate being made at $^1/_2$-in. intervals up to a height of 15 in. Figure 1 is a plot of these data. The effect of horizontal displacement of the point source was determined at the bottom of the sample well and 5 and 10 in. above the bottom. At each height, determinations were made at 1-in. intervals over the full length of the detector tank. Figure 2 is a plot of these data.

Body Composition Studies of Humans.—Total body potassium, lean body mass (LBM), and total body fat values of five different groups of humans have been calculated from the K^{40} content determined by liquid scintillation counting procedures. The average K^{40} activity of each subject was calculated from four 1-minute determinations. This value was corrected for background, background depression, and variation in efficiency of the detector. The total body potassium was determined by dividing the corrected activity in counts per minute by the counter efficiency and 177, the number of γ rays emitted per minute per gram of potassium. Using the factor 68.1 meq. of potassium per kilogram of LBM,[2] the LBM was calculated from the body potassium.

Based on the LBM concept described by Behnke (12), body fat was estimated by substracting the LBM from total body weight.

Reproducibility of Potassium, LBM, and Fat Measurements.—A group of eight subjects whose weight remained nearly constant was measured periodically (weekly when possible) for potassium, LBM, and fat content over a period of 12 weeks to establish reproducibility. The results are presented in Table I. The reproducibility of the measurements over the 12-week period was between 2 and 3%. The reproducibility measured on three of the subjects over an 8 to 10-month period was 3.1, 3.3, and 3.5%.

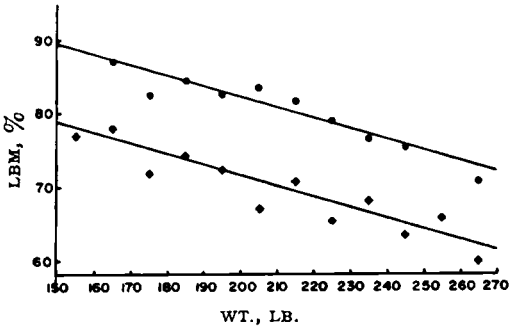


Fig. 4.—Relationships between per cent lean body mass and body weight of male humans of average and above average physical fitness. Key: ●, = above average physical fitness; ◆, average physical fitness.

Average Per Cent LBM of the Human Male As A Function of Age.—A group of 930 males ranging in age from 8 to 75 years was measured for potassium and LBM. Figure 3 shows the average per cent lean body mass as a function of their chronological age.[3]

The per cent LBM increases with age from 8 years up to a maximum at 16, then decreases sharply to about age 20 and assumes a gradual decline with increasing age. The structure of this curve, although not identical in detail, is similar to previously reported data by Anderson and Langham (13) and Onstead (14) in which potassium concentration (grams per kilograms) is reported *versus* age. Both groups report no significant differences between the male and female until approximately the age of puberty, after which the female curve drops rapidly to age 16. Beyond age 16, there is a gradual decline in potassium concentration with age paralleling the

[1] Model RT-2, Nuclear Chicago Corp.

[2] This is the average value of the published chemical analyses of the potassium content of human lean body mass (neutral fat extracted) (9–11).

[3] The age groups plotted contained from 14 to 113 persons (average group size, 46), except for the last four groups which had ten or less persons.

phenyl)-3-butanone (*B*, Fig. 1). In addition, very small amounts of 2-(*p*-chlorophenyl)-3-methylbutadiene-1,3 and *p*-chlorophenyl *tert*-butyl ketone were present. Any delay in fractionation allows the substituted butadiene to polymerize readily (probably catalyzed by peroxides) by a 1:4 addition to form an amorphous insoluble polymer.

A literature search produced no reference to *p*-chlorophenyl *tert*-butyl ketone. It was, therefore, necessary to synthesize this compound for comparison with the conjugated ketone detected in the reaction mixture. The first attempted synthesis of this ketone was from chlorobenzene and trimethyl-acetyl chloride *via* a Friedel-Crafts reaction. A ketone was separated by careful fractionation of the reaction product, but infrared absorption studies indicated that it was not a conjugated ketone. An alternate synthesis from *p*-chloroacetophenone and methyl iodide furnished the desired ketone.

Investigators have established rules for predicting the "migration aptitude" and percentage migration which can be expected for symmetrical pinacols (3), but no satisfactory rules have been formulated to predict the extent of migration of the groups of an unsymmetrical pinacol (4). Collins, in a recent review, states: "Every unsymmetrically substituted glycol can rearrange in two conceivable ways, depending upon which of the two hydroxyl groups is lost during reaction." (See Fig. 1.)

To confirm that 2-methyl-2-(*p*-chlorophenyl)-3-butanone was formed in almost quantitative yield and that it had not resulted from rearrangement during fractionation, a typical reaction mixture was subjected to a selective oxidation process for methyl ketones (5). A substantially quantitative yield of a mixture of *p*-chlorophenyl-dimethylacetic acid along with a small amount of *p*-chlorobenzoic acid was obtained. The mother liquor and washings from the oxidation process were worked up, and a small amount of nonoxidized ketone was isolated as the crystalline 2,4-dinitrophenylhydrazone. The powder X-ray diffraction pattern of this crystalline hydrazone was identical with that of the hydrazone prepared from the fractionated 2-methyl-2-(*p*-chlorophenyl)-3-butanone. The possibility that *p*-chlorophenyl *tert*-butyl ketone might form a 2,4-dinitrophenylhydrazone with difficulty or not at all was considered, but studies with the synthetic ketone prepared for comparison revealed that it formed the 2,4-dinitrophenylhydrazone even more readily than the unconjugated isomer.

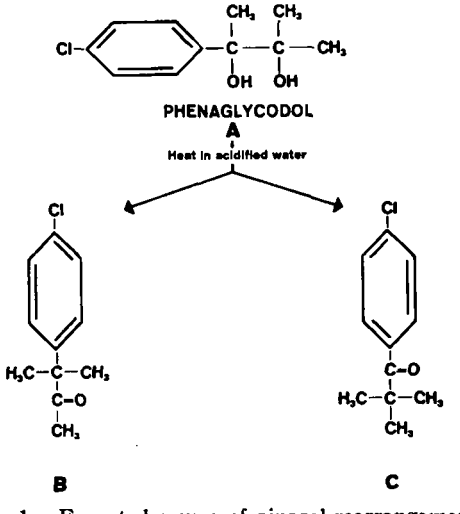


Fig. 1.—Expected course of pinacol rearrangement.

PHARMACOLOGICAL STUDIES

2-Methyl-2-(*p*-chlorophenyl)-3-butanone which had been purified by fractionation was studied in mice for neurosedative activity and in rats for anticonvulsant activity. Quite unexpectedly, this ketone displayed one-third to one-half the pharmacological activity of phenaglycodol. The acute toxicity (LD_{50} 1650 ± 50 mg. per Kg.) by mouth in white mice was about one-half that of phenaglycodol. Since *p*-chlorophenyl-dimethylacetic acid is a possible metabolite of phenaglycodol, the toxicities of the sodium salt by mouth and after intravenous injection in white mice were determined.

EXPERIMENTAL[2]

2-Methyl-2-(p-chlorophenyl)-3-butanone (B, Fig. 1).—One-hundred grams of commercial phenaglycodol was added to a solution of 20 ml. of concentrated sulfuric acid in 180 ml. of water in a round-bottomed flask. This mixture was refluxed gently with constant stirring for 16 hours. The heavier molten glycol separated to the bottom of the flask at first, but as rearrangement progressed the lighter ketonic layer rose to the surface.

The acidic mixture was cooled, transferred to a 500-ml. separator and the lower aqueous phase separated and discarded. The yellow, oily liquid was washed six times with about 80 ml. of 10% sodium chloride solution, then dried with 20 Gm. of anhydrous sodium sulfate. The dried product was filtered into a still pot which was attached to a spinning band reflux column. The column had a calculated efficiency of 28 theoretical plates. The liquid was fractionated at 3 mm. Hg and its composition was as indicated in Table I.

The fraction boiling at 105° was 2-methyl-2-(*p*-chlorophenyl)-3-butanone displaying characteristic infrared absorption bands at 5.83 μ for the unconjugated ketone, at 9.09 μ and 9.82 μ, characteristic of *p*-chlorophenyl, and at 11.96 μ, characteristic of *p*-substituted phenyl. Nuclear magnetic resonance studies supported the assigned structure.

Anal.—Calcd. for $C_{11}H_{13}ClO$: C, 67.17; H, 6.66; Cl, 18.03. Found: C, 67.18; H, 6.50; Cl, 17.62.

[2] All boiling points and melting points reported have not been corrected.

measurements emphasize the attractiveness of this method for research in the pharmaceutical sciences.

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Convenient Synthesis of 3-(γ-Aminopropyl)-5-ethoxyindole

By HUBERT W. MURPHY

3-(γ-Aminopropyl)-5-ethoxyindole has been synthesized from *p*-phenetidine and α-methyl-acetoacetate. This method involves fewer intermediates, with new and better methods for their purification.

THE PREPARATION of this indole in low yield by the use of the Japp-Klingmann reaction has been reported (1). A simpler and more satisfactory synthesis has been developed to prepare larger quantities. By this reaction the desired 4-ethoxyphenylhydrazone was prepared from 4-ethoxybenzenediazonium chloride and ethyl α-methyl-acetoacetate. The crude hydrazone was cyclized by heating in absolute alcohol containing 10% concentrated sulfuric acid to produce 5-ethoxy-2-carbethoxyindole in 40% yield. Saponification with methanolic potassium hydroxide gave an 82% yield of 5-ethoxy-2-carboxyindole. The latter was decarboxylated to furnish 5-ethoxyindole in 80% yield. 5-Ethoxyindole was reacted with acrylonitrile under pressure to yield about 80% of crude 5-ethoxy-3-indolepropionitrile. This can be crystallized from isopropanol before being reduced; however, it was found more expedient to reduce the crude nitrile. Reduction of the crude product furnished a mixture containing both primary and secondary amines from which the pure γ-(5-ethoxy-3-indolyl)-propylamine was separated by crystallization as a Schiff base from acetone. The yield, based on 5-ethoxyindole, was about 20%.

The structure of the isopropylidene was firmly established by infrared absorption: N—H 3.20; N=C, 6.01; aryl ring, 6.14, 6.27, and 6.69; and ethoxy, 8.03 and 9.56 μ. The assigned structure was supported by the nuclear magnetic resonance pattern. The isopropylidene derivative was converted practically quantitatively to an amine salt by hydrolyzing the Schiff base with a dilute aqueous solution of a strong acid.

This Schiff base can also be used as a starting material for the preparation of secondary amines (2). The ease with which the Schiff base is formed under alkaline conditions with acetone is remarkable. Since tryptamine and acetone readily furnish a similar Schiff base, this may be characteristic of 3-indolylamines.

A dicarboxylic acid was isolated from the acetone filtrate remaining from the crystallization of 5-ethoxy-2-carbethoxyindole. This acid is believed to be α-(5-ethoxy-2-carboxy-3-indolyl)-acrylic acid. Structure assignment has been made on the basis of titration data, ultraviolet and infrared absorption, nuclear magnetic resonance studies, melting point, and elemental analysis. The difference in pKa′ values (ΔpKa′, 1-2) in 66% dimethylformamide was 3.6 pH units for this acid. This is about halfway between that for succinic acid, representing an acid with free rotation of the carboxyl groups, and *o*-phthalic acid, an acid with somewhat limited rotation. It is distinctly different from maleic acid, an acid with restricted rotation. This is strong evidence against the alternative quinoline dicarboxylic acid structure. Infrared

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A dicarboxylic acid was isolated from the acetone filtrate remaining from the crystallization of 5-ethoxy-2-carbethoxyindole. This acid is believed to be α-(5-ethoxy-2-carboxy-3-indolyl)-acrylic acid. Structure assignment has been made on the basis of titration data, ultraviolet and infrared absorption, nuclear magnetic resonance studies, melting point, and elemental analysis. The difference in pKa′ values (ΔpKa′, 1-2) in 66% dimethylformamide was 3.6 pH units for this acid. This is about halfway between that for succinic acid, representing an acid with free rotation of the carboxyl groups, and *o*-phthalic acid, an acid with somewhat limited rotation. It is distinctly different from maleic acid, an acid with restricted rotation. This is strong evidence against the alternative quinoline dicarboxylic acid structure. Infrared

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and ultraviolet absorption spectra are consistent with the proposed structure. All hydrogen atoms can be accounted for by a careful comparative study of the nuclear magnetic resonance patterns of the free acid and its dimethyl ester.

Mixtures of 5-ethoxy-2-carboxyindole and this indole acrylic acid melt between the melting points of the pure compounds (179 and 221°, respectively). This may account for the different melting points that have been reported for 5-ethoxy-2-carboxyindole. Rydon and Siddappa (3) reported 202-203°, and Hoshino and Kotake (4) reported 204°. The mechanism by which this acrylic acid is produced is not known. It appears improbable that it results from an impurity in the initial reactants, since both were carefully purified and characterized before use.

EXPERIMENTAL[1]

5-Ethoxy-2-carbethoxyindole (I).—In a 4-L. beaker provided with a thermometer, mechanical stirrer, and an ice bath were placed 1400 Gm. of crushed ice, 180 Gm. (1.31 moles) of freshly distilled *p*-phenetidine, and 320 ml. of concentrated hydrochloric acid; this mixture was cooled to 4°. A solution of 89.6 Gm. of sodium nitrite in 320 ml. of water was added dropwise at such a rate that the temperature remained below 4°. Toward the end of the diazotization, the hydrochloride of *p*-phenetidine, which had precipitated, dissolved; a clear yellow solution was obtained. The completion of the diazotization was determined with starch-iodide test paper.

About 70 ml. cold 40% potassium hydroxide solution (320 Gm. in 800 ml. distilled water) was added, while stirring vigorously, to a mixture of 1600 Gm. of crushed ice and 190 Gm. (1.31 moles) of freshly distilled ethyl α-methyl-acetoacetate. About 140 ml. of the *p*-ethoxybenzene diazonium chloride solution was then added slowly, making sure that alkali was always present in excess. Vigorous stirring was provided to prevent the formation of localized areas of appreciable acidity. The remaining potassium hydroxide and *p*-ethoxybenzene diazonium chloride solutions were added in small quantities with sufficient crushed ice to keep the temperature of the reaction mixture near 0°.

The reaction mixture was stirred for an additional half hour and was then acidified by the addition of 256 ml. of concentrated hydrochloric acid in 1300 Gm. of crushed ice. A viscous red oil separated which was extracted from the aqueous phase by two 1200-ml. portions of benzene followed by a 1000-ml. portion of chloroform. The benzene and chloroform extracts were combined and filtered through a sintered-glass filter overlaid with 260 Gm. of anhydrous potassium carbonate. The filtrate was concentrated *in vacuo* at 30° and the residue was heated to 80° *in vacuo* for 2 hours to remove the last traces of volatile matter.

A mixture of 1600 ml. of absolute ethanol and 160 ml. of concentrated sulfuric acid, which had been cautiously mixed, was added to the residual oil and the solution obtained was refluxed gently for 3 hours. The alcohol was then removed at 5 mm. Hg without heating.

The residue was dissolved in 2 L. of chloroform and 1 L. of distilled water. The acidic aqueous layer was separated and discarded. The chloroform phase was washed with two 1-L. portions of distilled water, a 750-ml. portion of a 3% solution of sodium bicarbonate, and then with three more 750-ml. portions of distilled water. After drying the chloroformic phase with 200 Gm. of anhydrous sodium sulfate, the chloroform was removed at reduced pressure. The residue was dissolved in hot acetone. After filtration, acetone was removed from the filtrate at 5 mm. Hg to a volume of about 500 ml. The crystals which separated on cooling were collected and washed with about 70 ml. of cold acetone. The air-dried crystals weighed 120 Gm., m.p. 152°. Yield, 40% based on *p*-phenetidine.

Anal.—Calcd. for $C_{13}H_{15}NO_3$: N, 6.01. Found: N, 6.08.

5-Ethoxy-2-carboxyindole (II).—The ethyl ester (I) was added slowly to a solution of 100 Gm. of potassium hydroxide dissolved in 800 ml. of methanol. The mixture was stirred and heated gently until all the large particles had been dispersed. After stirring for an additional hour, the insoluble product was collected on a sintered-glass filter and washed with about 100 ml. of cold methanol then with 300 ml. of cold ether. The potassium salt was dissolved in about 1 L. of distilled water, filtered, and the filtrate was acidified to a pH of 2.2 with concentrated hydrochloric acid. The acidic mixture was cooled in the refrigerator; the acid which crystallized was collected and washed with about 150 ml. of cold distilled water.

This acid was purified by dissolving it in basic solution (pH 9.0) decolorized with 4 Gm. of carbon and 5 Gm. of talc. This mixture was heated to 70°, filtered, and the cooled filtrate acidified with hydrochloric acid (pH 2.2). The crystalline acid was collected as before and dried *in vacuo*. The yield was 83.0 Gm. (79% of theory), m.p. 179°. The pKa′ in 66% dimethylformamide was 6.4 and an apparent molecular weight of 205 (theory 205) was determined.

Anal.—Calcd. for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.63; H, 5.63; N, 6.53.

5-Ethoxyindole (III).—To a 50-ml. distilling flask containing 300 mg. of bronze powder, 30 Gm. of 5-ethoxy-2-carboxyindole (II) was added. The flask and its contents were heated in a silicone oil bath at 222° for 20 minutes to complete decarboxylation. The residue was distilled at reduced pressure (b.p. 162° at 4 mm., 194° at 11 mm. Hg) to furnish 18.0 Gm. (76.5% of theory) of 5-ethoxyindole.

Anal.—Calcd. for $C_{10}H_{11}NO$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.38; H, 6.75; N, 8.80.

5-Ethoxy-3-cyanoethylindole (IV).—This was prepared by a modification of a method described by Terent'ev, *et al.* (5). In a 100-ml. round-bottomed flask was placed 16.12 Gm. (0.1 mole) of 5-ethoxyindole (III), 21.2 Gm. of freshly distilled acrylonitrile, 10 ml. of thiophene-free benzene, 0.3 Gm. of glacial acetic acid, 0.2 Gm. of cupric acetate, and 70 mg. of boric acid. The flask was provided with a

[1] All melting points and boiling points reported are uncorrected.

ground-glass stopper and placed in a steel autoclave. About 50 ml. of benzene was placed in the autoclave. The mixture was heated under pressure for 6 hours at 180–190°, then allowed to cool. The flask was carefully removed from the autoclave and most of the contents dissolved in about 500 ml. of ether. The ether extract was washed with four 500-ml. portions of distilled water containing 15 ml. of glacial acetic acid, dried with 40 Gm. of anhydrous potassium carbonate, and decolorized with 2 Gm. of carbon. The ether was removed from the filtrate at reduced pressure to furnish 17.5 Gm. of gummy residue. This residue was dissolved in about 40 ml. of warm isopropanol and then chilled at −10° overnight. The crystals that separated were collected, washed rapidly with about 10 ml. of cold ether (−10°), and solvent removed *in vacuo*. Yield, 3.4 Gm., m.p. 89°. An additional 1.1 Gm. of product was obtained from the filtrate for a total yield of about 20%.

Anal.—Calcd. for $C_{13}H_{14}N_{2O}$: C, 72.87; H, 6.59; N, 13.08. Found: C, 72.74; H, 6.43; N, 12.81.

3-(γ-Isopropylideneaminopropyl)-5-ethoxyindole (V).—Two grams of 5-ethoxy-3-cyanoethylindole (IV) dissolved in 100 ml. of methanol, a mixture of 30 ml. of liquid ammonia, and 20 ml. of methanol, and 5 Gm. of Raney nickel catalyst which had been moistened with methanol were combined in a stainless steel bomb. The bomb was heated to 90° under hydrogen at 1500 p.s.i. for 4 hours with constant agitation. After cooling, the catalyst was removed by filtration and the ammonia and methanol removed with a stream of air in a hood. The residue was taken up in a mixture of 200 ml. of ether and 300 ml. of distilled water to which 10 ml. of concentrated nitric acid had been added. The aqueous portion was separated and washed twice with ether; it was decolorized with 1.25 Gm. of carbon. The filtrate was cooled and transferred to a separator and 250 ml. of ether and sufficient 40% sodium hydroxide solution to adjust the pH to 9.5 were added. The ether phase was separated and the alkaline aqueous portion was extracted with two more 150-ml. portions of ether. The ether extracts were combined, washed with four 250-ml. portions of distilled water, and dried and decolorized by adding 30 Gm. of anhydrous potassium carbonate and 2 Gm. of carbon. After filtration, the ether was removed from the filtrate at reduced pressure. The residue was dissolved in about 25 ml. of acetone and the product crystallized from the cooled solution. Yield, 1.14 Gm. (47% of theory), m.p. 128°.

Anal.—Calcd. for $C_{16}H_{22}N_{2O}$: C, 74.38; H, 8.58; N, 10.84. Found: C, 74.36; H, 8.81; N, 11.07.

It was not necessary to isolate crystalline 5-ethoxy-3-cyanoethylindole (IV). The crude gummy residue obtained, as described, was reduced to furnish the amine. Thus 17.4 Gm. of crude product from the acrylation procedure was reduced by the method described for the purified cyanoethylindole and gave 5.7 Gm. (22% based on 5-ethoxyindole) of the isopropylidene compound.

γ-(5-Ethoxy-3-indolyl)propylamine Hydrochloride (VI).—One gram of 3-(γ-isopropylideneaminopropyl)-5-ethoxyindole (V) was dissolved in 39 ml. of 0.1 *N* hydrochloric acid and the mixture filtered through an asbestos pad. The filtrate was frozen and dried from the frozen state to furnish 975 mg. of the desired hydrochloride, m.p. 196°.

α-(5-Ethoxy-2-carboxy-3-indolyl)acrylic Acid (VII).—The acetone was removed at reduced pressure from the filtrate and washings obtained after collecting the 5-ethoxy-2-carbethoxyindole (I); the residue was dissolved in 400 ml. of ethanol containing 22 Gm. of sodium hydroxide. This alcoholic solution was warmed to 50°, then allowed to stand at room temperature for 24 hours. A very small amount of precipitate separated which was removed with difficulty by filtration. The filtrate stood an additional 24 hours at room temperature, but no more precipitate appeared. The alcohol was removed at reduced pressure, and the residue was dissolved in about 600 ml. of distilled water. This aqueous solution was partially decolorized by several treatments with carbon. The filtrate was acidified (pH 2.2) with concentrated hydrochloric acid and chilled. The acidic product was collected and washed with cold distilled water. It was recrystallized from 150 ml. of methanol. Yield: 3.0 Gm., m.p. 221°.

A second crop was obtained by concentrating the methanolic filtrate. Yield: 1.5 Gm., m.p. 221°.

Anal.—Calcd. for $C_{14}H_{13}NO_5$: C, 61.09; H, 4.76; N, 5.09; O, 29.06. Found: C, 60.71; H, 4.89; N, 5.13; O, 28.68.

The pKa′ values in 66% dimethylformamide were 5.8 and 9.4, and in water, 3.9 and 5.3. The calculated molecular weight from titration was 282 (theory 275).

The dimethyl ester was prepared from absolute methanol with sulfuric acid catalyst in the usual manner in almost quantitative yield, m.p. 150.5°.

Anal.—Calcd. for $C_{16}H_{17}NO_5$: C, 63.36; H, 5.65; N, 4.62. Found: C, 63.15; H, 5.89; N, 4.59.

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Coumarins II

Structures of Columbianadin and Columbianin

By ROBERT E. WILLETTE† and TAITO O. SOINE

Columbianadin and columbianin were isolated from petroleum ether and alcoholic extracts, respectively, of *Lomatium columbianum*. Upon hydrolysis, these coumarins yielded tiglic acid and glucose, respectively, and an alcohol, columbianetin. This alcohol was dehydrated to dihydroöroselone and upon hydrogenation formed a product identical with a degradation product obtained from athamantin. This, in addition to ultraviolet, infrared, and nuclear magnetic resonance absorption studies, led to the establishment of columbianetin as (+)-8,9-dihydro-8-(1-hydroxy-1-methylethyl)-2H-furo[2,3-h]-1-benzopyran-2-one and columbianin as its glucoside. Columbianadin was established as its angelate by synthesis.

IN A CONTINUED search of western United States for plants of the *Umbelliferae* family containing coumarins and/or chromones exhibiting pharmacological activities, Call (1) investigated *Lomatium columbianum* Mathias & Const. From petroleum ether extracts of its root, he isolated a crystalline substance possessing spasmolytic properties which he named "columbianin." No structural studies were reported on this substance.

L. columbianum is an umbellifer growing in south central Washington and north central Oregon. The synonymy listed for this plant is *Leptotaenia purpurea* Coult. & Rose and *Ferula purpurea* Wats., but is not *Lomatium purpureum* (2).

The investigation reported here concerns a more exhaustive investigation of the root of *L. columbianum* and determination of the structures of the principal constituents.

Detailed pharmacological studies will be reported at a later date.

EXPERIMENTAL

Unless otherwise specified, melting points were determined in capillary tubes in an oil bath with a thermometer that read accurately for a set of standard samples. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined as Nujol mulls on a Perkin-Elmer spectrophotometer, model 137. Ultraviolet spectra were determined in 95% ethanol on Cary recording spectrophotometers, models 11 and 14.

Material.—The dried root of *Lomatium columbianum* Mathias & Const., collected near the Columbia River about 3 miles east of Bingen, Wash., was ground in a Jacobsen laboratory mill to an approximately No. 20 powder in preparation for extraction.[1]

Extraction.—The ground root (5 Kg.) was moistened with petroleum ether (b.p. 30–60°), allowed to macerate several hours, and extracted in a Soxhlet-type extractor continuously for 6 days with petroleum ether. The concentrated extract was allowed to stand, and the crystalline needles which formed were removed periodically by filtration. The combined needles were washed with a small portion of cold petroleum ether and air-dried to afford 43.0 Gm. (0.86%) of columbianadin.[2] Evaporation of the remaining extract left a dark viscous oil which possessed several fluorescent components when a small sample of it was developed on a chromatostrip[3] with 30% ethyl acetate in skellysolve B (b.p. 65–70°). Studies of this fraction will be reported at a later date.

The dried marc remaining from the petroleum ether extraction was next extracted continuously with methylene chloride for 4 days. Evaporation of this extract afforded 40 Gm. of a brown resin. A sample of this residue separated into several fluorescent spots on a chromatostrip developed as before. Studies of this fraction will be reported at a later date.

The dried marc remaining from the methylene chloride extraction was next extracted continuously with methanol for 11 days. The methanolic extract was concentrated to half its volume under reduced pressure and allowed to stand several days to afford 105 Gm. (2.1%) of columbianin as a light brown, microcrystalline powder. In an extraction of 400 Gm. of root with 95% ethanol, 16 Gm. (4.0%) of the same material was obtained.

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Previous paper: Willette, R. E., and Soine, T. O., THIS JOURNAL, 51, 149(1962).

[1] The authors are indebted to Dr. T. G. Call, 115 West Rancho Road, Corona, Calif., for collection and identification of the plant material, and to Dr. G. B. Ownbey, Botany Department, University of Minnesota for confirmation of the identity. A herbarium specimen has been retained by the Department of Botany, University of Minnesota.

[2] To follow customary coumarin nomenclature more closely, the authors recommend the names "columbianadin" (Call's "columbianin") for the ester and "columbianin" for the glucoside isolated in the present work. The name columbianetin is suggested for the alcohol resulting from hydrolysis of columbianadin and columbianin.

[3] See *Footnote 2* in the previous paper (3).

Columbianadin (I).—A portion of the crude fraction (9.5 Gm.) was recrystallized from aqueous methanol as long silky needles (5.6 Gm.) which melted at 113–115°; after drying at 100° in vacuum overnight, the needles melted at 121–122°. Repeated crystallizations from dilute methanol and drying at 100° in vacuum overnight gave columbianadin as long colorless needles, m.p. 121–122°; $[\alpha]_D^{27}$ + 26.5° (*c* 1.0, dioxane); ultraviolet spectrum (Fig. 1): $\lambda_{min.}$ 268 mμ (log ε 3.18); $\lambda_{max.}$ 219 (4.35) sh., 250 (3.56), 261 (3.59) and 327 mμ (4.19); infrared spectrum (Fig. 2): 1742–1727 (α-pyrone and α,β unsaturated ester C=O), 1631 (aromatic and α-pyrone ring C=C), 1587 (aromatic ring with conjugated C=C), 1272 (Ar—O—C), 1120 (Ar—O—CR_3), 925 [$(CH_3)_2C$—O], and 837 cm.$^{-1}$ (1,2,3,4-aromatic substitution).[4]

Columbianadin formed a yellow solution in aqueous and ethanolic alkali and was recovered from these solutions upon acidification. It gave a negative phenol test to 3% ethanolic ferric chloride and to the 2% phosphomolybdic acid–concentrated ammonium hydroxide test for hindered phenols. Refluxed in 0.247 *N* ethanolic sodium hydroxide for 6 hours, it gave a saponification equivalent of 172—calculated for two acids, 164.

Anal.[5]—Calcd. for $C_{19}H_{20}O_5$: C, 64.49; H, 6.14; C—CH_3, 9.2; mol. wt., 328.35. Found: C, 64.29; H, 6.08; C—CH_3, 9.2; mol. wt. (Rast), 323.

Columbianin (II).—A portion of the crude fraction (24.8 Gm.) was dissolved in water with the aid of heat; the brown solution was filtered, cooled, diluted with an equal volume of methanol, and evaporated under reduced pressure to a syrup. After standing several days, the syrup afforded very fine crystals. These were filtered off, rinsed with a little methanol, and dried at 100° in vacuum overnight, to give nearly white microcrystals (5.6 Gm.), m.p. 274.5 to 275.5°.

A portion of these crystals (250 mg.), digested in hot methanol and dried, was dissolved in water (5 ml.) and added to the top of a column of silica gel (25 Gm., Baker's chromatographic grade) prepared in a slurry with water. Elution of the column with water yielded a colorless eluate, which was concentrated under reduced pressure to a syrup. Crystallization from methanol, followed by drying at 100° in vacuum several days, gave 190 mg. of columbianin, m.p. 275–276°; $[\alpha]_D^{23}$ + 118 (*c* 0.25, H_2O); ultraviolet spectrum (Fig. 1): $\lambda_{min.}$ 269 mμ (log ε 2.98); $\lambda_{max.}$ 216 (3.97) sh. and 327 mμ (4.00); infrared spectrum (Fig. 3): 3584 (free OH), 3509–3425 (bonded OH) and 1730 cm.$^{-1}$ (α-pyrone C=O). It gave a positive Molisch test.

Anal.—Calcd. for $C_{20}H_{24}O_9 \cdot 2H_2O$: C, 54.07; H, 6.35. Found: C, 53.76, 54.05; H, 6.20, 6.04.

Columbianin Tetraacetate.—Acetylation of columbianin (1.0 Gm.) with acetic anhydride (15 ml.) and fused sodium acetate (1.0 Gm.) gave the tetraacetate (1.8 Gm.). Repeated crystallizations from dilute ethanol and drying at 100° in vacuum gave colorless needles (0.5 Gm.), m.p. 221–222°.

Anal.—Calcd. for $C_{28}H_{32}O_{13} \cdot 2H_2O$: C, 54.90; H, 5.88. Found: C, 55.11; H, 5.86.

Hydrolysis, Isolation of Columbianetin (III).—Columbianadin (5.0 Gm.) was refluxed for 3 hours with 2 *N* sodium hydroxide in 50% ethanol. Complete hydrolysis was determined by development of a neutralized sample of the reaction mixture on a chromatostrip with 30% ethyl acetate in skellysolve

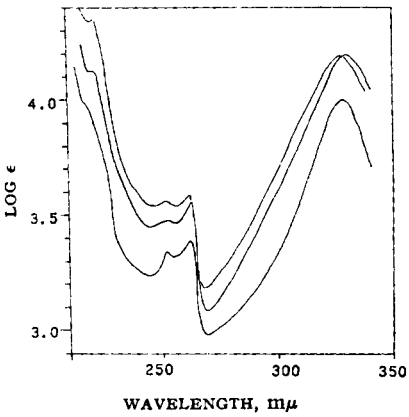


Fig. 1.—Ultraviolet absorption spectra in 95% ethanol. Upper curve, columbianadin; middle curve, columbianetin; lower curve, columbianin.

B. The cooled reaction mixture was diluted with water, partially concentrated under reduced pressure to remove ethanol, and acidified (pH5) with concentrated sulfuric acid. The large colorless needles which formed on standing were removed by filtration and air-dried to give 3.6 Gm. of columbianetin, m.p. 160–162°. Several recrystallizations from hot water and dilute methanol and drying at 100° in vacuum afforded long colorless needles, m.p. 164.5 to 165° (Kofler block); $[\alpha]_D^{27}$ + 20° (*c* 1.0, dioxane); ultraviolet spectrum (Fig. 1): $\lambda_{min.}$ 269 mμ (log ε 3.15); $\lambda_{max.}$ 219 (4.15) and 329 mμ (4.13); infrared spectrum (Fig. 4): 3610 (free OH), 1715 (α-pyrone C=O), 1410, 1149 (*tert*-OH, C—O stretching and OH deformation) and 871 cm.$^{-1}$ ($(CH_3)_2C$—O).

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73; mol. wt., 246.25. Found: C, 68.43; H, 5.65; mol. wt. (Rast), 222.

Columbianin (2.0 Gm.) heated on a steam bath with 5% hydrochloric acid (30 ml.) for 30 minutes also gave columbianetin (0.4 Gm.).

Isolation of Tiglic Acid.—The acidic aqueous filtrate from above was extracted with four 100-ml. portions of ether, which were combined and washed free of acid with 5% sodium carbonate solution. The carbonate wash was acidified with 10% sulfuric acid and extracted with four 50-ml. portions of ether, which were combined and evaporated. The residue was dissolved in skellysolve B, from which a small crop of columbianetin crystallized. The supernatant liquor was evaporated to an oil which crystallized upon standing to give 1.0 Gm. of tiglic acid as large prisms. Sublimation at atmospheric pressure afforded long, fine needles, m.p. 64–65°. The melting point was not depressed on admixture with an authentic sample of tiglic acid.

Identification of D-Glucose.—Columbianin (200 mg.) was refluxed with 2 *N* sulfuric acid in 30% ethanol (15 ml.) for 2.5 hours. The cooled mixture

[4] The assignment of groups responsible for some of the absorption bonds in the infrared spectra of the compounds discussed in this paper are in agreement with the findings of Halpern, Waser, and Schmid (4) for closely related structures.

[5] Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis; Drs. Weiler and Strauss, 164 Banbury Road, Oxford, England; or W. L. Johnson and associates, The Upjohn Co., Kalamazoo, Mich.

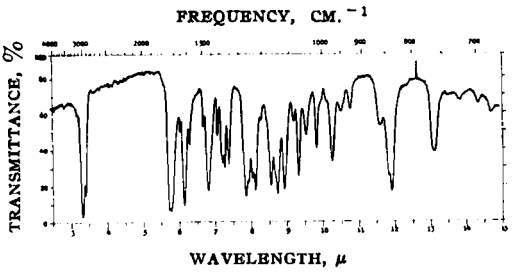


Fig. 2.—Infrared absorption spectrum of columbianadin (Nujol mull).

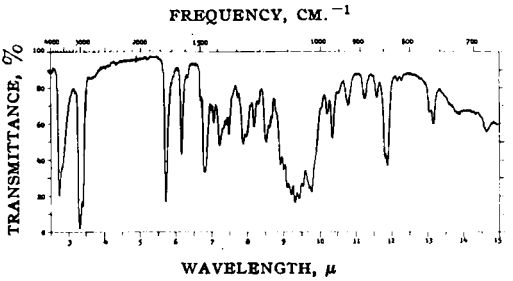


Fig. 3.—Infrared absorption spectrum of columbianin (Nujol mull).

was extracted with four 25-ml. portions of chloroform. The aqueous layer was neutralized with a saturated solution of barium hydroxide, filtered, and concentrated under reduced pressure to an amber syrup (250 mg.). The paper chromatographic behavior[6] was identical with that of authentic D-glucose and different from that of D-mannose or D-fructose.

The phenylosazone of a portion of the syrup (125 mg.) was prepared in the usual manner (6). The crystals, which formed in 7 minutes, were washed with acetone and recrystallized from aqueous methanol to give 80 mg. of phenyl-D-glucosazone as fine, reddish-brown needles, m.p. 205°; $[\alpha]_D^{27}$ − 6.6° (*c* 0.24, 1:1 pyr.-H_2O) [lit. m.p., 210°, $[\alpha]_D^{20}$ − 1.5° (7)]. The melting point was not depressed on admixture with a sample of glucosazone prepared from D-glucose.

Columbianetin Acetate.—Columbianetin (0.2 Gm.), acetic anhydride (5 ml.), and pyridine (5 ml.) were refluxed overnight; dilution with water precipitated fine needles (0.1 Gm.). These were recrystallized twice from dilute ethanol and dried at 100° in vacuum to give fine, colorless needles, m.p. 127.5 to 128.5° (Kofler block); ultraviolet spectrum: $\lambda_{min.}$ 268 mμ (log ε 3.07); $\lambda_{max.}$ 218 (4.11) and 327 mμ (4.12); infrared spectrum; 1727 (α-pyrone and ester C=O) and 1245 cm.$^{-1}$ (acetate C—O).

Anal.—Calcd. for $C_{16}H_{16}O_5$: C, 66.65; H, 5.59. Found: C, 66.64; H, 5.68.

Alkaline Permanganate Oxidation of Columbianetin.—A saturated solution of potassium permanganate (*ca.* 75 ml.) was added to a cold solution of columbianetin (0.5 Gm.) in 1% sodium hydroxide. The mixture was acidified with 10% sulfuric acid and steam distilled. *p*-Nitrophenylhydrazine (0.5 Gm.) in ethanol (20 ml.) was added to the distillate (200 ml.). The long, reddish-brown needles which separated overnight were filtered off and recrystallized twice from dilute ethanol to give 75 mg. (27% of theory, based on 0.5 Gm. of columbianetin) of acetone *p*-nitrophenylhydrazone, m.p. 147–149° [lit. m.p., 149–150° (8)]. The melting point was not depressed on admixture with the *p*-nitrophenylhydrazone prepared from acetone.

Dehydration of Columbianetin.—Columbianetin (0.5 Gm.) was dehydrated with glacial acetic acid (1.5 ml.) and concentrated hydrobromic acid (1.5 ml.) (9). Repeated crystallizations from hot water containing a small amount of ethanol gave 0.1 Gm. of dihydroöroselone (IV), m.p. 142.5 to 143.5° (Kofler block) [lit. m.p., 142° (4)]; ultraviolet spectrum: $\lambda_{min.}$ 231 (log ε 4.07) and 274 mμ (3.71); $\lambda_{max.}$ 224 (4.23) sh., 252 (4.45) and 302 mμ (4.06). The melting point was not depressed on admixture with an authentic sample of dihydroöroselone.[7] The infrared spectra of the two samples were identical.

Dihydrocolumbianetin (V).—Columbianetin (300 mg.) was dissolved in glacial acetic acid (10 ml.) and hydrogenated under 3 Atm. pressure in the presence of palladium black (100 mg.) for 8 hours. The filtered solution was evaporated under reduced pressure and the residue crystallized from 1:2 ether–skellysolve B. Repeated crystallizations from the same solvent gave 100 mg. of dihydrocolumbianetin, m.p. 111.5 to 112.5°; $[\alpha]_D^{24}$ +300° (*c* 0.33, CH_3OH) [lit. m.p. 112–113°, $[\alpha]_D^{19}$ +87° (*c* 0.65, CH_3OH)(4)]; ultraviolet spectrum: $\lambda_{min.}$ 260 (log ε 2.90) and 283 mμ (3.30); $\lambda_{max.}$ 281 (3.29) and 288 mμ (3.32); infrared spectrum: 1761 cm.$^{-1}$ (γ,δ-unsaturated δ-lactone).

Anal.—Calcd. for $C_{14}H_{16}O_4$: C, 67.73; H, 6.50. Found: C, 67.36; H, 6.53.

Columbianetin Tiglate.—Columbianetin (1.23 Gm., 0.005 mole) was powdered and suspended in dry benzene (20 ml.). The suspension was added slowly to a mixture of tigloyl chloride (0.60 Gm., 0.005 mole) in dry benzene (10 ml.). The mixture, protected by a calcium chloride drying tube was refluxed on a steam bath for 20 hours, during which the solid columbianetin dissolved completely. The course of the esterification was followed with chromatostrips which were developed with a 30% solution of ethyl acetate in skellysolve B after spotting with the reaction mixture directly. Inspection of the strip under ultraviolet light was used to determine when the slower moving columbianetin spot had disappeared completely in favor of the faster moving tiglate spot. The reaction mixture was then cooled and washed three times in a separator with 10-ml. portions of 5% aqueous sodium bicarbonate solution, followed by two 10-ml. portions of distilled water. The benzene solution was dried overnight over anhydrous sodium sulfate, filtered, and the solvent removed under reduced

[6] The procedure and solvent system used were essentially those of Jermyn and Isherwood (5). The method utilized a descending technique on Whatman No. 1 paper and detection of carbohydrate spots with potassium periodate solution followed by benzidine reagent. The paper was equilibrated with the lower layer and developed with the upper layer of a mixture of ethyl acetate-water-pyridine (2:2:1 by volume) prepared by shaking well and allowing to stand at room temperature overnight.

[7] Generously supplied by Dr. Hans Schmid, Chemisches Institut der Universität Zürich, Zürich, Switzerland.

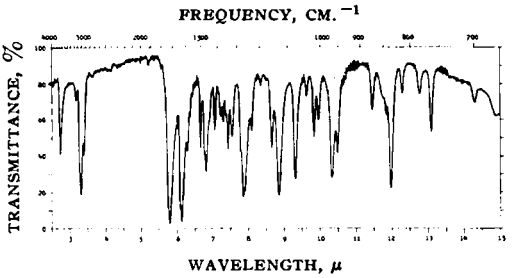


Fig. 4.—Infrared absorption spectrum of columbianetin (Nujol mull).

pressure to yield an oily residue that hardened and crystallized when rubbed with a little methanol. The crude product was recrystallized twice from methanol to yield 1 Gm. of fine, white needles, m.p. 107–108°. A mixed melting point with authentic columbianadin showed marked depression of the melting point and the infrared and nuclear magnetic resonance (NMR) spectra indicated the nonidentity of the two products. In the NMR spectrum, the vinyl proton of tiglate was denoted by a multiplet centering at 3.43 τ.

Anal.—Calcd. for $C_{19}H_{20}O_5$: C, 69.49; H, 6.13. Found: C, 69.43; H, 6.21.

Columbianetin 3-Bromoangelate.—This ester was prepared in exactly the same manner as columbianetin tiglate utilizing equimolar amounts of 3-bromoangeloyl chloride (16) and columbianetin. A reflux period of 30 hours, however, was required for completion of the reaction as determined by the chromatostrip method. The crude product was recrystallized twice from ethanol to yield white crystals, m.p. 135 to 137.5°.

Anal.—Calcd. for $C_{19}H_{19}BrO_5$: C, 56.06; H, 4.70. Found: C, 55.87; H, 4.86.

Columbianetin Angelate.—This ester was prepared by the method of Kupchan and Afonso (16) by atmospheric pressure hydrogenation of an ethanolic solution of columbianetin 3-bromoangelate in the presence of anhydrous sodium acetate and 10% palladium-on-carbon. After removal of catalyst and evaporation of solvent, the product was recrystallized twice from methanol and water to give long white needles. These were dried overnight at 100° in vacuum and gave a melting point of 115–118°. A mixed melting point with authentic columbianadin (m.p. 121–122°) was 117–119°. Infrared and NMR spectra of columbianetin angelate and columbianadin were identical. In the NMR spectrum of the angelate, the vinyl proton was denoted by a multiplet centering at 4.02 τ.

Anal.—Calcd. for $C_{19}H_{20}O_5$: C, 69.49; H, 6.13. Found: C, 69.36; H, 6.28.

DISCUSSION

The previously isolated (1) columbianadin (I) was obtained from petroleum ether extracts of the dried root of *L. columbianum* in 0.8 to 0.9% yields. Alcoholic extracts of the root afforded a crystalline substance in 2 to 4% yields, which gave a positive carbohydrate test (Molisch) and to which we assigned the name, columbianin (II).

Hydrolysis of columbianadin (I) with alkali and of columbianin (II) with acid in dilute ethanol yielded tiglic acid and D-glucose, respectively, and an alcohol, which we named columbianetin (III).

Dehydration of columbianetin (III) gave the known coumarin dihydroöroselone (IV), which conceivably could have arisen from the tertiary alcohol III or the secondary alcohol VI by rearrangement.[8] Hydrogenation of columbianetin (III) afforded the dihydroalcohol V, which is identical to a hydrogenation product of athamantin (4). Since a rearrangement is not possible during the hydrogenation, the location of the hydroxyl group in columbianetin is established.

Assignment of structure III to columbianetin was supported further by infrared and NMR spectra. In its infrared spectrum, columbianetin gives rise to peaks at 1410 and 1149 cm.$^{-1}$ attributable to tertiary alcohol O—H deformation and C—O stretching; whereas the spectrum of the secondary alcohol VI[9] shows analogous peaks at 1295 and 1090 cm.$^{-1}$ which are characteristic of a secondary alcohol (11).

The hydrogen attached to a secondary carbon carrying a hydroxyl group usually exhibits a peak in its NMR spectrum at a frequency about 60 c.p.s. higher than when the hydroxyl is acetylated (12). This is due to elimination of interaction between the two hydrogens of the HO—CH group. Conversion of the tertiary alcohol III to its acetate gives a shift of 23 c.p.s. in the absorption attributable to the hydrogen of the group: O—C—H. This agrees with the O—C—H group present in the furano ring of structure III. The NMR spectra of the secondary alcohol VI and its acetate[9] show a shift of 69 c.p.s. for this hydrogen, which agrees with their established structures. Also, the location of the peak for the proton of the hydroxyl group was at a higher frequency in the NMR spectrum of alcohol VI than alcohol III, which possibly is indicative of greater hydrogen bonding as is sometimes noted in spectra of acyclic secondary alcohols (13).[10]

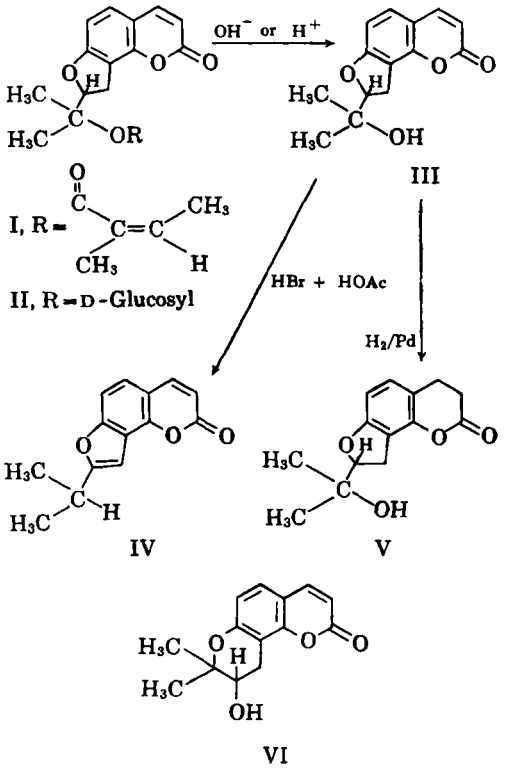
As previously indicated, tiglic acid was obtained by hydrolysis of columbianadin (I). Since angelic acid readily isomerizes to tiglic acid (14), it was necessary to determine whether columbianadin was an angelate. Kupchan and Afonso (15) have demonstrated that no validity can be placed on lack of isomerization of free angelic acid to tiglic acid when treated under the same reaction conditions used to hydrolyze the ester. They indicated that isomerization takes place either before or during the actual cleavage of the ester.

The tiglate of columbianetin was prepared by treatment with tigloyl chloride and the angelate from 3-bromoangeloyl chloride followed by hydrogenation (16). Columbianetin angelate showed no depression of melting point on admixture with columbianadin, although it should be pointed out that the melting point of the synthetic angelate was a few degrees lower than that of the natural product. Their infrared and ultraviolet spectra and chromatographic behavior were identical. The tiglate gave

[8] Bencze, *et al.* (10), minimized this possibility, however, in an analogous acid dehydration of visamminol.

[9] (±)-3′-Hydroxy-3′,4′-dihydroseselin (VI) and its acetate were obtained from pteryxin (3).

[10] The authors thank Dr. James N. Schoolery, Varian Associates, Palo Alto, Calif., Mr. George M. Slomp, The Upjohn Co., Kalamazoo, Mich., and Dr. Michael J. Martell, Jr., Lederle Laboratories, for assistance in determining and interpreting the NMR spectra.



dissimilar spectra and a melting point depression. Comparison of NMR spectra also confirmed the identity of the angelate and the nonidentity of the tiglate with columbianadin. It is of interest to note that the vinyl proton in the angelate (as well as in columbianadin) is at 4.02 τ (center of multiplet) and that of the tiglate is a multiplet centering at 3.43 τ. This agrees well with Fraser (17), who cites 4.03 τ for the vinyl proton of angelate and 3.28 τ for tiglate. Columbianadin is, thus, firmly established as the angelate ester of columbianetin.

The structure of columbiauin (*II*) as the D-glucoside of columbianetin follows from its identified hydrolytic products.

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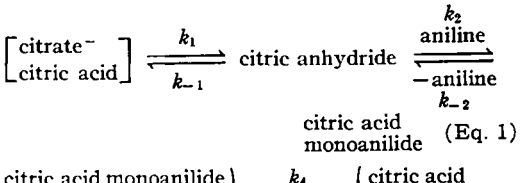
Facilitated Reversible Formation of Amides from Carboxylic Acids in Aqueous Solutions III

Reaction of Citric Acid with Aromatic Amines

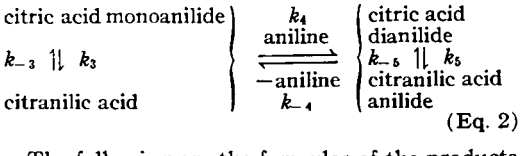
By T. HIGUCHI, SVEN O. ERIKSSON*, H. UNO, and J. J. WINDHEUSER‡

A detailed study of the rate of interaction of citrate buffers with aniline to form anilides and imides shows that the basic mechanism follows essentially that previously described. Results show in particular that the rate of anhydride formation by the polycarboxylic acid is faster than from the previously studied succinic system and probably involves both the undissociated acid and its singly charged anion. The overall equilibria and rates for the total reaction were determined and the approach to equilibria checked with an analog computer. The speed and nature of the reaction suggest that these interactions may often be responsible for loss of activity of some drugs formulated with this buffer.

IN PREVIOUS PUBLICATIONS (1, 2) it has been shown that certain dicarboxylic and polycarboxylic acids react with amines to form amides at unexpectedly fast rates. The significance of this reaction becomes apparent when one considers the wide use of some of these acids as buffers in numerous pharmaceutical systems. Because of the particular importance of citric acid in medicinal solutions, the behavior of this system has been explored in some detail with aniline as the other reactant. The reaction appears to be again mediated by the formation of cyclic anhydrides in aqueous solution which then form amido acids as described previously for the succinate system (2). The equilibria and reactions involved appear to be rather complex but may essentially be represented

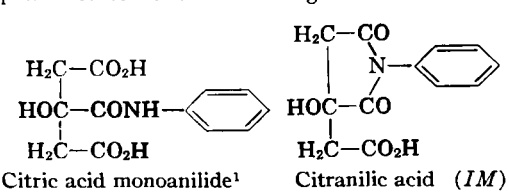


(Eq. 1)

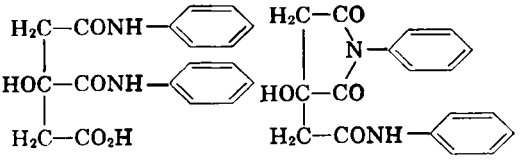


(Eq. 2)

The following are the formulas of the products presumed to be formed during the reaction



Citric acid monoanilide[1] Citranilic acid (*IM*)



Citric acid dianilide[1] Citranilic acid anilide

In further discussion the symbols (*CA*) for citric acid, (*AH*) for citric anhydride, (*AN*) for aniline, (*AM*) for citric acid monoanilide, and (*IM*) for citranilic acid will be used. k_n will denote apparent rate constants, with the subscript n being positive for the forward reactions and negative for the reverse.

Due to the polyfunctionality of the citric acid, other products such as the dianilide and a mixed anilide-imide are also possible. It was found from the experimental data that the latter two compounds were formed in relatively small concentrations, and Eqs. 1 and 2 represent the main reaction pathways in the concentration range studied.

RESULTS AND DISCUSSION

Identification of Reaction Products.—Sample solutions containing 0.05, 0.1, and 0.2 moles/L. of aniline were prepared in 0.5 *M* citrate buffer solutions, the pH at each concentration level being adjusted to 3.0, 3.5, 4.0, and 4.5. The solutions were heated for 100 hours at 85° and samples withdrawn at the end of 50 and 100 hours and analyzed with the *chromatographic separation* described under *Experimental*. Essentially no difference in composition could be demonstrated between the 50 and 100-hour samples, indicating the achievement of an equilibrium condition.

Based on separation of the reaction products by a partition chromatographic method with a pH 3.13 phosphate buffer as the fixed phase and mixtures of chloroform and butanol as the mobile phase, it was possible to establish the nature of the products. The identification was based on: (*a*) comparison of the elution characteristics of the products to known compounds treated in a duplicate manner; (*b*) ultraviolet absorption spectra of the unknown and known

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[1] The exact position, whether α, β, or γ of the amine groups in these structures has not yet been determined.

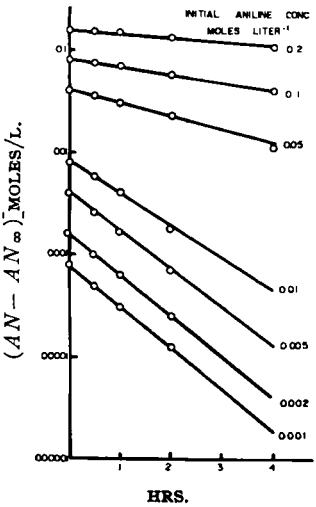


Fig. 1.—Plot showing logarithmic approach to equilibrium for aniline citrate systems. at pH 4. The several lines represent differing aniline concentration.

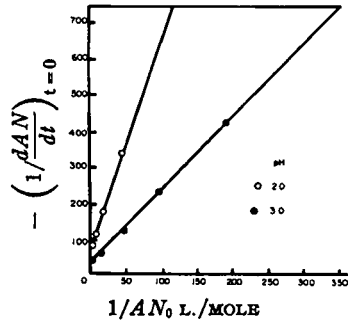


Fig. 2.—Plots of reciprocal initial rate of aniline disappearance in hours/liter/mole against reciprocal initial aniline concentration in liters/mole for aniline in presence of aqueous 0.5 *M* citrate buffer at pH 2.0 and 3.0 and at 85°. The relationship corresponds to Eq. 3.

compounds; and (*c*) equivalent weight and melting point comparison.

The results indicated that the main products of the reaction were the monoanilide (*AM*) and the imide (*IM*). Even under conditions of highest aniline concentration, less than 1% of the aniline was converted to the dianilide at pH 4.5. The amounts of citranilic acid anilide were higher than the dianilide at all pH values, the maximum being formed at pH 4.0. Even at this pH and highest aniline concentration, less than 3% of the aniline was present in the form of citranilic acid anilide.

The equilibrium ratios of the aniline: anilide: imide appeared to be independent of aniline concentration at a given pH and were in good agreement with the kinetically determined equilibrium values.

Kinetics of the Forward Reaction.—The rate of disappearance of aniline from mixtures of citric acid and aniline was studied at 85° using a 0.5 *M* citrate buffer system, the aniline concentration varying from 2×10^{-1} to 1×10^{-3} moles/L. Although the reaction appeared to follow a pseudo first-order kinetic mechanism in its approach to equilibrium, the rates of the reactions were dependent on initial aniline concentration. Figure 1 depicts the typical behavior observed at pH 4.0. Analysis of these curves suggested that at low aniline concentration the reaction became first-order in respect to aniline, but at high concentrations the reaction approached a zero-order dependency on aniline. This would be in accord with the mechanism postulated by Higuchi, *et al.* (2), for the reaction of succinic acid with aniline, which apparently is mediated by the formation of cyclic anhydrides in solution. Due to the complexity of the reaction, the kinetic analysis was limited to the initial rates utilizing the following assumptions: (*a*) the initial phase of the reaction can be represented by the upper half of the total scheme given above; (*b*) in this phase k_{-2} and lower half of the scheme can be neglected as the initial amide concentration is effectively zero; and (*c*) a steady-state assumption can be made for the anhydride concentration.

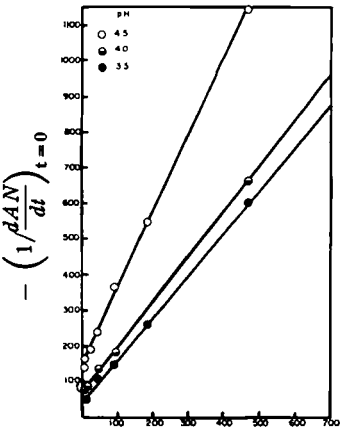


Fig. 3.—Plots of reciprocal initial rate of aniline disappearance in hours/liter/mole against reciprocal initial aniline concentration in liters/mole for aniline in presence of aqueous 0.5 *M* citrate buffer at pH 4.5, 4.0, and 3.5 and at 85°. The relationship corresponds to Eq. 3.

TABLE I.—EFFECT OF pH ON THE APPARENT CHANGE OF k_1 AND THE RATIO OF $\frac{k_2}{k_{-1}}$ AT 85°C.

pH	k_1 (hr.$^{-1}$)	$\frac{k_2}{k_{-1}}$ L./mole
2.0	4×10^{-2}	7
2.5	7.4×10^{-2}	7
3.0	7.2×10^{-2}	14
3.5	5.2×10^{-2}	33
4.0	3.1×10^{-2}	60
4.5	1.4×10^{-2}	70

These assumptions lead to the following reciprocal rate expression

$$-\frac{1}{\left(\frac{dAN}{dt}\right)_o} = \frac{1}{k_1 k_2 \frac{(AN)}{k_{-1}}(CA)} + \frac{1}{k_1(CA)} \qquad \text{(Eq. 3)}$$

During the initial phase we can substitute the initial aniline concentration (AN_o) and the initial citric acid concentration (CA_o) for (AN) and (CA), respectively. A plot of $[1/(dAN/dt)]_o$ *versus* $1/AN_o$ should yield a straight line with an intercept of $1/k_1(CA_i)$ and slope a of $\left\{1/\frac{k_1 k_2}{k-1}(CA_0)\right\}$. Figures

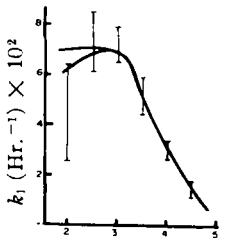


Fig. 4.—pH profile of rate of formation of citrate anhydride in citrate buffer at 85° as determined from intercepts of plots similar to Figs. 2 and 3. The lengths of the bars are approximate measures of the confidence limits of the individual values.

2 and 3 show the results of experiments conducted at pH 2.0, 3.0, 3.5, 4.0, and 4.5, treated in accord with Eq. 3. The nonzero intercept of the plots lend credence to the postulated mechanism.

From the intercept values of these plots, k_1 can be determined directly. Slope measurements were used to evaluate k_2/k_{-1} values as shown in Table I. The rate constants for the anhydride formation, k_1, measured in this fashion are shown in Table I and plotted in Fig. 4 for several pH values.

The shape of the profile strongly suggests that the species responsible for formation of the anhydride probably include both the uncharged, undissociated acid and its singly dissociated, singly charged, anionic form. If only the free acid underwent the reaction, the profile should exhibit a faster drop off at higher pH values. Unfortunately, reliable data under strongly acidic conditions were difficult to obtain because the ratio of rates of amide formation to hydrolysis of the anhydride becomes rather small as a result of protonation of the amine.

Due to the changing dependency of the reaction rate on the concentration of species, a simple pH rate profile of the total reaction at given concentrations is not fully informative regarding the expected overall rates under all conditions. A plot of the initial rate of aniline loss as a function of pH is shown in Fig. 5 at two levels of aniline. The difference in the shapes of the two curves is because at the higher aniline concentration the rate-limiting step is the formation of anhydride which is proportional to the concentration of undissociated citric acid (and, perhaps, the monocitrate anion as noted previously). Therefore, the decreasing rate at higher pH may be attributed to loss of the reactive, acidic species.

In the lower aniline concentration the reaction would be expected to show a first-order dependency on the unprotonated form of aniline. This is in accord with the mechanism previously postulated by Higuchi and Miki (1), which stated that kinetically the mechanism could be typified as $CA + NH_2R \rightleftharpoons$ amide. Such a system would exhibit the observed bell-shaped pH dependence.

The effects of temperature on these reactions were carried out in studies at 75°, 85°, and 95° at pH = 4.0. Figure 6 shows an Arrhenius-type plot for k_1 calculated from reciprocal plots as in Fig. 3. Higher temperatures favored the formation of the anhydride as was also observed for the succinic acid system (2). The apparent activation energy for the citrate reaction—based on the plot is 26.5 Kcal. mole^{-1}. The marked positive temperature dependence is of vital importance when considering the effect of heat sterilization of amine drugs in citrate buffer systems.

Kinetics of the Intermediate Reaction.—In this

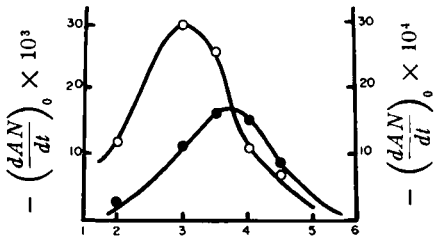


Fig. 5.—Apparent initial rate of disappearance of aniline in 0.5 M citrate buffer as a function of pH at two aniline concentrations. Key: ○, aniline = 0.2 mole/L. (left axis); ●, aniline = 0.002 mole/L. (right axis).

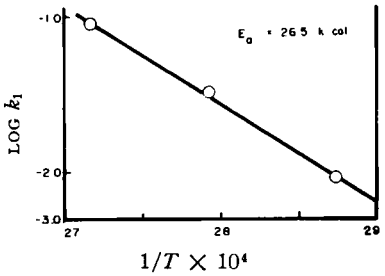


Fig. 6.—Arrhenius type plot for k_1 at pH 4.

phase of the work experiments were conducted to study the following equilibrium

$$\text{citrate}^- \text{ / citric acid} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{citric anhydride} \underset{k_{-2}}{\overset{\text{aniline } k_2'}{\rightleftharpoons}} \underset{-\text{aniline}}{} \text{citric monoanilide} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{imide}$$

where at low concentrations $k_2' = k_2 (k_1/k_{-1})(CA)$, a constant at fixed citrate concentration and pH. Starting with 2.5–3.0 mM solutions of the monoanilide, the formation of other products was followed as a function of time. It can be assumed from previously discussed chromatographic analyses that at these low aniline concentrations the formation of products containing 2 moles of aniline can be neglected. Also because of the low aniline concentrations the reactions can be expected to follow a first-order approach to equilibrium at a given pH.

Based on these assumptions, the rate of formation of aniline and imide can be described by

$$\frac{d\,AN}{dt} = k_{-2}(AM) - k_2'(AN) \quad \text{(Eq. 4)}$$

$$\frac{d\,IM}{dt} = k_3(AM) - k_{-3}(IM) \quad \text{(Eq. 5)}$$

At equilibrium Eqs. 6 and 7 are valid

$$\frac{(AN)_{eq}}{(AM)_{eq}} = K_2 = \frac{k_{-2}}{k_2'} \quad \text{(Eq. 6)}$$

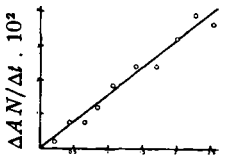
$$\frac{(IM)_{eq}}{(AM)_{eq}} = K_3 = \frac{k_3}{k_{-3}} \quad \text{(Eq. 7)}$$

where the subscript eq refers to the equilibrium concentration. Substitution of Eqs. 6 and 7 into Eqs. 4 and 5 yields

$$\frac{d\,AN}{dt} = k_{-2}\left[(AM) - \frac{1}{K_2}(AN)\right] \quad \text{(Eq. 8)}$$

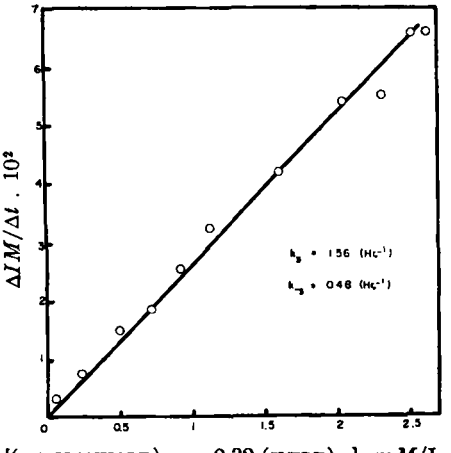
$$\frac{d\,IM}{dt} = k_3\left[(AM) - \frac{1}{K_3}(IM)\right] \quad \text{(Eq. 9)}$$

On the basis of Eqs. 8 and 9 the values of the rate constants can be estimated by plotting $\Delta AN/\Delta t$ *versus* $[(AM_{av}) - (1/K_2)(AN_{av})]$, where the subscript *av* refers to average concentrations during the time interval Δt. The slope of the line is equal to k_{-2}. k_3 can be estimated in a similar manner from a plot of $\Delta IM/\Delta t$ *versus* $[(AM_{av}) - (1/K_3)(IM_{av})]$. k_2' and k_{-3} can then be calculated by appropriate substitution into Eqs. 6 and 7. Figures 7 and 8 show



$[(\text{MONOANILIDE})_{av} - 0.63(\text{ANILINE})_{av}]$, m$M$/L.

Fig. 7.—Rate of aniline formation as a function of $[(AM)_{av} - 1/K_2\,(AN)_{av}]$ at pH = 3.0. k_2 = 0.96 (hours $^{-1}$); k_2' = 0.60 (hours^{-1}).



$[(\text{MONOANILIDE})_{av} - 0.32\,(\text{IMIDE})_{av}]$, m$M$/L.

Fig. 8.—Rate of imide formation as a function of $[(AM)_{av} - 1/K_3\,(IM)_{av}]$ at pH = 3.0.

the results obtained from plots of this type. The values obtained at various pH are recorded in Table II. As in the case of k_1 discussed earlier, they are apparent constants and are not corrected for species changes.

Since the reported values for the observed rate constants were not obtained by direct solution of the differential equations, their validity was checked by an analog computer. The system can be described in differential form by Eqs. 4 and 5 and the following equation for the change in amide concentration.

$$-\frac{d\,AM}{dt} = k_{-2}(AM) - k_2'(AN) + k_3(AM) - k_{-3}(IM) \quad \text{(Eq. 10)}$$

The analog computer circuit diagram representing Eqs. 4, 5, and 10 is shown in Fig. 9. When the experimentally determined constants were substituted, good agreements were found between the computer-calculated concentration-time curves for amide, imide, and aniline and the experimental values found. Figure 10 shows the computer-calculated curves and experimental values for the system at pH 3.0.

It was observed experimentally that at pH 3.0, 3.5, and 4.0 the aniline concentration passed through a maximum value. These results could not readily be anticipated by inspection of the differential equations, and consequently brought forth the speculation that some other mechanism might be active at these pH conditions. The computer-calculated concentration profile for aniline, based on the proposed reactions and rate constants, also exhibited a maximum which coincided with the experimentally observed peak, further supporting the validity of the proposed mechanism. This effect is apparently because the formation of aniline is kinetically more favored than would be expected from thermodynamic considerations.

Conclusions and Consideration of Possible Participation in Loss of Drug Activity in Citrate Buffers.—According to Table I the rate of presumed reactive intermediate (anhydride) formation is nearly two orders of magnitude greater for the cit-

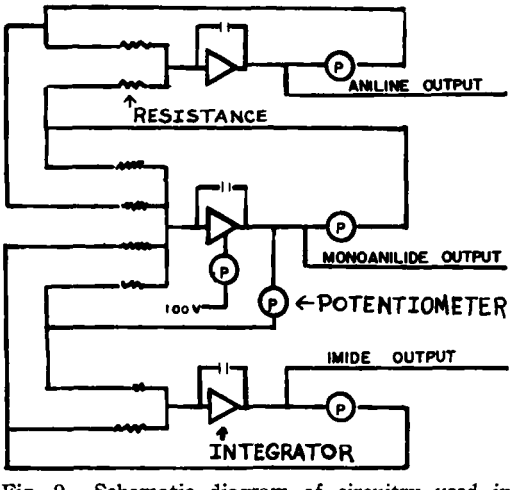


Fig. 9.—Schematic diagram of circuitry used in analog computation.

TABLE II.—EFFECT OF VARYING pH ON THE APPARENT PSEUDO FIRST-ORDER RATE CONSTANTS AND ON THE RATIOS OF k_{-2}/k_2' AND k_3/k_{-3}

pH	k_{-2} (Hr.$^{-1}$)	k_2' (Hr.$^{-1}$)	k_{-2}/k_2'	k_3 (Hr.$^{-1}$)	k_{-3} (Hr.$^{-1}$)	k_3/k_{-3}
2.0	1.3	0.23	5.5	1.3	0.24	5.5
3.0	0.96	0.60	1.6	1.6	0.48	3.1
3.5	0.81	0.94	0.9	1.6	0.55	2.9
4.0	0.55	0.88	0.6	1.1	0.59	1.9
4.5	0.29	0.43	0.7	0.84	0.70	1.2
5.0	0.12	0.13	0.9	0.44	0.77	0.6

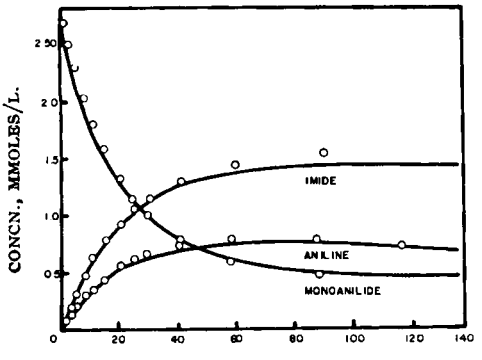


Fig. 10.—Curves obtained by computor analysis showing the concentrations of aniline, imide, and monoanilide at pH = 3 as a function of time. The points shown are experimental values. The computer curves were based on: $k_2' = 0.60$ (hours^{-1}); $k_{-2} = 0.96$ (hours^{-1}); $k_3 = 1.56$ (hours^{-1}); $k_{-3} = 0.48$ (hours^{-1}).

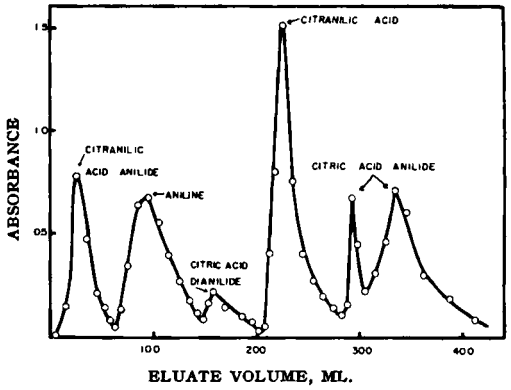


Fig. 11.—Elution chromatogram of a synthetic mixture of reaction products of aniline and citrate buffer readings in 1-cm. cells at 287 mμ for aniline and 241 mμ for the other fractions after extraction with their equal volume 0.1 *M* NaOH.

rate than for the succinate system (2). At 85° approximately 0.03 to 0.04 mole/liter of the suggested anhydride species would be expected to be formed per hour in a 0.5 *M* citrate buffer in the pH range 2.5 to 4.0. It would appear that this would be a significant factor during any autoclaving process. The effect of the buffer on shelf lives at room temperature would depend to a great extent on the concentration of the susceptible drug species and the value of k_2. Although k_1 may be markedly lower (approximately 1×10^{-5} hr.$^{-1}$ at 25° based on heat of activation of 26.5 Kcal./mole), the rate will still be expected to be significant for many drugs. In particular, any drug amine which may be readily acylated, including aromatic amines, hydrazine derivatives, etc., is suspect.

EXPERIMENTAL

Reagents

All reagents used were of analytical grade, except those synthesized and described below.

Citranilic Acid (N-Phenylcitric Acid Imide.)—This substance was prepared with some modifications according to a method originally described by Pebal (3) and later modified by Nau, Brown, and Bailey (4) and Leulier, Cier, and Drevon (5). The monoaniline salt, prepared by evaporating the ethanol from an equimolar solution of aniline and citric acid in ethanol, was heated in an open crucible to 150° for about 2 hours. The product was washed with ether and finally crystallized from chloroform. The equivalent weight was determined both by direct titration with sodium hydroxide solution and by adding sodium hydroxide solution in excess and back titration with hydrochloric acid. When using the latter method, the substance was hydrolyzed to the corresponding anilide. Both methods gave the molecular weight 255 (calculated 249), m.p. 186–187° (literature values vary from 185 to 189°).

Citric Acid Anilide.—This substance has not been isolated, but solutions of it have been prepared by adding an excess of sodium hydroxide solution to a solution of citranilic acid.

Citranilic Acid Anilide.—After boiling an aqueous solution containing 0.5 moles/L. aniline and 0.25 moles/L. citric acid for approximately 24 hours, citranilic acid anilide precipitated upon cooling the solution. The melting point was 181–182° after recrystallization from chloroform. Although the substance was prepared in a manner different from that reported by Bertram (6) and by Leulier, Cier, and Drevon (5), the observed melting point is in good agreement with that found by these investigators.

Citric Acid Dianilide.—This was prepared by adding an excess of sodium hydroxide to a solution of citranilic acid anilide which yielded the dianilide upon hydrolysis. Upon acidifying the solution with hydrochloric acid, the very sparingly soluble dianilide precipitated. The substance was also synthesized using a modification of the method originally described by Pebal (3). One mole of citric acid and 2 moles of aniline were heated at 170° for 1 hour in a test tube equipped with an air condenser. The dianilide product was extracted with sodium hydroxide solution and precipitated by acidifying with hydrochloric acid. The dissolution with alkali and precipitation with acid was repeated; finally the substance was recrystallized from ethanol. Both these methods of synthesis gave a substance with a melting point of 176° and an equivalent weight of 345 determined by titration (theoretical 342). The reported melting points in the literature vary from 180–184 (3, 6–8). The differences in the reported melting points might be attributed to the possibility of obtaining two different dianilides. Leulier, Cier, and Drevon stated that their compound with a melting point of 182° was the unsymmetrical dianilide.

Chromatographic Separation

The study of the reactions between aniline and citric acid required an assay method which permitted the determination of aniline and the four synthesized substances which might be formed. This was accomplished by the use of a partition chromatographic separation. Chromatographic columns with 18-mm. inner diameter were used. Twenty-five grams of silicic acid (Mallinckrodt, chromatographic grade) was used as supporting media, and 25 ml. of a pH 3.13 buffer of the following composition was used as the stationary phase:

Sodium phosphate monobasic, 75.90 Gm. (=0.55 mole)
Hydrochloric acid, 1 *M*, 30.00 ml.
Distilled water, *q.s.* 1000 ml.

The column was packed in the usual way using chloroform to make a slurry (9). A 2.00-ml. quantity of an aqueous solution containing approximately 0.05 mmole of aniline and its reaction products and with pH adjusted to 3.1 was adsorbed on 2 Gm. of silicic acid in a 30-ml. beaker. A slurry was made by adding 10 ml. of chloroform and then transferred to the column. To insure quantitative transfer of the sample to the column, another slurry was made in the same beaker using 1 Gm. silicic acid, 1 ml. stationary phase, and 5 ml. of chloroform. The second slurry was also added to the top of the column and packed tightly. The separation was carried out in the normal manner of gradient elution, collecting the fractions and changing solvent composition as follows.

The eluting solutions were chloroform and mixtures of chloroform and butanol according to the following compositions: eluant—0–100, 100–190, 190–270, and 270–425 ml.; butanol in chloroform—0, 1.5, 10, and 30%.

Figure 11 shows an elution chromatogram of a mixture of the five substances. Eluate was collected usually in fractions of 10 ml., but in some cases 5-ml. fractions were used. The final fractions used were 25 ml. each. The separated components are shown as separate peaks on the plot.

All the fractions were analyzed by U.V. spectrophotometry. Aniline was determined directly in the chloroform solution at 287 mμ (ϵ = 1760). The other fractions were extracted with 0.1 *M* sodium hydroxide solution and determined at 241 mμ. Citranilic acid anilide was determined as the citric acid dianilide (ϵ = 23,000), and the citranilic acid as the citric acid anilide (ϵ = 11,000).

As Fig. 11 shows, a very small amount of the citric acid dianilide was used because this substance is sparingly soluble in both water and chloroform. Figure 11 corresponds to 1.9 mg. of aniline and about 0.9 mg. of citranilic acid and citric acid monoanilide and 0.2 mg. of citranilic acid anilide. The citric acid monoanilide always gave two peaks which suggests that both of the two possible monoanilides were formed. No complete separation of the two monoanilides has been attempted.

Kinetics and Analytical Procedures Used in the Investigation of the Interaction of Citric Acid with Aniline

The test solutions were prepared by dissolving concentrations of aniline varying from 0.2 to 0.001 moles/L. in 0.5 *M* citric acid solution which had previously been adjusted to the desired pH with the addition of sodium hydroxide or hydrochloric acid. The solution was subdivided into 5-ml. ampuls and sealed. The samples were placed in a constant temperature bath maintained at 85 ± 0.10° and withdrawn at various time intervals. The reaction was quenched by chilling in an acetone–dry ice freezing mixture.

The progress of the reaction was determined by assaying the samples for residual free aniline. A suitable aliquot was transferred to a separator containing 25 ml. of chloroform and 5 ml. of 1 *N* NaOH solution. The aniline was extracted into the chloroform layer which was separated and placed into a 100-ml. volumetric flask. The extraction was repeated twice again using 20-ml. volumes of chloroform. The chloroformic extracts were combined, and the resultant solution brought to 100.0 ml. with chloroform. The aniline was determined spectrophotometrically at 287 mμ using a Cary model 11 spectrophotometer.

Procedure for Determining Rates of Formation of Aniline and Citranilic Acid from Citric Acid Monoanilide

A 175-ml. quantity of a 0.5 *M* citrate buffer in a 250-ml. volumetric flask was preheated to 85.0°, then placed in a constant temperature bath at 85.0°. Citranilic acid (0.5 to 0.6 mmole) was dissolved in 23 ml. of 0.1 *M* sodium hydroxide solution, preheated to 85°, and placed in the constant temperature bath at 85.0°. The imide is rapidly hydrolyzed to the monoanilide in the alkaline solution. The monoanilide solution was poured quickly into the buffer solution; 10-ml. samples were withdrawn at appropriate time intervals.

These samples were pipeted directly into 50-ml. volumetric flasks containing cold water and stored in an ice water mixture for later analysis. After appropriate dilution with water the absorbance (A) of this solution was determined at 241 mμ in 1-cm. cells. A concentrated solution of sodium hydroxide was then added to make the solution substantially alkaline, and aniline was extracted with chloroform. The volume of the chloroform extracts was adjusted to 50 ml. and the absorbance read at 287 mμ in 10-cm. or 1-cm. cells. The concentration of aniline was calculated from the absorbance.

For these systems a plot of log ($A - A_{eq}$) against time always exhibited a straight line relationship. The concentration of the monoanilide at zero time for each run was obtained from these plots. From this information together with molar absorptivity values of aniline, at the pH of the particular run and of the monoanilide and the imide all at 241 mμ, the concentrations of the latter two were calculated from the A values. Experiments showed that the absorptivities of the monoanilide and the imide were not influenced by pH over the range studied.

Computer Analysis

An Applied Dynamics, Inc., model AD-2-32PB analog computer was utilized for this portion of the work.

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Water Vapor Permeation of Selected Cellulose Ester Films

By M. PATEL, J. M. PATEL, and A. P. LEMBERGER

In a study of water vapor permeation of cellulose acetate propionate, cellulose acetate butyrate, and cellulose acetate stearate films it was observed that permeability constant decreased with increasing chain length of the acid moiety. Cellulose acetate hydrogen phthalate films were easily permeated by water vapor at temperatures below 30° and resembled films of cellulose acetate stearate in their permeability at higher temperatures. The rather abrupt change in permeability observed in the temperature range of 30–35° indicates that caution must be exercised in interpreting accelerated aging data at higher temperatures with these films. Mixed films of cellulose acetate hydrogen phthalate and polyethylene glycol 4000 show a minimum permeability constant when polyethylene glycol 4000 is 10–20 per cent by weight of the film.

IN THE LAST several years an increasing interest in and use of polymeric agents which have the unique property of forming films has developed in the pharmaceutical industry. One group of chemical compounds which has merited considerable attention is the group of cellulose derivatives, esters and ethers. The basic patent concerning the use of cellulose acetate hydrogen phthalate for enteric coating was granted to Hiatt in 1940–1941 (1). Antonides and DeKay in 1953 evaluated various types of carboxymethyl celluloses, cellulose acetate, cellulose acetate propionate, cellulose acetate butyrate, cellulose acetate stearate, and cellulose acetate hydrogen phthalate for their enteric properties (2).

The ability of agents, such as cellulose derivatives and other polymers, to form films when deposited from proper solvents is utilized not only for enteric coating but also in the deposition of protective films on tablets as an alternative to sugar coating. With the increasing use of cellulose derivatives as coating materials in the pharmaceutical industry, information on water permeation of such films has become increasingly important. This study reports on water vapor permeation of films of cellulose acetate propionate (CAP), cellulose acetate butyrate (CAB), cellulose acetate stearate (CAS), and cellulose acetate hydrogen phthalate (CAHP). Another aspect of this work deals with water vapor permeation of mixed films of CAHP and polyethylene glycol 4000 (PEG 4000).[1]

Barrer (3) has stated that membranes such as polystyrene (which adsorb little water) behave at low vapor pressures in accordance with Fick's law, and permeation velocity is inversely proportional to film thickness. At higher vapor pressures where increased sorption of water occurs, deviation from Fick's law is observed. In this work the data have been analyzed on the basis of Fick's linear diffusion law in a manner similar to that of Lovegren and Feuge (5). Thus, the permeability constant, P, is defined by

$$P = \frac{W a}{A t \Delta p} \qquad \text{(Eq. 1)}$$

where W is the weight of water in grams diffusing through a film of thickness, a, in centimeters and area, A, in square centimeters during time, t, in hours when the vapor pressure difference across the membrane is Δp in millimeters of mercury. The permeability constant represents the combined effect of diffusion and sorption and is given in units of grams per centimeter per hour per millimeter of mercury.

EXPERIMENTAL

Materials and Apparatus.—Cellulose acetate propionate (16% propionyl), cellulose acetate butyrate (16% butyryl), cellulose acetate stearate, and cellulose acetate hydrogen phthalate (30–40% phthalyl) were used as obtained from Eastman Organic Chemicals. Polyethylene glycol 4000 was used in preparing mixed films with cellulose acetate hydrogen phthalate. Reagent grade sodium chloride, nickel chloride, and magnesium chloride were used to prepare solutions to maintain the desired water vapor pressure within the permeation cell.

The permeation cell is shown in Fig. 1. It consisted of an approximately 20-ml. conical flask

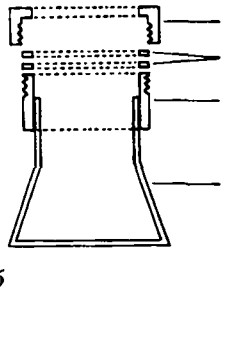


Fig. 1.—Permeation cell. Key: *1*, 20-ml. conical flask (29/12 outside ground glass top); *2*, tapered aluminum collar; *3*, *O-ring rubber gaskets*; *4*, aluminum cap.

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[1] Marketed as Carbowax 4000 by the Glyco Products Corp.

TABLE I.—PERMEABILITY CONSTANTS CALCULATED ACCORDING TO EQ. 1

Film Material	Film Thickness, cm. × 10^2	Permeability Constant, Gm. cm. $^{-1}$ hr. $^{-1}$ mm. Hg $^{-1}$ × 10^8
Cellulose acetate propionate (CAP)	1.40	73
Temp., 24.5° C.	2.15	97
Δp: 23.1 mm. Hg	3.45	96
	4.45	110
	5.58	110
CAP	1.35	51
Temp., 36.8° C.	1.75	56
Δp: 23.0 mm. Hg	2.75	62
	3.90	66
	4.25	67
CAP	1.40	39
Temp., 45° C.	1.80	44
Δp: 21.7 mm. Hg	2.75	50
	3.95	57
Cellulose acetate butyrate (CAB)	1.35	60
	1.70	66
Temp., 24.5° C.	2.50	77
Δp: 23.1 mm. Hg	3.45	75
	4.25	83
CAB	1.70	50
Temp., 36.8° C.	2.35	59
Δp: 23.0 mm. Hg	2.45	64
	3.70	65
	5.15	65
CAB	1.75	41
Temp., 46.5° C.	2.35	44
Δp: 23.0 mm. Hg	3.45	45
	3.75	49
	4.25	56
	5.15	58
Cellulose acetate stearate (CAS)	1.20	20
	2.10	22
Temp., 24.5° C.	3.50	24
Δp: 23.1 mm. Hg	4.50	26
	5.05	26
CAS	1.20	19
Temp., 36.8° C.	2.10	23
Δp: 23.0 mm. Hg	2.65	22
	3.50	24
	3.70	23
CAS	1.20	20
Temp., 46.5° C.	2.50	24
Δp: 23.0 mm. Hg	3.55	28
	4.50	27
	5.10	28
Cellulose acetate hydrogen phthalate (CAHP)	1.00	48
	1.90	71
Temp., 22.2° C.	2.95	89
Δp: 20.1 mm. Hg	3.80	100
CAHP	1.10	48
Temp., 23.5° C.	2.35	75
Δp: 21.7 mm. Hg	2.75	82
	4.40	96
CAHP	1.10	32
Temp., 27.5° C.	1.80	42
Δp: 21.0 mm. Hg	2.90	50
	3.70	54
CAHP	1.05	18
Temp., 35° C.	1.80	24
Δp: 21.5 mm. Hg	2.80	30
	3.65	33
CAHP	1.10	14
Temp., 36.8° C.	1.95	21
Δp: 23.0 mm. Hg	2.65	23
	3.30	28
	4.30	32
CAHP	1.25	16
Temp., 45° C.	2.10	24
Δp: 21.8 mm. Hg	3.35	32
	4.20	38
CAHP	1.45	16
Temp., 46.5° C.	2.45	24
Δp: 23.0 mm. Hg	3.40	33
	4.00	35

prepared by sealing the end of a male $^{29}/_{42}$ ground-glass joint cut to the proper size. The ground-glass connection was cut to $^{29}/_{12}$; a tapered threaded aluminum collar was fixed to the joint with white shellac. Films were held in a female threaded aluminum cap with a 2.8-cm. opening and sealed

TABLE II.—DATA FOR VARIOUS MIXED CAHP–PEG FILMS

Film Compn., % w/w	—Temp., 21° C., Δp 18.7 mm. Hg— Thickness, cm. × 10^2	Permeability Constant, Gm. cm. $^{-1}$ hr. $^{-1}$ mm. Hg $^{-1}$ × 10^8	—Temp., 35° C., Δp 21.5 mm. Hg— Thickness, cm. × 10^2	Permeability Constant Gm. cm. $^{-1}$ hr. $^{-1}$ mm. Hg $^{-1}$ × 10^8
CAHP, 95	1.00	43	0.80	15
PEG, 5	2.00	64	1.90	23
	3.00	79	2.70	28
	3.70	90	3.55	32
CAHP, 90	0.95	36	0.90	13
PEG, 10	1.70	49	2.15	22
	2.90	67	3.15	28
	3.70	79	3.60	30
CAHP, 80	0.80	31	0.75	15
PEG, 20	1.75	50	1.80	18
	2.70	66	2.50	21
	3.70	78	3.30	22
CAHP, 70	0.70	41	0.70	21
PEG, 30	1.70	70	1.50	29
	2.45	85	2.30	34
	3.30	100	3.00	36
CAHP, 60	0.70	50	0.80	24
PEG, 40	1.75	91	1.60	33
	2.50	110	2.50	38
	3.50	130	3.55	41

to the permeation cell with "O" ring rubber gaskets, 2.8 cm. inner diameter. In an experimental run the permeation cell was placed in a desiccator with magnesium perchlorate as desiccant. Dry air passed through concentrated sulfuric acid, and a calcium chloride tube was circulated through the desiccator during the run to provide agitation and insure that the water vapor pressure in the desiccator was negligible. The assembly was kept in a constant temperature room for determinations at temperatures up to 27°. For higher temperature runs the apparatus was placed in a constant temperature oven. Temperature control was within ±0.5°.

Procedure.—Films were prepared as described below. Sufficient solvent to make 100 ml. of solution was added to 10.0 Gm. of the desired cellulose derivative or to the proper weight ratio mixture of CAHP and PEG 4000. The solution was filtered through glass wool; a sufficient quantity of this solution was poured onto a clean mercury surface in a Petri dish to produce a film of the desired thickness. A perforated piece of filter paper was placed on the Petri dish, and an inverted 6-in. glass funnel was then placed over the assembly. The transparent film deposited as the solvent was permitted to evaporate slowly at room temperature. Solvent employed for CAP and CAB was a 1:1 volumetric mixture of acetone and dry chloroform. Solvent for casting films of CAS was a 3:1 volumetric mixture of acetone and dry chloroform. Acetone was used as solvent for casting CAHP and mixed CAHP–PEG films.

The deposited film was cut into circular disks approximately 3 cm. in diameter with a punch and dried 3–4 days in a desiccator containing magnesium perchlorate. The thickness of the film was measured at ten different locations with a caliper calibrated to 1×10^{-3} cm. The measurements on

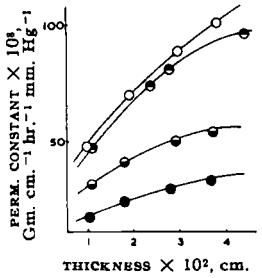


Fig. 2.—Typical curves for permeability constant as a function of thickness. CAHP films. Key: ○, 22.2° C.; ◓, 23.5°C.; ◒, 27.5°C.: ●, 35°C.

the film had to fall within $\pm 1.5 \times 10^{-3}$ cm. of the average to be acceptable.

Ten milliliters of distilled water (21 to 24.5° runs), 10.0 ml. of saturated sodium chloride solution (27°), 10.0 ml. of saturated nickel chloride solution (35 and 36.8°), or 10.0 ml. of saturated magnesium chloride solution (45 and 46.5°) were placed in the permeation cell; the film, mounted in the aluminum collar, was affixed to the cell. The cell was weighed and placed in the desiccator at the proper temperature. Steady state diffusion was achieved in 4–6 hours. Rate of diffusion under steady state conditions was determined by removing the cell from the desiccator periodically and measuring the weight loss. Usually five weighings were sufficient to give a reliable average.

The circular area through which permeation occurred was 6.1 cm.², and the vapor pressure differential under which it occurred, Δp, was equal to the vapor pressure of the solution in the cell. Desiccant was changed on alternate days to insure that the vapor pressure of water on the external side of the film was negligible. Vapor pressure gradient was held approximately constant for all films and at all temperatures studied.

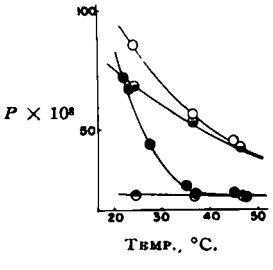


Fig. 3.—Permeability constant as a function of temperature for film thickness of 0.02 cm. Key: ○, CAP; ◒, CAB; ◓, CAS; ●, CAHP.

RESULTS AND DISCUSSION

Permeability constants calculated according to Eq. 1 are given in Table I. Temperature and vapor pressure gradient for each series of determinations are as indicated beneath the type of film. Similar data for the various mixed CAHP–PEG films are given in Table II. The order of magnitude of the permeability constants is in agreement with previously reported values (3–5) for similar films. Of the simple films studied, highest permeability constants were observed with CAP films, followed in decreasing order by CAHP, CAB, and CAS when compared at room temperature. At the higher temperatures (35–45°), permeability constants for CAHP films were lower than those obtained for CAB films and approached the magnitude of the permeability constants obtained for CAS films.

Influence of Film Thickness.—The data in Tables I and II show that the permeability constant increases with increasing thickness of film. The magnitude of the effect is greatest with CAP and CAHP films and falls off to a minimum with CAS films. This indicates that CAS films approach Fick's law behavior in their permeability to water vapor. Taylor, Hermann, and Kemp (6) have shown that nonwater absorbing materials offer uniform resistance to passage of water and follow Fick's law of diffusion. When absorption is strong,

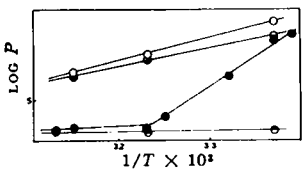


Fig. 4.—Arrhenius type plot for water vapor permeation of cellulose ester films at constant Δp for all temperatures. Key: ○, CAP; ◒, CAB; ⊔, CAS; ●, CAHP.

the pressure gradient across the membrane is no longer uniform, and deviation occurs (7). On the films reported here, CAHP, by virtue of its free carboxyl group could be expected to show significant ability to interact with water. In addition, the other films could be expected to show a decreasing water sorption in the order CAP, CAB, and CAS because of the increasing chain length of the acid

moiety. As the molecular weight of the acid residue increases, hydrophilic activity of the ester film should decrease, with a corresponding decrease in adsorption of water. The relative magnitude of change in permeability constant with increasing film thickness reported here is consistent with this view. Similar results have been reported by others (4, 5).

Influence of Temperature.—Although permeability constant increases with film thickness at all three temperatures studied, the magnitude of the effect falls off rapidly with CAHP, CAP, and CAB films; the decrease is particularly noticeable with CAHP films. On the other hand, permeation of CAS films seems relatively insensitive to temperature changes over the range reported in this work.

From the plots of permeability constant as a function of thickness, as illustrated for several CAHP films in Fig. 2, it was possible to obtain a value for the permeability constant of all films at a given thickness. By plotting permeability constants for these films at a thickness of 0.02 cm. as a function of temperature shown in Fig. 3, the effect of temperature is represented more effectively. It should be pointed out that Δp was held constant over the temperature range studied. The permeability constant for CAHP films reaches that of CAS films at a temperature of approximately 35°, while permeability constants for CAP and CAB films can be visualized as approaching the same limiting value. The abrupt fall in permeability of the CAHP films is interesting to note. That perhaps some change in the physical nature of the film occurs at 35° is further indicated in Fig. 4, where log permeability constant is plotted against the reciprocal of the absolute temperature. The actual significance of the plot is obscure at the present time, but the discontinuity obtained with CAHP films, while CAP, CAB, and CAS films behave linearly over the same region is interesting. Figures 3 and 4 show that accelerated aging studies utilizing higher temperatures must be approached with caution with cellulose derivative films to be sure the effect observed at high temperature is related to aging properties associated with lower temperatures.

Mixed CAHP–PEG 4000 Films.—Since various plasticizers are commonly used in combination with film forming materials, it was felt that it would be interesting to see what effects might be observed where such an agent was utilized. PEG 4000 was selected because, in addition to plasticizing the cellulose film, it would tend to form a film itself, and thus perhaps provide an alternate pathway for water vapor to permeate. Experimentally determined permeability constants at 21 and 35° for mixed films of CAHP and PEG 4000 of ratios 95:5, 90:10, 80:20, 70:30, and 60:40 on a weight basis are given in Table II. When these values were plotted against thickness of the film in the same manner as the simple films, permeability constants at a given thickness (0.02 cm.) could be read from the graph. In Fig. 5 these permeability constants are plotted as a function of weight per cent PEG 4000 in the film. At both 21 and 35°, a minimum permeability was observed when the weight per cent of PEG 4000 in the film was approximately 10–20%. Beyond this, minimum further increase in proportion of PEG 4000 in the film resulted in a marked increase in the permeability constant. Deeg and Frosch (4) also reported that plasticizers could enhance or retard permeation of polymer films. The pharmaceutical implication of this observation is that for a given mixture of film forming substances an optimum composition for maximum protective effect against atmospheric moisture may exist. In this particular instance, the optimum combination would be that corresponding to the minimum in the permeability–composition curve. Although it may be entirely coincidental, it is interesting that—calculated on an equivalent weight basis—at approximately 10% by weight of PEG 4000, a 1:1 ratio of ether oxygen to carboxyl exists.

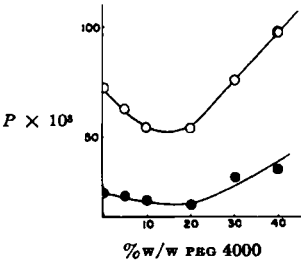


Fig. 5.—Plot of permeability constant against composition for mixed CAHP–PEG 4000 films. Key: ○, 21°C.; ●, 35°C.

Virtual Resistance to Permeation.—The increase in permeability constant observed upon increasing the thickness of the film suggests that a nonlinear concentration gradient exists across these films. If this is the case, it is expected that the rate of diffusion through a given segment, da, of the film will be dependent upon the location of the segment in the film. The reciprocal of the rate of permeation, $(w/t)^{-1}$, for a given film could be considered

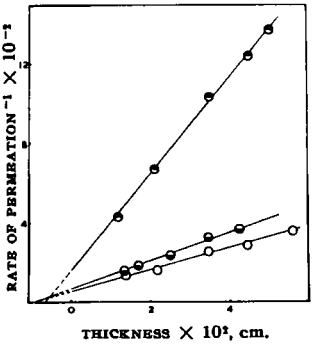


Fig. 6.—Reciprocal of the rate of permeation as a function of thickness for cellulose ester films (24.5° C.). Key: ○, CAP; ◒, CAB; ◓, CAS.

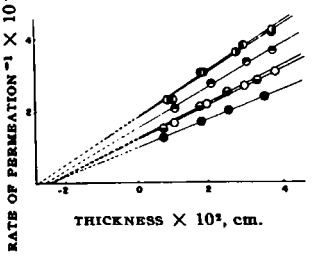


Fig. 7.—Reciprocal of the rate of permeation as a function of thickness for CAHP–PEG 4000 films (21°C.). Key: ○, 100% CAHP; ◓, 95% CAHP–5% PEG 4000; ◐, 90% CAHP–10% PEG 4000; ◑, 80% CAHP–20% PEG 4000; ◒, 70% CAHP–30% PEG 4000; ●, 60% CAHP–40% PEG 4000.

as the resistance of the film to permeation by water vapor. An interesting relationship is observed when $(w/t)^{-1}$ is plotted against thickness of film; although no theoretical basis for such a plot can be proposed at the present time, we feel it is worth presenting. Figure 6 shows such plots for the room temperature permeation of simple films of CAP, CAB, and CAS. Figure 7 depicts the curves obtained for CAHP–PEG 4000 films. Whether these relationships are fortuitous or actual remains to be established with future work, but rather good linear plots are obtained. Of particular interest is that these plots extrapolate to the same point on the abscissa within experimental error. Thus, the virtual resistance of these films at zero thickness could be considered equivalent to the resistance of an additional 0.006 to 0.010 cm. for the propionate, butyrate, and stearate films and to an additional 0.025 to 0.028 cm. for the CAHP–PEG 4000 films.

The physical significance (if any) of the virtual resistance observed is obscure. One possibility might be proposed, however. Other workers have reported that when permeation occurs under a gradient where both sides of the film are exposed to water vapor, permeability constants obtained are greater than when water vapor pressure is zero on one side of the film (5, 6). It is known that cellulose fibers in relatively dry conditions shrink, and that this results in a lower permeability constant (8). Since the external environment of our system was an atmosphere of relative humidity equal to zero, the external surface of the film may be dehydrated and offer a significant resistance to the passage of water vapor. The authors intend to explore this observation in future work.

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Studies on Cell Growth and Cell Division III

Action of Azaserine on Cell Division

By KWAN-HUA LEE and YOKO YUZURIHA

The inhibitory action of azaserine on cell division of heat treated *Tetrahymena pyriformis* in a nutrient-free medium is not reversed by either glutamine or formylglycinamide riboside, but is completely reversed by phenylalanine. These findings support our previous report that azaserine exerts its selective inhibitory action on cell division by some means other than by inhibition of any one of those intermediate steps involved in purine synthesis. Diazo-oxo-norleucine (DON), an antibiotic with a close structural analogy to azaserine, has essentially the same biologic activity on cell growth and cell division. Diazouracil, a pyrimidine derivative, inhibits both cell growth and cell division.

In a previous report (1), it was shown that azaserine (*o*-diazoacetyl-*l*-serine) has a selective inhibitory action on cell division of heat treated *Tetrahymena pyriformis* in a nutrient-free medium after the necessary ribonucleic acid and deoxyribonucleic acid have been accumulated. However, the net result of several studies (2,3) was impressively integrated evidence that azaserine exerted its primary inhibition by blocking the *de novo* synthesis of purine. This was further supported by the findings that glutamine reversed azaserine inhibition in a competitive fashion (4) and that azaserine inhibition could be reversed by formylglycinamide riboside (FGAR) and some purines (5–9). In recent years, a large number of natural amino acids and some of their metabolic products have been reported as reversal agents to the inhibitory action of azaserine in various biologic systems (6, 7, 10–12). These findings, together with the result we reported previously (1), indicate that azaserine may block more than one site or it may act as an antagonist of a metabolite involved in a number of biochemical transformations. In the present study we chose to use glutamine, FGAR, and phenylalanine as typical representatives in the study of their effects on the inhibitory action of azaserine on cell division in heat treated *T. pyriformis* and expect to shed some light on the mechanism of action of azaserine. We also studied the action of DON (6-diazo-5-oxo-1-norleucine) and diazouracil (5-

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diazo-2,4-dihydroxypyrimidine) on cell growth and cell division.

METHODS

T. pyriformis GL was maintained in a proteose-peptone, liver extract fraction L and salt medium as reported before (1), except that ferrous ammonium sulfate was omitted since this salt caused the formation of a black precipitate after autoclaving. Stock cultures were transferred daily with an inoculating loop to 10 ml. of medium in test tubes (18 × 150 mm.) in a slanted position and maintained at 28°. For the experimental culture, 20 ml. of medium in a 300-ml. Erlenmeyer flask was inoculated with 0.03 ml. of a 2-day-old culture (containing about 10^4 cells) and maintained at 28°. At the end of 18 hours, the culture contained about 35,000 cells/ml. and was subjected for temperature treatment in the temperature controlled system described before (14). Each cycle consists of 30 minutes at 34.3°, followed by 30 minutes at 28.5°. At the end of the seventh cycle, the cells were separated in a hand centrifuge and washed three times with salt buffer solution (1). These latter manipulations took about 3 minutes in total. The washed cells were suspended in a suitable amount of salt buffer solution and initial samples for cell count were taken. One milliliter portions of the cell suspension were transferred to a series of 50-ml. round-bottomed centrifuge tubes containing appropriate amounts of testing agents with and without azaserine in salt buffer solution. The final volume of each tube was 2 ml. Salt buffer solution was used in the blank. These tubes were then clamped on a shaking device which has a horizontal movement of 4 cm. displacement at 50 strokes per minute in the temperature controlled water bath at 28.5° for 5.5 hours. At the end of incubation time, 0.6 ml. of formaldehyde reagent was added to each tube, and the cells were counted in a Sedgwick-Rafter counting chamber, using a Whipple ocular micrometer disk (15).

The procedures used in the study of selective activity of DON and diazouracil were essentially the same as described in a previous report (1).

The formylglycinamide riboside (4-amino-5-imidazole carboxamide riboside) used is a product of the California Corp. for Biochemical Research; glutamine, phenylalanine, and diazouracil are prepared by the Nutritional Biochemicals Corp. Azaserine and DON were graciously supplied by Dr. E. L. Wittle, Parke Davis Research Laboratories, Detroit, Mich.

TABLE I.—EFFECT OF FGAR, GLUTAMINE, AND PHENYLALANINE ON THE INHIBITORY ACTION OF AZASERINE ON CELL DIVISION OF HEAT-TREATED *T. pyriformis*

Additions, mcg./ml.		Cell Count, 1,000/ml. Initial	Final
FGAR	Azaserine		
None	None		166.3
None	1.5	69.3	114.8
100	1.5		103.0
200	1.5		101.3
Glutamine	Azaserine		
None	None		245.5
None	1.5	80.1	171.7
200	1.5		167.0
200	None		243.0
Phenylalanine	Azaserine		
None	None		453.0
None	1.5		294.5
25	1.5		341.9
50	1.5	156.8	440.0
100	1.5		472.0
25	None		451.0
50	None		468.0
100	None		448.1

RESULTS

Effect of FGAR.—The reversal effect of FGAR to azaserine inhibition on purine synthesis has been studied in several laboratories (5, 7). It is well established that *T. pyriformis* requires pre-formed purines and pyrimidines (16); however, there is no information on the catabolism or the resynthesis of purine from its metabolic intermediates in these protozoa. For this reason, we studied the possible reversal effect of FGAR to azaserine inhibition. It was found that FGAR failed to reverse the cell division inhibited by azaserine. The results are shown in Table I. The results support the finding that *T. pyriformis* requires pre-formed purines and also indicate that purines in these protozoa are not degradated to simpler metabolites that can be used for synthesis of FGAR or purine. However, in pigeon liver preparations (3, 4) and in *E. coli* (17), it was shown that the primary site of inhibitory action of azaserine was at the step in which glutamine is used to amidinate formylglycinamide ribotide (FGARP) to formylglycinamidine ribotide (FGAMP). Since FGARP is not available, glutamine can be used for testing the above mentioned mechanism of action of azaserine.

Effect of Glutamine.—Azaserine blocks primarily the *de novo* synthesis of purine at the step in which glutamine is used to amidinate FGARP. At higher concentrations, azaserine also blocks the other two steps where glutamine is involved in purine synthesis (4, 5, 8). The ability to nullify azaserine inhibition and the close resemblance of the molecules of azaserine and glutamine have led several workers (3, 18) to believe that azaserine blocks the transfer of nitrogen from glutamine to a purine precursor. It has also been shown that azaserine inhibits the amination of xanthosine-5-phosphate to guanosine-5-phosphate in which glutamine is the donor of the amino group (19). It is possible that glutamine may reverse azaserine-inhibited nitrogen transfer reactions other than those reactions mentioned

TABLE II.—CELL DIVISION INHIBITORY ACTION OF AZASERINE IN THE PRESENCE OF CULTURE MEDIUM

Medium	Cell Count, 1,000/ml. Initial	Final	Final/ Initial
Salt buffer		175.7	3.1
Fresh medium[a]	56.7	306.3	5.5
Original medium with azaserine[b] (2 mcg./ml.)		213.7	3.8
Salt buffer		197.6	3.2
Fresh medium[c]	62.5	357.2	5.7
Fresh medium with azaserine (2 mcg./ml.)		234.1	3.5

[a] Heat treated cells with azaserine added at the beginning of heat treatment. [b] Medium from *fresh medium, footnote a*. [c] Heat treated cells with no azaserine added at the beginning of heat treatment.

above. Since the biologic system we used does not involve purine synthesis, it is a suitable system to test other possibilities. It was found that glutamine, even at higher concentrations failed to reverse azaserine inhibition on cell division. The results are shown in Table I.

Effect of Phenylalanine.—A large number of natural amino acids have been reported to be reversal agents to azaserine inhibition; phenylalanine was reported, in some biologic systems, to be a more potent reversal agent than glutamine (6, 7, 13). This fact suggests the possibility that phenylalanine may reverse azaserine inhibition on a site where glutamine is not involved. We found, indeed, that phenylalanine reversed completely the inhibitory action of azaserine on cell division in *T. pyriformis*. The results are shown in Table I.

Cell Division Inhibitory Action of Azaserine in the Presence of Culture Medium.—The reversal action of phenylalanine raised the question of whether the culture medium used has any reversal activity on the inhibitory effect of azaserine. To test this, the following experiment was performed. Azaserine was added to a culture of *T. pyriformis* at the beginning of temperature treatment (2.0 mcg./ml.), and the culture medium was separated at the end of the seventh temperature cycle; the heat treated organism was collected and washed with salt buffer. Aliquots of these heat treated cells, with known cell count, were then introduced to a series of centrifuge tubes and centrifuged. The supernatants were carefully removed. Measured portions of (*a*) original medium used in heat treatment, which contained 2 mcg./ml. of azaserine added at the beginning of heat treatment, (*b*) fresh culture medium, and (*c*) salt buffer were added to the series of tubes containing heat treated cells separately. These samples were then incubated at 28° for 5.5 hours. At the end of the incubation period, the cell counts of all samples were taken. A parallel control, without the addition of azaserine, was carried out at the same time. The culture medium of the control was also separated at the end of the seventh temperature cycle and the heat treated *T. pyriformis* was collected and washed. The cell division of these heat treated cells were studied essentially in the same way as described above in (*a*) fresh medium with the addition of azaserine, 2 mcg./ml. final concentration, (*b*) fresh medium, and (*c*) salt buffer. It was found that the cell counts of samples containing azaserine were practically the same and both were about 65% of that of the control. These results definitely indicated that the cell division inhibitory action of azaserine can be demonstrated in the presence of culture medium. It also suggested that azaserine is very stable during temperature treatment even in the presence of *T. pyriformis*. These experiments, with samples in duplicate, have been repeated three times, and the results obtained were practically the same. The results of a representative experiment are shown in Table II.

Selective Activity of DON.—DON is another antibiotic isolated from a streptomyces with a close structure analogy to azaserine. Both DON and azaserine are potent inhibitors of sarcoma 180, but with different inhibitory capacities. In the present study we intended to see if there was a difference in biologic action between these two antibiotics, and we found that DON acted essentially in the same way as azaserine. The results are shown in Fig. 1.

Effect of Diazouracil on Cell Growth and Cell Division.—Diazouracil is an analog of both uracil and thymine. It may also be considered as an alkylating agent. Diazouracil inhibited both cell growth and cell division. The results are shown in Fig. 2.

DISCUSSION

In an earlier report (1), we were able to demonstrate for the first time with experimental evidence the selective inhibitory action of azaserine on cell division in *T. pyriformis*. It should be mentioned again that the protozoa we used does not synthesize purine and is suitable to study the actions of azaserine and its inhibitory action on purine synthesis. It is a system in which cell growth is not involved, so any inhibitory action of drug on heat treated *T. pyriformis* in a nutrient-free medium is most probably on cell division only.

There is no doubt that azaserine, in several biologic systems, exerts its action on the *de novo* synthesis of purine at the steps whereby glutamine is involved (4, 5, 8). The competitive relationship of azaserine and glutamine in pigeon-liver enzyme system (4) and the reversal effects of glutamine and FGAR on azaserine inhibition in microorganisms (5–9) further support this proposed mechanism of action. The hypothesis of inhibition of purine synthesis by azaserine may explain its action on the overall

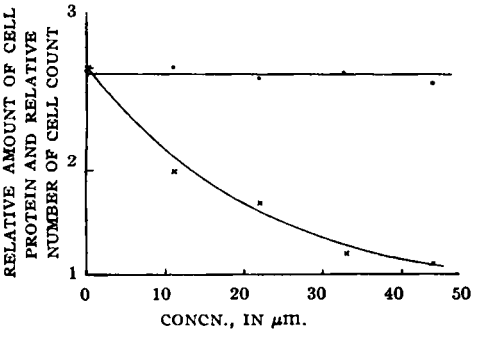


Fig. 1.—Effect of various concentrations (μM. per liter) of DON on cell protein and cell count. Key: ●, relative amount of cell protein at the end of heat treatment period in nutrient medium; X, relative cell count 5.5 hours after heat treatment in nutrient-free medium.

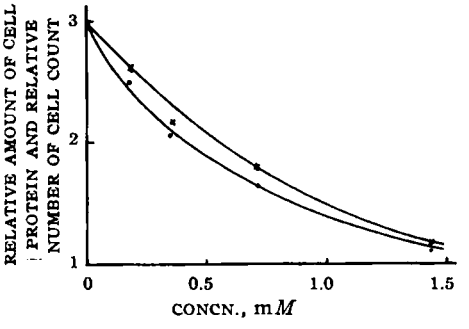


Fig. 2.—Effect of various concentrations (m*M* per liter) of diazouracil on cell protein and cell count. Key: ●, relative amount of cell protein at the end of heat treatment period in nutrient medium; X, relative cell count 5.5 hours after heat treatment in nutrient-free medium.

growth of microorganisms tested in mass cultures, but it does not explain the inhibitory action of azaserine on cell division of heat treated *T. pyriformis*, since these protozoa require pre-formed purines. Formylglycinamide riboside does not reverse azaserine inhibition on cell division in *T. pyriformis* which suggests that purines, if ever metabolized, do not catabolize beyond FGAR stage or the protozoa do not resynthesize purine from their metabolic products.

Gots and Gollub (6) failed to reverse azaserine inhibition by using naturally occurring purines in a purine requiring mutant strain of *Escherichia coli* and concluded that inhibition of purine biosynthesis is not sufficient in itself to account for the inhibition of growth.

It has been shown that azaserine inhibits the amination of xanthosine-5-phosphate to guanosine-5-phosphate by glutamine (19). This finding suggests that azaserine may inhibit other nitrogen transfer reactions in which glutamine is involved. In some cases, the glutamine-sparing mechanism has been applied in explaining the reversal effects of amino acids and other related compounds. It is not surprising that glutamine does not reverse the inhibitory action of azaserine on cell division in *T. pyriformis*, since glutamine has also been found without reversal effect in several other biologic systems (20–22). In some cases, an actual increase in glutamine pool by the addition of azaserine has been reported (7, 23).

Inhibition of transamination reaction in *Scenedesmus sp.* (23) has been suggested, but it was not reproduced in the cell-free preparations of *Azotobacter agilis* (24). Pyridoxal phosphate and several of its analogs do not affect azaserine inhibition as one would expect if inhibition of transamination reaction is involved (13).

The reversal effect of phenylalanine is of interest. From structural considerations, it would be difficult to assign to azaserine the role of a competitive inhibitor of phenylalanine.

It is possible that inhibition is brought about not by azaserine, but by a metabolic product of serine. This idea finds support in the observations that during inhibition of yeast systems, azaserine disappeared, and serine, a possible product of azaserine catabolism, appeared (12). Leucine, which prevented azaserine inhibition, also prevented the catabolism of azaserine. Azaserine was also found to be deaminated in mouse liver preparation (10) and in bacteria (13). Whether phenylalanine has anything to do in azaserine metabolism remains to be proved.

The possibility of a strictly chemical effect on the stability of azaserine was ruled out by Tomisek, *et al.* (7).

A hypothesis of lessened cell permeability toward azaserine in the presence of a reversal agent was shown to be inapplicable in *E. coli*, although an active transport of azaserine mechanism was observed for ascitic cells (25), neoplastic plasma cells, and lymphocytes (26). Whether phenylalanine has an effect on the permeability of *T. pyriformis* to azaserine remains to be studied.

The actual explanation of the reversal effect of phenylalanine is not available. It appears to act by preventing the inhibitory action of azaserine on cell division rather than by compensating for the inhibition. It is justified to suggest that a demonstration of the reversal effect of phenylalanine to azaserine inhibition in biologic system probably is related to cell division mechanism.

That azaserine does inhibit cell division in culture medium at 28° strongly supports the validity of the findings we reported earlier (1). The present results indicate that the failure of azaserine to inhibit cell growth in culture medium at the concentration it inhibits cell division in nutrient-free buffer is not due to any reversal agent present in the culture medium. It should be mentioned that when the selective activity of any drug on cell growth and cell division is to be tested with the system we described (1), one must first test the possible interference of the drug and the components in the culture medium.

The inhibitory actions of DON and diazouracil further demonstrate the usefulness of the biologic system we described (1) in the study of drug actions.

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Simplified Assay of Vitamins A and E in Mixtures

By WILLIAM T. FISHER, NANCY M. EDWARDS, and ROBERT W. LEHMAN

Assay for vitamins A and E in mixtures has required separate saponifications; the extraction for vitamin A had to be carried out from the alkaline mixture, while the extraction for vitamin E had to be from an acidic one. Furthermore, vitamin A interferes with the ferric chloride-dipyridyl determination of vitamin E, and vitamin E interferes with the spectrophotometric determination of vitamin A. Ascorbic acid in the alkaline medium was found to permit a common saponification and extraction for both vitamins A and E. The extraction of the unsaponifiable fraction can be shortened by using a single extraction with a large volume of ether instead of three or four extractions with smaller volumes. Hydrogenation of portions of the unsaponifiable fraction was shown to eliminate the mutual interference. These three improvements have been incorporated into a proposed procedure which is valid for the determination of vitamin A and vitamin E in the presence of each other.

THE ASSAY for vitamins A and E in mixtures using official procedures is lengthy and may yield inaccurate results. Separate saponifications are necessary, and several ethyl ether extractions are required to isolate the unsaponifiable fraction. In addition, vitamin E interferes in the spectrophotometric determination of vitamin A, often lowering the assay result, and vitamin A interferes in the colorimetric assay of vitamin E, often raising the assay result.

EXPERIMENTAL

Three simplifications in the assay of mixtures of vitamins A and E were considered in the course of this work. These are: (*a*) a common saponification, (*b*) a single extraction, and (*c*) elimination of the mutual interference so that accurate assay values can be obtained.

Common Saponification.—Adaptations of "The National Formulary" assays (1) for vitamin E in pharmaceutical products would usually require alkaline saponification, acidification of the saponification mixture, and extraction of the unsaponifiable fraction with three portions of ethyl ether. The U.S.P. assay (2) for vitamin A requires alkaline saponification and extraction of the unsaponifiable fraction with four portions of ethyl ether. A common saponification is not possible for both vitamins unless modifications are made in one of the official procedures. Unesterified tocopherol is very unstable to air in an alkaline medium. Therefore, the N.F. procedure calls for acidification of the saponification mixture with hydrochloric acid. Vitamin A, on the other hand, is unstable in the presence of strong acids, dehydrating to form anhydrovitamin A (3).

To prevent destruction of vitamin A by the acid added to the saponification mixture to preserve vitamin E, ascorbic acid was substituted for the hydrochloric acid. After several levels were tried, an amount of 4.5 Gm. was found satisfactory. Smaller quantities did not afford complete protection to the vitamin E; larger quantities might have caused anhydrovitamin A formation. It was necessary to use ascorbic acid U.S.P. having a minimum purity of 99%. Inadequate protection of vitamin E was obtained using a less pure preparation. If a pharmaceutical product has a high pH, more ascorbic acid may be required. This could be determined by titration of the sample.

Simplified Extraction.—Another simplification considered in this work was the reduction in number of extractions. Various proposals for a single extraction of the unsaponifiable portion of oil-soluble vitamins have appeared in the literature. These utilize a variety of solvents, including benzene (4), chloroform (5), and ethyl ether (6, 7).

To reduce the number of extractions required in working up the saponification solution, two of the

TABLE I.—EVALUATION OF SIMPLIFIED EXTRACTION PROCEDURES FOR ASSAYING COMMERCIAL VITAMIN A PALMITATE

Extraction Procedure	Vitamin A Content, u./Gm.	Morton-Stubbs Factor
U.S.P.	1,565,440	1.005
U.S.P.	1,562,710	1.014
Av.	1,564,080	1.010
Mulder	1,528,050	0.955
Mulder	1,556,260	0.974
Av.	1,542,160	0.965
Napoli, *et al.*	1,543,750	1.000
Napoli, *et al.*	1,531,230	0.988
Av.	1,537,490	0.994

TABLE II.—EVALUATION OF SIMPLIFIED EXTRACTION PROCEDURES FOR ASSAYING *d*-α-TOCOPHERYL ACID SUCCINATE N.F.

Extraction Procedure	Vitamin E Content, u./Gm.
N.F.	1190
N.F.	1220
Av.	1205
Mulder	1180
Mulder	1240
Av.	1210
Napoli, *et al.*	1200
Napoli, *et al.*	1230
Av.	1215

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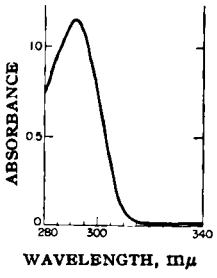


Fig. 1.—Ultraviolet absorption spectrum of *d*-α-tocopherol.

methods (4, 7) were compared with the official methods (1, 2). Results of these assays are shown in Tables I and II. In the assay of mixtures of vitamins A and E, considerable reduction in solvent volume is required prior to final measuring. Although both procedures evaluated appeared satisfactory, the Napoli, *et al.* (7), procedure was chosen because it uses ethyl ether, and the more volatile extracting solvent leads to shorter work-up time.

Elimination of Mutual Interference.—Vitamins A and E interfere with each other in the colorimetric or spectrophotometric steps when an attempt is made to apply the present official assay procedures to nonofficial preparations.

The U.S.P. method for vitamin A uses a formula originally derived by Morton and Stubbs (8) to correct for linear irrelevant absorption. The present formula (9) is: corrected $A_{(325)} = 6.815\ A_{(325)} - 2.555\ A_{(310)} - 4.26\ A_{(334)}$. Although its absorption maximum is at 292 mμ, α-tocopherol has some absorption at 310, 325, and 334 (Fig. 1). Its effect on the U.S.P. vitamin A assay is to increase slightly the uncorrected $A_{(325)}$, but to decrease corrected $A_{(325)}$, since its absorption is nonlinear in the region 310 to 334 mμ.

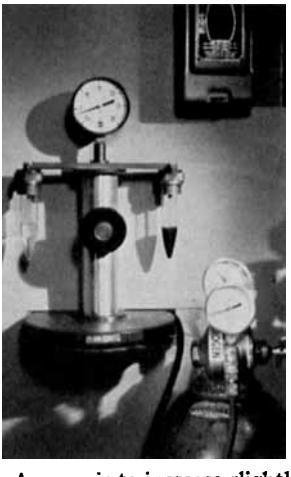


Fig. 2.—Hydrogenation apparatus.

Two general methods have been proposed for eliminating tocopherol interference so that vitamin A can be estimated accurately. Actual removal of vitamin E by partition chromatography on a column of Celite-polyethylene glycol 600 has been suggested (10). In another approach (11, 12), vitamin A is destroyed by washing an aliquot with 60% v/v sulfuric acid for use as a spectrophotometric blank.

Tocopherol reduces ferric chloride, and the ferrous ions react with dipyridyl to form a red complex. However, vitamin A also reduces ferric chloride. When vitamin A is present, vitamin E assay results tend to be too high.

Several methods have been suggested for destroying or removing vitamin A so that vitamin E can be determined accurately. These include washing with 85% v/v sulfuric acid (13), destruction of vitamin A with antimony trichloride (14), and hydrogenation (15). A number of procedures for chromatographically separating vitamins A and E have been described in the literature. These have used stannous chloride treated Floridin (16), alkaline alumina (17), and kaolin (18).

The use of hydrogenation in destroying vitamin A in the assay of vitamin E was described by Quaife and Biehler (15). Fox and Mueller (11) had used sulfuric acid to destroy the vitamin A in part of the sample to give a spectrophotometric blank used to cancel the extraneous (nonlinear) absorption interfering in the assay of vitamin A. We employed hydrogenation for this purpose instead of sulfuric acid.

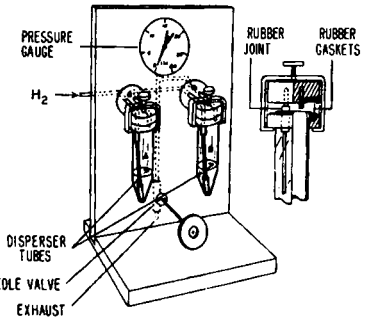


Fig. 3.—Construction and operating details of hydrogenation apparatus.

The apparatus we used is the same as that previously described (15) with differences only in external design. The apparatus and a hydrogen flow diagram are shown in Figs. 2 and 3. Hydrogen gas is bubbled through alcohol in the blank tube using a microporous dispersion tube. The gas saturated with alcohol vapor is bubbled through the sample in the sample tube. The exhaust gas passes through a pressure gauge and needle valve, allowing control of pressure in the system. In our work, 2 to 3 lb. hydrogen pressure was effective with 5 minutes hydrogenation time.

Two aliquots are taken for hydrogenation and determination of the two vitamins. Vitamin E is measured directly in one hydrogenated aliquot by ferric chloride-dipyridyl. The other hydrogenated aliquot is used for a blank in determining vitamin A spectrophotometrically; the sample for vitamin A determination is an unhydrogenated portion of this aliquot. Absorbance curves are shown in Fig. 4 for:

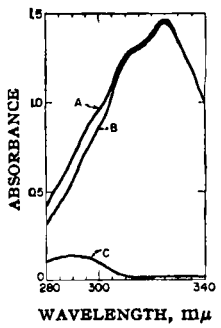


Fig. 4.—Absorbance curves for: (A) a mixture of vitamins A and E against an isopropanol blank; (B) the vitamin mixture against a hydrogenated blank; and (C) the hydrogenated aliquot against isopropanol.

(*a*) a mixture of vitamins A and E against an isopropanol blank, (*b*) the vitamin mixture against a hydrogenated blank, and (*c*) the hydrogenated aliquot against isopropanol.

Several mixtures of vitamins A and E were prepared and assayed, using a common saponification and single extraction, followed by hydrogenation to avoid interference from each vitamin during the measurement of the other. These mixtures contained both oil and dry vitamins A and E at various ratios of A to E. Results are shown in Tables V–XI. Recovery of the vitamins was based on assay values of the input materials shown in Tables III and IV.

PROPOSED PROCEDURE

In sampling for assay, use at least 10 I.U. of vitamin E since this facilitates dilution during later steps. Vitamin A concentration is less critical.

Transfer the sample for assay to a 125-ml. low actinic saponification flask. Add 3 ml. of 50% w/v KOH solution, displace the air in the flask with nitrogen, attach an air condenser, and swirl the saponification flask over a steam bath for 2 to 3 minutes. This aids in the release of vitamins from the solid matrix if present in a dry form. Add 50 ml. of ethanol through the condenser and saponify for 20 minutes. Remove from the steam bath and add 4.5 Gm. ascorbic acid U.S.P. through the condenser. (*Caution:* do not open the system until acidified!) Wash any ascorbic acid adhering to the sides of the condenser into the saponification flask with a small amount of water. Cool under tap water and transfer to a 500-ml. low actinic separator with 100 ml. of distilled water. Rinse the saponification flask with three 50-ml. portions of peroxide-free ethyl ether, combining these in the separator. Stopper, and shake vigorously to mix the layers thoroughly. Allow the layers to separate; draw off and discard the aqueous layer.

Wash the ether layer with 100-ml. portions of distilled water to neutrality as determined with phenolphthalein. Filter the ether extract through anhydrous sodium sulfate and reduce the volume to 10 to 20 ml. in a stream of nitrogen. Warm gently to aid solvent removal, but avoid overheating. Dilute the extract to 50 ml. with ethyl ether in a volumetric flask. Dilute aliquots from the master solution for assay as follows.

Vitamin E.—Prepare a solution of the unsaponifiable fraction in ethanol containing approximately 0.2 units vitamin E per milliliter. Transfer about 10 ml. of this solution to a 50-ml. centrifuge tube, and add approximately 20 mg. of catalyst (5% palladium on calcium carbonate.[1]) Stir with a glass rod, and hydrogenate for 5 minutes at 2 to 3 lb.

[1] Baker and Co., Inc., Newark 5, N. J.

TABLE III.—ASSAY RESULTS ON VITAMIN A USED IN PREPARING VITAMIN A-E MIXTURES

U.S.P. XVI u./Gm.	Morton-Stubbs Factor
Commercial Dry Vitamin A Palmitate	
560,430	1.005
561,030	1.025
Av. 560,730	1.015
Commercial Vitamin A Palmitate	
1,565,440	1.005
1,562,710	1.014
Av. 1,564,080	1.010

TABLE IV.—ASSAY RESULTS ON VITAMIN E[a] USED IN PREPARING VITAMIN A-E MIXTURES

d-α-Tocopheryl Acid Succinate, u./Gm.	*d*-α-Tocopheryl Acetate, u./Gm.	Dry *d*-α-Tocopheryl Acetate (Acacia Carrier), u./Gm.
1190	1370	354
1220	1370	354
Av. 1205	1370	354

[a] Vitamin E determined by ferric chloride-dipyridyl method on unsaponifiable fraction.

TABLE V.—MIXTURE OF 50,000 UNITS COMMERCIAL VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
1,612,600	...	1.012	2754	...
1,640,500	...	1.022	2730	...
Av. 1,626,550	104.0	1.017	2742	200.1
With Hydrogenation				
1,558,460	...	1.012	1391	...
1,540,050	...	1.004	1401	...
Av. 1,549,260	99.1	1.008	1396	101.9

TABLE VI.—MIXTURE OF 25,000 UNITS COMMERCIAL VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
1,559,580	...	0.964	2130	...
1,501,520	...	0.930	2350	...
Av. 1,530,550	97.9	0.947	2240	163.5
With Hydrogenation				
1,573,540	...	1.009	1396	...
1,564,130	...	1.038	1453	...
Av. 1,568,840	100.3	1.024	1425	104.0

TABLE VII.—MIXTURE OF 50,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS DRY *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
538,980	...	0.967	385	...
539,990	...	0.958	466	...
Av. 539,490	96.2	0.963	426	120.3
With Hydrogenation				
544,730	...	0.989	370	...
553,810	...	0.993	357	...
Av. 549,270	98.0	0.991	364	102.8

TABLE VIII.—MIXTURE OF 25,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS DRY *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
553,890	...	0.974	534	...
522,810	...	0.939	470	...
Av. 538,850	96.1	0.957	502	141.8
With Hydrogenation				
529,840	...	1.004	333	...
543,190	...	1.038	371	...
Av. 536,520	95.7	1.021	352	99.4

TABLE IX.—MIXTURE OF 50,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACID SUCCINATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
561,010	...	0.992	2856	...
527,880	...	0.954	3096	...
Av. 544,450	97.1	0.973	2976	247.0
With Hydrogenation				
551,620	...	1.055	1277	...
552,890	...	1.049	1302	...
Av. 552,260	98.5	1.052	1290	107.1

TABLE X.—MIXTURE OF 25,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACID SUCCINATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
467,990	...	0.919	2336	...
551,110	...	0.965	2097	...
Av. 514,050	91.7	0.942	2217	184.0
With Hydrogenation				
551,810	...	1.045	1270	...
529,900	...	0.984	1210	...
Av. 540,860	95.6	1.015	1240	102.9

pressure. Centrifuge until the solution is clear and proceed with the determination of vitamin E by ferric chloride-dipyridyl assay.[2]

Vitamin A.—Prepare a solution of unsaponifiable fraction in isopropanol containing approximately 10 units vitamin A per milliliter. Hydrogenate a portion of this solution as above. Centrifuge and use this as a reagent blank. Use an unhydrogenated portion of the isopropanol solution as the sample for assay. Determination and calculation of vitamin A from this point is by the U.S.P. procedure (2). Since vitamin E is not hydrogenated at the conditions used here, both sample and hydrogenated blank contain the same amount of vitamin E.

[2] Adaptations of official vitamin E assay procedures for "unofficial" products are discussed by Lehman (19).

Vitamin E in the blank cancels out the vitamin E in the sample, allowing accurate measurement of the vitamin A. This is illustrated in Fig. 4.

RESULTS AND DISCUSSION

The results obtained using the proposed procedure on a variety of mixtures of vitamins A and E representing combinations that might be encountered in finished pharmaceutical dosage forms are presented in Tables V–XI.

The modifications introduced into the saponification and extraction technique (use of ascorbic acid instead of hydrochloric and use of a single ether extraction instead of three or four) have not prevented quantitative recovery of both vitamins. Hydrogenation was effective both for preventing interference of vitamin A in the dipyridyl assay for vitamin E and the interference of vitamin E in the spectrophotometric assay for vitamin A.

The need for hydrogenation and its degree of effectiveness is seen by comparing the data on per cent recovery in Table XII with those in Table XIII. This comparison shows that without hydrogenation, assays for vitamin E appear to be falsely high when vitamin A levels are high, and that assays for vitamin A tend to be falsely low when vitamin E levels are high. Both defects are overcome by the hydrogenation step in the procedure as outlined.

CONCLUSIONS

The assay of vitamins A and E in mixtures has been simplified by employing a common saponifica-

TABLE XI.—MIXTURE OF 5,000 UNITS COMMERCIAL VITAMIN A PALMITATE AND 100 UNITS *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
1,056,530	...	0.656	1390	...
1,066,490	...	0.660	1390	...
Av. 1,061,510	67.9	0.658	1390	101.5
With Hydrogenation				
1,564,850	...	1.070	1390	...
1,564,850	...	1.098	1400	...
Av. 1,564,850	100.1	1.084	1395	101.8

TABLE XII.—RECOVERY OF VITAMINS A AND E WITHOUT HYDROGENATION

Input: Vitamin A Units	Input: Vitamin E Units	% Recovery: Vitamin A	% Recovery: Vitamin E
50,000 (oil)	10 (oil, acetate)	104.0	200.1
25,000 (oil)	10 (oil, acetate)	97.9	163.5
50,000 (dry)	10 (dry, acetate)	96.2	120.3
25,000 (dry)	10 (dry, acetate)	96.1	141.8
50,000 (dry)	10 (succinate)	97.1	247.0
25,000 (dry)	10 (succinate)	91.7	184.0
5,000 (oil)	100 (oil, acetate)	67.9	101.5

TABLE XIII.—RECOVERY OF VITAMINS A AND E USING PROPOSED PROCEDURE

Input		% Recovery	
Vitamin A Units	Vitamin E Units	Vitamin A	Vitamin E
50,000 (oil)	10 (oil, acetate)	99.1	101.9
25,000 (oil)	10 (oil, acetate)	100.3	104.0
50,000 (dry)	10 (dry, acetate)	98.0	102.8
25,000 (dry)	10 (dry, acetate)	95.7	99.4
50,000 (dry)	10 (succinate)	98.5	107.1
25,000 (dry)	10 (succinate)	96.5	102.9
5,000 (oil)	100 (oil, acetate)	100.1	101.8

tion which allows good recovery of both vitamins. The key change from previous procedures is the substitution of ascorbic acid for hydrochloric acid in acidifying the saponification mixture before extraction.

Hydrogenation is effective in removing the mutual interference of vitamins A and E upon each other.

These two new improvements have been combined with one previously described (using a single large volume of ether for extraction rather than several smaller ones) in a simplified procedure for the assay of these two vitamins in mixtures. The proposed assay is valid for a number of typical mixtures.

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Pinacol Rearrangement of Phenaglycodol I

Characterization of Products Produced

By HUBERT W. MURPHY

PHENAGLYCODOL[1] is one of a series of anticonvulsant ethylene glycols described by Mills, *et al.* (1), which has been evaluated in the clinic as an anticonvulsant and neurosedative agent by Gruber and Mosier (2). This compound is characterized by a somewhat persistent bitter taste. In the hope of obtaining a product with a sufficiently improved taste to permit the convenient administration of phenaglycodol in liquid preparations, several attempts were made to prepare derivatives of this glycol.

All attempts to esterify one or both of the hydroxyl groups with acid anhydrides or acid chlorides were fruitless, even when special methods developed for tertiary hydroxyl groups were carried out. Likewise, all efforts at transesterification with a number of commonly used catalysts and attempted etherifications were unsuccessful. It was observed that a characteristic odor developed in those instances in which acidic conditions were maintained during attempted esterification. Moreover, it was difficult to recover the unreacted phenaglycodol in crystalline form from these experiments. The possibility of a pinacol rearrangement was considered, and a typical odoriferous reaction mixture was treated with 2,4-dinitrophenylhydrazine reagent. The presence of a ketone was revealed by the separation of an orange, crystalline 2,4-dinitrophenylhydrazone.

Further experimentation furnished a substantially quantitative method of converting phenaglycodol into a ketone by refluxing the glycol with 10% sulfuric acid. The ketonic liquid obtained was immediately subjected to careful fractionation at reduced pressure. The main component was 2-methyl-2-(*p*-chloro-

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[1] Marketed as Ultran by Eli Lilly and Co.

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Input		% Recovery	
Vitamin A Units	Vitamin E Units	Vitamin A	Vitamin E
50,000 (oil)	10 (oil, acetate)	99.1	101.9
25,000 (oil)	10 (oil, acetate)	100.3	104.0
50,000 (dry)	10 (dry, acetate)	98.0	102.8
25,000 (dry)	10 (dry, acetate)	95.7	99.4
50,000 (dry)	10 (succinate)	98.5	107.1
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tion which allows good recovery of both vitamins. The key change from previous procedures is the substitution of ascorbic acid for hydrochloric acid in acidifying the saponification mixture before extraction.

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Further experimentation furnished a substantially quantitative method of converting phenaglycodol into a ketone by refluxing the glycol with 10% sulfuric acid. The ketonic liquid obtained was immediately subjected to careful fractionation at reduced pressure. The main component was 2-methyl-2-(*p*-chloro-

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phenyl)-3-butanone (*B*, Fig. 1). In addition, very small amounts of 2-(*p*-chlorophenyl)-3-methylbutadiene-1,3 and *p*-chlorophenyl *tert*-butyl ketone were present. Any delay in fractionation allows the substituted butadiene to polymerize readily (probably catalyzed by peroxides) by a 1:4 addition to form an amorphous insoluble polymer.

A literature search produced no reference to *p*-chlorophenyl *tert*-butyl ketone. It was, therefore, necessary to synthesize this compound for comparison with the conjugated ketone detected in the reaction mixture. The first attempted synthesis of this ketone was from chlorobenzene and trimethyl-acetyl chloride *via* a Friedel-Crafts reaction. A ketone was separated by careful fractionation of the reaction product, but infrared absorption studies indicated that it was not a conjugated ketone. An alternate synthesis from *p*-chloroacetophenone and methyl iodide furnished the desired ketone.

Investigators have established rules for predicting the "migration aptitude" and percentage migration which can be expected for symmetrical pinacols (3), but no satisfactory rules have been formulated to predict the extent of migration of the groups of an unsymmetrical pinacol (4). Collins, in a recent review, states: "Every unsymmetrically substituted glycol can rearrange in two conceivable ways, depending upon which of the two hydroxyl groups is lost during reaction." (See Fig. 1.)

To confirm that 2-methyl-2-(*p*-chlorophenyl)-3-butanone was formed in almost quantitative yield and that it had not resulted from rearrangement during fractionation, a typical reaction mixture was subjected to a selective oxidation process for methyl ketones (5). A substantially quantitative yield of a mixture of *p*-chlorophenyl-dimethylacetic acid along with a small amount of *p*-chlorobenzoic acid was obtained. The mother liquor and washings from the oxidation process were worked up, and a small amount of nonoxidized ketone was isolated as the crystalline 2,4-dinitrophenylhydrazone. The powder X-ray diffraction pattern of this crystalline hydrazone was identical with that of the hydrazone prepared from the fractionated 2-methyl-2-(*p*-chlorophenyl)-3-butanone. The possibility that *p*-chlorophenyl *tert*-butyl ketone might form a 2,4-dinitrophenylhydrazone with difficulty or not at all was considered, but studies with the synthetic ketone prepared for comparison revealed that it formed the 2,4-dinitrophenylhydrazone even more readily than the unconjugated isomer.

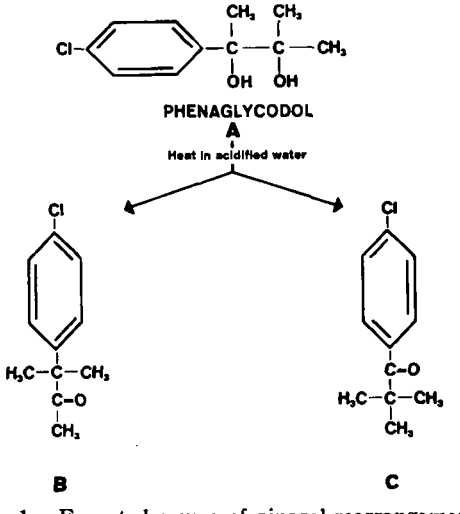


Fig. 1.—Expected course of pinacol rearrangement.

PHARMACOLOGICAL STUDIES

2-Methyl-2-(*p*-chlorophenyl)-3-butanone which had been purified by fractionation was studied in mice for neurosedative activity and in rats for anticonvulsant activity. Quite unexpectedly, this ketone displayed one-third to one-half the pharmacological activity of phenaglycodol. The acute toxicity (LD_{50} 1650 ± 50 mg. per Kg.) by mouth in white mice was about one-half that of phenaglycodol. Since *p*-chlorophenyl-dimethylacetic acid is a possible metabolite of phenaglycodol, the toxicities of the sodium salt by mouth and after intravenous injection in white mice were determined.

EXPERIMENTAL[2]

2-Methyl-2-(p-chlorophenyl)-3-butanone (B, Fig. 1).—One-hundred grams of commercial phenaglycodol was added to a solution of 20 ml. of concentrated sulfuric acid in 180 ml. of water in a round-bottomed flask. This mixture was refluxed gently with constant stirring for 16 hours. The heavier molten glycol separated to the bottom of the flask at first, but as rearrangement progressed the lighter ketonic layer rose to the surface.

The acidic mixture was cooled, transferred to a 500-ml. separator and the lower aqueous phase separated and discarded. The yellow, oily liquid was washed six times with about 80 ml. of 10% sodium chloride solution, then dried with 20 Gm. of anhydrous sodium sulfate. The dried product was filtered into a still pot which was attached to a spinning band reflux column. The column had a calculated efficiency of 28 theoretical plates. The liquid was fractionated at 3 mm. Hg and its composition was as indicated in Table I.

The fraction boiling at 105° was 2-methyl-2-(*p*-chlorophenyl)-3-butanone displaying characteristic infrared absorption bands at 5.83 μ for the unconjugated ketone, at 9.09 μ and 9.82 μ, characteristic of *p*-chlorophenyl, and at 11.96 μ, characteristic of *p*-substituted phenyl. Nuclear magnetic resonance studies supported the assigned structure.

Anal.—Calcd. for $C_{11}H_{13}ClO$: C, 67.17; H, 6.66; Cl, 18.03. Found: C, 67.18; H, 6.50; Cl, 17.62.

[2] All boiling points and melting points reported have not been corrected.

TABLE I.—COMPOSITION OF REACTION MIXTURE FROM PHENAGLYCODOL REARRANGEMENT

Reflux Ratio	B.p., °C.	Volume, ml.	$n_D^{25°}$	$d_4^{24°}$	
9:1	80–82	0.62	...	...	
5:1	82–84	2.56	1.5564	...	
7:1	84–87	0.67	...	...	
7:1	87–90	0.33	...	...	
8:1	90–94	0.77	...	...	
8:1	94–98	0.67	...	...	
8:1	98–102	0.67	...	...	
8:1	102–105	0.67	...	...	
2:1	105	75.8	1.5250	1.0964	
5:1	106–140	1.3	...	...	
Residue	...	...	...	...	6.3 Gm.

2,4-Dinitrophenylhydrazone (6) recrystallized from isopropanol, m.p. 199°.

2-(p-Chlorophenyl)-3-methylbutadiene-1,3.—The fraction boiling at 82–84° (Table I) consisted principally of a butadiene with displayed infrared absorption peaks at 6.25, 6.68, 9.09, 9.82, 11.03, and 11.96 μ. The 9.09 and 9.82-μ bands are characteristic of *p*-chlorophenyl; the 11.96-μ band is characteristic of the *p*-substituted-phenyl group. The absorption at 11.03 μ is characteristic of a terminal methylene group. There was also present in this fraction small amounts each of an unconjugated ketone which displayed carbonyl absorption at 5.83 μ and a conjugated ketone which displayed absorption at 5.92 μ, respectively. The butadiene structure of the principal component was confirmed by nuclear magnetic resonance studies.

Upon standing at room temperature, this fraction became very viscous; most of it became insoluble in acetone and in methanol. An insoluble, amorphous solid was obtained by repeated washing with boiling methanol. The infrared absorption of this solid indicated a complete loss of all terminal methylene absorption, but the absorption band at 6.68 μ and the two bands for *p*-chlorophenyl and that for *p*-substituted phenyl were still present, although all absorption peaks were shifted to slightly longer wavelengths.

Anal.—Calcd. for $(C_{11}H_{11}Cl)_n$: C, 73.95; H, 6.21; Cl, 19.85. Found: C, 74.07; H, 6.24; Cl, 19.56.

Trimethyl-acetyl Chloride.—Meyer (7) has reported that this chloride was easily made by using thionyl chloride, but he gave no details. A 33.4-ml. quantity of thionyl chloride (0.47 mole, 56 Gm.) was placed in a round-bottomed flask provided with a magnetic stirrer and a water cooled reflux condenser. This was heated on a water bath at 65° while being stirred, and trimethyl-acetic acid, 40.85 Gm. (0.4 mole), was added in small portions through the condenser during the course of 1 hour. The fumes from the condenser were collected in a water trap. When all the acid had been added, the reaction mixture was refluxed for 0.5 hour. The reaction mixture was then distilled from a glycerin bath and 25 ml. of a fraction boiling at 102–105° at 757 mm. Hg was collected [Butlerov (8) 105–106° at 760 mm.]. Some unconverted acid, 8 ml., was collected 62–64° at 5 mm. Hg.

Attempted Synthesis of p-Chlorophenyl tert-Butyl Ketone by Friedel-Crafts Reaction.—This reaction was carried out by a slight modification of the procedure described by Schweitzer (9, 10) for bromobenzene and acetyl chloride. The reaction product from chlorobenzene and pivalyl chloride was fractionated on a spinning band column and a ketonic fraction boiling at 109° at 2 mm. Hg was isolated; $n_D^{25°} = 1.5250$. Infrared absorption studies revealed absorption bands at 5.83 μ (unconjugated ketone), the *p*-chlorophenyl bands at 9.09 μ and 9.82 μ, and the *p*-substituted phenyl band at 11.96 μ. However, the phenyl absorption bands were of much lower intensity with respect to the carbonyl absorption band than they were in the spectrum of 2-methyl-2-(*p*-chlorophenyl)-3-butanone. There were other spectral differences which left little doubt that this ketone was different from the one isolated from the rearrangement of phenaglycodol. Nuclear magnetic resonance studies confirmed this difference, but the structure of the synthesized ketone was not established. A 2,4-dinitrophenylhydrazone of this ketone was not obtained by the usual method of preparation.

TABLE II.—COMPOSITION OF REACTION MIXTURE FROM METHYLATION OF *p*-CHLOROACETOPHENONE

Reflux Ratio	B.p., °C.	Volume, ml.	$n_D^{25°}$	$d_4^{24°}$
5:1	85–88	0.33	...	...
3:1	88–90	11.86	1.5497	1.1787
5:1	90–94	2.0	...	...
5:1	94–98	2.0	...	...
7:1	98–100	2.7	...	...
3:1	100–102	21.9	1.5288	1.1196

No conjugated ketone was detected by infrared absorption studies in any of the fractions separated from the reaction mixture or in the considerable amount of high boiling residual liquid which remained in the still pot.

p-Chlorophenyl tert-Butyl Ketone (C, Fig. 1).—This ketone was prepared by exhaustive methylation of *p*-chloroacetophenone by a modification of the method of Haller and Bauer (11). One-hundred-fifty milliliters of benzene, which had been dried over sodium, was placed in a round-bottomed three-necked flask provided with a water bath, a magnetic stirrer, a reflux condenser, and a dropping funnel. Sodium amide (35.2 Gm., 0.9 mole) was added to the dry benzene and mixed well. A mixture of 45.5 Gm. (0.295 mole) of *p*-chloroacetophenone and 149.5 Gm. (1.05 moles) of methyl iodide was added slowly from the dropping funnel. The rate of addition was adjusted so that the reaction never became violent. After all of the *p*-chloroacetophenone and methyl iodide had been added, the reaction mixture was gently refluxed with constant stirring for 3

hours. The reaction mixture was allowed to stand at room temperature for 12 hours, then 50 ml. of distilled water was added slowly and carefully from the dropping funnel to decompose any excess of sodium amide. The reaction mixture was transferred to a separator, 100 ml. of distilled water was added, and the benzene phase was extracted. The aqueous layer was separated and discarded. Extraction with 150 ml. of distilled water was repeated five times; then the benzene phase was separated and dried with 20 Gm. of anhydrous sodium sulfate. The sodium sulfate was removed by filtration, and the filtrate was fractionated in a spinning band column at 4 mm. Hg. The composition was as indicated in Table II.

The fraction collected at 88 to 90° was *p*-chloroacetophenone; the fraction boiling at 100 to 102° was a mixture containing about 45 mole per cent of the desired *p*-chlorophenyl *tert*-butyl ketone. The infrared absorption pattern displayed, in addition to the typical *p*-chlorophenyl bands at 9.09 and 9.82 μ and *p*-substituted phenyl band at 11.96 μ, the conjugated carbonyl absorption at 5.92 μ. Nuclear magnetic resonance studies of this fraction indicated that three species of ketones were present. A monomethylated ketone was present in about 7 mole per cent concentration, a dimethylated ketone (isopropyl) in about 48 mole per cent concentration, and the trimethylated ketone (*tert*-butyl ketone) in 45 mole per cent concentration. Since these ketones could not be separated by fractionation, a small amount was converted easily to a mixture of the 2,4-dinitrophenylhydrazones. These were separated into an isopropanol insoluble fraction with a melting point of 213° and a fraction that was recrystallized from isopropanol, m.p. 121°. Nuclear magnetic resonance studies revealed that the 2,4-dinitrophenylhydrazone melting at 121° was the dimethylated ketone (isopropyl), and the 2,4-dinitrophenylhydrazone melting at 213° was that of the trimethylated (*tert*-butyl) ketone.

Anal.—Calcd. for (2,4-dinitrophenylhydrazone of *p*-chlorophenyl isopropyl) $C_{16}H_{15}ClN_4O_4$: Cl, 9.77. Found: Cl, 9.86.

Anal.—Calcd. for (2,4-dinitrophenylhydrazone of *p*-chlorophenyl *tert*-butyl ketone) $C_{17}H_{17}ClN_4O_4$: Cl, 9.41. Found: Cl, 9.56.

p-Chlorophenyl-dimethylacetic Acid.—A mixture of 930 ml. of 3 *N* sodium hydroxide solution and 67 ml. of bromine was placed in a 2-L. three-necked, round-bottomed flask provided with a magnetic stirrer and a water bath. This mixture was stirred until homogeneous; 50 Gm. of the crude washed reaction mixture consisting primarily of 2-methyl-2-(*p*-chlorophenyl)-3-butanone was added all at once. The flask was heated to 68–70° while the contents were constantly stirred until bromine ceased to be liberated when a few drops of the reaction mixture was acidified with acetic acid. This required about 4 hours. Sulfur dioxide was then passed into the reaction mixture until no more bromine was liberated when a few drops of the reaction mixture was made strongly acidic with concentrated hydrochloric acid. The reaction mixture was filtered; the filtrate was made strongly acidic by the addition of 75 ml. of concentrated hydrochloric acid. The acidified liquid was stirred for 10 minutes, then stored overnight at 5°. The separated crystals were collected on a filter and washed with about 200 ml. of cold distilled water. The filtrate and washings were saved for the recovery of nonoxidized ketone. The washed crystals of precipitated acid were dried in a vacuum desiccator at 5 mm. Hg for 12 hours at 25°. Yield, 48 Gm. (Theory, 50.50 Gm.)

This product when examined by nuclear magnetic resonance proved to be a mixture of about 90% *p*-chlorophenyl-dimethylacetic acid and 10% *p*-chlorobenzoic acid. This benzoic acid was produced by oxidation of some unconverted phenaglycodol. Its formation from phenaglycodol was confirmed in a separate experiment.

Pure *p*-chlorophenyl-dimethylacetic acid was prepared by oxidizing a like amount of the fractionated 2-methyl-2-(*p*-chlorophenyl)-3-butanone by the same method. The yield of dried product was 50 Gm., m.p. 126°. The oxidation of the purified ketone proceeded at a much slower rate than that of the crude reaction mixture.

Anal.—Calcd. for $C_{10}H_{11}ClO_2$: C, 60.46; H, 5.58; Cl, 17.85. Found: C, 60.15; H, 5.62; Cl, 17.58.

Infrared and ultraviolet absorption spectra and a nuclear magnetic resonance study supported the assigned structure. The solubility in water at 26° was 2 mg. per ml. The pKa' in 66% dimethylformamide was 7.0, and the calculated molecular weight was 195 (theory 198.65). The acute toxicities for the sodium salt determined in fasted white mice after 7 days' observation were: LD_{50} by mouth, 1052 ± 129 mg. per Kg.; LD_{50} intravenous, 553.9 ± 67.6 mg. per Kg.

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Stereochemical Studies on Medicinal Agents I

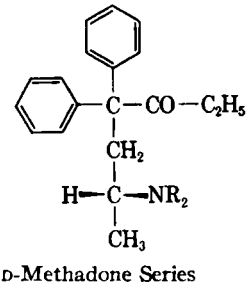
Absolute Configuration of Certain Analgesic (−)-*N*-[2-(Phenalkylmethylamino)propyl]propionanilides

By PHILIP S. PORTOGHESE and DENNIS L. LARSON†

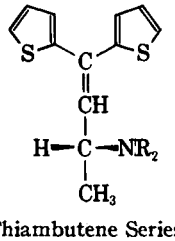
The absolute configuration of certain (−)-*N*-[2-(phenalkylmethylamino)propyl]-propionanilides has been determined by relating them to D-(−)-alanine. The compounds corresponding to the L-configuration have greater analgesic potency than those with the D-configuration. An interpretation of the significance of the relationship of absolute configuration to analgesic activity is discussed in terms of an analgesic receptor surface.

THE RELATIONSHIP of the absolute spatial geometry of medicinal agents to their pharmacological potency has received serious attention only in the past 10 years. There are numerous observations recorded in the literature on the optical specificity of enzymes and on the unequal distribution of pharmacological activity between (+)- and (−)-enantiomers of many types of drugs (1).

One of the few classes of drugs which has been systematically investigated from a three-dimensional point of view is the narcotic analgesic group. Though there has been a considerable research effort devoted to the elucidation of the optimum structural requirements for analgesic molecules, only the methadone-type (including its isosteres) and thiambutene-type compounds have been studied from an absolute configurational approach. In such experiments Beckett and co-workers have shown that most of the analgesic activity resides in the enantiomorphs possessing the D-configuration. A hypothesis



D-Methadone Series



D-Thiambutene Series

was advanced in an attempt to explain this antipodal specificity on the basis of three point contact with a receptor in which only one antipode can fit properly (1).

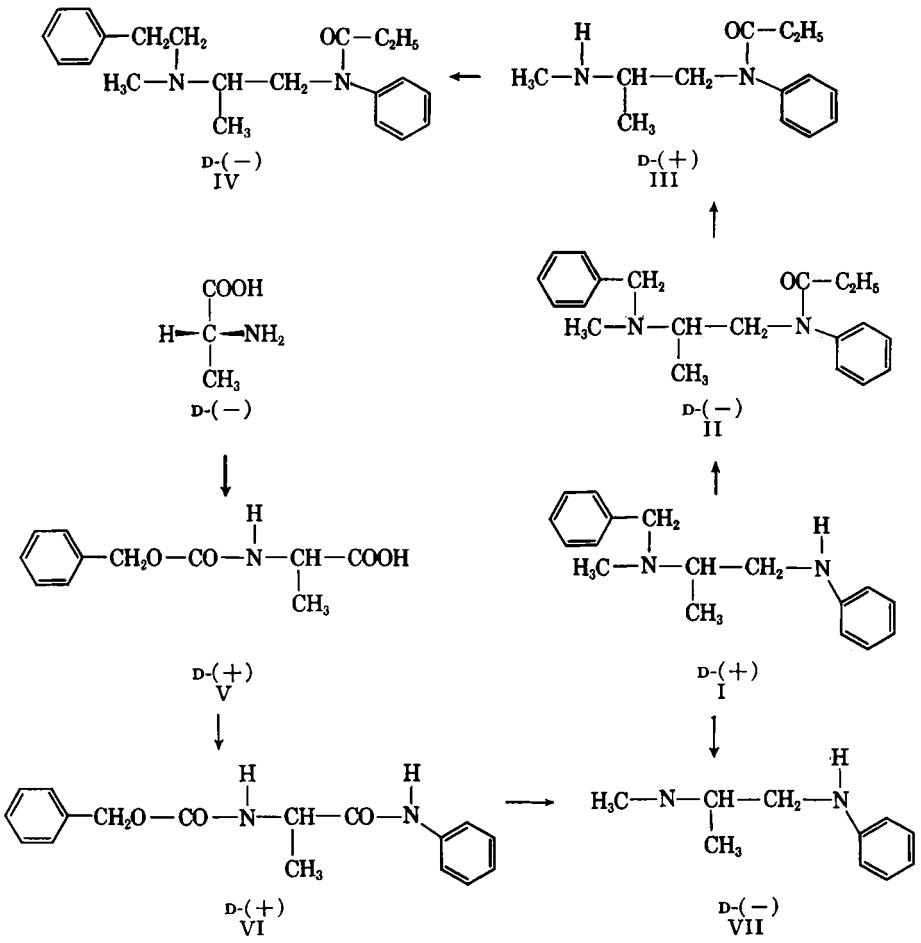
The need for more information concerning the relationship of configuration to analgesic activity has prompted us to determine the absolute configuration of certain potent analgesics of the basic anilide type (II and IV) (2). We have reported some of our experiments in prior preliminary communications (3, 4) and now wish to present a detailed description and interpretation of our results.

The configuration of the (−)-enantiomers of II and IV have been related to D-(−)-alanine by an unambiguous sequence in which the stereochemical integrity of the asymmetric carbon in question was at all times preserved.

In preliminary experiments, attempts at direct optical resolution of racemic II and IV were not successful. Therefore, we turned to the resolution of the (±)-diamine (I) which is an intermediate in the synthesis of racemic basic anilide analgesics containing the phenalkyl moiety (2). This was accomplished by mixing equivalent amounts of the (±)-diamine (I) and (+)-tartaric acid in 95% ethanol. Several recrystallizations afforded a salt of high purity which, on treatment with aqueous sodium hydroxide, yielded optically pure (+)-diamine (I). The enantiomeric (−)-diamine (I) was obtained in pure form by triangular crystallization of a second crop of crystals obtained from the alcoholic mother liquor. The (+)-diamine (I) was converted to the (−)-propionanilide (II) by heating in propionic anhydride. Catalytic hydrogenolysis of (−)-II produced the debenzylated (+)-intermediate (III) which was subsequently phenethylated to give the (−)-propionanilide (IV).

Catalytic hydrogenolysis of the resolved (+)-diamine (I) produced the debenzylated (−)-diamine (VII). This compound was shown to have the D-configuration through an unambiguous synthetic procedure. D-(−)-Alanine was converted to (+)-carbobenzoxy-D-alanine (V) (5) by treating with benzyl chloroformate. This intermediate (V) was transformed to the (+)-anilide (VI) in high yield by allowing a tetrahydrofuran solution of compound V, aniline, and dicyclohexylcarbodiimide to stand for several hours. Reduction of VI with lithium alumi-

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num hydride afforded D-(−)-N^2-methyl-N^1-phenyl-1,2-propanediamine, whose physical properties are identical with the (−)-diamine (VII) derived from the catalytic hydrogenolysis of (+)-I.

The preceding sequence of transformations, therefore, unequivocally demonstrates that the (−)-enantiomers of II and IV are stereochemically related to D-(−)-alanine.

The (+)- and (−)-enantiomers of analgesics II and IV have been tested by others (6) on rats by a modification of the rat tail heat response method. As illustrated in Table I, the L-isomers of II and IV possess activity comparable to that of morphine, whereas the D-isomers are less active. These pharmacological results are quite unexpected, as it has been demonstrated that the more active enantiomorphs of methadone-type and thiambutene-type analgesics have the D-configuration (1). The addictive properties of II and IV (7) and their antagonism by nalorphine (8) suggest that they are acting on the same analgesic receptor through which methadone and other potent analgesics exert their activity, despite the fact that the more active enantiomorphs of these compounds (II and IV) are related to L-(+)-alanine.

These results are in disagreement with the hypothesis that all highly active analgesics possessing an asymmetric carbon have the D-configuration (1). Moreover, the apparent inversion of antipodal specificity in an analgesic receptor is not without precedent. It has been reported that (−)-α-methadol derived from the catalytic reduction of L-(+)-methadone is seven times more active than (+)-α-methadol obtained from D-(−)-methadone (9). Interestingly, the sodium-in-propanol reduction of D-(−)-methadone affords a preponderance of the diastereomeric (−)-β-methadol which has seven times greater potency than (+)-β-methadol derived by reducing L-(+)-methadone (9). These results are summarized in Table II.

The preceding example is highly significant because it serves to illustrate how a reversal in potency ratio between D- and L-isomers of diastereomeric methadols can be effected by merely altering the stereochemistry of the hydroxyl group.

It has been postulated (1) that an aromatic ring, basic nitrogen, and a hydrocarbon moiety interact with the analgesic receptor in a certain conformation. Accordingly, the receptor can make contact with analgesics of the D-configuration in such manner as to accommodate all three of the aforementioned groups, whereas the L-configuration could not easily fit the receptor because the hydrocarbon moiety no longer can fill a suitably oriented "cavity" on the receptor surface.

Since the basic anilide analgesics (II and IV) and the α-methadols do not conform to the above hypothesis, there may be certain additional require-

TABLE I.—RELATIONSHIP OF ABSOLUTE CONFIGURATION TO ANALGESIC POTENCY

Configuration	Compd.	AD_{50}[a,b] (mg./Kg.)
D	II[c]	Inactive at 50
L	II[c]	4.3
D	IV[d]	11.7
L	IV[d]	3.6
	Meperidine	11.0
	Morphine	3.0

[a] We thank Dr. W. B. Wright and Dr. R. A. Hardy for forwarding us their pharmacological data prior to publication. [b] AD_{50} = the subcutaneous dose which elevates the rat tail radiant heat response time by 100% in 50% of the animals. [c] Tested as the hydrochloride salt. [d] Tested as the free base.

ments which must be considered in the depiction of an analgesic receptor surface.

In comparing the potency ratios of the D- and L-isomers of the basic anilide type (II and IV) to those in the methadone series, it is tempting to speculate that, as in the case of the optically active methadols, the reversal in potency ratio on going from the anilides to the methadones is due to a difference in the orientation of the oxygen function. Inspection of Dreiding stereomodels of anilide analgesics (II and IV) and methadone-type compounds reveal that there is a distinct difference in the position of the carbonyl oxygen relative to the rest of the molecule. The amide moiety is planar due to partial sp^2 character in the bond connecting the nitrogen and carbonyl carbon. In contrast to compounds II and IV, the quaternary carbon in the methadone series is tetrahedral. The planarity of the amide moiety consequently causes the orientation of the amide carbonyl oxygen (relative to the rest of the molecule) to differ from that of the ketonic oxygen in the methadone compounds.

If the relative position of the oxygen function does influence the optical specificity of an analgesic receptor, the stereochemical course of binding of certain analgesic molecules to the active site may in part be governed by the orientation of the oxygen function (hydroxyl, amide carbonyl oxygen, and ketonic oxygen) which would be capable of interacting with a secondary site of the analgesic receptor.

We are presently engaged in stereochemical studies on the methadols, methadones, and basic anilides in order to gain further insight into factors which may alter the stereochemical course of molecular binding to an analgesic receptor.

EXPERIMENTAL

(+)-N²-Benzyl-N²-methyl-N¹-phenyl-1,2-propanediamine (I).—A solution of 34.66 Gm. (0.136 mole) of (±)-diamine (I) (2) in 70 ml. of 95% ethanol was mixed with 280 ml. of an ethanolic solution containing 20.48 Gm. (0.136 mole) of (+)-tartaric acid. The combined solution was allowed to stand at 25° for 24 hours. The crystals were collected and recrystallized four times from 95% ethanol to afford 17.3 Gm., m.p. 101–103°,[1] of the tartrate salt, $[\alpha]_D^{27}$ − 16.0° (*c* 5% in water).

Anal.—Calcd. for $C_{21}H_{28}N_2O_6 \cdot H_2O$: C, 59.98; H, 7.17; N, 6.64. Found: C, 60.01; H, 7.13; N, 6.72.

[1] All melting points are uncorrected and were obtained with a "Hoover" capillary melting point apparatus.

TABLE II.—ANALGESIC ACTIVITY OF ISOMERIC METHADOLS (9)

Parent Methadone	ED_{50}[a]	Methadol Isomer	ED_{50}[a]
L-(+)	25.7	(−)-α	3.5
D-(−)	0.8	(+)-α	24.7
L-(+)	...	(+)-β	63.7
D-(−)	...	(−)-β	7.6

[a] Values are mg./Kg. administered subcutaneously in mice.

To 16.4 Gm. of the above salt there was added 80 ml. of 1 *N* sodium hydroxide and 100 ml. of water; the mixture was extracted with ether and dried over sodium sulfate. On removal of the solvent *in vacuo*, 9.1 Gm., m.p. 59–61°, of (+)-I crystallized, $[\alpha]_D^{27°}$ + 31.2° (*c* 5% in ethanol). This compound was analyzed as the monopicrate, m.p. 168–169°.

Anal.—Calcd. for $C_{27}H_{29}N_5O_7$: C, 57.11; H, 5.21; N, 14.48. Found: C, 56.84; H, 5.33; N, 14.38.

(−)-N²-Benzyl-N²-methyl-N¹-phenyl-1,2-propanediamine.—The combined mother liquor from the preceding resolution was concentrated and allowed to stand 12 hours at 25°. The crystalline product was collected and purified by triangular crystallization from water. There was obtained 8.5 Gm., m.p. 82–84°, of tartrate salt, $[\alpha]_D^{25°}$ + 30.0° (*c* 2% in water).

Anal.—Calcd. for $C_{21}H_{28}N_2O_6 \cdot 2H_2O$: C, 57.29; H, 7.12; N, 6.37. Found: C, 57.18, H, 7.39; N, 6.47.

The (+)-salt (7.6 Gm.) was converted to 3.8 Gm. of (−)-*N*²-benzyl-*N*²-methyl-*N*¹-phenyl-1,2-propanediamine, m.p. 59–60°, $[\alpha]_D^{30°}$ − 30.8° (*c* 5% in ethanol) by the procedure described for the (+)-diamine (I).

(−)-N-[2-(Benzylmethylamino)propyl]propionanilide (II).—A mixture of 5.08 Gm. (0.02 mole) of (+)-diamine (I) and 10 ml. of propionic anhydride was heated on a steam bath for 2 hours. The propionic acid and propionic anhydride were removed by distillation and the residue fractionated through a spinning band column to give 4.9 Gm. (80% yield) of (−)-anilide (II), b.p. 152–157° (0.3 mm.), $[\alpha]_D^{32°}$ − 45.7° (*c* 5% in ethanol). This compound exhibited an infrared spectrum and gas chromatographic retention time which are identical with that of racemic II (2).

(+)-N-[2-(Methylamino)propyl]propionanilide (III).—The preparation of (+)-III was similar to the procedure employed in the synthesis of racemic III (2). A mixture of 18.0 Gm. (0.058 mole) of (−)-anilide (II), 14.5 ml. of 4 *N* HCl, 115 ml. of 95% ethanol, and 1.2 Gm. of 10% palladium-on-carbon was shaken in a hydrogen atmosphere at a pressure of 40 p.s.i. for 24 hours. The mixture was filtered, evaporated *in vacuo* to an oil, basified with aqueous sodium hydroxide, and extracted with ether. After the ether extract was dried over sodium sulfate, the solvent was removed *in vacuo* and the residue distilled. The yield of (+)-III, b.p. 110–114° (0.1 mm.), $n^{25°}$ 1.5214, $[\alpha]_D^{25°}$ + 14° (*c* 5% in ethanol), was 8.1 Gm. (69%). The infrared spectrum of (+)-III is identical with that of racemic III.

(−)-N-[2-(Phenethylmethylamino)propyl]-propionanilide (IV).—A mixture of 1.75 Gm. (0.008 mole) of (+)-III, 1.48 Gm. (0.008 mole) of phen-

ethyl bromide and 0.85 Gm. of anhydrous sodium carbonate was refluxed in 12 ml. of benzene for 48 hours. The mixture was extracted with water and the benzene solution concentrated *in vacuo*. Distillation through a spinning band column gave 2.0 Gm. (77%) of product, b.p. 159–163° (0.1 mm.), $n^{28°}$ 1.5440, $[\alpha]_D^{28°}$ − 26.4° (*c* 5% in ethanol). The infrared spectrum and gas chromatographic retention time are identical with that of racemic IV (2).

Preparation of (−)-N²-Methyl-N¹-phenyl-1,2-propanediamine (VII) from (+)-N²-Benzyl-N²-methyl-N¹-phenyl-1,2-propanediamine (I).—A mixture of 2.54 Gm. (0.01 mole) of (+)-diamine (I), 2.5 ml. of 4 *N* HCl, 20 ml. of 95% ethanol, and 0.2 Gm. of 10% palladium-on-carbon was shaken in an atmosphere of hydrogen at a pressure of 40 p.s.i. When the theoretical amount of hydrogen was absorbed, the mixture was filtered and the ethanol removed *in vacuo*. The remaining liquid was made basic with aqueous sodium hydroxide, extracted with ether, and dried over sodium sulfate. Removal of the solvent afforded an oil which on distillation through a spinning band column yielded 1.2 Gm. (73%) of hygroscopic (−)-VII, b.p. 75–78° (0.3 mm.), m.p. 34–35°, $[\alpha]_D^{27°}$ − 29.5° (*c* 5% in ethanol). The dipicrate, infrared spectrum, and gas chromatographic retention time of this compound (VII) are identical with those of the (−)-diamine derived from D-(−)-alanine.

(+)-Carbobenzoxy-D-alanine (V).—This was prepared (5) by reacting D-(−)-alanine and benzylchloroformate in sodium bicarbonate solution (10).

(+)-Carbobenzoxy-D-alanine Anilide (VI).—To a mixture of 30 Gm. (0.13 mole) of (+)-carbobenzoxy-D-alanine and 12.1 Gm. (0.13 mole) of aniline dissolved in 200 ml. of tetrahydrofuran there was added a solution of 29.5 Gm. (0.143 mole) of dicyclohexylcarbodiimide in an equal volume of tetrahydrofuran. After allowing the mixture to stand 6 hours, the solution was concentrated to 200 ml. Five milliliters of acetic acid was added and the solution was again filtered. Removal of all the solvent produced 36 Gm. (93%) of crude (+)-anilide (VI). Recrystallization from 95% ethanol produced crystals, m.p. 162–164°, $[\alpha]_D^{30°}$ + 28.4°. An infrared spectrum of the product exhibited bands at 3.0 μ (—NH), 5.92 μ (—OCO—N), and 6.03 μ (amide).

Anal.—Calcd. for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.33. Found: C, 68.19; H, 6.13; N, 9.66.

Synthesis of D-(−)-N²-Methyl-N¹-phenyl-1,2-propanediamine (VII) from (+)-Carbobenzoxy-D-alanine Anilide (VI).—To a stirred mixture of 10.64 Gm. (0.28 mole) of lithium aluminum hydride and 200 ml. tetrahydrofuran, there was added (over a 30-minute period) a solution of 20.88 Gm. (0.07 mole) of VI in an equal volume of tetrahydrofuran. The mixture was refluxed for 5.5 hours, cooled, and treated successively with 11 ml. of water, 28 ml. of 15% sodium hydroxide, and 28 ml. of water. The mixture was filtered, dried over sodium sulfate, and the solvent removed *in vacuo*. Ethyl acetate was added to the residue, and it was extracted with 100 ml. 1 *N* HCl. The aqueous solution was made basic with 1 *N* sodium hydroxide and extracted with ethyl acetate. After removal of solvent *in vacuo*, the residue was distilled through a spinning band column. There was collected 9.0 Gm. (78% yield) of D-(−)-diamine (VII), b.p. 67–71° (0.1 mm.), $[\alpha]_D^{30}$ − 29.2° (*c* 5% in ethanol), which crystallized, m.p. 35–36.5°, on cooling. This compound is very hygroscopic and quickly liquifies on exposure to moist air. The product was analyzed as the dipicrate.

Anal.—Calcd. for $C_{22}H_{22}N_8O_{14}$: C, 42.43; H, 3.56; N, 18.00. Found: C, 42.49; H, 3.60; N, 18.23.

The infrared spectrum, gas chromatographic retention time, and dipicrate were found to be identical with those of (−)-diamine VII derived from the catalytic hydrogenolysis of (+)-*N*²-benzyl-*N*²-methyl-*N*¹-phenyl-1,2-propanediamine (I).

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Thin-Layer Chromatography of Ergot Alkaloids

By J. L. McLAUGHLIN, J. E. GOYAN, and A. G. PAUL

A technique for qualitative identification and quantitative determination of micro quantities of ergot alkaloids has been developed. Using glass plates spread with a layer of silica gel G and a solvent system composed of benzene:dimethylformamide (13:2) or of ethyl acetate:dimethylformamide:ethanol (13:1.9:0.1), one can separate ergonovine, ergotamine, ergosine, ergocristine, and a mixed zone composed of ergocornine and ergokryptine. The mixed zone is removed from the plate, the alkaloids eluted with 10 per cent methanol in chloroform, and filtered or centrifuged to remove the silica gel. The eluant is evaporated, and the mixture of the alkaloids is dissolved in a standard volume of methanol-chloroform. The respotting of this solution on glass plates spread with a layer of aluminum oxide G and chromatographed with a solvent system composed of chloroform:ether:water (3:1:1) results in the separation of ergocornine and ergokryptine. These solvent systems can be utilized to separate the isomers of the six alkaloids with equal facility. The technique has been extended to the resolution and quantitative estimation of the alkaloids.

THESE INVESTIGATIONS were undertaken to develop a rapid technique for the qualitative identification and quantitative estimation of 12 ergot alkaloids in micro amounts. The technique of thin-layer chromatography (TLC) was chosen because of its advantages over paper partition chromatography in requiring shorter time of equilibration and shorter time of development. In addition, 18 spots can be chromatographed on one plate, 8 × 8 in., and resolved components of mixtures are easily recovered from the developed chromatogram by elution.

A procedure reported by Teichert, *et al.* (1), combines some of the advantages of TLC with the versatility of paper partition chromatography using cellulose layers for the separation of the water insoluble ergot alkaloids. This procedure is useful for qualitative identification of ten of the 12 alkaloids. However, the R_f values of ergocorninine and ergocristinine, and of ergokryptine and ergocornine are almost identical. This prohibits use of this technique as a quantitative tool. Other reports using adsorption TLC show only group-wise separation of the alkaloids (1, 2).

The clavine types of ergot alkaloids, which are found in saprophytic cultures and in certain species of the fungus, have been successfully separated by TLC and paper chromatography (3–5).

EXPERIMENTAL

Apparatus.—The RSCo model A-200 chromatofilm assembly for TLC was used in these investigations.[1] Glass plates were 8 × 8 in.

Reagents.—Chemicals used in these investigations were reagent grade or equivalent, except for the following: *N,N*-dimethylformamide and *p*-dimethylaminobenzaldehyde were obtained from Eastman Organic Chemicals, and silica gel-G (SGG) and aluminum oxide-G (Al_2O_3G) were obtained from the Research Specialties Co.[1]

Preparation of Plates.—A slurry was prepared by mixing 25 Gm. of the adsorbent (SGG or Al_2O_3G) with 35 ml. of deionized water to a uniform consistency. An additional 15 ml. of deionized water was added to the slurry and the mixture stirred. The final slurry was poured into the spreader, and this in turn was rapidly drawn over the glass plates arranged on the aligning tray.[2] The plates were permitted to air-dry for approximately 5 minutes, placed in a storage rack, and activated for at least 30 minutes at 105°. Plates were stored at 105° until used.

Just prior to use, 7 mm. of adsorbent was scraped from the starting edge of each plate with a razor blade, using a plastic template as a guide.

Plates used for the determination of R_f values were scored with a sharp stylus in the direction of solvent development at 1-cm. intervals. This scoring of plates yields 18 individual strips of adsorbent and reduces the drift of zones due to irregularities in thickness of the adsorbent layer.

Standard Solutions.—The alkaloids[3] used in these investigations included ergonovine, ergometrinine, ergotamine, ergotaminine, ergosine, ergosinine, ergocristine, ergocristinine, ergokryptine, ergokryptinine, ergocornine, and ergocorninine. Solutions were prepared by dissolving 1.00 mg. of each alkaloid, weighed with a Cahn model M-10 electrobalance,[4] in 10% methanol in chloroform and diluting to 1.0 ml. in a volumetric flask. Mixtures of ergocornine and ergokryptine and of all 12 alkaloids were prepared in the same concentration as well. The solutions of alkaloids were protected from light and stored at refrigerator temperature. Solutions of alkaloids used for quantitative studies were prepared just prior to use.

Solvent Systems.—Of the 111 solvent systems tested, the following were most effective in separat-

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[1] Research Specialties Co., Richmond, Calif.

[2] The fixed-thickness spreader employed delivers a layer of slurry approximately 250 μ thick.

[3] Ergotamine obtained from Mann Research Laboratories, New York, N. Y. All other alkaloids obtained from Sandoz Pharmaceuticals, Hanover, N. J.

[4] Cahn Instrument Co., Paramount, Calif.

ing the ergot alkaloids tested: ethylacetate:*N,N*-dimethylformamide:ethanol, 13:1.9:0.1 (*A*); benzene:*N,N*-dimethylformamide, 13:2(*B*); chloroform:ethyl ether:water, 87.5·12.5:25(*C*); and chloroform:ethyl ether:water, 3:1:1(*D*). The first two solvent systems were mixed at 21 ± 0.5° and used immediately. Solvent systems *C* and *D* were mixed, shaken vigorously, and allowed to equilibrate for 18 to 24 hours at 21 ± 0.5°. The organic phase was separated from the aqueous phase just prior to use.

Application of Test Solutions.—Solutions of the alkaloids were applied to the plates 2 cm. from the starting edge, using Kirk micropipets. For studies of qualitative separation of the alkaloids, 2 μl. of each solution was spotted in successive portions to assure a zone no greater than 5 mm. in diameter using a template as a guide. In the studies of quantitative separations of the alkaloids and in qualitative confirmation of ergokryptine and ergocornine, solutions were applied as a series of spots, using a 10-μl. Kirk micropipet, resulting in a band of the test compound. Again, the rate of application was controlled to assure a band width of no greater than 5 mm.

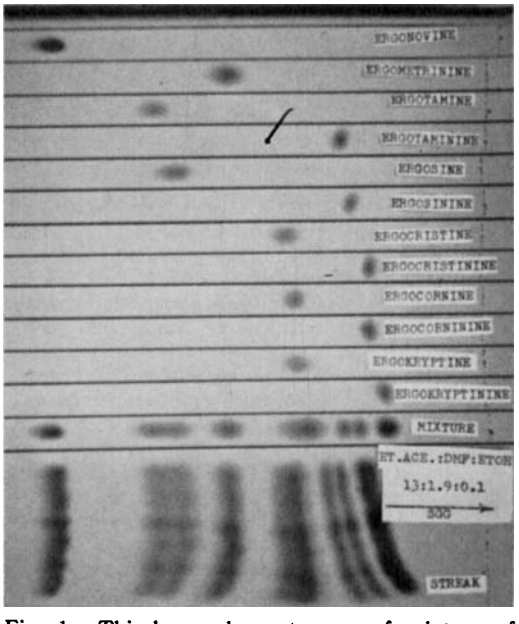


Fig. 1.—Thin-layer chromatogram of mixture of ergot alkaloids. (Solvent system *A*.)

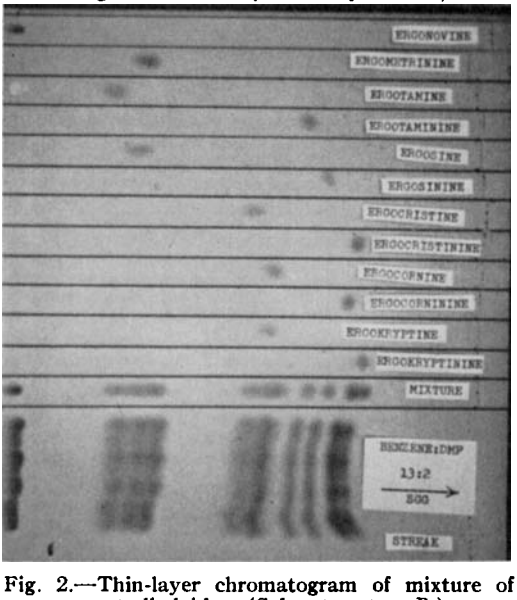


Fig. 2.—Thin-layer chromatogram of mixture of ergot alkaloids. (Solvent system *B*.)

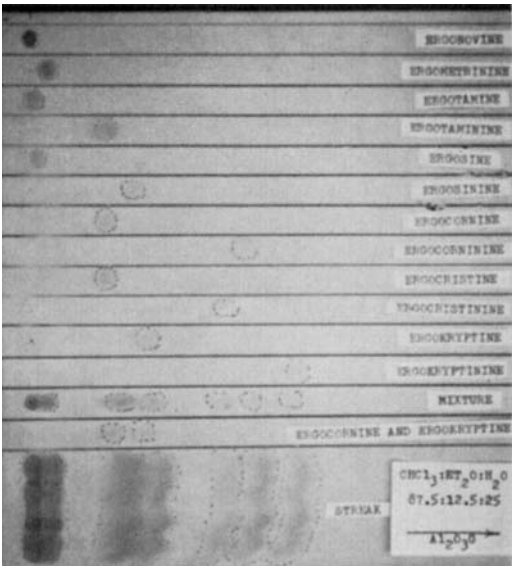


Fig. 3.—Thin-layer chromatogram of mixture of ergot alkaloids. (Solvent system *C*.)

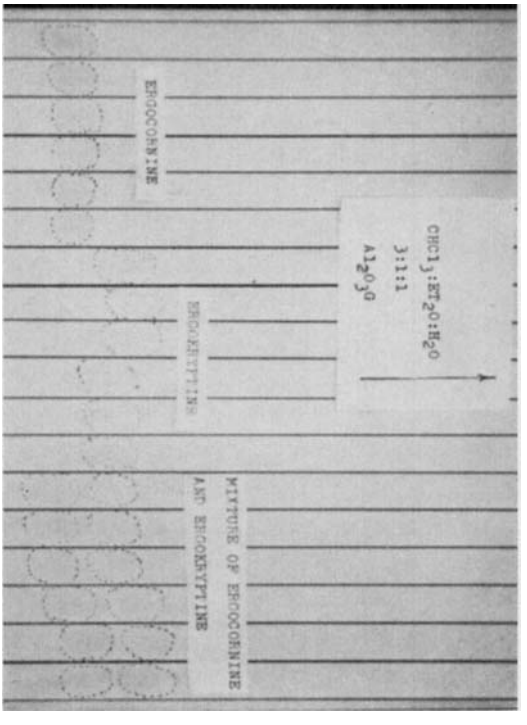


Fig. 4.—Thin-layer chromatogram of ergokryptine and ergocornine. (Solvent system *D*.)

Preparation of Developing Chambers.—Glass

TABLE I.—R_f VALUES OF ERGOT ALKALOIDS

Alkaloid[a]	Solvent System A on SGG		B on SGG		C Al_2O_3G		D Al_2O_3G	
	Mixture	Individually	Mixture	Individually	Mixture	Individually	Mixture	Individually
1	0.166 ± 0.008	0.173 ± 0.002	0.084 ± 0.002	0.120 ± 0.002	0.000	0.000	...	...
2	0.433 ± 0.013	0.441 ± 0.005	0.285 ± 0.012	0.384 ± 0.005	0.019 ± 0.002	0.008 ± 0.002	...	...
3	0.303 ± 0.008	0.313 ± 0.002	0.244 ± 0.011	0.308 ± 0.003	0.019 ± 0.002	0.010 ± 0.001	...	...
4	0.659 ± 0.012	0.682 ± 0.012	0.601 ± 0.023	0.638 ± 0.009	0.113 ± 0.010	0.065 ± 0.003	...	...
5	0.340 ± 0.009	0.350 ± 0.004	0.273 ± 0.012	0.314 ± 0.002	0.019 ± 0.002	0.016 ± 0.002	...	...
6	0.701 ± 0.011	0.747 ± 0.005	0.649 ± 0.022	0.677 ± 0.022	0.113 ± 0.010	0.115 ± 0.002	...	...
7	0.529 ± 0.009	0.544 ± 0.009	0.472 ± 0.017	0.558 ± 0.022	0.113 ± 0.010	0.093 ± 0.004	...	...
8	0.749 ± 0.013	0.796 ± 0.005	0.700 ± 0.023	0.743 ± 0.014	0.225 ± 0.016	0.379 ± 0.004	...	...
9	0.566 ± 0.011	0.579 ± 0.016	0.518 ± 0.019	0.588 ± 0.004	0.113 ± 0.010	0.095 ± 0.003	0.217 ± 0.017	0.243 ± 0.013
10	0.749 ± 0.013	0.833 ± 0.009	0.700 ± 0.023	0.726 ± 0.014	0.278 ± 0.020	0.310 ± 0.008	...	...
11	0.566 ± 0.011	0.598 ± 0.005	0.518 ± 0.019	0.554 ± 0.015	0.113 ± 0.010	0.148 ± 0.012	0.273 ± 0.019	0.299 ± 0.009
12	0.749 ± 0.013	0.854 ± 0.003	0.728 ± 0.021	0.746 ± 0.023	0.332 ± 0.028	0.457 ± 0.019	...	...

[a] *1*, Ergonovine; *2*, ergometrinine; *3*, ergotamine; *4*, ergotaminine; *5*, ergosine; *6*, ergosinine; *7*, ergocristine; *8*, ergocristinine; *9*, ergocornine; *10*, ergocorninine; *11*, ergokryptine; and *12*, ergokryptinine.

jars, 8¼ × 3 × 9 in. (internal dimensions) were lined with Whatman No. 1 filter paper as wicks. The solvent system (250 ml.) was added, the jars covered, leveled, and allowed to equilibrate for at least 30 minutes at 21 ± 0.5°.

Development of Chromatograms.—Plates were placed on a stainless steel support rack, immersed in the solvent pool, covered, and development allowed to proceed to within 1 cm. of the upper edge of the plate, unless otherwise specified. Ordinarily, development to this height required 80 minutes on SGG plates and 60 minutes on Al_2O_3 plates.

Detection of Alkaloids.—Short wavelength U.V. light and reaction with Ehrlich's reagent (5% *p*-dimethylaminobenzaldehyde in concentrated HCl) were used to locate the positions of alkaloids on the chromatograms.

Quantitative Determination of Alkaloids.—Solutions of alkaloids were assayed by mixing 1 part of the solution with 2 parts *p*-dimethylaminobenzaldehyde T.S. (U.S.P.), permitting reaction to occur for 30 minutes, and determining absorbance using a Beckman model DU spectrophotometer at a wavelength of 590 mμ with a 0.06-mm. slit width.

RESULTS AND DISCUSSION

Qualitative Analysis of Alkaloids.—Using solvent system *A* and SGG, the following alkaloids and mixtures of alkaloids (in order of increasing R_f values), were separated: ergonovine, ergotamine, ergosine, ergometrinine, ergocristine, ergocornine plus ergokryptine, ergotaminine, ergosinine, and a mixture of ergocristinine, ergocorninine, and ergokryptinine. Figure 1 shows a chromatogram of these results.

Using solvent system *B* and SGG, the same order of separation of the alkaloids occurred, with minor variations in resolution. The R_f values of ergometrinine and ergosine varied only slightly, but some resolution of ergokryptinine from ergocorninine and ergocristinine was achieved (Fig. 2).

With solvent system *C* and Al_2O_3G, poor resolution of the alkaloids occurred, except for ergocristinine, ergocorninine, and ergokryptinine. These three alkaloids were well separated from the other nine alkaloids and well separated from one another (Fig. 3).

Resolution of ergocornine from ergokryptine occurred in solvent system *C*, but separation could not be achieved in mixture with the other alkaloids because of the close proximity of ergotaminine, ergosinine, and ergocristine. Solvent system *D*, using Al_2O_3G, gave better resolution of ergocornine and ergokryptine, but again the separation was obscured by ergotaminine, ergosinine, and ergocristine.

Using either solvent system *A* or *B* and SGG, 50 μl. of the mixture of 12 alkaloids was applied to the

TABLE II.—SOLVENT SYSTEMS USED FOR RESOLUTION OF ALKALOIDS FOR QUANTITATIVE DETERMINATIONS

Alkaloid Combination	Solvent Systems
Ergonovine	*A* on SGG
Ergotamine + ergosine	*A* on SGG
Ergometrinine	*A* on SGG
Ergocristine + ergokryptine + ergocornine	*A* on SGG
Ergokryptine + ergocornine	*D* on Al_2O_3G
Ergotaminine + ergosinine	*A* on SGG
Ergocristinine + ergokryptinine + ergocorninine	*C* on Al_2O_3G

TABLE III.—STATISTICAL ANALYSIS OF THE ASSAYS[a]

Alkaloid	a ($\times 10^2$)	b ($\times 10^2$)	b^2 ($\times 10^6$)	n	$\bar{y}$	$\Sigma (x - \bar{x})^2$	s^2 ($\times 10^6$)
Ergonovine	−3.35	14.8	219.0	15	0.262	750	3.85
Ergometrinine	−5.26	14.8	219.0	10	0.243	500	9.63
Ergotamine	−2.40	7.92	62.72	10	0.134	500	3.86
Ergotaminine	−3.25	6.58	43.23	20	0.099	1000	7.77
Ergosine	−2.99	7.50	56.25	10	0.120	500	74.5
Ergosinine	−4.29	6.63	43.96	20	0.090	1000	15.8
Ergocristine	−3.36	7.83	61.31	25	0.123	1250	1.32
Ergocristinine	−3.45	7.31	53.33	25	0.117	1134	12.3
Ergocornine	−5.79	9.23	85.19	10	0.127	500	15.1
Ergocorninine	−1.76	4.73	22.40	25	0.081	1134	20.0
Ergokryptine	−5.40	7.56	57.18	10	0.098	500	13.9
Ergokryptinine	−2.31	6.17	38.08	24	0.104	1116	12.7
Ergocornine + ergokryptine	−5.77	7.93	62.82	25	0.259	5000	77.4

[a] y = Absorbance. x = Concentration (micrograms per milliliter). n = Total number of determinations. s^2 = Variance about the regression.

$$x = \frac{y - a}{b} \qquad \text{(Eq. 1)}$$

$$V(x) = \frac{s^2}{b^2}\left\{\left(\frac{1}{m} + \frac{1}{n}\right) + \left(\frac{Y - \bar{y}}{b}\right)^2 \frac{1}{\Sigma(x - \bar{x})^2}\right\} \qquad \text{(Eq. 2)}$$

where $V(x)$ = variance of a prediction of concentration from a new absorbance value, Y = absorbance of a new sample, and m = number of absorbance determinations of the new sample.

adsorbent in a line 7.5 cm. in length (Figs. 1 and 2). The chromatograms were examined under U.V. light, and the band corresponding to the mixture of ergocornine and ergokryptine was outlined. This area was scraped from the plate, the powder added to a test tube (9.5 × 1.5 cm.), and the alkaloids eluted with two 5-ml. portions of 10% methanol in chloroform. The eluates were filtered, combined in a 50-ml. round-bottomed flask, and the solvent removed under vacuum at 40°, using a Rinco model VE-1000B rotating evaporator.[5] Sufficient 10% methanol in chloroform was added to the flask to give a total volume of 50 μl.

This solution was spotted in the usual manner on Al_2O_3G, and the chromatogram developed with solvent system *D* (Fig. 4). Confirmation of the presence of one or both of these alkaloids was achieved in this manner.

Table I lists the average R_f values of the 12 ergot alkaloids in the various solvent systems. The R_f values of the alkaloids chromatographed as single compounds (individually) are the averages of six determinations. The R_f values determined when a mixture of all 12 alkaloids were chromatographed represent the averages of 36 determinations. Standard deviations were calculated in the usual manner. Reproducibility of the values differ significantly with each development. The order of separation and the resolution of the mixtures is constant, however.

Quantitative Analysis of Alkaloids.—With successful resolution for qualitative identification, the investigations were extended to studies of the resolution and quantitative estimations of 20 to 60 mcg. quantities of each of the 12 alkaloids. Because the ergot alkaloids are easily decomposed by light, the following manipulations were carried out in subdued light.

Solutions of the alkaloids were prepared in the following combinations: ergonovine; ergotamine and ergosine; ergometrinine; ergocristine, ergokryptine, and ergocornine; ergotaminine and ergosinine; and ergocristinine, ergokryptinine, and ergocorninine. Each solution contained 1 mcg./μl. of each alkaloid of the combination. Solutions were spotted on the adsorbents with a 10-μl. pipet to form a band as described previously. Each 10 μl. of solution was restricted in the length of spotting to 1.5 cm. on SGG and to 3.0 cm. on Al_2O_3G. After emptying the pipet, 10 μl. of 10% methanol in chloroform was drawn into it; this rinse was superimposed on the area just spotted. Using the pipet 20, 30, 40, 50, and 60-μl. quantities of the solutions were applied to the chromatograms as bands, varying in length from 3 to 9 cm. on SGG and 6 to 18 cm. on Al_2O_3G. The chromatograms were developed in the appropriate solvent system as shown in Table II. Chromatograms on SGG were developed to within 1 cm. of the top edge of the plate. Better resolution was obtained with chromatograms on Al_2O_3G by permitting continuous development for 120 minutes.

The chromatograms were examined under U.V. light and the bands outlined with a sharp stylus. The adsorbent within an outlined area was scraped from the plates with a razor blade onto glassine paper. The powder was then carefully transferred to a 9.5 × 1.5-cm. centrifuge tube; 2 ml. of a solvent composed of methanol:deionized water:glacial acetic acid (4.5:4.5:1.0) was added to the tube to affect desorption (3).

The solvent-adsorbent mixture was stirred with a Vortex Jr. mixer, model K-500J,[6] during an 8–10-minute period. Four milliliters of *p*-dimethylaminobenzaldehyde T.S. was added, the time noted immediately, and the mixture stirred with a glass stirring rod. The suspension was centrifuged at 4500 r.p.m. for approximately 7 minutes, and the supernatant was then decanted into a 1-cm. quartz spectrophotometer cell. Absorbance was determined 30 minutes after the addition of the *p*-dimethylaminobenzaldehyde T.S. as previously described.

The absorbance values of each alkaloid chromatographed were tabulated in terms of the original concentrations. These data were analyzed by the

[5] Rinco Instrument Co., Inc., Greenville, Ill.

[6] Scientific Industries, Inc., Queens Village, N. Y.

method of least squares, and the variance about the regression was determined (6). The statistical data are given so that concentration and variance for any given absorbance value may be determined by the equations in Table III.

Investigations were undertaken to study the effect on quantitative recovery of rechromatographing the ergokryptine-ergocornine mixture obtained by resolution with solvent system *A*, using solvent system *D* on Al_2O_3G. The earlier studies indicated that quantitative recovery of this mixture from chromatograms developed with solvent system *A* was possible. Quantitative recoveries of the separate alkaloids by chromatography of the mixture with solvent system *D* on Al_2O_3G were also successful. These data are presented in Table III.

Mixtures of several concentrations of ergocristine-ergokryptine-ergocornine were chromatographed with solvent system *A* in the usual manner. The bands corresponding to the ergokryptine-ergocornine mixture were removed, the alkaloids eluted, and the solution concentrated in the manner described previously. The residues were taken up in the appropriate volumes of 10% methanol in chloroform and applied to Al_2O_3G plates as bands. The chromatograms were developed with solvent system *D*. On examination of the chromatograms with U.V. light, several fluorescent bands were revealed. Two corresponded to the alkaloids chromatographed, two to isomers—ergokryptinine and ergocorninine—and at least three bands of unknown composition.

The bands corresponding to ergokryptine and ergocornine were outlined, the adsorbent removed, and the compounds eluted and analyzed in the usual manner. The absorbance values determined for replicate concentrations and between the several concentrations tested were too erratic to make the method useful.

CONCLUSIONS AND SUMMARY

1. The 12 ergot alkaloids investigated can be qualitatively identified in a mixture in concentrations of 2 mcg. by the thin-layer chromatographic procedures outlined.

2. Chromatography of the mixture on SGG using solvent system *A* resolves ergonovine, ergometrinine, ergotamine, ergotaminine, ergosine, ergosinine ergocristine, a mixture of ergokryptine and ergocornine, and a mixture of ergocristinine, ergokryptinine, and ergocorninine.

3. Ergokryptine and ergocornine can be identified by removing the mixture of them from SGG plates developed with solvent *A*, eluting the alkaloids from the adsorbent, and rechromatographing the eluate on Al_2O_3G with solvent system *D*.

4. Chromatography of the mixture on Al_2O_3G using solvent system *C* resolves ergocristinine, ergokryptinine, and ergocorninine from each other and the other alkaloids of the mixture.

5. All of the alkaloids, except for ergocornine and ergokryptine, can be resolved from a mixture of the 12 alkaloids and concentrations of 20 mcg. or more quantitatively recovered.

6. Quantitative recovery of a mixture of ergocornine and ergokryptine from chromatograms developed with solvent system *A* was demonstrated. The separate alkaloids can be resolved and quantitatively recovered from chromatograms developed with solvent system *D*.

7. The quantitative data have been analyzed statistically. The variance of the data about the regression is given so that the relative precision of the different assays is available. The other statistical parameters are presented so that the concentration and its variance may be determined when using the method as an assay procedure.

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Investigation of Drug Release from Solids V

Simultaneous Influence of Adsorption and Viscosity on the Dissolution Rate

By DALE E. WURSTER and GERALD P. POLLI†

The simultaneous influence of several factors including chemical reaction, adsorption, and viscosity on the dissolution rate of a slightly-soluble acidic solid was investigated using bentonite, a montmorillonite clay. Adsorption isotherms, determined by a dialysis procedure, were developed for a benzoic acid and bentonite system. The viscosity of various bentonite and neutralized bentonite suspensions was determined.

THE INFLUENCE OF adsorption on the dissolution rate was shown in a previous report from this laboratory (1). Experimental evidence obtained from a diffusion controlled dissolution process indicated that an adsorbent, when used in adequate amounts, was capable of increasing the dissolution rate obtained under a decreasing concentration gradient to the maximum rate obtained at a constant concentration gradient by removing solute molecules from solution. In the case of the viscosity parameter, the current study showed the dissolution rate for 96% of the total viscosity range studied (1 to 260 cps.) to be a function of the viscosity raised to the -0.5 power. However, a nearly linear relationship was obtained at low viscosities (1 to 5 cps.).

In most pharmaceutical and *in vivo* systems it would not be possible to separate the viscosity influence from that caused by the adsorbent. Therefore, a study of the simultaneous influence of these factors on the dissolution rate was attempted.

EXPERIMENTAL

Apparatus and Procedure.—The apparatus, tablet production, and general experimental procedure for determining the dissolution rate were identical to that used in previous investigations (1, 2).

Adsorption Isotherms.—Adsorption isotherms were determined at 25 ± 1° for benzoic acid by bentonite U.S.P. (3–7) using a dialysis procedure. A 50-ml. quantity of distilled water was added to a 125-ml., wide mouth, ground glass-stoppered container, containing 750 mg. of bentonite. A cellulose bag[1] containing 25 ml. of a standard benzoic acid solution was placed in the bentonite suspension. Control runs using distilled water instead of benzoic acid solutions were also included in order to correct the alkalinity of bentonite. The containers were closed and shaken until equilibrium was reached. Although preliminary studies demonstrated equilibrium after only 4 hours, each flask was shaken for 24 hours. A sample of the clear fluid from inside the dialysis bag was taken for analysis at the end of this time. The amount of benzoic acid in solution was determined by titrating with a standard sodium hydroxide solution under an atmosphere of nitrogen using phenolphthalein as the indicator. The amount of benzoic acid adsorbed by the bentonite was calculated by difference. The amount of benzoic acid adsorbed was verified by assaying the suspension outside the dialysis bag for benzoic acid using a back titration technique.

Dissolution Studies in Bentonite Suspensions.—The bentonite suspensions (2 L. of freshly boiled distilled water plus an accurately weighed quantity of bentonite) were prepared 2 days before using. Agitation of the solvent medium was started 15 minutes before zero dissolution time. This was done to allow any shearing of the suspension by the stirrer to occur before the initial viscosity was measured.

Using an Epprecht rheometer, the viscosity was determined at zero dissolution time and after completion of each run. Bentonite suspensions, at sufficient concentrations, exhibit thixotropic flow. A standard method for measuring viscosity was necessary since various concentrations were to be studied; therefore, the following method was used throughout. The shear rate was increased until the pointer was deflected approximately half scale in order to remove entrapped air; the instrument was then stopped. After 2 minutes, the instrument was again started and the appropriate shear rate selected so that the pointer was deflected approximately half scale. The initial reading was recorded and the bob was allowed to rotate for 30 seconds. All lower shear rates were then measured in rapid succession. This was repeated twice, at 2-minute intervals. Cup and bob "O" were used for all measurements.

The dissolution rates of benzoic acid tablets in bentonite suspensions of increasing concentration were determined as previously described (2).

Dissolution Studies in Neutralized Bentonite Suspensions.—The procedure was the same as given above, except for the following. The alkalinity introduced by the bentonite was eliminated by titrating to pH 7.0 using a standard HCl solution. Twenty-four hours were allowed for the neutralization. The suspensions were brought to their final volume at the end of the neutralization period.

The dissolution rates of benzoic acid tablets in

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Abstracted in part from a dissertation submitted by Gerald P. Polli to the Graduate School, University of Wisconsin, Madison, in partial fulfillment of Doctor of Philosophy degree requirements.
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[1] Visking Co.

neutralized bentonite suspensions of increasing concentration were then determined.

RESULTS AND DISCUSSION

The method of calculating the dissolution rate including the surface-weight and shape-volume factors have been discussed previously (1, 2).

Adsorption Isotherms

Before the adsorption isotherms were determined, several exploratory experiments were carried out. Adsorption of benzoic acid by the cellulose dialysis bag did not occur. This was determined by placing a standard benzoic acid solution in the glass container and distilled water in the dialysis bag and allowing the system to equilibrate. All the benzoic acid was accounted for by assaying the inside and outside solutions. Also, significant amounts of bentonite did not pass through the wall of the dialysis bag. Experimentally, this was determined by gravimetric analysis of the solids content when a dialysis bag containing distilled water was immersed in a bentonite suspension and allowed to come to equilibrium. The osmotic pressure effect was negligible, too. This was determined by measuring the volume of the solution inside the dialysis bag after attaining equilibrium with a bentonite suspension. A significant difference in the volume of the solution was not found.

A typical adsorption isotherm is shown in Fig. 1. The adsorptive capacity ranged from 26.1 to 28.9 mg. of benzoic acid per 750 mg. of bentonite. Thus, while bentonite did adsorb benzoic acid from aqueous solution, the adsorption was only moderate. The adsorption of this relatively small amount was, however, readily reproducible.

Viscosity

The viscosity was determined with a rheogram; the viscosities of various bentonite and neutralized bentonite suspensions are shown in Fig. 2. The average scale reading at each rate of shear was used to determine total τ. The bentonite suspensions exhibited both Newtonian and plastic flow; the tendency for plastic flow increased as the bentonite concentration increased. The yield values ranged from 0 to 1 dyne/cm.^{2}, whereas the neutralized bentonite suspensions gave yield values from 0 to 7.6 dynes/cm.^{2}

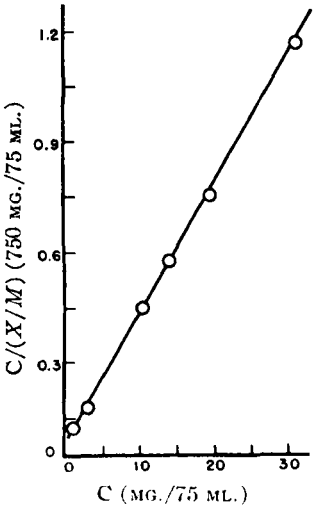


Fig. 1.—Typical adsorption isotherm for benzoic acid by bentonite.

Dissolution Studies in Bentonite and Neutralized Bentonite Suspensions

Chemical Reaction, Adsorption, and Viscosity.—Figure 3 shows the dissolution rate plotted against the concentration of the suspensions for both bentonite and neutralized bentonite. The point where both curves intersect the ordinate is, of course, the dissolution rate in water under conditions of a decreasing concentration gradient. This figure shows that an initial increase, followed by a leveling off, and subsequently, a decline in the dissolution rate occurred. The initial increase in rate in the upper curve is a result of the combined effects of the alkalinity of the bentonite and its adsorbent property; both of these properties result in an increased dissolution rate. The theoretical aspects of these effects are thoroughly treated in previous papers from this laboratory (1, 8). The decrease in rate results from the increase in viscosity. As stated previously, a near linear relationship existed between the decrease in dissolution rate of benzoic acid and the viscosity of methylcellulose solutions varying between 1 to 5 cps. The more rapid decline of the dissolution rate in the case of the neutralized bentonite resulted from the slight increase in viscosity over that of the bentonite suspensions at the same concentration, as shown in Fig. 2. However, the main difference between the two curves in Fig. 3 is due to the alkalinity of bentonite.

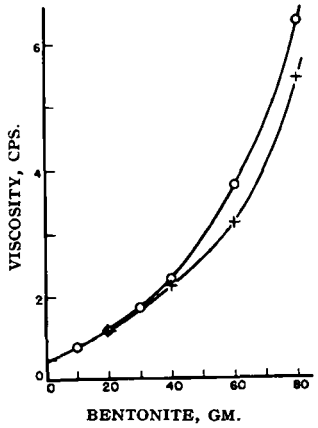


Fig. 2.—Viscosity of bentonite suspension *vs.* concentration of bentonite in 2 L. of distilled water. +, bentonite supensions; O, neutralized bentonite suspensions.

Curves *A* and *B* in Fig. 4 were obtained from the data given in Fig. 3. Here the dissolution rate is plotted as a function of viscosity. The viscosity is that of the corresponding bentonite concentration given in the previous figure. Curve *C* was obtained from the dissolution rate studies in methylcellulose solutions and shows only the effect of viscosity on the dissolution rate. In general, the explanation given for Fig. 3 also explains curves *A* and *B;* however, several other points should be mentioned.

In a previous study (1) it was shown that an adsorbent is capable of increasing the dissolution rate up to a maximum. The maximum is the rate obtained in pure water under such conditions that no appreciable buildup of the solute occurs, and a constant concentration gradient (Nernst-Brünner film theory) is maintained. When the concentration of the solute increases to the point where the concentration gradient decreases significantly, the

dissolution rate is also decreased. However, an adsorbent in adequate concentration is capable of increasing the rate to the maximum by removing solute molecules from solution and thereby maintaining the concentration gradient at the maximum. A further increase in the adsorbent concentration will not increase the dissolution rate above this maximum. This is apparently the explanation for the plateau region in curve *B*. The maximum dissolution rate of benzoic acid in these neutralized bentonite suspensions was 0.0462 Gm./cm.²/hour. This compares favorably to the value of 0.0459 Gm./cm.²/hour obtained with Norite (1) and the rate of 0.0456 Gm./cm.²/hour in water.

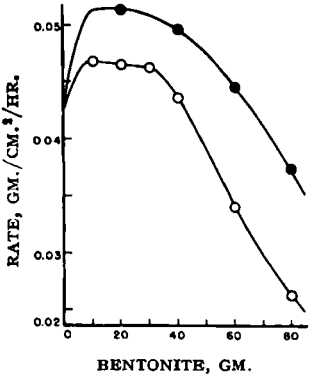


Fig. 3.—Dissolution rate of benzoic acid *vs.* concentration of bentonite in 2 L. of distilled water. Key; ●, bentonite suspensions; O, neutralized bentonite suspensions.

At low viscosities adsorption appeared to be the dominant factor; ultimately the influence of viscosity overcomes this factor. Above 3.5 cps., for the viscosity range studied, curves *B* and *C* are nearly identical. This indicates that viscosity is the controlling factor for these concentrations of neutralized bentonite. This behavior would be expected, theoretically, since a linear relationship between the amount adsorbed and the concentration of the adsorbent would exist. A linear relationship between the dissolution rate and viscosity over the range of 1 to 5 cps. also exists; however, the viscosity increases at a faster rate with an increase in the bentonite concentration.

The difference between curves *A* and *B* can be accounted for by the alkalinity of the untreated bentonite. Also, since the influences of alkalinity and adsorption are superimposed upon the viscosity influence, the dissolution rate of a solid weak acid will be greater in bentonite suspensions than that indicated solely by the viscosity of the medium.

Salt Effect.—In the case of neutralized bentonite suspensions another possible effect on the dissolution rate should be mentioned. This is the salt effect. Again it was shown previously (2) that the dissolution rate decreased in a linear manner as the equilibrium concentration decreased. The latter concentration decreases as the concentration of various salts increases and thus decreases the water concentration (1, 8). For a suspension containing 80 Gm. of bentonite in 2 L. and neutralized with HCl, the amount of salt formed in terms of NaCl was approximately 7×10^{-3} moles/L. On the basis of the above-mentioned experiments this salt concentration would not cause a significant change in the dissolution rate of the benzoic acid. For example, a 0.154 *M*/L. concentration of NaCl caused the dissolution rate under the existing conditions to change from 0.0426 to 0.0414 Gm./cm.²/hour (8).

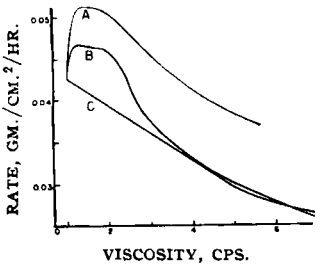


Fig. 4.—Simultaneous influence of chemical reaction, adsorption, and/or viscosity on dissolution rate. Key: A, chemical reaction, adsorption, and viscosity; B, adsorption and viscosity; C, viscosity.

Viscosity.—The measured viscosity of all bentonite suspensions was essentially the same after the dissolution experiment as before except for the neutralized suspensions containing 60 and 80 Gm. of bentonite. For these suspensions the final viscosity was 0.9 and 2.3 cps. higher, respectively, than the viscosity at zero dissolution time. In these two cases the average viscosity was used.

Change in pH.—The pH of the suspensions following the dissolution experiments varied with the bentonite concentration, the pH being higher for those suspensions of greater concentration. The final pH varied from 4.6 to 7.3 over the range of bentonite concentrations studied (10 to 80 Gm./2 L.). The increased pH was due to both increased chemical reaction and adsorption as the bentonite concentration increased.

The pH of the neutralized bentonite suspensions was followed as a function of dissolution time. The results indicated that neutralized bentonite had little or no buffer capacity toward benzoic acid. The final pH varied from 4.0 to 4.6 for the range of neutralized bentonite concentrations studied (10 to 80 Gm./2 L.). The increased pH was due to the increased adsorption of benzoic acid as the bentonite concentration increased which then resulted in a decrease in the equilibrium concentration of the acid.

SUMMARY

Using bentonite U.S.P., a montmorillonite clay, the simultaneous influence of several factors on the dissolution rate of compressed benzoic acid tablets was investigated.

The adsorption isotherms, determined by a dialysis procedure, showed bentonite to have a moderate and reproducible adsorption capacity for benzoic acid.

When the dissolution rate was determined in bentonite suspensions, the controlling factors were characterized as chemical reaction, adsorption, and viscosity; when the dissolution rate was determined in neutralized bentonite suspensions, only adsorption and viscosity were the controlling factors.

For both bentonite and neutralized bentonite suspensions, the dissolution rate increased, leveled off, and finally decreased as the concentration of bentonite increased.

In methylcellulose solutions of various concentrations where a correction was made for the increased solubility of benzoic acid in this medium, viscosity was made the only variable affecting the rate of solution. A linear relationship between the rate

and the viscosity was found for viscosities of 1 to 5 cps. The dissolution rate was a function of the viscosity raised to the −0.5 power for the rest of the viscosity range studied (1 to 260 cps.).

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(6) *Ibid.*, p. 34.
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Technical Articles

Continuous Production of Tablet Granulations in a Fluidized Bed I

Theory and Design Considerations

By MORTON W. SCOTT*, HERBERT A. LIEBERMAN, ALBERT S. RANKELL, and JOSEPH V. BATTISTA

A single step process for the production of tablet granulations on a continuous basis is described. The process is based on the use of a modified fluidized bed dryer. Important considerations in the design and operation of the equipment are derived from material and energy balances and from heat and mass transfer relationships. The report illustrates the usefulness of the pharmaceutical engineering approach in the analysis of new process developments.

FLUIDIZATION TECHNIQUES have been used in various process industries for over 20 years (1, 2). The unit operation has found applications in areas as diverse as roasting processes (3), refining uranium ores (4), gasification of carbon (5), and ion exchange procedures (6). Fluidized bed drying has been studied extensively (7–9). The usefulness of fluidization techniques for the drying of tablet granulations was also discussed recently (10); rapid rates of drying and reduced operating costs were some of the advantages reported for the process.

Procedures for coating particulate solids in fluidized beds have been developed by Wurster (11) and applied to a variety of pharmaceutical products. When the coating agents have adhesive qualities, the technique also can be used for agglomeration and for the preparation of tablet granulations (12). The Wurster apparatus operates in a batchwise fashion and thus finds major usefulness when numerous small batches of different formulations must be processed.

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* Present address: Maradel Products, Inc., New York, N. Y.

The present report introduces a new fluidization process capable of high-volume production of tablet granulations on a continuous basis. The fundamental pharmaceutical engineering relationships which have been applied successfully in designing and operating the fluidized bed granulator are developed in this report. A brief discussion of conventional batch and continuous fluidization processes is presented as background material. An accompanying report reviews the process performance levels and product characteristics obtained in batchwise and continuous operation of the equipment (13).

The analysis of the fluidized bed granulation

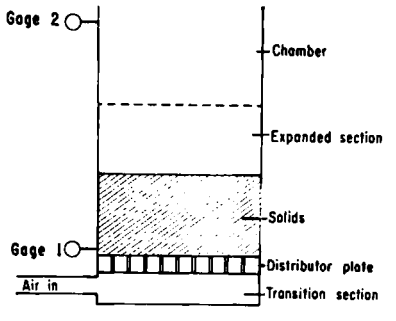


Fig. 1. —Schematic drawing of a simple fluidized bed.

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The analysis of the fluidized bed granulation

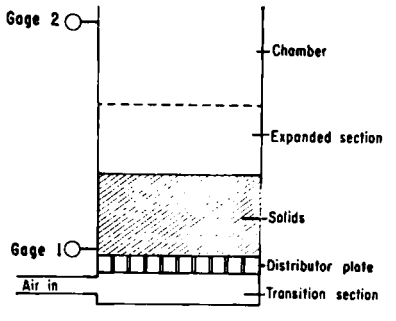


Fig. 1. —Schematic drawing of a simple fluidized bed.

process is derived from the statements of the material balance and the heat and mass transfer requirements of the system. The value of this method of attack in defining the interrelationships of process variables has not been illustrated previously in the pharmaceutical literature. However, the approach is not limited to the specific fluidization system described here, and will be applied to film coating operations and other pharmaceutical processes in subsequent reports.

SIMPLE FLUIDIZATION

Figure 1 is a schematic drawing of a simple fluidized bed. Three essential components are involved: (*a*) a vertical chamber for containing the solids to be fluidized, (*b*) a source of air (such as a blower), and (*c*) an air distributor device for obtaining the proper velocity profile for the inlet air. The air distributor may be a perforated plate, a fine mesh screen, or a honeycomb plate, etc., (1). A short transition section may be included beneath the distributor.

The unit is operated by charging the feed solids to the fluidization chamber and admitting an increasing flow of air through the distributor plate. When correct flow conditions are established, fluidization of the solids will occur. At this point, the bed of solids expands appreciably and has an appearance similar to a boiling fluid. Pressure readings generally are taken at gage locations 1 and 2 (Fig. 1) during the operation in order to follow the progress of fluidization.

Figure 2 is a generalized diagram of the relationship between pressure drop (between gages 1 and 2) and the air velocity in the bed. At low flow rates, air passes between the particles of solid without causing motion. As the velocity of air is increased, the pressure drop ($P_1 - P_2$) rises until eventually it equals the force of gravity on the particles. This occurs at point A, and the particles begin to move. Increasing the air velocity to point B causes the particles to separate, and the bed expands. Point B represents the start of true fluidization. Much of the air passing through the fluidized bed at this stage is in the form of "bubbles" and "pockets" containing little or no solids. In the bed itself, the solids move in more or less distinct aggregates (14). Air velocities higher than that at point B result in further bed expansion with little increase in the pressure drop. Turbulence and overall bed turnover, however, become more marked. At point C, the bed height equals the height of the container and pneumatic transport occurs.

When heated air is used for fluidization, high heat transfer rates are obtainable because of the turbulence and efficient mixing occurring in the bed. The advantages of the fluidized bed dryers now commercially available for drying tablet granulations stem from these considerations (10). In well designed systems, heat transfer between the inlet air and the fluidized solids may be complete within less than one quarter of an inch above the air distributor plate (15). Therefore, in drying applications, the temperature of the bulk solids may be kept relatively low regardless of the inlet air temperatures used.

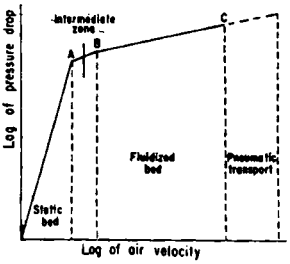


Fig. 2.—Influence of air velocity on pressure drop in fluidized beds.

CONTINUOUS FLUIDIZATION SYSTEMS

The batch equipment shown in Fig. 1 may be converted to a continuous system by the simple addition of a feed pipe and an outlet pipe. A dust collector may be added to catch any entrained solids in the exit air stream. This type of equipment is shown schematically in Fig. 3. Other continuous systems have been illustrated in the literature (1, 2).

When the rate of adding solids to the fluidized bed equals the rate of withdrawal, no net accumulation or loss of solids will occur. Therefore, the weight of solids in the bed remains constant. Since the pressure drop across the bed is determined by the weight of solids it contains (1), pressure drop values can be used as a convenient indicator of bed inventory. In continuous operations, feed rates and/or product withdrawal rates are controlled and adjusted in accordance with the pressure drop across the bed.

CONTINUOUS GRANULATION IN FLUIDIZED BEDS

A system for rapid production of tablet granulations can be designed around the basic components of the continuous fluidized bed unit shown in Fig. 3. For this purpose, an atomizing nozzle is installed in the fluidization chamber to apply a spray of liquid binding agent onto the fluidized solids. Heated inlet air, as employed in the fluidized bed dryers, is used to fluidize the solids and for simultaneous evaporation of the atomized solvent.

The flow of materials to and from such equipment is shown in the material balance diagram in Fig. 4. Relationships between the various flow streams can be developed readily and can be used to uncover a number of important operating and design criteria.

The following primary material balances apply when an aqueous granulating solution (containing no active ingredient) is used:

for air $$A_i = A_o \qquad \text{(Eq. 1)}$$

for solids $$S_i + CG_i = P_o \qquad \text{(Eq. 2)}$$

for active ingredients $$X_iS_i = X_oP_o \qquad \text{(Eq. 3)}$$

for moisture $$M_iS_i + H_iA_i + (1 - C)G_i = H_oA_o + M_oP_o \qquad \text{(Eq. 4)}$$

where A = air flow rate (dry basis), Kg. per minute; C = concentration of solids in granulating liquid, Kg. per Kg.; G = granulating liquid flow rate, Kg. per minute; H = absolute humidity, Kg. water per Kg. dry air; M = moisture content of solids (dry basis), Kg. per Kg.; P = product removal rate (dry basis), Kg. per minute; S = solids feed rate (dry basis), Kg. per minute; X = concentration of active ingredient

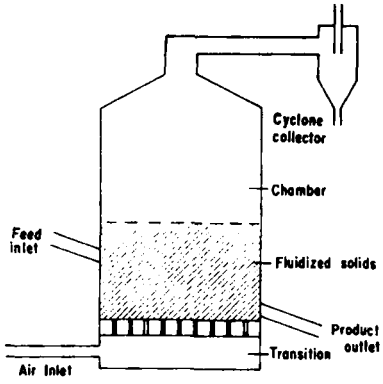


Fig. 3.—Continuous fluidized bed.

(assay value on a dry basis), Kg. per Kg.; i = inlet; and o = outlet.

Maintenance of Product Assay.—Equation 2 shows that solids derived from the liquid granulating agent are included in the granulated product. To attain a given product assay value (X_o), the concentration of active ingredient in the powder feed cannot be made equal to that desired in the product, but must be adjusted in accordance with Eq. 3. The relationship between the desired product assay and the feed concentration for various flow rates and concentrations of the granulating agent is established by substitution of Eq. 2 into Eq. 3

$$X_i = X_o \left[1 + \frac{CG_i}{S_i}\right] \qquad \text{(Eq. 5)}$$

For the special case when water is used as the granulating liquid, C equals 0, and X_i will equal X_o. No adjustment in feed assay, therefore, would be required. Similarly, it can be shown that modifications in feed assay would not be necessary when a slurry of the feed solids (in a volatile solvent) is used as the granulating agent.[1] However, for all other cases Eq. 5 establishes the changes required in feed concentration in order to achieve desired product assays.

Influence of Entrainment Losses.—Equations 1–5 assume no loss of solids in the effluent air stream, A_o. In general practice, however, some entrainment losses do occur from fluidized beds (1, 2). Extensions of the material balance relationships can be developed, as shown below, to predict the influence of these losses on product assay. The need for adequate dust collection systems thereby can be established as an early design requirement.

When entrainment losses occur, the material balance for solids and for the active ingredients are given by Eqs. 6 and 7, respectively

$$\text{for solids} \qquad S_i + CG_i = P_o + L \qquad \text{(Eq. 6)}$$

for active ingredients

$$X_iS_i = X_oP_o + X_LL \qquad \text{(Eq. 7)}$$

where L = entrainment losses, Kg. per minute; and X_L = concentration of active ingredient in entrainment solids, Kg. per Kg.

[1] For this case, Eq. 3 must be modified to account for the addition of active ingredient to the system by the granulating agent. Thus

$$X_iS_i + X_iCG_i = X_oP_o \qquad \text{(Eq. 3A)}$$

Substitution of the value for P_o from Eq. 2 into Eq. 3A establishes that X_i will equal X_o.

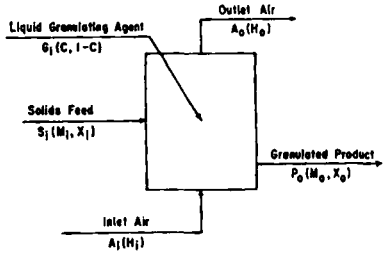


Fig. 4.—Material balance diagram for fluidized bed granulator. Key: A = air flow rate, dry basis, Kg. per hour; H = absolute humidity, Kg. (H_2O) per Kg. (dry air); S = solids feed rate, dry basis, Kg. per hour; P = product exit rate, dry basis, Kg. per hour; M = moisture content of solids, dry basis, Kg. (H_2O) per Kg. (dry solids); X = concentration of active ingredient, Kg. (A.I.) per Kg. (dry solids); G = liquid feed rate, Kg. per hour; C = concentration of granulating agent, Kg. (solids) per Kg. (liquid); i = inlet conditions; o = outlet conditions.

Rearrangement of Eq. 7 after substitution for P_o as defined in Eq. 6 yields the desired relationship between entrainment losses and product assay

$$X_i = X_o \left[1 + \frac{CG_i}{S_i}\right] + [X_L - X_o]\frac{L}{S_i} \qquad \text{(Eq. 8)}$$

For the special case in which the losses consist only of product granules ($X_L = X_o$), the last term of Eq. 8 vanishes, and the relationship reduced to Eq. 5. Under these conditions, the entrainment loss, L, does not influence the feed concentration, X_i, required to achieve a given product assay.

When the entrained solids consist of only unprocessed feed powder, X_L will equal X_i, and Eq. 8 can be simplified

$$X_i = \frac{X_o}{S_i - L}(S_i + CG_i - L) \qquad \text{(Eq. 9)}$$

For this condition, Eq. 9 shows that the magnitude of the solids loss will alter final product assays. The necessary adjustments in feed concentration required to maintain a desired product assay can be calculated from Eq. 9, however, providing that reliable estimates of the losses are available.

Equations 5 and 9 represent the limiting cases. In normal operation, it is likely that the losses from the fluidized bed granulator will contain varying proportions of feed powder together with finished granules and partially processed materials. Since each type of loss exerts a different effect on product assay, it is apparent that adequate control of these losses must be a prerequisite for consistent production of granules meeting assay specifications. This in turn suggests that efficient cyclone collectors (or other dust collection units), preferably operating with continuous recycling of the elutriated solids to the fluidized bed, should become an early design consideration.

Allowable Liquid Flow Rates.—The relationship between the flow rates of liquid granulating agent and inlet air is obtained by substituting the value of A_o (from Eq. 1) and P_o (from Eq. 2) into Eq. 4. Rearrangement of terms leads to

$$G_i = K[A_i(H_o - H_i) - S_i(M_i - M_o)] \qquad \text{(Eq. 10)}$$

where $K = \dfrac{1}{1 - C - M_oC}$.

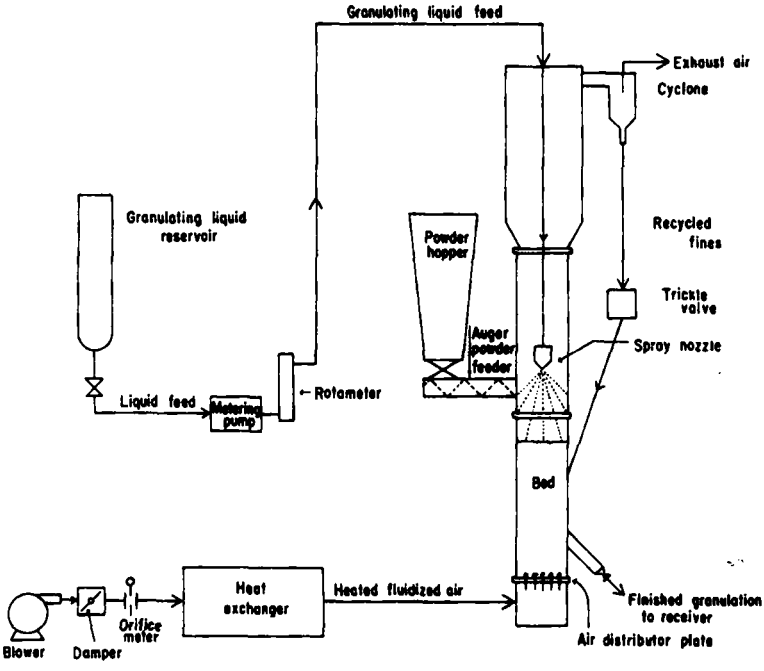


Fig. 5.—Fluidized bed granulator.

Certain simplifying restrictions can be imposed on Eq. 10 as a result of process requirements. For example, the moisture content of the product, M_o, will be fixed by the required flow and compression characteristics for the granulated material. Generally, the moisture content of the raw materials will be close to that of the product granules, and M_i will approximate M_o. Under these conditions the last term of Eq. 10 disappears and the relation reduces to

$$G_i = KA_i(H_o - H_i) \qquad \text{(Eq. 11)}$$

For maintenance of steady state conditions, Eq. 11 shows that the allowable granulating liquid feed rate varies directly with the inlet air flow rate. Therefore, increasing the air flow rate will permit operation with a higher liquid rate giving, in turn, an overall increase in the production rate.

Influence of Outlet Air Temperature and Humidity.—Equation 11 shows that permissible liquid flow rates will be directly proportional to the difference in moisture content between the outlet and inlet air. However, for any outlet air temperature, the outlet moisture content, H_o, cannot exceed that obtained in saturated air at that temperature. If liquid flow rates, G_i, exceed those established by Eq. 11 in process, material can be expected to become increasingly overwet. This condition may not be fully detected by inlet and outlet humidity measurements alone. When the outlet air is partially saturated (less than 100% R.H.), a fluctuation in liquid or air flow rate will be reflected in the outlet humidity level. Instrumentation for these psychrometric measurements, therefore, should be provided in the design of the granulating unit.

For optimum thermal efficiency, it is desirable to raise the level of the moisture in the outgoing air as high as possible. However, when the outlet air is saturated, condensation of water in the dust collection systems (cyclones, etc.) will occur readily. The relative humidity of the outlet air must be kept under 100% to avoid this condensation.

High outlet air temperatures also would appear desirable in increasing thermal efficiency since the moisture content of saturated air (100% R.H.) increases with increasing temperature. The thermal stability characteristics of the product impose a practical limitation on the outlet temperature, since the temperature of the solids will be close to the outlet air temperature (7) under good fluidization.

In the absence of other data, however, this temperature need be no lower than that selected for drying the product by conventional tray drying techniques.

Thermal Energy Balances.—Simplified thermal energy balances can be established as shown in Eq. 12 for the fluidized bed granulator[2]

$$A_{iw}C_{pa}(T_{ai} - T_{ao}) = \lambda G_i(1 - C) + S_iC_{ps}(T_{so} - T_{si}) + E_L \qquad \text{(Eq. 12)}$$

where A_{iw} = air flow rate (wet basis),[3] Kg. per minute; C_{pa} = humid heat capacity of air, Kcal. per Kg. per °C.; C_{ps} = heat capacity of solids, Kcal. per Kg. per °C.; T = temperature, °C.; λ = heat of vaporization of water, Kcal. per Kg.; C = concentration of solids in granulating liquid, Kg. per Kg.; G_i = flow rate of granulating liquid, Kg. per minute; S_i = solids feed rate, Kg. per minute; E_L = thermal energy losses, Kcal. per minute; a = air; s = solid; i = incoming conditions; and o = outgoing conditions.

The thermal energy losses, E_L, in Eq. 12 primarily arise as a result of radiation and convection heat transfer from the fluidization chamber. These losses will be negligible when the outlet air temperature is maintained close to the ambient temperature.

[2] This equation neglects the energy requirements for heating the granulating solids and the vaporized granulating liquid to the outlet temperatures. Heats of solution and wetting also are neglected.

[3] Air flow rate on a wet basis is

$$A_{iw} = A_i + H_iA_i \qquad \text{(Eq. 12A)}$$

Similarly, the term $C_{ps}(T_{so} - T_{si})$, representing losses associated with the heating up of the solids, becomes negligible when the outlet temperature is held near the incoming feed temperature. Under these conditions, the evaporation process in the fluidized bed granulator occurs adiabatically and will be similar to that found in an adiabatic humidifier or spray dryer. The process can be defined and traced more or less completely on psychrometric charts (16).

When air flow rate (A_i), concentration of granulating agent (C), and product temperature (T_{so} equal to T_{ao}) are fixed, Eq. 11 shows that maximum liquid flow rates will be directly proportional to the inlet air temperature.

Increasing the inlet air temperature, therefore, represents a convenient and predictable method for increasing production rates.

Rates of Evaporation and Drying.—The foregoing analysis has concerned the heat and material balance requirements. Analyses of some of the factors governing the rates of evaporation in a fluidized bed granulator are also useful in setting design and operating criteria.

For maintenance of steady state conditions, the rate of evaporation must equal the rate of addition of liquid granulating agent. The rate of evaporation is

$$R_e = A_i(H_o - H_i) \qquad \text{(Eq. 13)}$$

where R_e = rate of evaporation, Kg. water per minute.

The required relationship is shown in Eq. 14 and is obtained by rearrangement of Eq. 10 and combination with Eq. 13

$$R_e = \frac{G_i}{K} + S_i(M_i - M_e) \qquad \text{(Eq. 14)}$$

When the liquid flow rate exceeds the rate of evaporation, overwetting of the product will occur. If sufficient evaporative capacity is available in the incoming air, this condition will be evidenced by rising outlet air temperatures. Excessive rates of evaporation will not be detectable by a corresponding decrease in air temperature but will be reflected by a decrease in product moisture content. Measurements of outlet air temperature and product moisture content, therefore, can serve as convenient indices of equipment performance; suitable instrumentation should be provided for these measurements.

The rate of evaporation of the atomized granulating liquid can be put in general form (15)

$$R_e = \frac{h\,S(T_{ai} - T_d)}{\lambda} \qquad \text{(Eq. 15)}$$

where R_e = rate of evaporation in Kg. (of water) per minute; h = overall heat transfer coefficient, Kcal. per minute per °C. per cm.^{2} surface area; S = total surface area of droplets created by atomization of liquid granulating agent, cm.^{2}; T_{ai} = temperature of inlet air, °C.; T_d = temperature at surface of droplet, °C.; and λ = heat of vaporization of water, Kcal. per Kg.

Equation 15 shows that an increase in the surface area of the liquid droplets will increase the rate of evaporation. This can be accomplished by reducing the average droplet particle size in the spray. Factors which improve the degree of atomization (such as decreasing the liquid feed rate, decreasing the liquid viscosity,[4] or increasing the nozzle pressure) will improve the rate of evaporation. Measurements of the flow rate, temperature, and pressure of the liquid (and air pressure, if pneumatic nozzles are used) will be useful in controlling rates of evaporation.

Equation 15 shows further that increased rates of evaporation are obtainable by increasing the inlet air temperature. Since the heat transfer coefficient, h, improves with high air velocities (14), increased rates of evaporation also can be expected with increasing air flow rates.

Factors Related to Fluidization Quality.—Under proper fluidizing conditions, the solids in the bed undergo efficient mixing, and little or no segregation will occur (1, 2). All particles will tend to move in more or less random directions and have equal opportunity to enter the zone near the top where the concentration of droplets of granulating agent will be highest. Some "short circuiting" between the solids inlet and outlet positions may be expected, but this will be minimized in the well fluidized bed. Proper fluidization is evidenced by the "surging" in the manometer lines which results from the formation and collapse of air bubbles in the bed (1, 2). Uniform temperatures will also be observed throughout the bed when good fluidization has been achieved.

Bed expansion will occur with increasing inlet air velocities. Under these conditions, the momentum of the particles will increase, and increased degrees of particle attrition may result. Bed losses will also increase with increasing air velocity; however, improved collector efficiency may compensate for these effects.

Empirical correlations are available for first estimates of required air velocities for fluidization. The selection of optimum velocities is best determined by experimental tests and will represent a compromise between bed loss, attrition effects, collector efficiency, and heat transfer considerations. Once the air velocity has been selected, the maximum value of liquid flow rate, G_i, can be determined (for any given inlet air temperature) from the previous equations.

Possible Mechanism for Agglomeration.—The exact mechanisms by which size enlargement and agglomeration can occur in the fluidized bed granulator are difficult to establish on the basis of theoretical considerations alone. It is probable that small granules will be formed when two or more solid particles impinge with a droplet of liquid granulating agent. As the solvent evaporates, these particles will be cemented together. Further size enlargement may occur when these small granules impinge with other liquid droplets and particles. Rates of agglomeration can be expected to be influenced by the probability of droplet-particle collision. This will in turn be governed by bed height, droplet and particle, size and size distribution, rate of evaporation, and nozzle position among other factors.

For a given nozzle operating at a fixed position and with fixed inlet air conditions to the granulator,

[4] This may be accomplished by increasing the temperature of the granulating liquid or decreasing its solids content.

the length of the spray cone will determine the number of liquid droplets entering the fluidized bed. Lowering the nozzle height will increase the probability of droplet-particle contacts. An increase in droplet size will also improve this probability. Elevating the nozzle high above the bed can be expected to result in premature evaporation of the granulating solvent. This will lead to the formation of spray dried particles of the granulating agent, and little agglomeration of the feed solids will occur. Finally, control of the length and angle of the nozzle spray cone will be required so that the walls of the fluidized bed are not wetted by the liquid granulating agent.

CONTINUOUS FLUIDIZED BED GRANULATOR

Figure 5 is a schematic diagram of the continuous fluidized bed granulator. The system was developed from a commercially available fluidized bed reactor[5] which was suitably modified and instrumented for granulating operations in line with the preceding theoretical analyses.

The fluidization section of the unit consists of a 12 in. diameter, vertical, steel pipe with an expansion section at the top. The overall height of the unit is approximately 15 ft.

Air is supplied to the fluidized bed by a blower fitted with an adjustable control damper on its outlet. A calibrated orifice meter to measure the air flow rate is installed in the air line past the blower. The pressure drop across the orifice is measured by appropriate manometers.

The fluidizing air is heated to the desired temperature by passage through a steam heat exchanger. The temperature of the air leaving the exchanger is controlled by adjustment of inlet steam conditions. The heated air then passes through an air distributor plate into the bottom of the bed.

The solids to be granulated are fed into the bed from a powder hopper discharging into a screw conveyor. The inlet for the feed is located below the expansion section.

Air leaving the fluidized bed passes through a cyclone collector which removes the entrained solids. These solids are returned automatically to the fluidization section through a one-way trickle valve and connecting pipe inlet. All lines carrying solids are set at sharp angles with the vertical to insure good flow.

Liquid granulating agent is sprayed onto the fluidized solids through a pneumatic atomizing nozzle. The nozzle enters the unit from the top and is aimed in a downward direction. The liquid granulating agent is fed to this nozzle by a calibrated pump. The flow rate is measured by a calibrated rotameter installed in the liquid feed line.

Granulated product is removed near the bottom of the bed through an outlet pipe located slightly above the distributor plate; the rate of product removal is controlled by adjustment of an outlet valve.

Manometer taps are located directly above the distributor plate and at the approximate midpoint of the fluidization section. Pressure drop values between these points are obtained from respective manometer readings and are used to determine the weight of solids in the fluidized bed; additional manometer taps are located below the distributor plate and near the top of the unit. Thermocouples are placed below and above the distributor plate and at three additional elevations within the fluidized bed granulator.

The relative humidities of the ambient and outlet air are obtained with a sling-type psychrometer held near the blower inlet and the cyclone exhaust; the relative humidity of the heated air entering the fluidized bed is obtained by tracing the heating process in the steam heat exchanger on appropriate psychrometric charts.

[5] Fluo-Solids Reactor, Dorr-Oliver, Inc., Stamford, Conn.

FEASIBILITY STUDY

An introductory experiment with the fluidized bed granulator was completed to demonstrate the feasibility of the process. For this run, a 30-Kg. preblend of the components of a standard tablet formulation was used as the solid charge to the unit. The liquid granulating agent was syrup U.S.P. and was sprayed into the bed at a rate of 7.5 L. per hour. The fluidizing air velocity was set at approximately 73 c.f.m. with an inlet air temperature of approximately 240° F. Under these conditions a bed temperature of approximately 100° F. was maintained. The relative humidity of the outlet air was close to 100%. The agglomerated product obtained after approximately 40 minutes of operation showed an increase in average particle size from approximately 75 μ (feed) to approximately 275 μ (product) and was dry. This experiment established the feasibility of the process for agglomerating operations. Further, the operating conditions (outlet humidity, bed temperature, air temperature) obtained in this run were in accord with those predicted from the equations established in the previous sections of this report.

Further experiments have been completed to confirm and extend the value of this system in the continuous, high-speed production of tablet granulations. These studies are presented in an accompanying report (13).

SUMMARY AND CONCLUSIONS

A fluidized bed granulator has been designed and constructed. The unit allows continuous addition of powder feed and granulating liquid and continuous removal of dry granulated product.

The basic design criteria were developed by analysis of material and energy balances and consideration of rates of heat and mass transfer. The equations developed by this approach were used to predict the relationships among the important process variables.

This report also is intended to illustrate the value of applying pharmaceutical engineering techniques in the analysis of both new and conventional processes in pharmacy.

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Continuous Production of Tablet Granulations in a Fluidized Bed II

Operation and Performance of Equipment

By ALBERT S. RANKELL, MORTON W. SCOTT*, HERBERT A. LIEBERMAN, FRANK S. CHOW, and J. V. BATTISTA

Data collected from batch runs in a 1-ft. diameter fluidized bed granulator were employed in preliminary evaluation of process performance. Moisture content, granule screen analysis, and compressibility tests were used to evaluate the granulated product. Process variables such as powder and liquid feed rates, inlet air temperature, and nozzle location influenced the characteristics of the product. Losses from the unit were related to cyclone collector efficiency. Data obtained from replicate continuous runs under selected operating conditions illustrated the ability of the process to maintain product uniformity from run to run. Finished granulations from the continuous runs were evaluated on a rotary press. Tablets made from these granulations conformed to tablets made from identical ingredients granulated by conventional techniques.

SIGNIFICANT ADVANCES have been made recently in the development of direct compression techniques for the production of tablets. However, until the physical and chemical principles of compression are more fully understood, wet granulation techniques will be required for numerous formulations.

New procedures for preparing tablet granulations have been reported in the recent literature. These techniques include a method for vapor phase granulation (1) and an air suspension granulation method (2); procedures for preparing granulations in coating pans have also been described (3, 4).

An accompanying report (5) describes the theory and design of a continuous fluidized bed granulation technique. The present report represents an extension of this study, and is an evaluation of the performance of a pilot model fluidized bed granulator, including a study of process variables. The apparatus used has been adapted from a fluidized bed dryer (of the type commonly used in the chemical process industry)[1] which was specifically modified for the production of tablet granulations. The unit is adaptable for batch processing, but is particularly useful in the continuous granulating of large volumes of raw materials.

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* Present address: Maradel Products, Inc., New York, N. Y.

EXPERIMENTAL

Materials.—The product granulated in these experiments was an antacid mixture based primarily on aluminum hydroxide. A preblend of all formula components (except lubricants) was used as the feed powder. The particle size of the feed was less than 200 mesh (74 μ); loss on drying (L.O.D.) was approximately 5%.

The feed powder contained sucrose. To maintain constant assay levels in the granulated product, the concentration of sucrose in the feed was varied from run to run as required by the changes in the sucrose content of the granulating liquid. The granulating agents included in this study were water, diluted syrup (43% w/v), simple syrup (85% w/v), and a 10% w/v aqueous gelatin solution. A mixture of water soluble dyes (FD&C Red No. 2 plus FD&C Red No. 4) was dissolved in the granulating liquid (syrup) in one experiment.

Equipment.—The general design of the fluidized bed granulator used in these studies was discussed

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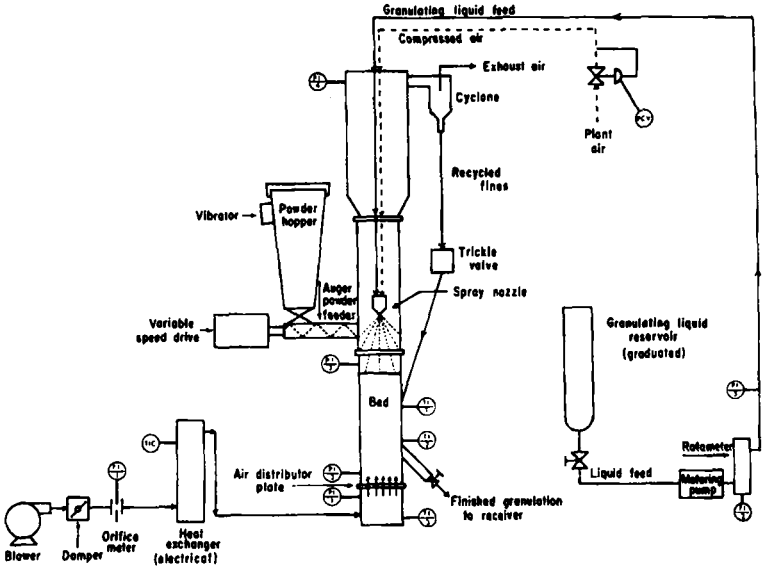


Fig. 1.—Diagram of fluidized bed granulator. FI = flow indicator; PCV = pressure control valve; PI = pressure indicator; TI = temperature indicator; TIC = temperature indicator controller.

in a previous report (5); a schematic diagram of the equipment is shown in Fig. 1. The granulator consists of a 12-in. diam. column (expanded to 18 in. at the top) with provision for the introduction of heated air and the feed and removal of solid materials. Fine particles entrained in the exhaust air are removed in a cyclone collector and are recycled to the bed through a trickle valve.

A pneumatic spray nozzle for atomizing the granulating liquid is vertically positioned within the fluidization section. The spray is directed in a downward direction, and the vertical position of the nozzle can be changed. The feed powder is supplied to the fluidized bed by an auger type feeder with a variable rate of rotation. The feeder is modified to form a positive gas check against blow back of fluidizing air by insertion of a rubber-lined butterfly valve between the feed hopper and the auger, and the addition of a removable airtight cover to the top of the hopper; a vibrator is attached to the hopper to prevent bridging of the powder.

Instrumentation.—Fluidizing air flow rate is controlled by a butterfly valve damper and measured by an orifice meter (FI-1) [designations refer to components in Fig. 1]. The air temperature is set by a thermostat (TIC) on the electric air heater and measured by a thermocouple below the air distributor plate (TI-3).

Bed temperature is measured by two thermocouples (TI-1 and TI-2) placed at one-third and two-thirds the estimated bed height. The actual bed height is determined by the pressure drop across the bed (PI-2 minus PI-3) The pressure at the top of the expansion section (PI-4) is compared to PI-3 to determine if the bed height has gone above the PI-3 location.

TABLE I.—POWDER COLLECTION

Air Flow Rate, c.f.m.	Efficiency at Different Air Flow Rates: Product Entrainment, Kg./hr.	Cyclone Efficiency, %	Losses, %
37	8.0	42.5	7.3
73	8.8	73.3	5.3
107	19.6	91.5	3.7

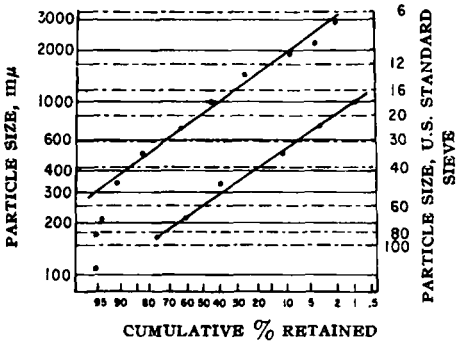


Fig. 2.—Comparison of particle size distribution of a typical fluidized bed granulation (O—O) and a conventional wet granulated product (●—●).

The granulating liquid flow rate is controlled by a gear type metering pump and is measured by a rotameter (FI-2). The total quantity delivered is measured by reading the liquid level in the reservoir. The degree of atomization of the liquid spray is determined by the pressure in the compressed air line (PCV).

A pressure gage (PI-5) in the granulating liquid line between the metering pump and the nozzle gives early warning of nozzle clogging. Clogging in the fluidizing air distributor plate is indicated by increasing pressure drop across the plate (PI-1 minus PI-2).

Procedure for Batch Runs.—Batchwise operation of the equipment was employed for the study of several important process variables such as air flow rate, inlet air temperature, liquid flow, residence time, and composition of granulating agent. For these runs 30 Kg. of feed powder was used as charge; this was added to the bed after air flow rate and inlet air temperatures were stabilized. Atomization of the granulating agent then was started. Thereafter, samples of product and instrument readings were taken at 10–20-minute intervals until the run was completed (90 minutes or more). At the end of the run, the product was removed by opening the

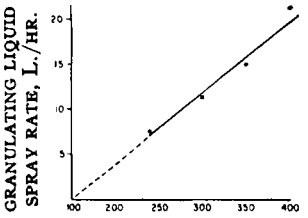


Fig. 3.—Relationship of granulating liquid spray rate to inlet air temperature to maintain bed temperature at 100–110° F.

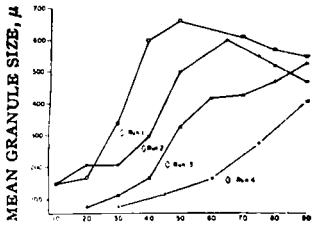


Fig. 4.—Influence of granulating liquid spray rate on mean granule size. Granulating liquid flow rates: run 1, 21.4 L./hr; run 2, 15.0 L./hr.; run 3, 11.2 L./hr; run 4, 7.5 L./hr.

bottom outlet port and allowing the bed to flow into a receiving vessel.

Procedure for Continuous Runs.—In the continuous process, 30 Kg. of granulation (obtained from a previous run) was used as a starting bed in order to shorten the time required to reach steady state conditions. Powder feed and liquid granulating agent then were added continuously to the bed for the entire run. The bottom outlet valve was partially open to allow product removal. The valve position was periodically adjusted to maintain a constant bed height (indicated by a constant pressure drop across the bed).

All batch and continuous experiments in this study were run at a bed temperature of 100–110°F. The flow rate of granulating liquid was adjusted from run to run to maintain this temperature. Complete material balances were established for each run.

Sampling and Evaluation Procedures.—Samples of approximately 250 Gm. of product were taken at short periodic intervals throughout each run. These samples were generally obtained through the outlet port at the bottom of the fluidized bed. In several experiments additional samples were taken from an auxiliary upper port. Particle size analysis was performed on all samples by sieving through a nest of U. S. standard sieves (Nos. 6, 12, 16, 20, 30, 40, 60, 80, 100, and 200) on a Cenco-Meinzer sieve shaker at setting 4 for 10 minutes. The particle size distribution data so obtained were plotted on logarithmic probability coordinates. The size corresponding to 50% on the cumulative percentage axis was taken as the geometric mean diameter. Moisture content on all samples was determined with a Cenco moisture balance set at 90 v. to constant L.O.D. Assays for alumina were performed on representative samples from the continuous runs.

After blending with lubricant, the compression characteristics of the product prepared in each run were tested on both a single punch tablet press (Stokes model E) and a rotary press (Stokes model RD-3). Tablets compressed from the fluidized bed granulation were compared to the same product prepared by the conventional wet granulation method. Compression characteristics (absence of capping, sticking, binding, and picking) as well as tablet characteristics such as hardness, thickness,

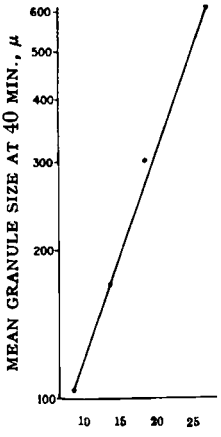


Fig. 5.—Correlation of mean granule size with granulating liquid spray rate during constant agglomeration rate period.

weight variation, and friability were the comparison criteria.

DISCUSSION OF BATCH OPERATIONS

Preliminary Evaluation.—As a starting point for these studies, batch experiments were conducted to establish optimum velocities for the fluidizing air. These experiments were run using 30 Kg. of powder feed. Liquid granulating agent was not added to the bed during the tests.

Three air flow rates of 37, 73, and 107 c.f.m were studied. Adequate fluidization was obtained at each level, but significant differences in entrainment and cyclone efficiencies were discernible, as summarized in Table I. Cyclone collection efficiency was best at the highest air velocity (107 c.f.m.), but the entrainment of solids in the effluent air stream was more than double that obtained at the lower velocities. Since this velocity would result in a large inventory of recirculating material, with increased opportunities for attrition, it was rejected. At the lowest air velocity (37 c.f.m.), excessive losses were obtained as a result of poor cyclone efficiency. Heat and mass transfer also was expected to be influenced adversely by decreasng the air velocity to this level. On this basis the intermediate fluidizing air velocity (73 c.f.m.) was established for all further runs.

The batchwise granulating operation was studied using an inlet air temperature of 235 ±5°F. and a 30-Kg. bed of feed powder. Syrup was used as the granulating agent and was sprayed into the bed at a rate of 7.5 L. per hour. Under these conditions, approximately 90 minutes was required for formation of granules with satisfactory tablet compression characteristics. The particle size distribution of such a typical batch product is illustrated in Fig. 2. It will be observed that the fluidized bed granulation has a smaller average particle size and higher degree of particle size uniformity than that obtained by the conventional wet granulation process.

The minimum bed temperature, which was obtainable with the given ambient air conditions (70°F. and 40% R.H.), was approximately 98°F., the dew point temperature, as determined from psychrometric charts. This indicated that the outlet air was close to saturation when the bed temperature was maintained, as previously stated, at 105 ±5°F.

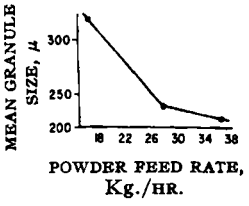


Fig. 6.—Relationship of powder feed rate to mean granule size.

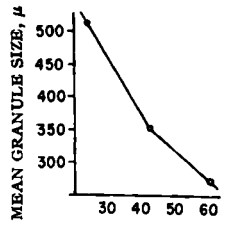


Fig. 7.—Influence of nozzle position on mean granule size.

In one experiment, the air flow rate was reduced 15%. Inlet air temperature and liquid flow rates were maintained at their previous levels. The temperature of the bed was maintained at 105°F., but the product granules were moist to the touch and not acceptable for tablet compression. The insufficient degree of drying attained in this experiment suggests that the process is essentially adiabatic with outlet air at saturation.

Influence of Inlet Air Temperature.—Experiments were conducted using various inlet air temperatures. For each run the flow rate of liquid granulating agent was adjusted to maintain a constant bed temperature of 105 ±5°F. and a constant product L.O.D. of 5%. The results of this study are shown in Fig. 3 and indicate that allowable liquid flow rates are directly proportional to the inlet air temperature. The curve shows an intercept value of 100°F. when extrapolated to the inlet air temperature axis. This value agrees with that predicted by theory—when the inlet air temperature is the same as the bed temperature, no latent heat of vaporization of the granulating liquid is needed.

Influence of Granulating Liquid Flow Rate.—The change in mean granule size with increasing residence time in the fluidized bed for different flow rates of granulating liquid is shown in Fig. 4. The data indicate that the size tends towards a maximum, regardless of the granulating liquid flow rate. This may indicate that at a certain point the rate of agglomeration is offset by attrition effects.

The data in Fig. 4 show that the rate of agglomeration is increased as the granulating liquid flow rate is raised. This is shown more clearly in Fig. 5 which correlates the mean granule size after 40 minutes residence time as a function of granulating liquid flow rate.

Influence of Composition of Granulating Liquid.—Water, diluted syrup, and a 10% aqueous gelatin solution also were tested as granulating agents. The flow rate of these agents was adjusted so that

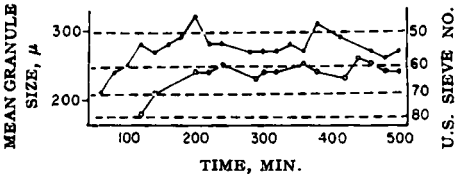


Fig. 8.—Uniformity of product granule size during continuous runs. Key: —●—●—●—, run 1; —○—○—○—, run 2.

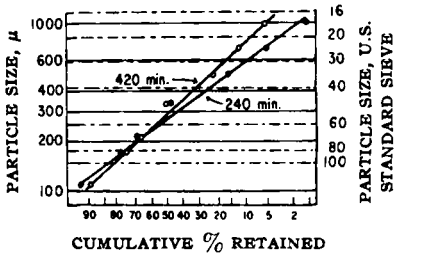


Fig. 9.—Uniformity of granule size distribution during continuous run.

the rate of addition of water to the bed was equal to that obtained when granulating with simple syrup. When water was used as the granulating liquid, no perceptible agglomeration occurred. Some increase in particle size was produced with the use of diluted syrup as the granulating material. Under the conditions of the test, however, granules of adequate size for compression could not be obtained even after 5 hours of processing. Properly sized granules were formed when the gelatin solution was used. The moisture content of the resulting product was higher than that required for successful compression. Since the total water added in these tests was equal to that used successfully in previous tests with simple syrup, the poor results suggest that heat transfer rates were probably too low in the run using gelatin solution.

DISCUSSION OF CONTINUOUS OPERATIONS

Influence of Powder Feed Rate.—Conditions for continuous operation of the fluidized bed were: inlet air temperature at 240°F., liquid feed rate (simple syrup) at 7.5 L. per hour, and bed temperature at 105 ± 5°F.

Figure 6 shows the relationship between the rate of powder feed and the mean granule size of product produced in the continuous runs. An increase in the powder feed rate resulted in a decreased mean particle size. This would be expected considering the feed powder dilution effects alone. The mean granule size obtained at any feed rate was constant over long periods of operation, suggesting that new steady state conditions of operation are reached for each powder feed rate.

Influence of Nozzle Location.—The mean granule size of product under constant feed rate and temperature conditions could be altered merely by changing the location of the liquid spray nozzle. This is shown in Fig. 7. The largest mean granule size was obtained when the nozzle was at its lowest test position—23 in. above the air distributor plate. At this point the nozzle was actually in the bed.

Process Evaluation.—The process was further evaluated by continuous granulation runs of 8 or more hours duration. Figure 8 shows the mean granule size of samples taken at periodic intervals during two such continuous runs. The ability of the process to produce a product with closely controlled mean granule size is apparent. The slight difference in particle size between the runs can be attributed to an increase (1.9 Kg./hour) in the powder feed rate in run 2 compared to run 1. Figure 9 compares the particle size distribution curves for representative samples taken at 3-hour intervals during the run. The similarity in the slopes of these lines indicates that the particle size distribution and the mean granule size were closely controlled.

Uniformity of product also was indicated by alumina assay for several runs; the values obtained were all within 7% of the theoretical value (after correction for losses).[2]

The rate of granulation was approximately 40 lb. per hour. It is believed that the rate of granulation can be increased to 120 lb. per hour by increasing the inlet air temperature and granulating liquid spray rate, thus allowing a decreased residence time.

Evaluation of Batch and Continuous Process Products.—Granulations from batch and continuous runs compressed without difficulties. The tablets produced from these granulations were identical in appearance to those prepared from conventional granulations. Thickness, weight, hardness, and friability of the tablets made from the fluidized bed granulation also were identical to the wet granulated product.

Addition of Color.—The suitability of the process for preparing a colored granulation was tested by granulating with dye dissolved in the granulating liquid. The granules produced showed a high degree of color uniformity and compressed into tablets with better color distribution than those prepared from conventional granulations.

SUMMARY AND CONCLUSIONS

The results reported in this paper clearly illustrate several advantages for preparing tablet granulations by the fluidized bed method. The equipment described is capable of both batch and continuous operation. The granulation produced by either technique has a high degree of particle size uniformity and a controllable moisture content. Tablets prepared from the fluidized bed granulation meet or exceed all criteria established for tablets prepared from granules conventionally prepared.

Granule size and production rate of the granulated product can be controlled by adjusting each or all of the following process variables: (*a*) powder and liquid feed rates, (*b*) inlet temperature, and (*c*) lowering the position of the spray nozzle. Since each of these are readily adjustable in the pilot size fluidized bed granulator, this unit is judged suitable for small-scale production of granulations as well as for use in scale-up procedures. The experimental results are in direct agreement with those previously derived from theoretical considerations.

The materials granulated by this process are subjected to temperatures only slightly above room temperature. As illustrated in this report, processing can be done at bed temperatures of only 100–110°F. Thus, the fluidized bed granulator appears to have particular usefulness for granulating heat sensitive materials.

The fluidized bed granulator combines into one process several of the individual steps normally required in wet or dry granulating methods. The ability of the apparatus to produce granules with controlled size, either by batch or continuous processing, eliminates the need for any grinding operations conventionally required. Moisture content of the final product can be closely controlled, eliminating the need for separate drying procedures. In batch operations pre- and post-blending may be accomplished in the fluidized bed, eliminating the need for additional mixing equipment.

[2] Low collection efficiencies (77%) were obtained in the continuous runs described above. The loss of powder reflected by this inefficiency could be corrected by the use of auxiliary bag collectors or multiple cyclones.

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Comparative Neurotoxicity and Antipentylenetetrazol Activity of Some Piperidinediones

By GREGORY B. FINK and MONT R. JUCHAU

A new piperidinedione, DAPD, has been compared with four other piperidinediones—glutethimide, amino-glutethimide, methyprylon, and bemegride—and also phenobarbital. With two techniques that measured neurotoxicity and pentylenetetrazol seizure threshold, the onset, peak, and duration of activity of each drug was plotted. DAPD and glutethimide reached peak activity by 5 minutes and exhibited the same neurotoxicity. DAPD had the shortest duration of action and produced the least increase in seizure threshold of the piperidinediones studied. Amino-glutethimide resembled phenobarbital with a slow onset but a long duration of activity. Methyprylon and bemegride reached peak activity by 2.5 minutes; the former increased seizure threshold, whereas the latter was the only agent to decrease seizure threshold. Bemegride was the most toxic drug and amino-glutethimide the least toxic.

SEVERAL USEFUL therapeutic agents have been found in the piperidinedione series. Glutethimide[1] and methyprylon[2] are sedatives and hypnotics, amino-glutethimide[3] is an anticonvulsant, and bemegride[4] is an analeptic. Our interest in these compounds was prompted by the synthesis of a new piperidinedione (1), 3-(N,N-diethylamino)-3-phenyl-2,6-piperidinedione, hereafter called DAPD. To compare the pharmacology of DAPD with that of closely related agents, a comprehensive comparative pharmacologic study of several piperidinediones has been initiated in our laboratories. This report deals with the onset and duration of activity, the neurotoxicity, and the antipentylenetetrazol activity of DAPD, four piperidinediones, and one barbiturate. The structural formulas of the agents investigated are shown in Fig. 1.

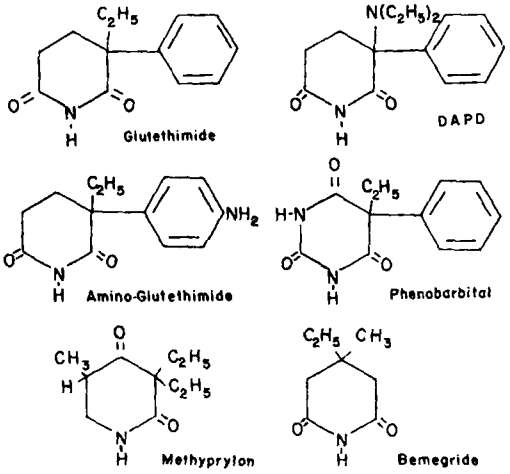


Fig. 1.—Structures of the compounds investigated. Glutethimide (3-ethyl-3-phenyl-2,6-piperidinedione), DAPD [3-(N,N-diethylamino)-3-phenyl-2,6-piperidinedione], amino-glutethimide (3-ethyl-3-(*p*-aminophenyl)-2,6-piperidinedione), phenobarbital (5-ethyl-5-phenyl-barbituric acid), methyprylon (3,3-diethyl-5-methyl-2,4-piperidinedione), and bemegride (4-ethyl-4-methyl-2,6-piperidinedione).

METHODS

The experimental animals were adult male albino mice (CF No. 1) obtained from the Carworth Farms. The drugs investigated (listed in Fig. 1) were given by the oral route as suspensions in 1% methylcellulose solution. Activity-duration curves were determined for each drug by two techniques that measured neurotoxicity and pentylenetetrazol seizure threshold. The neurotoxicity test was based on muscular incoordination; mice that could not remain for 1 minute on a horizontal rod (diameter, 2.5 cm.) rotating at 6 r.p.m. were considered toxic. The mice were tested for neurotoxicity at 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes and thereafter at hourly intervals until no mice were toxic. Groups of six to 12 mice were given various doses of the drugs until at least three points were established in the range between 0 and 100% neurotoxicity at the indicated time intervals. The dose of each drug required to produce toxicity in 50% of mice (TD_{50}) at the indicated times were determined and 95% confidence limits calculated by the method of Litchfield and Wilcoxon (2). The second technique measured seizure threshold. Pentylenetetrazol[5] was used as the convulsant stimulus; seizure threshold was measured by the intravenous infusion technique of Orloff, *et al.* (3), as modified by MacQuarrie and Fingl (4). The mice were randomly divided into groups of ten or more and given equivalent doses ($^1/_2$ of the TD_{50}) of the drugs to be tested, except

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[1] Marketed as Doriden by Ciba Pharmaceutical Products, Inc., Summit, N. J.
[2] Marketed as Noludar by Hoffmann-La Roche, Inc., Nutley, N. J.
[3] Marketed as Elipten by Ciba Pharmaceutical Products, Inc., Summit, N. J.
[4] Marketed as Megimide by Abbott Laboratories, North Chicago, Ill.
[5] Marketed as Metrazol by the Knoll Pharmaceutical Co., Orange, N. J.

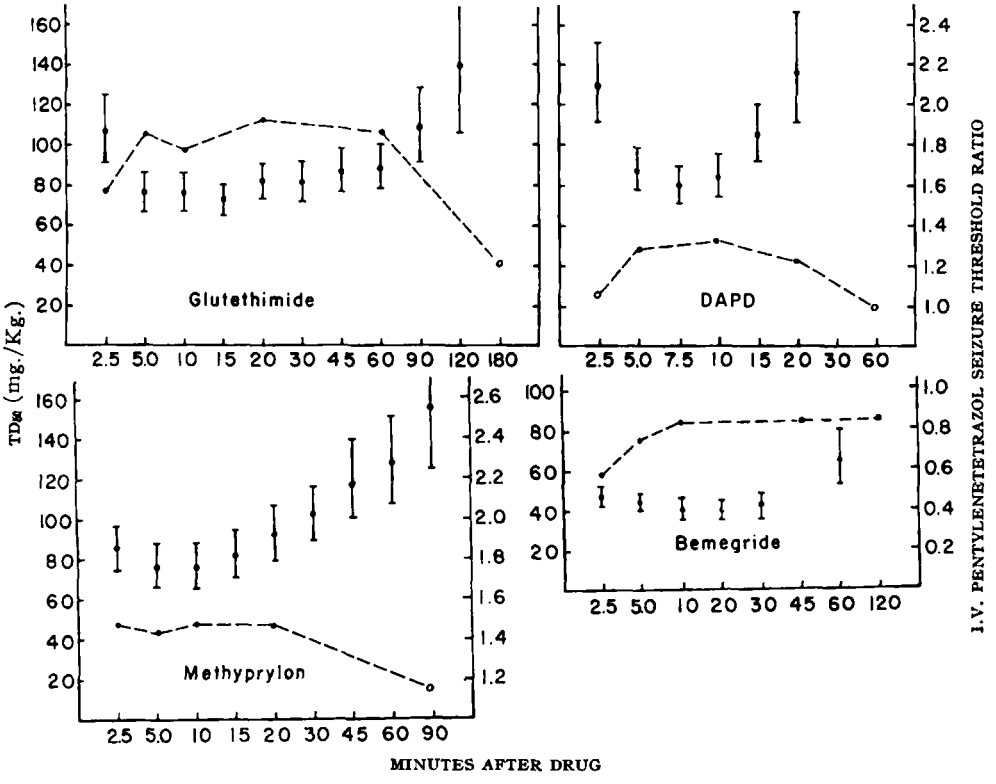


Fig. 2.—Neurotoxicity (TD_{50}) of some piperidinediones and their effect on intravenous pentylenetetrazol seizure threshold (PST) in mice. The vertical bracketed lines represent 95% confidence limits. Solid symbols at a PST ratio (mean threshold of drug-treated mice/mean threshold of control mice) indicate a significant change ($p < 0.05$) from 1.0, normal threshold. The PST ratios were determined at the indicated times after oral administration of 40 mg./Kg. of glutethimide, DAPD, and methylprylon, and after 20 mg./Kg. of bemegride.

that one of the groups was given the requisite volume of vehicle only and served as the control. At selected times after oral drug administration seizure thresholds were determined and the results expressed as threshold ratios (mean threshold of drug-treated group/mean threshold of concurrently tested control group).

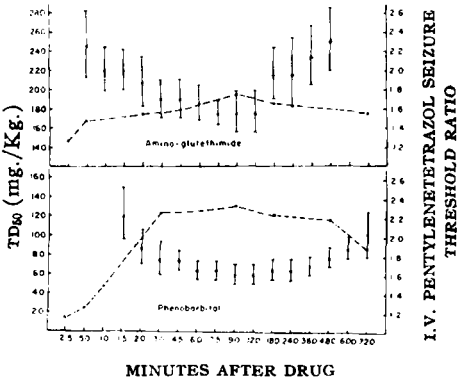


Fig. 3.—Neurotoxicity (TD_{50}) of amino-glutethimide and phenobarbital and their effect on intravenous pentylenetetrazol seizure threshold (PST) in mice. The vertical bracketed lines represent 95% confidence limits. Solid symbols at a PST ratio (mean threshold of drug-treated mice/mean threshold of control mice) indicate a significant change ($p < 0.05$) from 1.0, the normal threshold. The PST ratios were determined at the indicated times after oral administration of 90 mg./Kg. of amino-glutethimide and 30 mg./Kg. of phenobarbital.

RESULTS

The onset of action, time of peak activity, and duration of activity of the six drugs investigated are illustrated in Figs. 2 and 3. Glutethimide reached peak toxicity within 5 minutes, maintained this activity about 1 hour, then toxicity began to wane. The pentylenetetrazol seizure threshold (PST) ratio was 1.84 at 5 minutes, 1.88 at 60 minutes, but not significantly different from 1.0 at 3 hours. DAPD, in a similar manner, reached peak toxicity within 5 minutes, but toxicity was decreasing within 15 minutes. PST ratios were 1.05 at 2.5 minutes, 1.29 at 5 minutes, 1.31 at 10 minutes, 1.22 at 20 minutes, and 0.99 at 60 minutes. The peak activity of methyprylon, however, was reached by 2.5 minutes, but was waning by 30 minutes. PST ratios were 1.48 at 2.5 minutes, 1.47 at 20 minutes, but not significantly different from 1.0 at 90 minutes. Bemegride also reached peak activity by 2.5 minutes. This was the only compound which significantly lowered PST ratios to less than 1.0. It was difficult to delineate the complete toxicity curve of bemegride since its TD_{50} (40 mg./Kg.) approached the dose that was

lethal for 50% of the mice (80 mg./Kg.). Amino-glutethimide and phenobarbital took longer to reach peak activity—approximately 30 minutes. Both drugs exhibited antipentylenetetrazol activity before toxicity was evident. Although both drugs exhibited little neurotoxicity after 12 hours, they still exhibited significant PST ratios.

The peak TD_{50}s in mg./Kg. (with 95% confidence limits) of the six agents arranged in ascending order were: bemegride 40 (36–45), phenobarbital 60 (50–72), glutethimide 74 (67–81), methyprylon 77 (67–88), DAPD 80 (72–89), and amino-glutethimide 175 (164–187). The maximum PST ratios of the compounds in ascending order were: DAPD, 1.31; methyprylon, 1.49; amino-glutethimide, 1.75; glutethimide, 1.92; and phenobarbital, 2.31. Bemegride, a stimulant drug, lowered seizure threshold and exhibited PST ratios less than 1.0.

DISCUSSION

The minimal overt evidence of neurotoxicity (muscular incoordination) following the oral administration of five piperidinediones and one barbiturate to mice has been utilized to plot comparative activity-duration curves of these agents. In addition, intravenous pentylenetetrazol seizure threshold (PST) ratios were determined at selected times after drug administration and another set of activity-duration curves of the six drugs plotted. The PST ratios were determined at dose levels representing one-half of the TD_{50} of each compound; none of the mice at this dosage level of the six agents exhibited neurotoxicity. In general, the data obtained by the two techniques showed good correlation. Thus the time of onset and duration of activity, the TD_{50}, and the effect on PST was delineated for each compound.

Glutethimide, DAPD, and amino-glutethimide are closely related piperidinediones whose structural formulas resemble that of phenobarbital (Fig. 1). The only difference between glutethimide and phenobarbital is that a CH_2-CH_2 group in the piperidine ring of the former compound has replaced a NH-CO group in the pyrimidine ring of the latter. The substitution of a diethylamino group for an ethyl group of glutethimide produces DAPD and the addition of an amino group on phenyl ring of glutethimide produces amino-glutethimide. These four compounds, however, varied considerably in onset and duration of action and in their effects on PST.

It is probably best to compare DAPD with glutethimide. Although both drugs reached peak activity within 5 minutes, DAPD was active only a few minutes, while glutethimide was active more than 1 hour. DAPD appeared to have less depressant properties than glutethimide and appeared to excite the mice during the time of peak activity. Furthermore, DAPD had less antipentylenetetrazol activity and only raised seizure threshold 27% (based on an average of the PST ratios obtained during peak activity), whereas glutethimide raised seizure threshold 86%. Both compounds exhibited the same TD_{50}—about 80 mg./Kg.

Amino-glutethimide had a slower onset of action than glutethimide and DAPD but also had a much longer duration of action. In this respect it resembled phenobarbital more than the other piperidinediones. It was intermediate between DAPD and glutethimide in antipentylenetetrazol activity since it increased seizure threshold an average 66%. The high TD_{50}, 175 mg./Kg., exhibited by amino-glutethimide may indicate that this drug has less sedative properties than glutethimide. Clinical studies have confirmed its comparative lack of sedative-hypnotic effects (5–8).

Two other piperidinediones studied, methyprylon and bemegride, exhibited a very rapid onset of action after oral administration. Both compounds reached peak activity within 2.5 minutes by both techniques. Methyprylon, a sedative-hypnotic, increased seizure threshold; whereas bemegride, an analeptic agent, added to the convulsant effect of pentylenetetrazol and decreased the seizure threshold. Methyprylon exhibited the same TD_{50} as glutethimide and DAPD, 80 mg./Kg. Bemegride, with a TD_{50} of 40 mg./Kg., was the most toxic piperidinedione studied. Phenobarbital had a slower onset and a longer duration of activity and also greater antipentylenetetrazol activity than the five piperidinediones studied.

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Molecular Weight Determination in Aqueous and Nonaqueous Solvents I. Instrumentation

By FRANK M. GOYAN and RICHARD D. JOHNSON

The apparatus previously used by Goyan and Johnson to study colligative properties has been improved. Provisions have been made for introducing and removing the sample without disturbing the closed system, and techniques have been developed for using aqueous and nonaqueous solvents interchangeably with the same equipment. The results presented show improvement in rapidity of operation and reliability. Circuitry is basically the same but has been somewhat improved.

THE THERMOELECTRIC METHOD for measuring colligative properties of solutes is becoming increasingly popular for the routine determination of molecular weights (1–3). Modern instrumentation favors the use of thermistors instead of thermocouples for these measurements. Recent studies by the authors (4) suggested a new development in this field which permits the addition of sample solutions without direct observation and without opening the equilibrium chamber. Provision is also made for withdrawing old samples, and the instrument is designed so that only glass and platinum come in contact with solvents or solutions.

The results show that the new instrument makes possible more rapid and accurate determinations than previously. Water and organic solvents may be used with a minimum of difficulty in changing from one solvent to another.

EXPERIMENTAL

The circuitry and method of measurement used were described under *Phase II* in the previous paper (4). A 6-v. battery was used throughout to power the bridge, and the switches were always arranged in order to utilize the reference thermistor. The only significant improvement in the circuitry was the substitution of a proportional temperature control device[1] for the on-off control previously described; it was modified in order to use a thermistor probe which was inserted in the Dewar flask used as a water bath. All measurements were made at 25.00°. Commercially available chemicals of reagent or equivalent quality were used without purification; ratio-arm settings were 10,000 ohms.

The essential new feature of the instrument is shown in Fig. 1. It consists of two parts—the thermistor holder and the equilibrium chamber. The thermistor holder is one piece of glass made by sealing a tapered capillary tube to the upper part of the outer tube of the holder with a ring seal. This capillary is prepared before the sealing operation so that the tip is small enough to fit alongside the thermistor in the narrow lower part of the holder. The upper part of the capillary where the ring seal is made is a shaped part of the tube from which the capillary was drawn; the shaping is accomplished with a carbon rod sharpened in a pencil sharpener before blowing the bulb needed for the seal. The tapered tip of a special medicine dropper is ground into this socket after the glass blowing is finished. The thermistor is inserted from the bottom after threading its insulated leads through the outer tube and the upper opening. An open spiral of platinum wire (0.013 in. in diameter) is wound around the lower part of the thermistor and allowed to extend slightly below the end; the tip of the capillary is arranged to deliver liquid along the side of the thermistor just above the platinum spiral where it is drawn into the spiral by capillary action. The spiral and capillary are self-cleaning either with solvent or by rinsing with the next solution to be used. A 5-ml. sample is adequate for several determinations; 0.5 ml. would be sufficient if the spiral were first washed with solvent, dried, and

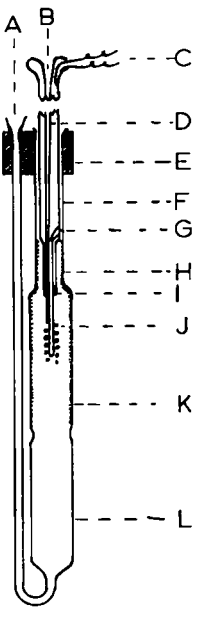


Fig. 1.—Thermistor chamber and holder. Key: A = ground-glass socket to receive ground-glass tip of medicine dropper used for removing waste liquid; B = same for adding sample through inner capillary; C = electric leads from thermistor; D = inner capillary, terminating in tip just above coils on thermistor; E = stopper for holding chamber in water bath; F = narrow portion of chamber; G = wide portion of thermistor holder selected to fit part F; H = platinum gauze for holding solvent and supporting thermistor holder; I = narrow portion of thermistor holder showing dental amalgam seal of thermistor and capillary in holder; J = platinum wire (0.013 in. in diam.) coiled in an open spiral from the tip of the filling capillary to beyond the tip of the thermistor; K = platinum gauze for holding solvent by capillary action; L = lower part of chamber for isolating waste from gauze. The figure is drawn to approximate scale. The overall length to the top of the stopper is 18 cm.

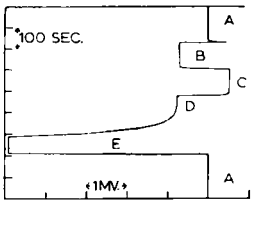


Fig. 2.—Tracing of a typical recording Key: A = switch open; B = final equilibrium; C = displacement produced by changing the decade resistance box by 100 ohms; D = first indication of equilibrium; E = start.

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Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1963.

[1] Electron-O-Therm Sr., model 148, Technical Equipment Co., Walnut Creek, Calif.

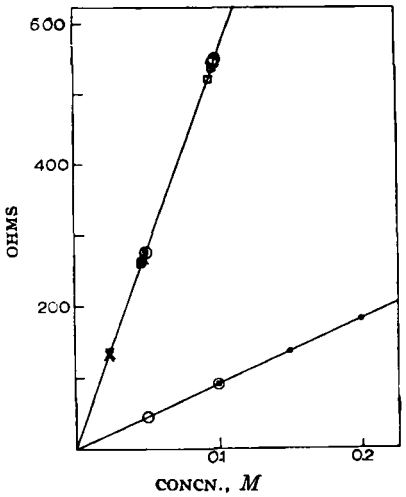


Fig. 3.—Calibration chart. The steeper line represents readings with benzene. Key: ● = benzil and sucrose; O = diphenyl and dextrose; X = anthracene; □ = naphthalene.

allowed to come to temperature equilibrium in the chamber.

The equilibrium chamber is made with an enlarged portion to provide free fall of drops from the thermistor. A smaller upper portion is just large enough to accept the thermistor holder. In making this chamber, platinum gauze (three wires per millimeter) is fitted to the inside of the upper part of the large tube before sealing the lower end; it is held in place by a constriction made after the gauze is inserted. The gauze lining the lower part of the smaller tube is inserted after the glass blowing is finished and serves to check the downward slipping of the thermistor holder at a point which places the tip of the thermistor near the geometrical center of the larger cylinder of gauze.

In use, a sample is drawn into the special medicine dropper which fits the upper ground-glass attachment to the capillary. The sample is forced through the capillary and excess allowed to drop to the bottom of the equilibrium chamber. The excess can be withdrawn through the small tube (Fig. 1) which also terminates with a ground-glass connection to a different medicine dropper used to withdraw liquid that accumulates in the bottom of the chamber. While these operations are performed, all parts of the apparatus below the stopper are in the Dewar flask covered with water. Inspection of this operation is seldom necessary.

RESULTS

Figure 2 shows the results obtained in a typical case. Millivolt readings are converted to ohm readings by the method described earlier (4). Equilibrium is attained in 400 seconds and in half that time significant readings can be taken.

TABLE I.—RELATIVE RESISTANCES OF THE THERMISTOR[a]

Substance	Concn., *M*	Ohms
Benzil (in benzene)	0.100	554
	0.050	277
	0.025	138
Naphthalene (in benzene)	0.100	538, 523
	0.050	265, 262
Anthracene (in benzene)	0.050	270, 270
	0.025	137, 135
Diphenyl (in benzene)	0.100	550, 546
	0.050	277, 277
Sucrose (in water)	0.200	184
	0.150	138
	0.100	92
Dextrose (in water)	0.100	92
	0.050	46

[a] Readings subtracted from solvent readings.

TABLE II.—RESULTS OF MOLECULAR WEIGHT CALCULATIONS[a]

Substance	*M* Concn. (graph)	Mol. wt. (calcd.)	% Error
Naphthalene, mol. wt. = 128.2	0.0975	131.2	2.3
	0.0950	134.5	4.9
	0.0481	133.0	3.7
	0.0475	134.5	4.9
Anthracene, mol. wt. = 178.2	0.0488	182.0	2.1
	0.0488	182.0	2.1
	0.0248	179.0	0.4
	0.0245	182.0	2.1
Diphenyl, mol. wt. = 154.2	0.0994	155.2	0.6
	0.0988	156.0	1.2
	0.0500	154.2	0.0
	0.0500	154.2	0.0
Dextrose, mol. wt. = 180.2	0.1000	180.2	0.0
	0.0500	180.2	0.0

[a] Solvents given in Table I.

Figure 3 and Table I show the results obtained with two different solvents—water and benzene. Table II shows these results converted to molecular weights; the required calibration was established with benzil in benzene and sucrose in water. In Table II, the column headed—*Concn. (graph)* represents the molar concentration of the reference material corresponding to the reading obtained. In Table I, *Concn. M* refers to the nominal molar concentration of each solution weighed out on the basis of the theoretical molecular weight. The calculated molecular weight shown in Table II is simply weight per liter (known from weight and volume) divided by moles per liter read from the calibration chart.

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Effect of the Trifluoromethyl Group on Biological Activity of Certain Arylmercurials

By M. TIN MAUNG, J. J. LAGOWSKI, F. P. COSGROVE, and J. N. DELGADO

The effect of the trifluoromethyl group on the biological properties of four new isomeric (trifluoromethyl) phenylmercuric chlorides was evaluated with a representative strain of *Micrococcus pyogenes* (var. *aureus*) using the Vincent paper disk modification of the Oxford cup method. This extension of the chemical investigations was motivated by numerous reports in the literature which indicate that there are certain relationships between the physico-chemical properties of organo-metallic compounds and their respective biological activities. The data given reveal some interesting relationships between the chemical structure and antibacterial activity among some of these new organo-mercury compounds. Results with the above method of evaluation and concentrations of 1:1,000 indicate that *ortho* and *para* (trifluoromethyl) phenylmercuric chlorides are more effective than the corresponding *meta* compound. The bis (trifluoromethyl) mercury compound and the *meta* (trifluoromethyl) phenylmercuric chloride showed activities comparable to that of phenylmercuric chloride.

THIS PRELIMINARY EVALUATION of the antibacterial activity of some new isomeric (trifluoromethyl) phenylmercuric chlorides was conducted as a part of the general investigation of the electronic effect of the trifluoromethyl group on the properties of certain organo-mercury compounds (1). This extension of the chemical study was motivated by numerous reports in the literature which indicate that certain relationships between the physico-chemical properties of organo-metallic compounds and their respective biological activities (2) exist.

Inorganic mercury compounds such as mercuric chloride, mercury oxycyanide, and potassium tetraiodomercurate have long been known as antiseptic agents. The chief objections to their therapeutic use are their toxicity and irritating action upon tissue (3).

Various theories for the mechanism of action of these mercurials have been proposed (3). Fildes (4) suggested that the antibacterial activity of mercurials is due to the interference with sulfhydryl (S-H) containing compounds which are essential cellular metabolites. This suggestion led to the use of phenylmercuric salts—notably the acetates, borate, and nitrate. The acid radical influences the solubility and other physical properties of the cation but does not alter its antibacterial activity (3). Since organo-mercuric compounds do not readily liberate mercuric ions, they are reported to be less toxic, less irritating, less corrosive to surgical instruments, and retain a higher degree of antibacterial activity in the presence of organic matter than inorganic mercuric compounds (3).

The antibacterial activity of various substituted phenylmercurials has been investigated and compared (5); the antibacterial activity of some fluorine-substituted aromatic mercurials also has been reported (6).

A survey of the literature reveals that in many areas of medicinal and pharmaceutical chemistry, one of the approaches often employed in searching for new drugs is the synthesis of structural modifications of prototype compounds which are known to possess interesting biological properties. During the past decade many efforts have been devoted to the incorporation of the trifluoromethyl group into such prototype molecules—in some instances in place of a methyl group, and in some cases instead of a chlorine atom. It is generally anticipated that such trifluoromethyl analogs might be acceptable as medicinal agents in view of the known unique chemical and physiological stability of the trifluoromethyl group (7).

Since it has been shown that the trifluoromethyl group affects the physico-chemical properties of certain organo-mercurials of this type, it was of interest to determine the effect of the trifluoromethyl group on the biological properties of these agents.

EXPERIMENTAL

Many methods have been developed and used to conduct antibacterial tests (3). The Oxford cup method (8), with several modifications (9), has been a widely accepted procedure because of its commensurate accuracy, speed, and minimum of labor. The filter paper disk modification of the Oxford method also has been successfully used. For expediency, this method is considered very practical in preliminary evaluations; consequently, the Oxford method as modified by Vincent (10) was employed in this study.

MATERIAL

The representative strain, *Micrococcus pyogenes* (var. *aureus*), used in the investigation was provided by the Microbiology Department, University of Texas, Austin. The nutrient agar and nutrient broth employed were from Difco Laboratories, Detroit, Mich.

Phenylmercuric chloride was prepared according to the method of Gilman, *et al.* (11), and trifluoromethyl phenyl mercuric chlorides were prepared by the method described by Maung and Lagowski (12). The bis (trifluoromethyl) mercury was prepared by the method described in the literature (13, 14). All the compounds were purified by sublimation *in vacuo* prior to use.

PROCEDURE AND RESULTS

Since the mercurials used were practically insoluble in water (except the bis (trifluoromethyl) mercury), test samples of 1:1,000, 1:10,000, and 1:100,000 were prepared in the following manner. The accurately-weighed quantity of required mercurial

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TABLE I.—ZONES OF INHIBITION OBSERVED WITH AQUEOUS SUSPENSIONS OF SOME FLUOROARYLMERCURIALS

Compd.	Concn.	Diam. of Inhibition, Zones in mm.	Av. Diam. Inhibition Zones in mm. ± A. D.
C_6H_5HgCl	1:1,000	21, 22, 20, 20, 20	20.6 ± 0.72
	1:10,000	19, 18, 17, 17, 18	17.8 ± 0.64
	1:100,000	16, 15, 15, 15, 17	15.6 ± 0.72
o-$CF_3C_6H_4HgCl$	1:1,000	30, 30, 30	30.
	1:10,000	19, 20, 20, 20	19.8 ± 0.35
	1:100,000	16, 16, 17, 16	16.2 ± 0.35
m-$CF_3C_6H_4HgCl$	1:1,000	22, 22, 22	22
	1:10,000	19, 19, 19	19
	1:100,000	18, 17, 17	17.3 ± 0.43
p-$CF_3C_6H_4HgCl$	1:1,000	24, 24, 23	23.7 ± 0.43
	1:10,000	19, 19, 19	19
	1:100,000	16, 17, 17	16.7 ± 0.43
$(CF_3)_2Hg$	1:100	22 (37)[a], 23 (39)[a], 24 (38)[a]	23.0 ± 0.67 (38.0 ± 0.67)[a]
	1:1,000	20 (27)[a], 20 (27)[a], 20 (26)[a]	20.0 (26.7 ± 0.43)[a]
	1:10,000	15, 15 (d)[b], (d)[b]	15

[a] Values in parentheses indicate practical inhibition. [b] d = Diffuse secondary zone.

for each sample was dissolved in a minimum amount of acetone, then poured into 75 ml. of 0.5% tragacanth mucilage. This mixture was stirred for approximately 30 minutes, warmed in a water bath to remove the acetone, and then made up to the required volume (100 ml.) with distilled water. Each test sample of the bis (trifluoromethyl) mercury was prepared by dissolving the drug, accurately weighed, in the required amount of distilled water.

Each plate was prepared by distributing 0.1 ml. of the inoculated broth (broth incubated for 24 hours with test organism) in 15 ml. of warm Difco agar, then pouring the mixture into the Petri dish.

Paper disks, 12 mm. in diameter, were saturated with the test samples, superimposed on the media, and the plates incubated at 37° for 24 hours. All tests were performed three or more times. Distilled water and a tragacanth mucilage prepared in the manner described above without the drugs were used as controls; results expressed as zones of inhibition appear in Table I.

DISCUSSION AND SUMMARY

All the test mercurials gave zones of inhibition with the dilutions employed. The tragacanth control showed some diffusion of material around the paper disks; however, no distinct zones of inhibition could be observed with any of the controls.

It is apparent from Table I that with 1:1,000 dilution, the *ortho* and *para* (trifluoromethyl) phenylmercuric chlorides were the most active of the compounds studied; the *meta* compound was the least active among the isomeric (trifluoromethyl) phenylmercuric compounds; the bis trifluoromethyl mercury and the *meta* trifluoromethyl compound exhibited activities comparable to that of phenylmercuric chloride.

Considering these concentrations, it is of interest to note the relationship between the position of the trifluoromethyl group on the ring and the relative antibacterial activity. A similar relationship has been reported among the halogenomercury compounds (XC_6H_4HgCl, X = F, Cl, Br, and I), where the *ortho* and *para* compounds are more active than the corresponding *meta* compound (5, 6).

It might be expected from the consideration of ionization constants and solubility products shown in Table II that the trifluoromethyl analogs would be less effective than phenylmercuric chloride. However, extensive hydrolysis of the (trifluoromethyl) phenylmercuric compound and the phenylmercuric compound will tend to minimize differences in the tendency of the chloride to ionize; no justifiable conclusion can be made regarding a relationship between ionization constants or solubility products of the compounds and their antibacterial activity. But if the antibacterial activity of mercury compounds is postulated to be due to the complexing ability of the RHgX, *i.e.*, to its ability to combine with the sulfhydryl (S-H) groups on certain enzyme systems (3), it is not surprising that $CF_3C_6H_4HgCl$ and XC_6H_4HgCl (X = F, Cl, Br and I) are more active than C_6H_5HgCl.

With the trifluoromethyl and the halogen analogs, the electron attracting trifluoromethyl group and the halogen might alter the electronic environ-

TABLE II.—IONIZATION CONSTANTS AND SOLUBILITY PRODUCTS OF TRIFLUOROMETHYL PHENYL MERCURIC COMPOUNDS (12)

Cation	Constant	OH^-	Cl^-	Br^-	I^-
p-$CF_3C_6H_4Hg$	K_i	8.3×10^{-11}	...	...	...
	K_{sp}	...	1.2×10^{-10}	1.0×10^{-12}	7.7×10^{-16}
o-$CF_3C_6H_4Hg$	K_i	9.1×10^{-11}	4.6×10^{-6}	...	...
	K_{sp}	...	2.6×10^{-10}	6.3×10^{-12}	3.5×10^{-15}
m-$CF_3C_6H_4Hg$	K_i	1.2×10^{-10}	6.0×10^{-6}	...	...
	K_{sp}	...	4.8×10^{-10}	8.3×10^{-12}	3.9×10^{-5}
C_6H_4Hg	K_i	1.3×10^{-10a} 1.0×10^{-10b}	...	...	...
	K_{sp}	...	5.0×10^{-10}	1.8×10^{-12}	1.0×10^{-15}

[a] Paritch, S. S., and Sweet, T. R., *J. Phys. Chem.*, **65**, 1909(1961). [b] Waugh, T. D., Walton, H. F., and Laswich, J. A., *ibid.*, **59**, 395(1955).

ment of the mercury atom and make it a more effective electron-pair acceptor (15).

If the antibacterial activity changes with the electronic effect of the halogen and the trifluoromethyl groups, substitution in the *ortho* and *para* positions would affect the electronic environment of the mercury atom more than substitution in the *meta* position, and the activity of former compounds would be greater than the latter chemical. This was observed with the high concentrations.

It might be postulated that with very dilute samples certain factors such as the dispersion of the drug, the media, or the dilution factor might overshadow or interfere in some manner with the electronic effect of the trifluoromethyl group. Perhaps other testing techniques which are more sensitive should be employed to test the antibacterial activity of the very dilute samples.

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Colorimetric Determination of Chlorpheniramine Maleate

By JOSEPH HUDANICK

A sensitive colorimetric method has been developed for determining chlorpheniramine maleate in various pharmaceutical preparations. The method is especially suitable as a rapid control method.

CHLORPHENIRAMINE MALEATE, a pyridine derived antihistamine, is presently marketed in a variety of pharmaceutical preparations. Its determination is especially difficult when present in small amounts and in combination with certain other antihistamines and related compounds. Ordinary methods (1–3) such as ultraviolet, chromatographic, and gravimetric techniques, are time consuming and relatively nonselective. Jones and Brady (4) describe a general colorimetric method for determining pyridine derived antihistamines which is a modification of the Koenig reaction (5, 6). This method in its present form is of low sensitivity and unsuitable for mixtures of antihistamines. By changing the reaction conditions and using sulfanilic acid instead of aniline and an acetate buffer instead of phthalate, chlorpheniramine formed a very intense transient yellow suitable for quantitative analysis. The intensity of the color (absorption peak at 480 mμ) is approximately 40 times that obtained by the Jones and Brady method and about three times as sensitive as the ultraviolet method. In addition, the color is very reproducible (standard deviation 0.4%) and unaffected by the presence of methapyrilene HCl and pyrilamine maleate, two pyridine derived antihistamines which are commonly associated with chlorpheniramine in many preparations. The very closely related compounds, pheniramine maleate and brompheniramine maleate, react the same as chlorpheniramine maleate. The method has been found suitable for determining chlorpheniramine in many varied tablet combinations and timed release pellets.

Received June 5, 1963, from the Rexall Drug Co., St. Louis, Mo.
Accepted for publication July 20, 1963.

EXPERIMENTAL

Buffered Sulfanilic Acid Solution.—Dissolve 2.5 Gm. of sulfanilic acid and 4.00 Gm. of anhydrous

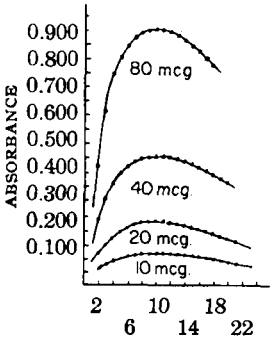


Fig. 1.—Plot of absorbance *vs.* time for specific concentrations of chlorpheniramine maleate.

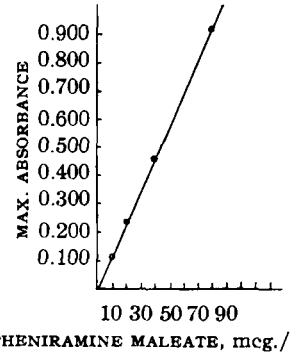


Fig. 2.—Plot of maximum absorbance of developed color *vs.* concentration.

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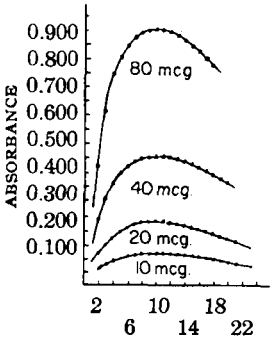


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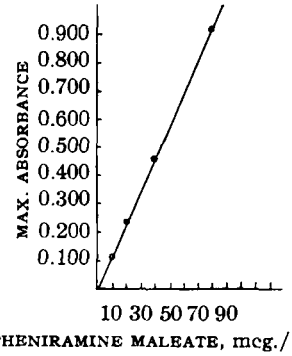


Fig. 2.—Plot of maximum absorbance of developed color *vs.* concentration.

TABLE I.—EFFECT OF DIVERSE AMINE COMPOUNDS ON ABSORBANCE

40 mcg. Chlorpheniramine Maleate +	Compd. Added, mcg.	Absorbance
	...	0.461, 0.460
+ 800	Methapyrilene HCl	0.460, 0.462
+ 800	Pyrilamine maleate	0.461, 0.459
+2000	Phenylpropanolamine HCl	0.463, 0.459
+2000	Phenylephrine HCl	0.464, 0.460
+2000	Racephedrine HCl	0.458, 0.461
+2000	Dextromethorphan HBr	0.460, 0.459

sodium acetate into 40 ml. water and dilute to 175 ml. with 95% ethanol.

Aqueous Hydrochloric Acid 0.25 *N*.—The normality was adjusted to ±2.0%.

Cyanogen Bromide Solution.—Dissolve 2.0 Gm. cyanogen bromide into 50 ml. water and keep under refrigeration.

Stock Standard Solution.—This is a solution of chlorpheniramine maleate in 0.25 *N* HCl, concentration = 1.00 mg./ml. Prepare solutions containing 0.01, 0.02, 0.04, and 0.08 mg./ml. chlorpheniramine maleate in 0.25 *N* HCl from this solution.

Pipet 7.0 ml. of buffered sulfanilic acid solution into each of five 50-ml. glass-stoppered Erlenmeyer flasks. Pipet 1.0 ml. of the serial dilutions into the flasks; pipet 1 ml. of 0.25 *N* HCl for a reagent blank into one flask. Add 3.0 ml. cold cyanogen bromide solution to each with swirling. Determine the absorbance of the solutions relative to the reagent blank at 1-minute intervals at 480 mμ on a Beckman DU spectrophotometer. A plot of absorbance *versus* time is shown in Fig. 1; a plot of maximum absorbance *versus* concentration is shown in Fig. 2. The maximum absorbance is linear with respect to concentration.

Interference from Other Amines

To determine the interference from certain other amines, solutions of chlorpheniramine maleate (concentration = 0.040 mg./ml. in 0.25 *N* HCl) were prepared containing added amounts of the various compounds. As previously described 1 ml. of each solution was used for the color reaction and the maximum absorbance recorded. The results of duplicate runs are shown in Table I. No significant interference was observed at the stated concentrations. Normal tablet excipients and compounds such as aspirin, phenacetin, caffeine, and ascorbic acid do not cause interference.

Chromatographic Identification

A paper chromatographic separation is used to ascertain which of the three similarly reacting antihistamines (pheniramine, brompheniramine, or chlorpheniramine maleate) is present in a sample. The sample and control spots (pipeted as ether extracts from aqueous alkaline solutions) are chromatographed using an ascending technique on Whatman No. 1 paper which has been pretreated by soaking in 2.0% aqueous ammonium sulfate and thoroughly air drying. The developing solvent is made by shaking 100 ml. of *n*-butanol with 100 ml. aqueous 6.0% citric acid. After separation of the phases, the butanol layer is used as the mobile solvent and the aqueous layer as the immobile solvent. After chromatographing for 15 hours, the paper is air dried and dipped into Dragendorff's reagent (2) to locate the spots. The R_f values for pheniramine, brompheniramine, and chlorpheniramine are 0.25, 0.58, and 0.53, respectively.

SUMMARY

A colorimetric method based upon the Koenig reaction has been described for determining chlorpheniramine maleate in certain pharmaceutical preparations. The method requires very little time to perform and has very good precision. (Standard deviation is 0.4%.)

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Solubility and Dissolution Rates in Reactive Media

By W. I. HIGUCHI, EINO NELSON†, and J. G. WAGNER‡

The relationship between the diffusion-controlled dissolution rate of a substance in a reactive medium and the solubility of the substance in the medium has been analyzed. The results reconcile the total solubility method and the simultaneous diffusion and chemical reaction method of interpreting data on dissolution rates.

THE PROBLEM OF dissolution rates of solids in reactive solutions has been recently examined by the total solubility method (1–3) and by the simultaneous chemical reaction and diffusion (SCRD) method (4). Because of the differences between the two concepts and because the problem is important, it appears that a clarification of the data is necessary.

Both theories are based on the diffusion layer (or film) model; therefore, they both assume that the dissolution rate is controlled by diffusion rates of the important species through this layer. The model assumed in the SCRD method takes into account simultaneous rapid reversible chemical reaction and diffusion of all the important species. The resulting equations for dissolution rate, G, in this case are relatively complicated. On the other

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TABLE I.—EFFECT OF DIVERSE AMINE COMPOUNDS ON ABSORBANCE

40 mcg. Chlorpheniramine Maleate +	Compd. Added, mcg.	Absorbance
	...	0.461, 0.460
+ 800	Methapyrilene HCl	0.460, 0.462
+ 800	Pyrilamine maleate	0.461, 0.459
+2000	Phenylpropanolamine HCl	0.463, 0.459
+2000	Phenylephrine HCl	0.464, 0.460
+2000	Racephedrine HCl	0.458, 0.461
+2000	Dextromethorphan HBr	0.460, 0.459

sodium acetate into 40 ml. water and dilute to 175 ml. with 95% ethanol.

Aqueous Hydrochloric Acid 0.25 *N*.—The normality was adjusted to ±2.0%.

Cyanogen Bromide Solution.—Dissolve 2.0 Gm. cyanogen bromide into 50 ml. water and keep under refrigeration.

Stock Standard Solution.—This is a solution of chlorpheniramine maleate in 0.25 *N* HCl, concentration = 1.00 mg./ml. Prepare solutions containing 0.01, 0.02, 0.04, and 0.08 mg./ml. chlorpheniramine maleate in 0.25 *N* HCl from this solution.

Pipet 7.0 ml. of buffered sulfanilic acid solution into each of five 50-ml. glass-stoppered Erlenmeyer flasks. Pipet 1.0 ml. of the serial dilutions into the flasks; pipet 1 ml. of 0.25 *N* HCl for a reagent blank into one flask. Add 3.0 ml. cold cyanogen bromide solution to each with swirling. Determine the absorbance of the solutions relative to the reagent blank at 1-minute intervals at 480 mμ on a Beckman DU spectrophotometer. A plot of absorbance *versus* time is shown in Fig. 1; a plot of maximum absorbance *versus* concentration is shown in Fig. 2. The maximum absorbance is linear with respect to concentration.

Interference from Other Amines

To determine the interference from certain other amines, solutions of chlorpheniramine maleate (concentration = 0.040 mg./ml. in 0.25 *N* HCl) were prepared containing added amounts of the various compounds. As previously described 1 ml. of each solution was used for the color reaction and the maximum absorbance recorded. The results of duplicate runs are shown in Table I. No significant interference was observed at the stated concentrations. Normal tablet excipients and compounds such as aspirin, phenacetin, caffeine, and ascorbic acid do not cause interference.

Chromatographic Identification

A paper chromatographic separation is used to ascertain which of the three similarly reacting antihistamines (pheniramine, brompheniramine, or chlorpheniramine maleate) is present in a sample. The sample and control spots (pipeted as ether extracts from aqueous alkaline solutions) are chromatographed using an ascending technique on Whatman No. 1 paper which has been pretreated by soaking in 2.0% aqueous ammonium sulfate and thoroughly air drying. The developing solvent is made by shaking 100 ml. of *n*-butanol with 100 ml. aqueous 6.0% citric acid. After separation of the phases, the butanol layer is used as the mobile solvent and the aqueous layer as the immobile solvent. After chromatographing for 15 hours, the paper is air dried and dipped into Dragendorff's reagent (2) to locate the spots. The R_f values for pheniramine, brompheniramine, and chlorpheniramine are 0.25, 0.58, and 0.53, respectively.

SUMMARY

A colorimetric method based upon the Koenig reaction has been described for determining chlorpheniramine maleate in certain pharmaceutical preparations. The method requires very little time to perform and has very good precision. (Standard deviation is 0.4%.)

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hand, the total solubility method assumes that G, the rate of dissolution per unit area of solid may be expressed by the conventional Noyes-Whitney law (5)

$$G = k(C_s - C) \qquad \text{(Eq. 1)}$$

Here C_s is the total solubility of the solid in the medium, C is the solute concentration in solution, k is a function including the diffusion coefficients, and h, the thickness of the diffusion layer. For a single component solid dissolving in a nonreactive medium

$$k = \frac{D}{h} \qquad \text{(Eq. 2)}$$

where D is the diffusion coefficient of the solute in the solvent.

The problem in this communication is the meaning of k and C_s in Eq. 1 in terms of the SCRD model and the ramifications thereof. To accomplish this best, let us take two specific cases: (a) a weak acid dissolving in a basic medium and (b) a salt of a weak acid and a strong base dissolving in an acidic medium. For sake of simplicity we shall examine only the initial dissolution rates.

Weak Acid Dissolving in Basic Medium.—Consider the situation in which a solid weak acid, HA, is dissolving in a buffer solution of a base, B^-, and its conjugate acid, HB. Equation 10 in *Reference 4* gives[1] the following initial dissolution rate expression based on the SCRD method for this case

$$G = \frac{D_{HA}(HA)_o}{h} - \frac{D_{HB}(HB)_h}{2h} - \frac{D_{HB}D_AK(HA)_o}{2D_Bh} + \frac{1}{2D_Bh}[D_{HB}^2D_A^2K^2(HA)_o^2 + 4D_B^2D_{HB}D_AK(HA)_o(B^-)_h + D_B^2D_{HB}^2(HB)_h^2 + 2D_AD_BD_{HB}^2K(HB)_h(HA)_o]^{1/2} \qquad \text{(Eq. 3)}$$

Here the D's are the diffusion coefficients of the species indicated by the subscripts, $(HA)_o$ is the unionized acid concentration in equilibrium with the solid, and $(B^-)_h$ and $(HB)_h$ are, respectively, the base concentration and the conjugate acid concentration in solution. The concentration equilibrium constant K is defined

$$K = \frac{K_{HA}}{K_{HB}} = \frac{(HB)(A^-)}{(HA)(B^-)} \qquad \text{(Eq. 4)}$$

Now let us compare this equation with Eq. 1. The total solubility, C_s, of the weak acid in a buffer solution of initial concentrations $(B^-)_h$ and $(HB)_h$ may be calculated from Eq. 4

$$K = \frac{[(HB)_h + x][x]}{(HA)_o[(B)_h - x]} \qquad \text{(Eq. 5)}$$

where x is the concentration of A^- at equilibrium. Solving Eq. 5 we obtain

$$C_s = (HA)_o + x = (HA)_o - \frac{(HB)_h}{2} - \frac{K(HA)_o}{2} + 1/2\,[K^2(HA)_o^2 + 4K(HA)_o(B^-)_h + 2K(HA)_o(HB)_h + (HB)_h^2]^{1/2} \qquad \text{(Eq. 6)}$$

Now if Eq. 6 is substituted into Eq. 1 with $C = 0$ and $k = D/h$, it is clear that Eqs. 1 and 3 become identical when all diffusion coefficients are equal to D.

If the base happens to be the hydroxide ion, Eq. 12 in *Reference 4* may be used[2] for the initial dissolution rate of a weak acid solid. We have in this case then the SCRD method giving

$$G = \frac{D_{HA}(HA)_o}{h} + \frac{D_B^2(OH^-)_h}{h} \times \left(\frac{D_A\frac{K_a}{K_w}(HA)_o}{D_B^2 + D_AD_B\frac{K_a}{K_w}(HA)_o}\right) \qquad \text{(Eq. 7)}$$

where K_w is the ion product for water, $K_w = (H^+)(OH^-)$. In this instance the total solubility, C_s, is

$$C_s = (HA)_o + x$$

where x again is the concentration of A^- at equilibrium and is given by

$$\frac{K_a}{K_w} = \frac{x}{(HA)_o[(OH^-)_h - x]}$$

therefore

$$C_s = (HA)_o + \frac{\frac{K_a}{K_w}(HA)_o(OH^-)_h}{1 + \frac{K_a}{K_w}(HA)_o} \qquad \text{(Eq. 8)}$$

It is again clear that substitution of Eq. 8 into Eq. 1 with $k = D/h$ and $C = 0$ will give the same expression as Eq. 7 when all the diffusion coefficients are set equal to D.

Sodium Salt of a Weak Acid Dissolving in Acid.—Consider the dissolution of a salt, NaA, in a solution containing hydrogen ions at a concentration, $(H^+)_h$. The SCRD method gives (6) the following implicit expression for the initial dissolution rate, G, in this case[3]

$$\frac{D_AD_{HA}}{D_HK_A}\left(\frac{D_{Na}K_{sp}}{Gh}\right)^2 + \left(\frac{D_{Na}K_{sp}}{Gh}\right)\left(D_A + D_{HA}\frac{(H^+)_h}{K_A}\right) - \frac{D_{HA}D_{Na}K_{sp}}{D_HK_A} - Gh = 0 \qquad \text{(Eq. 9)}$$

The expression for the total solubility, C_s, for this case may be set up according to

$$K_{sp} = C_s(C_s - x) \qquad \text{(Eq. 10)}$$

and

$$K_a = \frac{(C_s - x)[(H^+)_h - x]}{x} \qquad \text{(Eq. 11)}$$

where K_{sp} is the solubility product for the salt, K_a is

[1] There were two minor typographical errors in the original equation. The corrected equation is

$$C_1 = \frac{D_B(B)_h - D_{HA}(HA)_h + D_{HA}(HA)_o}{h} - \frac{D_B(L_1 + L_2) + D_{HB}D_AK(HA)_o \pm \{[D_B(L_1 + L_2) + D_{HB}D_AK(HA)_o]^2 - 4L_1L_2D_B^2\}^{1/2}}{2D_Bh}$$

where C_1 is the same as the present G. Note that this is a general expression; Eq. 3 applies to initial rates only, *i.e.*, $(A)_h = (HA)_h = 0$.

[2] While Eq. 12 of *Reference 4* involved uncharged species, the same equation may be used here. It is only important that the form of the equilibrium constant expression is the same.

[3] Assumes that solubility of weak acid itself is not exceeded anywhere in the system under the conditions of the dissolution experiment. If such is not the case or if another solid phase is formed during dissolution, the present equation may not apply. (See *References 7–9* for examples of the precipitation of a weak acid onto the surface of the salt during dissolution.)

as before the dissociation constant for the weak acid, HA, and X is the amount of HA formed per unit volume at equilibrium. Solving Eqs. 11 and 12 we get

$$\frac{K_{sp}^2}{C_s^2} - K_{sp} + \frac{K_{sp}(H^+)_h}{C_s} + \frac{K_a K_{sp}}{C_s} - K_A C_S = 0 \quad \text{(Eq. 12)}$$

Now substitution of Eqs. 1 and 2 with $C = 0$ into Eq. 12 will show that Eqs. 12 and 9 are again identical when all the diffusion coefficients are set equal to D.

DISCUSSION

The present analysis demonstrates that within the framework of the diffusion layer model the total solubility method and the SCRD method give the same results when all of the diffusion coefficients may be set equal to the same value. Because diffusion coefficients of solute molecules do not differ[4] much in general and other uncertainties—such as variation of dissociation constants and solubilities with ionic strength and other solute interaction effects—are frequently the overriding factors, it would be expected that the total solubility method should explain experimental results as well as the SCRD method. Nelson found this to be the case in many instances (1–3).

Where the two methods will significantly differ would be primarily those situations in which the reacting agent is a colloid, *e.g.*, micellar surfactants which solubilize the solute and nonionic polymers and polyelectrolytes which react with and bind the solute. In these instances, the relatively small diffusion coefficients of colloids will lead to much smaller dissolution rates for the SCRD theory under certain conditions.

[4] Neglecting effects of solvation, Stokes-Einstein law predicts the diffusion coefficients vary approximately as the cube root of the molecular weight for materials of the same density.

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Use of Solubility Analysis in Pharmaceutical Stability Studies

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Purity determined by solubility analysis was used to evaluate the efficacy of other analytical methods for measuring thermal degradation. Techniques for filtration and a method for calculation of confidence interval for solubility analysis are described.

The validity of a stability assay procedure may be confirmed in different ways. If the assays on samples stored at elevated temperatures decrease with time, the method is confirmed. The method of analysis may also be checked by a comparison with a method which is known to measure stability. Several tests of purity are available to the analysts in such cases. Those commonly used include vapor phase, thin layer, and paper chromatography. Garrett (1) reviewed other tests for solvolytic stability of drugs. The purpose of this study was to investigate the possibility of using solubility analysis as a reference assay to evaluate a proposed assay procedure on thermally degraded drug substances. It is not practical to use solubility analysis as the stability assay because of cost, time, and the fact that solubility analysis is only applicable to the drug substance free of excipients. If the proposed or conventional assay procedure is confirmed by the purity test, the procedure may then be used with a fair degree of certainty on the finished pharmaceutical if thermal instability is the main consideration.

EXPERIMENTAL

Three newly developed and two older drug substances were stored at varying temperatures and were periodically withdrawn and assayed by solubility analysis and by an alternate procedure.

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Solubility Analysis Procedure (2)

Varied amounts of the material being tested were allowed to equilibrate at 25° with 2 ml. of an appropriate solvent. After equilibrium was reached, the solution was filtered with a Swinny filter, using S and S No. 740 E filter pads. The filtrate was transferred to a previously tared drying flask and weighed. (The drying flasks were prepared by Ace Glass Co. with an average weight of 6.0 Gm.) The solvent was removed under vacuum at 40°. All weights were recorded to the nearest 0.02 mg. Ratios of mg. solute per Gm. solvent and mg. residue per Gm. solution were calculated for each amount of solute. The data were plotted as mg. residue per Gm. solution (ordinate) *versus* mg. solute per Gm. solvent (Fig. 1). The per cent purity and 95% confidence interval about this purity were calculated by a variation of the method of least squares (3).

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Maximum and minimum values for per cent purity corresponding to a level of confidence of 95% are

$$\text{max. \% purity} = 100 - 100 \tan\left[\arctan\theta - \frac{\arcsin\phi}{2}\right]$$

$$\text{min. \% purity} = 100 - 100 \tan\left[\arctan\theta + \frac{\arcsin\phi}{2}\right]$$

$$\phi = \frac{\sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2] - [n\Sigma xy - \Sigma x\Sigma y]^2}\, t}{\sqrt{\frac{([n\Sigma x^2 - (\Sigma x)^2] - [n\Sigma y^2 - (\Sigma y)^2])^2}{4} + [n\Sigma xy - \Sigma x\Sigma y]^2}\sqrt{n-2}}$$

where x = mg. solute/Gm. solvent, y = mg. residue/Gm. solution, λ = 1.0 for linear bivariant data, and t values (student t) were chosen to correspond to a level of confidence of 95% and (n-2) degrees of freedom.

Alternate Assays

α - d - 4 - Dimethylamino - 1,2 - diphenyl - 3-methyl-2-propionoxy Butane Hydrochloride.—Sixty-five milligrams of the sample was dissolved in distilled water, and the free base was precipitated with 4 drops of 50% NaOH. The free base was extracted with four 25-ml. portions of chloroform; the chloroform was evaporated to dryness. The residue was dissolved in exactly 2 ml. of chloroform; the absorbance of this solution was measured on a Beckman I.R. spectrophotometer at 5.8 μ in 0.1 mm. rocksalt cells at a slit width of 0.3 mm. These absorbances were compared with those obtained in the same manner on 65 mg. of standard material.

α - 1 - 4 - Dimethylamino - 1,2 - diphenyl - 3-methyl-2-propionoxy Butane-N-oxide Hydrochloride.—The alternate assay for this compound was the same as the one for α-*d*-4-dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane hydrochloride.

Ergonovine Maleate.—This material was assayed per the directions for the standard in the procedure for ergonovine maleate injection in the U.S.P. XVI.

Ascorbic Acid.—The U.S.P. XVI method was used to analyze 100-mg. samples.

N-(p-Acetylphenylsulfonyl)-N-cyclohexyl Urea.—The absorbance of the sample and standard were measured at 249 mμ, at a concentration of 0.01 mg./ml., on a Beckman DU spectrophotometer. The absorbances were compared with those obtained on the original starting material in the same manner as the sample.

The data obtained by solubility analysis and by the alternate procedure were plotted as ln concentration *versus* time (Fig. 2). The results in Table II were obtained from these plots. By plotting the data in this way the assumption is made that the reaction occurring is first order. This was found to be true for the compounds used over the range investigated. The slope of the best fitting line for this type of plot will be the reaction rate constant (k). The k values and the confidence intervals about these values were calculated by the method of least squares.

t values (student t) were chosen to correspond to a level of confidence of 95% and (n-2) degrees of freedom.

One of the major advantages in using a technique such as solubility analysis as a reference assay was that the values obtained were figures of absolute purity. Table II lists the k values and the 95% confidence intervals about these values on five different drug substances. The k values are shown for both solubility analysis and for an alternate procedure. The 95% confidence interval appears to be quite large compared with the k values. The major consideration, however, is to observe the difference between the two methods. If the confidence intervals of the two methods do not overlap, it is apparent that the results of the two methods are significantly different.

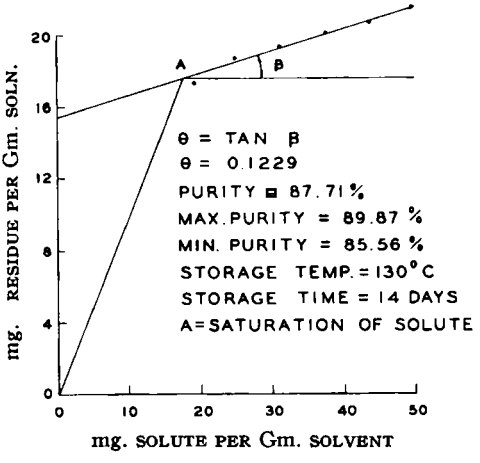


Fig. 1.—α-*d*-4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane hydrochloride.

RESULTS

Neither solubility analysis nor the I.R. spectrophotometric assay detected degradation of α-*d*-4-dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane hydrochloride. The solubility analysis and the I.R. spectrophotometric data showed approximately the same amount of degradation on α-1,4-

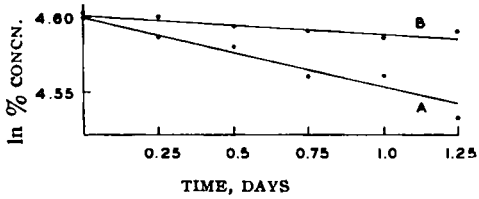


Fig. 2.—Ascorbic acid storage temperature, 130° C. Key: A, solubility analysis data; B, U.S.P. data. Slope A = −0.051 ± 0.021; slope B = −0.0158 ± 0.0079.

$$k = \frac{n\Sigma xy - \Sigma x\Sigma y}{n\Sigma x^2 - (\Sigma x)^2} \pm t\sqrt{\frac{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2] - [n\Sigma xy - \Sigma x\Sigma y]^2}{[n\Sigma x^2 - (\Sigma x)^2]^2\,(n-2)}}$$

TABLE I.—SOLVENT SYSTEMS OF SOLUBILITY ANALYSIS

Compd.	Solvent System, %	Solubility at 25°C., mg./Gm.
α-d-4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane hydrochloride	100 ETOAc	7.5
α-1,4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane-N-oxide hydrochloride	65 ETOAc 35 CH_3OH	16.0
Ergonovine maleate	100 CH_3OH	10.5
Ascorbic acid	50 $(CH_3)_2CHOH$ 50 CH_3CH_2OH	15.0
N-(p-Acetylphenylsulfonyl)-N-cyclohexyl urea	40 $CHCl_3$ 60 CH_3OH	23.0

N-(p-Acetylphenylsulfonyl)-N-cyclohexyl urea was measured by an ultraviolet spectrophotometric assay and by solubility analysis. The data obtained indicated that the U.V. assay was incapable of completely measuring the thermal degradation that had occurred. The reaction rate constant (k) of the solubility analysis plot on the samples stored at 130° was ten times greater than that obtained from the U.V. data.

By using solubility analysis as the criteria for purity in the above examples, the conventional methods for measuring thermal degradation were confirmed for one compound, shown to be questionable for three, and verified the thermal stability for one drug substance.

There are many practical considerations involved in the interpretations of these data for pharmaceutical stability studies. Thermal instability may be inconsequential in reference to other modes of degradation. In such cases assay procedures must be designed and verified by other procedures. The use of a method that will measure purity—such as solubility analysis for short term accelerated aging studies—does give valuable information to the pharmaceutical analyst. It provides an estimate of the efficacy of the proposed method and also provides preliminary evaluation of the thermal stability of the drug substance.

TABLE II.—REACTION RATE CONSTANTS

Drug Substance	Storage Temp., °C.	Sol. Anal., $k \times 10^{-3}$	Conf. Int., 95% ± 1×10^{-3}	Alt. Assay, $k \times 10^{-3}$	Conf. Int., 95% ± 1×10^{-3}	Alt. Assay Method
α-d-4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane hydrochloride	80.0	−0.03	0.01	0.01	0.07	I. R.
	105.0	−0.04	0.30	−0.01	0.11	I. R.
	130.0	−0.76	6.70	0.09	3.40	I. R.
α-1,4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionoxy butane-N-oxide hydrochloride	80.0	−0.49	0.70	−0.53	1.20	I. R.
	105.0	−4.83	7.10	−5.7	7.64	I. R.
	125.0	−109.0	72.5	−105.0	57.5	I. R.
Ergonovine maleate	65.0	0.48	1.07	−0.10	1.04	Colorimetric
Ergonovine maleate	80.0	−0.50	0.67	−0.28	0.71	Colorimetric
Ergonovine maleate	100.0	−3.98	1.76	−1.61	0.47	Colorimetric
Ascorbic acid	80.0	−0.98	1.59	−0.88	0.44	I_2 titr.
Ascorbic acid	105.0	−9.10	1.93	−1.06	0.91	I_2 titr.
Ascorbic acid	130.0	−51.0	21.3	−15.8	7.88	I_2 titr.
N-(p-Acetylphenyl sulfonyl)-N-cyclohexyl urea	80.0	−0.06	0.57	−0.49	0.87	U. V.
	105.0	−4.09	1.28	−1.19	1.10	U. V.
	130.0	−123.0	14.7	−13.7	18.8	U. V.

dimethylamino-1, 2-diphenyl-3-methyl-2-propionoxy butane-N-oxide hydrochloride.

The solubility analysis on ergonovine maleate indicated that more degradation had occurred than was shown by the colorimetric assay. The colorimetric assay was therefore not capable of measuring completely the thermal degradation that had occurred.

Ascorbic acid was measured by iodometric titration and by solubility analysis. The iodometric assay was incapable of completely measuring the thermal degradation that had occurred.

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Stability of Certified Dyes in Tablets II. Fading of FD&C Red No. 3 and FD&C Red No. 3 Aluminum Lake in Three Tablet Formulas

By F. W. GOODHART, M. E. EVERHARD, and D. A. DICKCIUS

The fading rates of FD&C Red No. 3 and its aluminum lake were determined in three tablet formulas which differed only in the excipient employed. In tablets of calcium sulfate the dye faded at about twice the rate of the same dye in either dicalcium phosphate or lactose tablets. However, the use of the corresponding lake in the calcium sulfate formulation reduced the fading rate significantly. No significant differences in fading rates were demonstrated between the dye and the lake in tablets of dicalcium phosphate or lactose. The method of size reduction of the dyed powder did not significantly affect the fading rate.

IN A PREVIOUS COMMUNICATION (1), a method was described by which the approximate color shelf life of a tablet formulation can be predicted on the basis of high intensity fading. This study demonstrated that FD&C Red No. 3 possessed a fading rate independent of the dye concentration in the tablet. The first-order rate equation which described the fading was

$$\ln\theta_t = -kIt + \ln\theta_{t_o} \qquad \text{(Eq. 1)}$$

where $\theta_t = \left(\frac{1 - R_t}{2R_t}\right)^2 = AC$, in which R_t is the reflectance at 543 mμ (the absorption peak) at time t, A is a constant, C is the concentration of dye in weight per cent, k is the first-order rate constant, I is the intensity of light, and t_o is time zero.

The present work was concerned with three variables. The first of these was the nature of the colorant, dye or lake. Enhanced stability to ultraviolet radiation was indicated for several lakes (2), but quantitative data comparing fading rates for FD&C Red No. 3 and its lake were not reported. The influence of tablet diluents on dye stability has not been investigated; since the diluent itself is sometimes a source of incompatibility, this factor was included. Another factor investigated was the method of preparation of the dyed powder used in preparing the tablets. While the affinity of the dye for different diluents varies, this property alone did not determine the color uniformity. Some formulas appeared to be uniformly dyed, but an unusual degree of mottling was noted when pressed into tablets. Therefore, three methods of size reduction were included to improve color uniformity and to determine whether this variable altered the rate of fading.

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EXPERIMENTAL

Materials.—Lactose U.S.P., calcium sulfate dihydrate, anhydrous dibasic calcium phosphate N F., acacia U.S.P., magnesium stearate U.S.P., FD&C Red No. 3, and FD&C Red No. 3 aluminum lake were employed.

Equipment.—Low intensity light was provided by a bank of G.E. F40CW fluorescent tubes; high intensity light was provided by a light cabinet previously described (3, 4). Reflectance measurement, light intensity measurement, and preparation of the tablets were the same as described in our first study (1), except that some formulas were reduced by comminuting or ball milling rather than micropulverizing. Comminuting was carried out on the Fitzpatrick model D comminutor and micropulverizing on the Pulverizing Machine Co. micro-sample mill.

Storage of Tablets.—Prepared tablet samples were placed in a light cabinet under a flux of 518 f.-c. and under a bank of fluorescent light at 195, 74, 50, and 11 f.-c. Samples were withdrawn periodically for reflectance measurement.

RESULTS AND DISCUSSION

Results.—A summary of the first-order rate

TABLE I.—RATE CONSTANTS FOR THE FADING OF FD&C RED NO. 3

Diluent	Nature of Colorant	Dye Dispersion Method	$k \times 10^5$ (f.-c. hr.)$^{-1}$
$CaSO_4 \cdot 2H_2O$	Dye	Comminuted[a]	11.3 ± 1.1
$CaSO_4 \cdot 2H_2O$	Dye	Micropulverized[a]	11.4 ± 0.8
$CaSO_4 \cdot 2H_2O$	Lake	Comminuted or ball milled[b]	7.4 ± 0.8
$CaHPO_4$	Dye	Micropulverized[a]	6.7 ± 0.6
$CaHPO_4$	Lake	Comminuted or ball milled[b]	7.8 ± 0.3
Lactose	Dye	Micropulverized[a]	6.1 ± 0.3
Lactose	Lake	Micropulverized[b]	4.6 ± 0.9

[a] Average of three slopes, each a different dye concentration. [b] Average of two slopes, each the same dye concentration.

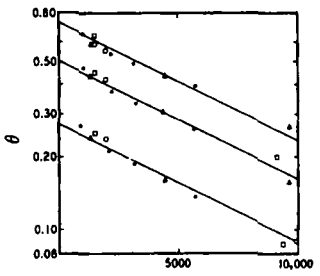


Fig. 1.—Plots of θ_t *vs.* the product of time x intensity for the dye on $CaSO_4 \cdot 2H_2O$. Key: ●, 518 f-c.; △, 195 f.-c.; ○, 74 f.-c.; □, 50 f.-c.; and ×, 11 f.-c. Upper line, 0.060% w/w dye; middle line, 0.030% w/w dye; lower line, 0.015% w/w dye. Method of size reduction was micropulverization.

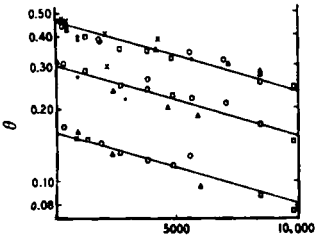


Fig. 2.—Plots of θ_t *vs.* the product of time x intensity for the dye on $CaHPO_4$. Key: ●, 518 f.-c.; △, 195 f.-c.; ○, 74 f.-c.; □, 50 f.-c.; and ×, 11 f.-c. Upper line, 0.060% w/w dye; middle line, 0.030% w/w dye; lower line, 0.015% w/w dye. Method of size reduction was micropulverization.

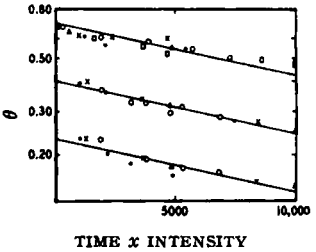


Fig. 4.—Plots of θ_t *vs.* the product of time x intensity for the lake on lactose. ●, 518 f.-c.; △, 195 f.-c.; ○, 74 f.-c.; □, 50 f.-c.; and ×, 11 f.-c. Upper line, 0.060% w/w dye; middle line, 0.030% w/w dye; lower line, 0.015% w/w dye. Method of size reduction was micropulverization.

TABLE II.—EFFECT OF CALCIUM ION ON FADING OF FD&C RED NO. 3 AT 25° AND 900 F.-C.

Soln.	Initial mM of Calcium Ion	mg./ml. of Dye Initial[c]	Initial pH	Final pH	% Dye Remaining After 17 Hr. Exposure
Control	None	0.78	6.8	4.9	85
$CaSO_4 \cdot 2H_2O$	0.12	0.71	7.1	5.5	85
$CaHPO_4$[a]	0.15	0.76	7.8	6.4	82
$CaCl_2$	0.90	0.76	6.9	5.0	67
$CaSO_4 \cdot 2H_2O$[b]	1.5	0.40	7.1	5.3	55
$CaCl_2$	9.0	0.14	7.3	4.3	46

[a] Saturated solution of calcium phosphate at 23°C. [b] Saturated solution of calcium sulfate at 23°C. [c] All solutions were prepared to contain 0.78 mg./ml. of dye. Precipitation of calcium salt of dye is responsible for reduction of initial dye concentration in all experiments except the control.

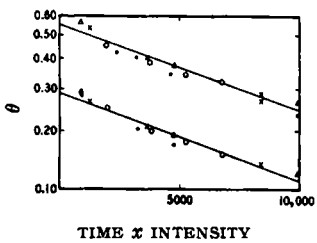


Fig. 3.—Plots of θ_t *vs.* the product of time x intensity for the lake on $CaSO_4 \cdot 2H_2O$ and $CaHPO_4$. Upper line, $CaSO_4 \cdot 2H_2O$, 0.060% w/w dye; lower line, $CaHPO_4$, 0.060% w/w dye. Key: ●, 518 f.-c.; △, 195 f.-c.; ○, 74 f.-c.; □, 50 f.-c.; X, 11 f.-c. Method of preparation was micropulverization.

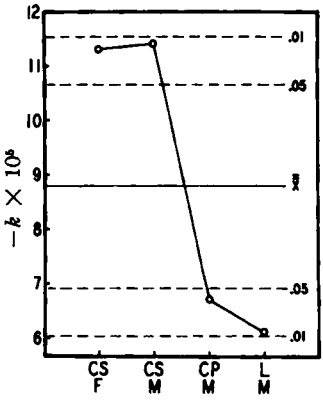


Fig. 5.—Statistical analysis of means illustrating significant differences of rate constants for the dye in tablets of calcium sulfate dihydrate, dibasic calcium phosphate, and lactose. $\bar{X}$ is overall average of 12 rate constants and dotted lines give 95 and 99% limits. Key: CS, calcium sulfate dihydrate; CP, dicalcium phosphate; L, lactose; F, comminuted; and M, micropulverized.

constants, k, calculated by Eq. 1, along with their average deviations is given in Table I for all systems studied.

The rate constants listed in Table I were determined by fitting the best straight line using regression analysis. Correlation coefficients ranged from 0.90 to 0.99. The standard deviation of the rate constant was also calculated to determine whether any rate constants were statistically different from others at 95% confidence limits.

Effects of Size Reduction.—While the dye seems to have an affinity for all three diluents employed, the calcium sulfate tablets were noticeably more mottled than those made with either dicalcium phosphate or lactose. Dispersion by comminuting rather than micropulverization did not decrease this effect. As seen in Table I the rate constants for dye in calcium sulfate were the same regardless of the kind of size reduction employed. Figure 1 illustrates the fading of the dye on calcium sulfate tablets prepared by micropulverization.

Marked speckling occurred with both calcium salts when the lake was employed. Therefore, these blends were subjected to ball milling to produce a uniform color. Although the fading rate was slightly lower for the tablets prepared by ball milling compared to comminuting, the difference between the rates was not statistically significant at the 95% level. An average value is therefore reported in Table I.

Effect of Diluent on the Fading Rate of the Dye.—Figures 1 and 2 show the fading of the dye on calcium sulfate and calcium phosphate, respectively. As seen in Table I, the rate constant for calcium sulfate was nearly double that found for either calcium phosphate or lactose. A statistical treatment of the rates (5) is shown in Fig. 5. The significant differences demonstrate an incompatibility of the dye with calcium sulfate.

To substantiate that calcium was the cause of the faster fading, a series of dye solutions was prepared and exposed to high intensity fluorescent light (900 f.-c.) for 17 hours. The results of the experiment

are shown in Table II. As the concentration of calcium increases, the dye deteriorates more rapidly. It is apparent that no correlation exists between degree of fading and the initial or final pH. Therefore, it can be concluded that the higher solubility of calcium sulfate dihydrate (0.2 Gm./100 ml. at 25°) is the cause for the more rapid fading. The solubility of dicalcium phosphate is about 0.05 Gm./100 ml. at 25°, an amount apparently insufficient to cause a significant increase in the fading rate.

Effect of Colorant Nature.—No significant differences in the fading rates existed between the dye and the lake on either dicalcium phosphate or lactose. However, in the case of calcium sulfate a marked reduction in the rate was afforded by use of the lake over the dye as seen in Table I and Figs. 1 and 3. The reason for increased stability with the lake can be attributed to the adsorption of the dye to its substrate. In this form the dye is not free to react freely with available calcium ion.

SUMMARY AND CONCLUSIONS

The usefulness of semilogarithmic plots of the Kubelka-Munk function and the product of time x intensity has been shown applicable to tablet systems other than those originally employed. The rate constants for tablets of calcium sulfate dihydrate lake, dibasic calcium phosphate-dye or lake, and lactose-dye or lake are not statistically different. However, tablets of calcium sulfate dihydrate and dye show a fading rate significantly higher than other tablet formulas studied. The method of size reduction did not significantly alter the fading rate in those tablets that were mottled or speckled when prepared by micropulverization.

It is believed that better control of pressure and the selection of more closely matched tablets will reduce experimental error due to tablet-to-tablet variation. This factor along with the use of color differences calculated from tristimulus values will no doubt lead to new interpretations of color fading.

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In Vitro Evaluation of Sustained-Release Tablets by Dual Channel Scintillation Counting

By K. O. MONTGOMERY, C. V. FLEMMING, M. H. WEINSWIG†, R. F. PARKE†, and H. A. SWARTZ†

A rapid procedure was developed for the study of the *in vitro* release characteristics of two radiolabeled drugs in a tertiary drug system. The use of dual channel scintillation spectroscopy allowed for simultaneous determinations of per cent release of each labeled drug from the single sustained-action core tablet.

RADIOISOTOPES have been used extensively in the testing of various pharmaceutical dosage forms (1). The literature contains several reports of evaluative methodology regarding *in vivo* and *in vitro* uses of radioisotopes with the sustained-release dosage form (2–5). This report will be restricted to the determination of the release characteristics of Cl-36 labeled phenylephrine hydrochloride and C-14 labeled aspirin in the presence of chlorpheniramine hydrochloride *in vitro* from the single sustained-release core tablet. The method employed was that of dual channel scintillation spectroscopy which permitted the simultaneous determination of the percentage released of the individual radiolabeled drugs.

EXPERIMENTAL

Preparation of Labeled Compounds.—Chlorine-36 labeled phenylephrine hydrochloride was prepared by dissolving 3 Gm. of compound in distilled water and crystallizing out the free base from the solution rendered alkaline with ammonia. The base was filtered, washed, and dried. Fifty microcuries of chlorine-36 labeled hydrochloric acid was then added to a mixture of 1.23 Gm. of the base and 4 ml. of distilled water. One drop of methyl red T.S. was added; the solution was neutralized with dilute hydrochloric acid. This solution was later added to the mass during the preparation of the tablet cores. Carbon-14 labeled aspirin was synthesized using 50 μc. of carbon-14 labeled acetic anhydride and salicylic acid A.R. using routine procedures (6). The labeled aspirin was then blended with about 25 Gm. aspirin C.P. by dissolving both in an ether–petroleum ether mixture. The blend was then crystallized and dried. The specific activities for the respective compounds were 15 μc./Gm. for phenylephrine hydrochloride and 45 μc./Gm. for aspirin prior to dilution.

Preparation of Tablets.—A batch of 100 core tablets, each made to weigh 405 mg., was prepared by using a single punch tablet press fitted with a $^{11}/_{32}$-in. die and standard concave punches. Each tablet contained 180 mg. of labeled aspirin, 1.3 mg. of labeled phenylephrine hydrochloride, and 8.3 mg. of chlorpheniramine hydrochloride after preparation by the methods of Nash and Jeffries (7, 8). The cores were not compression coated. An immediate release portion—additional quantities of drugs in lactose—would lend nothing to the evaluation of the sustained-action formula and, therefore, was not used.

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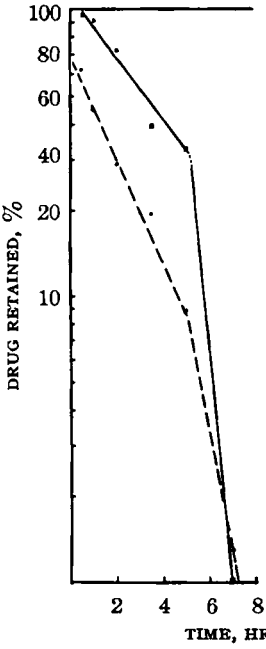


Fig. 1.—Release *in vitro* of aspirin and phenylephrine hydrochloride in simulated gastric juice T.S. Key: ——, aspirin; - - -, phenylephrine hydrochloride.

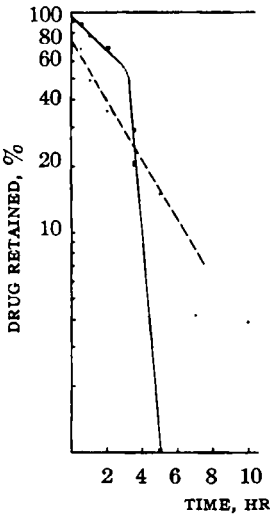


Fig. 2.—Release *in vitro* of aspirin and phenylephrine hydrochloride in simulated intestinal fluid T.S. Key: ——, aspirin; - - -, phenylephrine hydrochloride.

Dissolution and Sampling Procedure.—The rotating bottle apparatus of Souder and Ellenbogen (9) was placed in a water bath with a temperature of 37.5 ± 1°. Into three separate bottles was added 60 ml. of simulated gastric fluid T.S. Each of three more bottles received 60 ml. of simulated intestinal fluid T.S. One core tablet was placed in each bottle and the instrument rotated with a speed of 44 r.p.m. Samples ranging from 0.1 ml. to 0.2 ml. were withdrawn from each bottle at periodic intervals. Each sample was added to a counting bottle[1] which contained 10 ml. of the liquid scintillation solvent (0.4% 2,5-diphenyloxazole, 0.01% 2,2-*p*-phenylene bis-5-phenyloxazole, 5% naphthalene, 20% ethanol, *q.s.* to 100% with toluene). The counting bottles were then refrigerated at −5° for 24 hours for dark adaptation and temperature stability. A blank consisting of 10 ml. of liquid scintillation solvent and 0.2 ml. of distilled water served as the sample for background determinations.

Counting of Samples.—All vials were placed in the detector[2] at −5° for a 10-minute count. The scaler[3] and pulse height analyzer[3] were adjusted to permit simultaneous measurement of the carbon-14 and chlorine-36 by means of dual channel counting. At the selected settings the counting efficiencies were determined using standards of known activity (10). Each sample was counted for 10 minutes. The three count determinations for each sampling time were averaged for each channel. Because each isotope used possesses an energy spectrum and is not monoenergetic, it was necessary to correct the preliminary results to eliminate the contribution of activity of each isotope in each channel.

[1] Wheaton glass counting vials, T. C. Wheaton, Milville, N. J.
[2] Ekco universal counter No. N664, American Tradair Corp., Long Island City, N. Y.
[3] Ekco scaler No. N610 A, American Tradair Corp., Long Island City, N. Y.

RESULTS AND DISCUSSION

The sensitivity of the isotope tracer method allows for selection of extremely small samples. The data presented consist of averages of runs for only three tablets. It could well have been only one tablet because the range of gross counts at each time interval did not exceed ±5% of the mean gross count.

In graphically recording drug release it was most convenient to plot the log of the per cent of drug retained *versus* time. In this study the highest corrected activity was arbitrarily chosen as 100% release. Plots of the release patterns of aspirin and phenylephrine hydrochloride in two media may be seen in Figs. 1 and 2, which illustrate that the release in both media assumes typical pseudo-first order behavior (11, 12). Thus, it appears that this procedure may have merit in studying the rate and reliability of release of single tablets at the stage of formulation.

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Relationship Between Elimination Rate of Drugs and Rate of Decline of Their Pharmacologic Effects

Sir:

Recent advances in the mathematical analysis of the kinetics of drug absorption, metabolism, and excretion make it timely to consider the relationship between the time course, after drug administration, of drug content in the body and the time-intensity course of the pharmacologic effect elicited by the drug. Of particular interest is the nature of the relationship between drug elimination rate and the rate of decline of a pharmacologic effect. It has been pointed out previously (1) that several factors can account for appreciable differences in rates of these two processes; the rate of decline of a pharmacologic effect may be related, among other factors, to the rate of reformation of an essential enzyme, to the rate of drug diffusion from a target site, to the rate of elimination of a pharmacologically active metabolite, or it may even be characteristic of the effect itself rather than of the drug (2). The rate of decline of certain types of pharmacologic activity can be modified also by physiologic compensatory (homeostatic) mechanisms. It is proposed to examine here the relatively uncomplicated case in which the administered drug is pharmacologically active as such, its biotransformation products are inactive, and the intensity of pharmacologic activity at any given time is some function of the amount of drug in the body at that time. It is assumed, for the sake of simplicity, that drug absorption is instantaneous (as would be the case upon intravenous administration).

It is found frequently that the intensity of a pharmacologic effect is related linearly, over a considerable range, to the logarithm of the administered amount of drug (3). Thus

$$E = m \log A + e \qquad \text{(Eq. 1)}$$

where E is the intensity of the pharmacologic effect, A is the amount of administered drug, m is the slope of the line when E is plotted *versus* log A, and e (which usually is a negative quantity) is the intercept of the line on the E axis. Equation 1 may be expressed

$$\log A = \frac{E - e}{m} \qquad \text{(Eq. 2)}$$

Considering the common case where drug elimination follows first-order kinetics

$$\log A = \log A_0 - \frac{K}{2.3} t \qquad \text{(Eq. 3)}$$

where A is the amount of drug in the body at time t, A_0 is the intercept at zero time of the extrapolated linear portion of a plot of the logarithm of A *versus* time, and K is the first-order elimination rate constant for the drug. Substituting for log A and log A_0 in Eq. 3 from Eq. 2 yields

$$\frac{E - e}{m} = \frac{E_0 - e}{m} - \frac{K}{2.3} t \qquad \text{(Eq. 4)}$$

which, upon multiplication of each term by m and rearrangement, results in

$$E = E_0 - \frac{Km}{2.3} t \qquad \text{(Eq. 5)}$$

Also

$$E = E_0 - \frac{0.3m}{t_{1/2}} t \qquad \text{(Eq. 6)}$$

where $t_{1/2}$ is the biologic half-life of the drug. Significantly, Eqs. 5 and 6 are zero order and, under these conditions, the rate of decline of pharmacologic activity is a function of drug elimination rate and of m, with the latter value reflecting quantitatively (over the applicable range) the proportionality between intensity of the pharmacologic effect and the logarithm of the amount of drug in the body.

It is not uncommon to find apparent zero-order

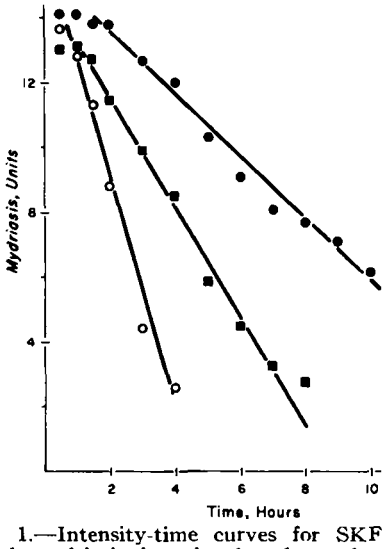


Fig. 1.—Intensity-time curves for SKF 5516-induced mydriasis in mice based on data from Sturtevant (5). Differences in slopes suggest variation of K with dose (see text). Key: ●, 3 mg./Kg.; ■, 1 mg./Kg.; ○, 0.3 mg./Kg., intravenously.

rates of decline of pharmacologic activity described in the pharmacologic literature. For example, the time course of mydriatic response by mice to atropine is apparently zero order; this is particularly interesting in view of the excellent linearity of the response *versus* log dose plot obtained in the same study (4). The time course of mydriatic response to two other anticholinergic drugs has been described recently in terms of first-order kinetics (5), yet the data are, in fact, much more representative of zero-order kinetics (for example, see Fig. 1). Naturally, the occurrence of apparent zero-order kinetics in the decline of pharmacologic activity, while consistent with the kinetic relationship developed here, cannot prove its existence. Moreover, none of these comments should be interpreted as ruling out the theoretical basis for the occurrence of first- or second-order kinetics with respect to the rate of decline of some types of pharmacologic activity.

Perhaps the most important conclusion to be derived from the present discussion is this: the frequent reasoning (see, for instance, the references cited by Schaumann and Stoepel (6)) that the pharmacologic effect is likely to decline exponentially because the body drug content does is intrinsically wrong unless the intensity of a pharmacologic effect is a linear function of the dose. The latter occurs quite infrequently, although we have recently reported one such case (7). It seems desirable, therefore, to be circumspect in depicting activity *versus* time data and to consider the possibility of a linear decline of activity with time.

The relationship expressed in Eq. 5 has interesting implications. It indicates that, despite a direct relation between body drug content and intensity of the elicited pharmacologic effect, rates of decline of these two quantities can follow different kinetics and, therefore, not be parallel. Since not only K but also m can vary significantly in a series of similar pharmacologic agents (8), the possibility presents itself, through the design and choice of agents with different K/m ratios, of modifying the relationship between the time course of body drug content and the time-intensity course of a pharmacologic effect. This may be a means of reducing certain side effects associated with a given class of chemotherapeutic agents. The K/m ratio may also be a useful additional parameter for characterizing the properties of certain drugs.

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Tumor Inhibitors III. Monocrotaline, the Active Principle of *Crotalaria spectabilis*

Sir:

In the course of a continuing screening program for tumor inhibitors from plant sources, an alcoholic extract of the fruits of *Crotalaria spectabilis* Roth,[1] was found to have reproducible activity against adenocarcinoma 755 in mice.[2] We report herein the fractionation of the active extract and the isolation and characterization of the active principle, monocrotaline.

Preliminary studies of the alcoholic extract indicated that partition between chloroform and water resulted in concentration of the activity in the water phase. Upon treatment of the aqueous solution with dilute aqueous alkali, the active principle was liberated from its salt form and became extractable into chloroform. The systematic procedure which was subsequently developed for isolation of the active alkaloid started with extraction of dried ground plant material in a Soxhlet extractor with 95% ethanol. After concentration of the extract under water pump pressure, a thick brown resinous solid was obtained. Partition between

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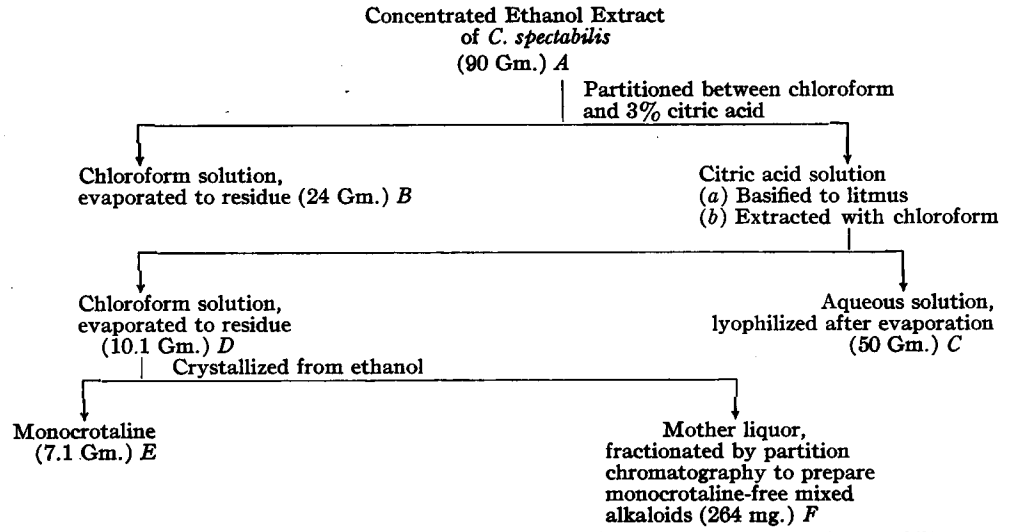


Fig. 1.—Flow sheet for the isolation of the tumor-inhibiting principle from *C. spectabilis*.

TABLE I.—ACTIVITY OF FRACTIONS FROM *C. spectabilis* IN ADENOCARCINOMA 755

Fraction	Control No.	Dose mg./Kg.	Survivors	Animal Wt. Change Diff. (T-C)	Tumor Wt., mg. (Test/Control)	T/C
A	1536	500	6/6	−1.4	393/1294	30
		250	5/6	−1.1	798/1294	61
		125	5/6	−0.4	1523/1294	117
B	1552	300	6/6	−2.7	553/974	56
		150	5/6	−0.2	861/974	88
		75	6/6	−0.6	1293/974	132
C	1552	1000	6/6	−1.1	925/974	94
		500	6/6	−0.8	1001/974	102
		250	5/6	−1.4	1228/974	126
D	1552	200	6/6	−1.9	418/974	42
		100	6/6	−0.9	835/974	85
		50	6/6	−0.1	1169/974	120
E	1552	140	6/6	−1.8	274/974	28
		70	6/6	−1.8	352/974	36
		35	6/6	−0.1	600/974	61
F	1574	100	6/6	−0.2	779/907	85
		50	5/6	−0.5	690/907	76
		25	6/6	+1.0	1033/907	113

chloroform and 3% citric acid solution, followed by basification of the acid solution and extraction with chloroform effected concentration of the active material into a single crude alkaloidal fraction. The latter fraction was readily crystallized from absolute alcohol to afford a high yield of monocrotaline (I).

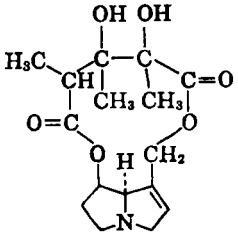
The flow sheet for the isolation is given in Fig. 1; the biological data for the fractions obtained in a typical experiment are reported in Table I. The evaluation of assay results by CCNSC on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less.[3] The data in Table I indicate that only subfractions *D* and *E* showed tumor-inhibitory activity.

Paper chromatographic examination of the mother liquors after crystallization of monocrotaline (using Whatman No. 2 paper and a solvent system of 1 part 5% acetic acid equilibrated with 1 part *n*-butanol (1)) revealed the presence of two other alkaloids of lower R_f value. Preparative partition chromatography on Whatman paper powder (standard) with the same solvent system afforded a monocrotaline-free alkaloid mixture (*F*). The absence of significant tumor-inhibitory activity from fraction *F*, coupled with the high yield of monocrotaline isolated from the active concentrate *D*, support the conclusion that monocrotaline was principally —if not solely—responsible for the tumor-inhibitory activity of the alcoholic extract of *C. spectabilis*.

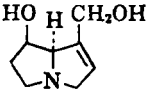
Characterization of the active principle as monocrotaline (I) was effected by comparing the melting points of the alkaloid and its picrate and methiodide derivatives with reported values (2) and the infrared spectrum of the alkaloid with the recorded spectrum (3). In addition, hydrolysis with barium hydroxide solution afforded retrone-

[3] For further details compare protocols described in the reference in *Footnote 2*.

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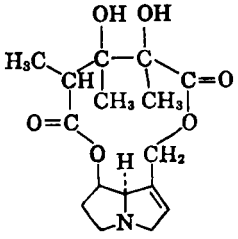
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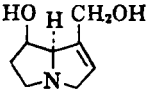
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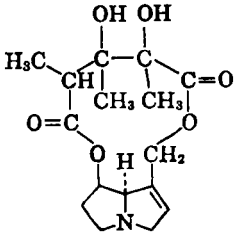
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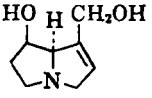
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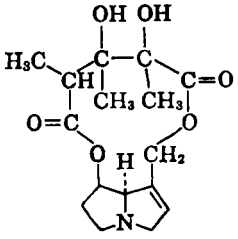
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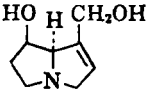
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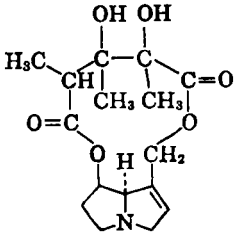
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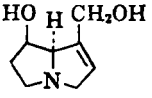
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Review Article

Factors in the Design of Active-Site-Directed Irreversible Inhibitors

By B. R. BAKER

CLASSICAL ANTIMETABOLITES—those having only a small change in structure compared to the substrate—have such a limitation in the structural changes that can made (1), that utility is considerably restricted. In contrast, nonclassical antimetabolites—those having large but appropriate structural changes compared to substrate—should have much wider application (1, 2). For example, the concept of irreversible inhibition by the exoalkylation mechanism with nonclassical antimetabolites was proposed in 1959 (2). A properly designed inhibitor such as 4-(iodoacetamido)salicylic acid can reversibly complex with an enzyme such as GDH,[1] then become irreversibly bound within the complex by alkylation of the enzyme adjacent to the binding site; an expression more general than exoalkylation is "active-site-directed irreversible inhibition." This review will attempt to show the evidence for the phenomenon of active-site-directed irreversible inhibition, how the design of the required inhibitors can be experimentally approached, and the possible utility of the phenomenon in chemotherapy and protein structure studies.

CHEMICALLY REACTIVE FUNCTIONAL GROUPS ON ENZYMES

Since proteins in general and enzymes in particular are highly organized but heterogeneous polymers linked through a polyamide backbone, it has long been obvious that along the polymer chain there will be reactive functional groups; these reactive functional groups result from the third group in trifunctional amino acids such as the ϵ-amino group of lysine, the γ-carboxyl of glutamic acid, the mercapto group of cysteine, the hydroxyl group of serine and threonine, the methylthio group of methionine, the imidazole of histidine, the phenolic hydroxyl of tyrosine, and others. Over the years, many studies have been made with relatively common reagents that might be specific for one of these functional groups (3)—for example, heavy metal ions are apparently specific for thiol groups. However, not all the available thiol groups will necessarily react since some may be buried in the hydrophobic center of the enzyme. Furthermore, all thiol groups may not be necessary for the functioning of the enzyme; for example, 10 thiol groups of aldolase can be destroyed without loss of activity (4). Thus, it is clear that chemical interaction of simple reagents with enzymes by S_N2 type reactions would not have the specificity necessary to have relatively specific effects on one enzyme, let alone the specificity with a multiplicity of enzymes required for chemotherapy.

MAJOR MILESTONES IN ACTIVE-SITE-DIRECTED IRREVERSIBLE INHIBITION

Diisopropyl Fluorophosphate.—A major milestone in specificity of chemical reaction on an enzyme occurred with the discovery (3,

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[1] The following abbreviations are used: GDH, glutamic dehydrogenase; LDH, lactic dehydrogenase; DFP, diisopropyl fluorophosphate; DPN, diphosphopyridine nucleotide.

5, 6) that DFP[1] was a specific reagent for the active site of esterases and proteolytic enzymes.



R and R′ = alkyl
X = —F, —CN, —$OC_6H_4NO_2$

Many related compounds (I and II) have been found to have utility in clinical medicine and as insecticides (7). Although these compounds (I and II) will react with a number of esterases and proteolytic enzymes by formation of a phosphoryl serine in the active site, the action is limited only to this type of enzyme, and others are not inhibited (3). This specificity is clearly due to the unique arrangement in the active site and the mechanism of action of the enzymes that can be inhibited; in all cases an acylated enzyme is an intermediate in the enzymic hydrolysis of the substrate, and the acylated enzyme then undergoes further reaction to give the free enzyme and the acidic hydrolysis product. However, a properly substituted phosphate (II) can form a phosphorylated enzyme which may dephosphorylate to free the enzyme at such a slow rate that it results in selective inactivation at the active site; phosphonates can also interact in the same way. The interaction of DFP with cholinesterase (5, 6) can be considered to be the first example of *active-site-directed irreversible inhibition* that occurred by taking advantage of some unique property of an enzymic active site. It should be noted that of a total of 20 serine residues in chymotrypsin, only one reacts specifically with DFP (8), thus indicating the unique character of the single serine residue at the active site.

Azaserine.—The second milestone in specificity of chemical reaction with an enzyme occurred with the discovery that the L-glutamine (III) antagonists, L-azaserine (VII), and 6-diazo-5-oxo-L-norleucine (VIII) rapidly react chemically with the active site of the enzyme that converts formylglycinamide ribotide (V) to the corresponding amidine (VI) using L-glutamine (III) as the cofactor (9). Kinetic data indicated that L-azaserine (VII) was a competitive inhibitor for L-glutamine (III); rapid inactivation apparently occurred within the enzyme-inhibitor reversible complex by an internal alkylation reaction. Later work by Buchanan, *et al.* (10, 11), showed that a single cysteine residue had reacted specifically with azaserine, as determined by hydrolysis to S-carboxymethyl-L-cysteine. The internal alkylation reaction within the enzyme-azaserine complex can be considered the first example of *a properly constructed antimetabolite causing specific active-site-directed irreversible inhibition.* Azaserine chemically interacts with those enzymes that transfer the amide group of L-glutamine to substrate (12–14), but is not a general enzyme poison (2,10,11, 14). Thus, the unique specificity for this chemical interaction is dependent upon the nature of the active site.

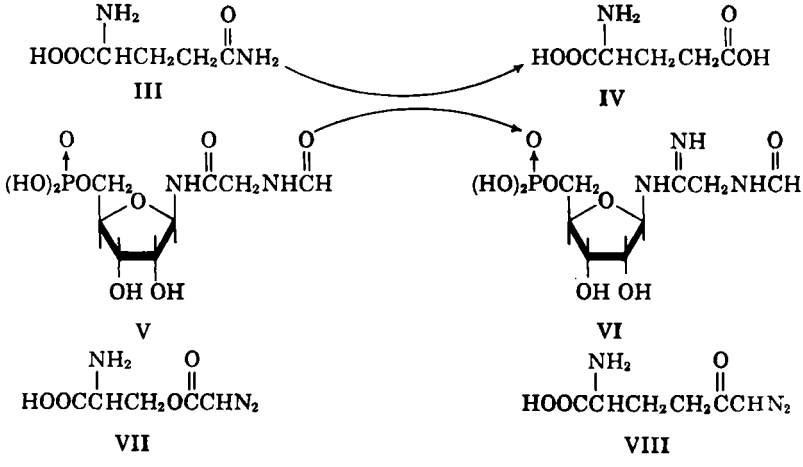
ENDO AND EXOALKYLATION CONCEPTS

Endoalkylation.—The observations by Buchanan, *et al.* (9), on the selective irreversible inhibition by azaserine (VII) led Baker (2) to propose the concept of endoalkylation. When Buchanan's observations were combined with observations of over-sized inhibitors (15, 16), the concept of exoalkylation was also proposed (2).

Enzymatic reactions utilizing a cofactor can be written in the following general form

$$S + FX \rightleftarrows SX + F \qquad \text{(Eq. 1)}$$

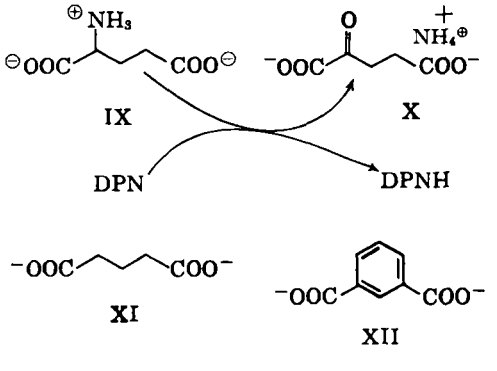
where S is the substrate and F is a cofactor bear-



ing a group X which is to be transferred to substrate. In order for the group X to be transferred to substrate, the group X must be held in juxtaposition between F and S by the active site of the enzyme as shown in Fig. 1; if more than a single interatomic distance exists between S and X, then new bond formation to form SX will be difficult. When the group, X, is replaced by B, as shown in Fig. 2, an inhibitor results if B is a group that cannot be transferred. If the group B is the diazomethyl group of azaserine (VII), then reaction with the SH group within the active site occurs as shown by the arrow; the formation of this new covalent bond stops the dissociation of the enzyme-inhibitor complex, and the active site becomes selectively denatured. Since the alkylating group, B, has replaced the transfer group, X, the alkylation most probably takes place *within the active site* (*endoalkylation*).

The active site may not be able to tolerate a group, B, that is bulkier than X since there may no longer be room for the juxtapositioning of S and FB; that such may be the case is indicated by the fact that the higher homolog of azaserine, α-diazopropionyl-L-serine shows no biological activity (17). This limitation on bulk greatly reduces the utility of the endoalkylation concept since only small changes in structure of the substrate or cofactor can be made. It was proposed (2) that another small B group that might be useful for endoalkylation would be the FCH_2CO- group; as will be described later, the $ClCH_2CO$- has been found useful for endoalkylation.

Exoalkylation.—To develop the concept of exoalkylation, the critical dimensions of an antimetabolite must be considered. Hellerman, *et al.* (16), observed that both glutarate (XI) and isophthalate (XII) effectively inhibited the GDH-catalyzed oxidation of L-glutamate (IX) to α-oxoglutarate (X)



by DPN. Since isophthalate (XII) can fit the active site of GDH as well as the substrate,

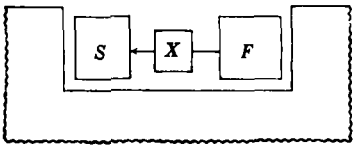


Fig. 1.—A simplified diagram of the enzyme-catalyzed transfer of the group X from a cofactor F to the substrate, S, both molecules being juxtapositioned at the active site of the enzyme.

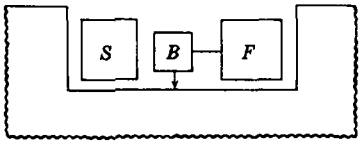
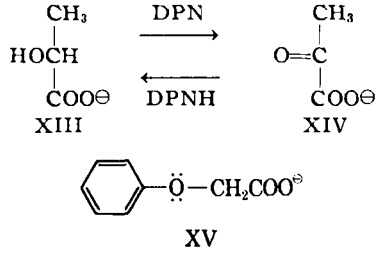


Fig. 2.—A simplified diagram of endoalkylation. A group, B, replaced the transfer group, X, in Fig. 1. B can then alkylate some nucleophilic group within (endo) the active site to form a covalent bond.

glutamate (IX), it is apparent that glutamate must assume the conformation equivalent to isophthalate when bound to the enzyme (16). It is also apparent that the remainder of the benzene ring of isophthalate (XII) constitutes a considerable change in gross measurement compared to the substrate, glutamate (IX) (2).

Inhibitors of the enzymatic oxidation of succinate to fumarate can vary considerably in gross measurements, providing the change is only at one α-position of succinate; thus α-alkylsuccinic acids, even with large alkyl groups, competitively inhibit succinic dehydrogenase (18).

Phenoxyacetic acid (XV) has been observed to be a competitive inhibitor of LDH;[1] it was proposed that phenoxyacetate (XV) binds to LDH[1] through its carboxylate group and an electron



pair of the ether oxygen the same as the substrate, lactate (XIII) (15). When bulky groups were placed on the benzene ring of phenoxyacetate (XV), little change in binding occurred (1), thus indicating that the benzene ring was not in contact with the enzyme surface (see Fig. 3).

If the alkylating group, B, (Fig. 3) is on the side of the inhibitor not in contact with the enzyme and is placed so that it can bridge to some nucleophilic group on the enzyme surface, then covalent bond formation might take place (shown

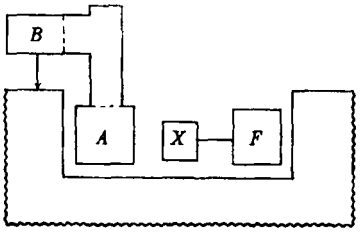


Fig. 3.—A simplified diagram of an oversize (nonclassical) inhibitor. Note that the excess size above the horizontal dotted line of substrate faces away from the enzyme surface. When the alkylating group *B* can bridge to a nucleophilic site on the enzyme surface adjacent to the active site, covalent bond formation (arrow) occurrs outside the active site (exoalkylation).

by arrow). The new bond formation outside the active site is termed exoalkylation; such bond formation can stop dissociation of the enzyme-inhibitor complex in the same fashion as the endoalkylation example with azaserine (VII).

EXAMPLES OF ENDO AND EXOALKYLATING IRREVERSIBLE INHIBITORS

After Buchanan's first announcement on the irreversible inhibition of an enzyme by azaserine (VII) (9), some 4 years elapsed before another example of an active-site-directed irreversible inhibitor appeared in the literature. In 1961, Baker, *et al.* (19), presented data that 4-(iodoacetamido)salicylic acid (XLVI) complexed with the active site of GDH, then caused irreversible inhibition by the exoalkylation mechanism. 4-(Iodoacetamido)salicylic acid is believed to be the first example of an active-site-directed irreversible inhibitor deliberately designed as such; the earlier examples, DFP and azaserine, were found to be active-site-directed irreversible inhibitors when their biological activity was investigated. In 1962, five other laboratories (20–24) announced their independently conceived work on active-site-directed irreversible inhibitors and in 1963 a seventh laboratory announced another example (25). Of these seven different examples, four were of the exoalkylation type and three were of the endoalkylation type. It is also of interest that five of the seven examples were irreversible inhibitors of chymotrypsin, one was an irreversible inhibitor of GDH, and one of a combining region of an antibody. Rather than discussing the seven examples in chronological order, the five examples with chymotrypsin will be discussed first.

Active-Site Labeling of Chymotrypsin.—The proteolytic enzyme, chymotrypsin (XVI), first complexes reversibly with the substrate (XVII, an ester or amide) to form XVIII; the reversible complex (XVIII) then undergoes an internal alcoholysis reaction with a serine residue to form the acyl chymotrypsin (XX) and an amine or alcohol (XVII*a*). Presumably through mediation of a nearby histidine residue, XX undergoes deacylation to give the acid (XIX) and regenerated enzyme (XVI). The enzyme is rather nonspecific and a variety of amides and esters, usually of L-configuration, can be hydrolyzed; the relative rates of acylation of XVI and deacylation can vary dependent upon the nature of XVII and the resultant seryl ester, XX. For example, pivalyl-chymotrypsin deacylates much slower than natural acyl chymotrypsins (26–28).

In an elegant study, Lawson and Schramm (22) selected *p*-nitrophenyl *N*-bromoacetyl-α-amino-isobutyrate (XXI) as a bifunctional reagent "so designed that it becomes covalently bound to an amino acid side chain at the active site, and then, fixed in position, reacts with another amino acid in the vicinity." The acyl group becomes bound to a serine residue at the active site (XX*a*), then attacks an adjacent amino acid (exoalkylation) which ultimately was shown to be a methionine residue (as in XXIII).

Perhaps fortuitously, but more likely by intent, Lawson and Schramm (22) selected an acylated chymotrypsin (XX*a*) that should deacylate slowly, analogous to pivalyl-chymotrypsin; such a slow deacylation gives a greater half-life for a molecule such as XX*a*, thus giving more time for a neighboring group reaction to take place with formation of XXIII. In 4 hours at ambient temperature, a 10:1 ratio of XXI to chymotrypsin (XVI) react to give a solution containing 20% of the original enzyme activity; this activity fails to decrease further with time. Evidence that the active site of chymotrypsin (XVI) is involved in this inactivation is as follows.

(*a*) The initial rapid "burst" of *p*-nitrophenol when XX*a* is formed is retarded by the presence of the reversible inhibitor, 3-indolepropionic acid, also retarding the rate of inactivation.

(*b*) *N*-Bromoacetyl-α-aminoisobutyric acid in a hundredfold molar excess causes no inactivation under the conditions used with XXI, thus giving evidence that an acyl-chymotrypsin (XX*a*) is an intermediate in the inactivation.

(*c*) When the serine residue of chymotrypsin is first blocked by conversion to the diisopropyl phosphoryl derivative with DFP (5, 6), no alkylation of methionine by XXI occurs (see Fig. 5), thus excluding a random alkylation reaction of the simple S_N2 type.

Lawson and Schramm (22) then showed by amino acid analysis that the 80% inactivated

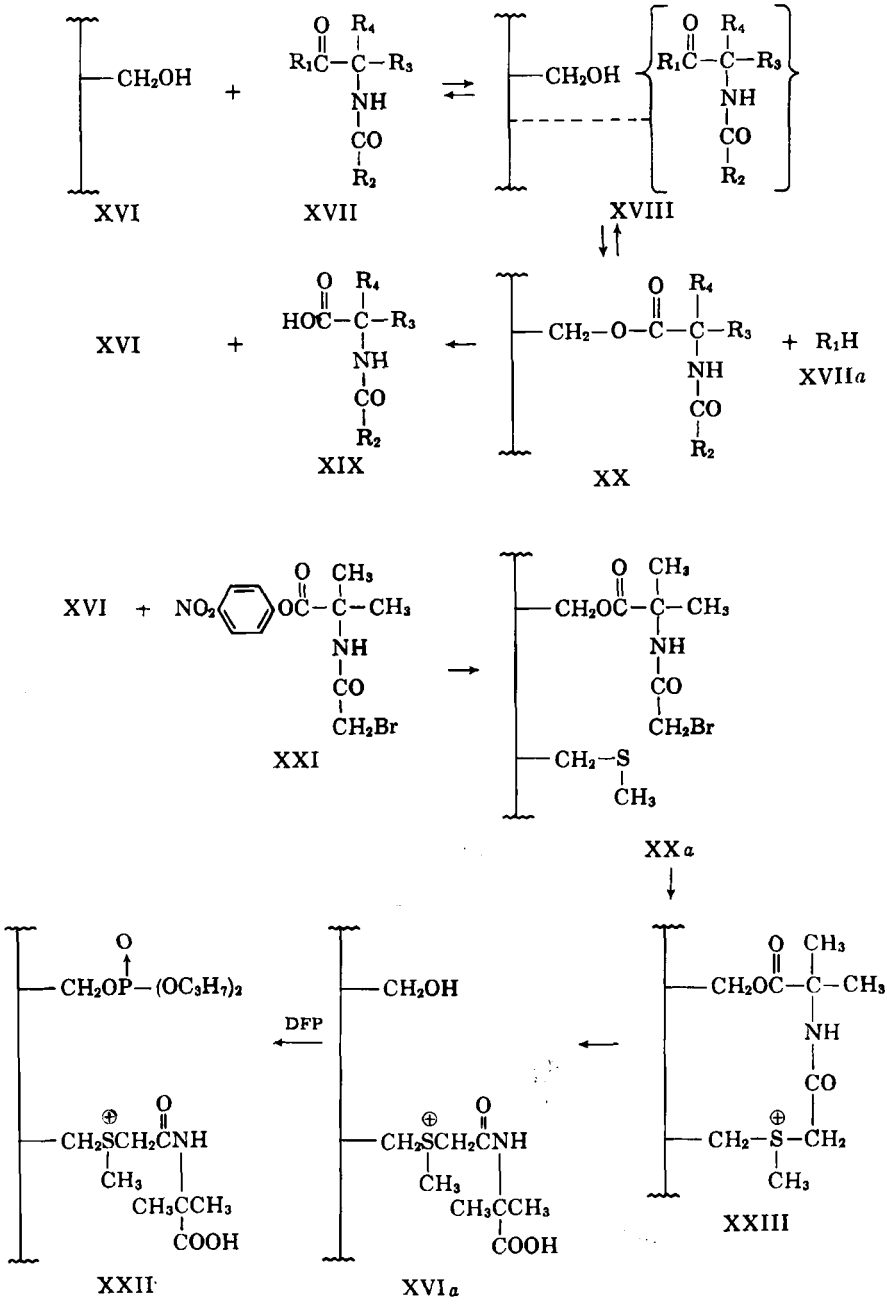


Figure 4.

chymotrypsin no longer contained one of its two methionine residues. The missing mole of methionine appeared as 0.2 mole of S-carboxymethyl-L-homocysteine (XXVI), 0.1 mole of homoserine (XXVII) and 0.3 mole of homoserine lactone (XXVIII); in addition, about 1 mole of α-aminoisobutyric acid (XXIV) was recovered that must have been bound (as in XVI*a*) to the 80% inactivated, but dialyzed, chymotrypsin. Such a splitting pattern of a carboxymethylated methionine residue in ribonuclease had been previously observed (28), and XVI*a* $\equiv$ XVI*b* could be expected to give a similar hydrolysis pattern.

That the inactivated chymotrypsin was a modified enzyme (XVI*a*) with only 20% of the activity of the original chymotrypsin (XVI) was also clearly demonstrated as follows.

(*a*) Further reaction of the modified chymotrypsin of 20% activity (XVI*a*) with DFP resulted in complete inactivation of the enzyme and 1 mole of phosphorus per mole of enzyme was introduced, as shown in structure XXII.

(*b*) The Michaelis constant (K_m) for tyrosine ethyl ester was increased by elevenfold; thus, the active site of XVI*a* is still capable of functioning, but offers some hindrance to reversible complexing with substrate in the normal fashion (XVIII).

At about the same time, Gundlach and Turba (23) reported a similar study with chymotrypsin (XVI*c*) and methyl iodoacetyl-phenylalaninate (XXIX). Since the conditions used by Gundlach and Turba for their inactivation studies are somewhat different than those reported by Lawson and Schramm (22), it is difficult to interpret the much slower rate of reaction of XXIX with chymotrypsin (XVI*c*) compared to XXI. In fact, the data of Gundlach and Turba could be interpreted to indicate that the two compounds inactivate by slightly different mechanisms, even though one methionine residue is attacked in both cases. Note the following information.

(*a*) The L-isomer of XXIX gives insignificant inhibition of chymotrypsin after 14 days at 0°, whereas the D-isomer of XXIX gives 35% inhibition under these conditions. Since *N*-acyl esters of D-phenylalanine are reversible inhibitors that do not form an acyl-chymotrypsin at a reasonable rate, it would appear that the inactivation by the D-isomer of XXIX might not occur through an acyl-chymotrypsin such as XX*a*, but might occur through the initial reversible noncovalent complex of type XXX with formation of XXXI without loss of the ester group. This point could be resolved by using a C^{14}-methyl ester group or a *p*-nitrophenyl ester (22).

(*b*) That inactivation may also occur through a reversible complex such as XXX is further supported by the fact that the rate of incorporation of nondialyzable C^{14} from C^{14} labeled XXIX parallels the rate of inactivation; if inactivation occurred *via* a covalent complex such as XX, labeling might be expected to proceed more rapidly than inactivation, particularly with a *p*-nitrophenyl ester.

(*c*) Iodoacetamide and iodoacetate gave no inactivation under the same conditions used for XXIX, excluding a random S_N2 inactivation mechanism.

Since the inactivation of chymotrypsin by XXI (Fig. 4) and by XXIX (Fig. 5) may proceed by slightly different mechanisms, it cannot be certain at this time that the same methionine is attacked in both cases. Needless to say, the experiments of Lawson and Schramm (22) are more clean-cut mechanistically than those of Gundlach and Turba (23); the latter workers could certainly clarify the situation by using some of the techniques of Lawson and Schramm.

Gundlach and Turba (23) consider the alkylation reaction that forms XXXI to be an introduction of "a reactive residue into the 'active center' of the enzyme." Since we consider the formation of XXXI (Fig. 6) and of XXIII (Fig. 4) to occur by the exoalkylation mechanism "adjacent to the active site" (1, 2, 19), some redefinition may be necessary, as will be discussed later.

The reports from the other three laboratories (20, 24, 25) on inactivation of chymotrypsin meet

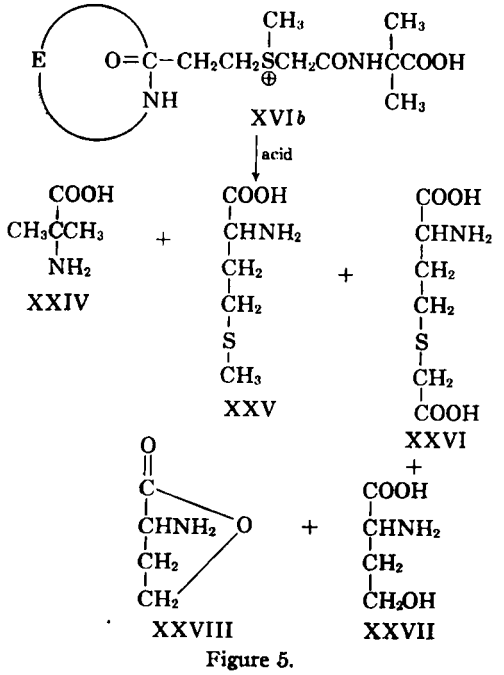


Figure 5.

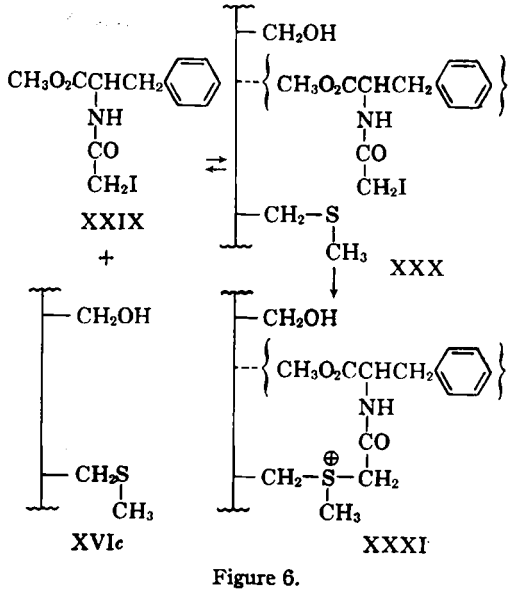


Figure 6.

our earlier criteria (2) for alkylation within the active site, so-called "endoalkylation."

Schoellmann and Shaw (20) wished "to develop new, specific reagents for locating the active centers of enzymes by designing molecules which combine two kinds of structural features—namely, those which provide affinity to the active center and, in addition, a chemically reactive grouping which may anchor irreversibly to the enzyme. . . ." Based on the observation that *N*-tosyl-L-phenylalanine ethyl ester was a substrate for chymotrypsin (29), they synthesized and evaluated the related chloromethyl ketone (XXXII) as an irreversible inhibitor (see Fig. 7) with the following results.

(*a*) The chloromethyl ketone (XXXII) gave 50% inactivation of chymotrypsin (XVI*d*) in 50 minutes at pH 6 and 37°. The rate of inactivation was slowed in the presence of the reversible inhibitor, β-phenylpropionic acid, indicating that the active site was involved.

(*b*) When C^{14}-chloromethyl ketone (XXXII) was employed, a 1:1 ratio of nondialyzable C^{14} to chymotrypsin was observed after inactivation was complete. In the presence of 8 *M* urea, less than 3% binding of C^{14}-XXXII took place under the same conditions, showing that the loss of the tertiary structure and active site caused by the urea also caused loss of chemical reaction with XXXII, implicating a complex with the active site (XVIII*a*) as being a necessary prerequisite for inactivation.

(*c*) *N*-Methylation has been shown to decrease greatly the ability of the modified derivatives to serve as substrates; the *N*-methyl derivative of XXXII gave no inactivation of chymotrypsin under the conditions that XXXII did, thus implicating a reversible complex with the active site (XVIII*a*) as being an intermediate to inactivation.

(*d*) The inactivation of chymotrypsin by XXXII showed a pH profile of dependence with a peak near pH 7.2, the same as the pH profile of the enzymatic reaction; these data also implicate participation of the active site in the inactivation.

(*e*) Amino acid analysis of chymotrypsin inactivated by XXXII showed the loss of one of the two histidine residues originally present in the enzyme; thus, the inactivated chymotrypsin probably has a partial structure of type XXXIII or the corresponding N_1-imidazolyl derivative.

(*f*) When DFP inactivated chymotrypsin was treated with XXXII, no loss of histidine was found, indicating that the reversible complex XVIII*a* is an intermediate to inactivation.

(*g*) That XXXII was not a general enzyme poison was shown by a comparable incubation with trypsin; no loss in activity occurred.

Even though the type of inactivation ("endoalkylation") given by the chloromethyl ketone (XXXII) has precedent in the azaserine (VII) inactivation discussed earlier, the beautiful work of Schoellmann and Shaw (20) stands as a milestone in the progress toward design of active-site-directed irreversible inhibitors. Kallos (25) has independently found a closely related "endoalkylation" of chymotrypsin with "phenylalaninol ditosylate" (XXXIV). The data are meager, and the

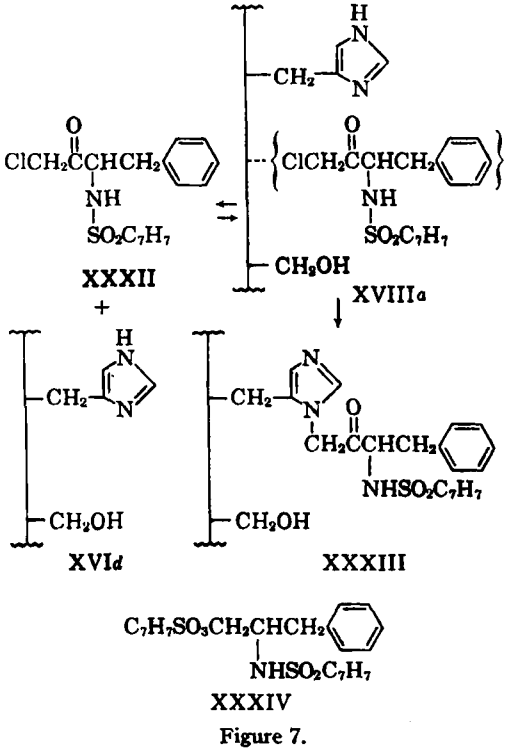


Figure 7.

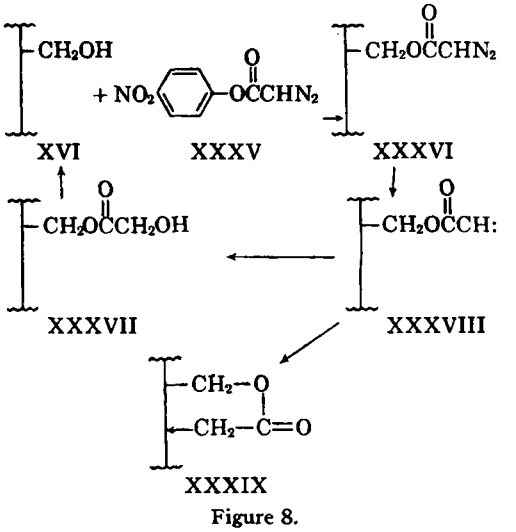


Figure 8.

only evidence that a simple S_N2 inactivation was not the mode of inactivation was that the D-isomer of XXXIV leveled off at 40% inactivation, whereas the L-isomer leveled off at 80% inactivation. Without the thorough work of Schoellmann and Shaw (20) preceding Kallo's work, XXXIV could not even be implicated as an active-site-directed irreversible inhibitor; certainly further criteria such as those used by Schoellmann and Shaw (20) will have to be applied by Kallos before XXXIV can be reasonably accepted as an active-site-directed irreversible inhibitor.

Westheimer, *et al.* (24), have used a photolytic method for introducing a C^{14}-carbene into chymotrypsin (XVI). When the enzyme was reacted with *p*-nitrophenyldiazoacetate (XXXV), (Fig. 8), an acyl-chymotrypsin (XXXVI) was obtained that was stable to deacylation, as previously observed with pivalyl-chymotrypsin and XX*a* (22); however, chymotrypsin (XVI) could be regenerated by treatment of XXXVI with hydroxylamine, then assayed. Since the diazoacetyl chymotrypsin (XXXVI) had a weak absorption band at 370 mμ, this wavelength was used in the photolytic decomposition to minimize photolysis of the parent enzyme. Unfortunately, the resultant carbene (XXXVIII) underwent solvolysis to glycolyl-chymotrypsin (XXXVII) in 80% yield and only 20% inactivation by an insertion reaction (symbolized by XXXIX) took place; the 20% loss of enzyme by formation of XXXIX paralleled a 20% uptake of C^{14} from C^{14}-labeled XXXVI. An investigation of the position of attack by the carbene is being actively pursued, and results will be awaited with interest.

Affinity Labeling of Antibodies.—Wofsy, Metzger, and Singer (21) have used an approach similar to "exoalkylation" for attaching covalently bound groups to antibody molecules, which they termed "affinity labeling." It had been known for a number of years that when a protein is coupled with a diazonium salt such as diazotized arsanilate (XL*a*), then injected into a rabbit, antibodies (XLI*a*) were produced that had specific combining regions dependent upon the character of the attached azo grouping, called a hapten (XL). The use of other compounds attached to the same protein (XL) gave antibody molecules (XLI) that were specific for the particular haptenic determinate in forming insoluble complexes (XLII). It was also known that the hapten (XL*a*)-antibody (XLI*a*) complexing to give the insoluble XLII*a*, could be decreased if the combining region of XLI*a* was complexed with a small molecule such as *p*-nitrobenzenearsonic acid. Wofsy, *et al.* (21), chose this system for studying the nature of the combining regions by covalently attaching specific reagents that were related in structure to the hapten; this system has the advantage that haptenic combining regions can be designed more or less to order.

They reasoned that the diazonium salt (XLIII), from which the original modified protein (XL) was prepared, could combine with the specific antibody (XLI) to form a reversible complex (XLIV), then undergo irreversible reaction by formation of a covalent linkage as depicted in XLV. That affinity labeling (XLV) of the combining region (XLIV) of an antibody could take place is demonstrated in Figs. 9 and 10.

(*a*) The antibody for azobenzenearsonic acid (XLI*a*) reacted rapidly at 0° with the arsonic diazonium salt (XLIII*a*) to form a colored azo derivative (XLV*a*), the rate of formation being followed spectrophotometrically. Of the three types of azo compounds that can be formed in a protein—namely, azohistidine, azolysine, and azotyrosine—only the latter was formed with a peak at about 475 mμ. No azohistidine peak was formed.

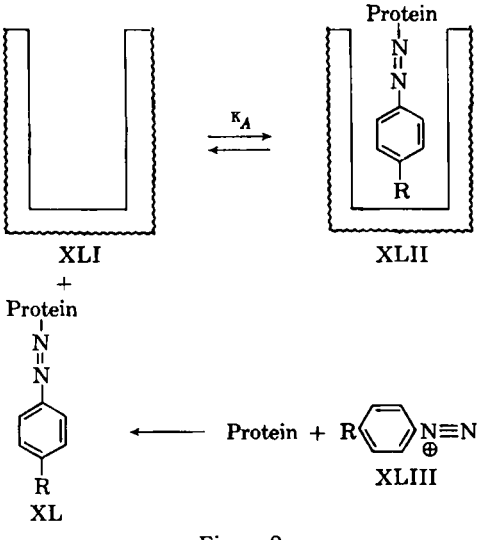
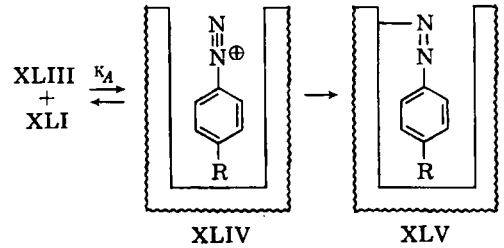


Figure 9.



a series, R = 4-AsO_3H_2
b series, R = 4-COOH
c series, R = 2,4-$(NO_2)_2$-

Figure 10.

(*b*) Normal γ-globulin reacted with the arsonic diazonium salt (XLIII*a*) at one-four-hundredth the rate of XLI*a*. With the assumption that there was only one tyrosine in each of two active sites on the antibody molecule (XLI*a*), but that there were 60 tyrosine residues in a normal γ-globulin molecule, the enhancement of the rate of reaction with XLI*a* was about 10,000; "such a large enhancement is explicable only by the mechanism of initial reversible complex formation [XLIV*a*], according to the theory proposed."

(*c*) The heterologous reagent, *p*-carboxybenzene diazonium salt (XLIII*b*), reacted with the arsonic antibody (XLI*a*) at the same slow rate it reacted with normal γ-globulin, thus showing that there is not a uniquely reactive tyrosine in the arsonic antibody (XLI*a*); these results were consistent with the concept of affinity labeling; that is, when an initial reversible complex (XLIV) is formed, the rate of azo reaction is greatly accelerated by an anchimeric reaction.

(*d*) When the combining region (XLI*a*) of the arsonic antibody was protected by initial complexing with *p*-nitrobenzenearsonic acid, then XLI*a* reacted at the same slow rate with either the arsonic diazonium salt (XLIII*a*) or the carboxybenzene diazonium salt (XLIII*b*) at other regions than the haptenic site.

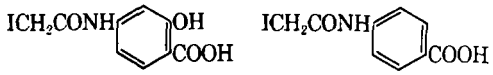
(*e*) An antibody (XLI*c*) specific for the 2,4-dinitrophenyl hapten reacted with a 5-mole ratio of 2,4-dinitrobenzenesulfonyl chloride to give 50% loss of antibody activity in 2 hours at 25°. In the presence of a 50-mole ratio of ε-(2,4-dinitrophenyl)lysine, no loss of antibody activity occurred due to the protective effect of the latter by complex formation.

(*f*) Treatment of the antiarsonic antibody (XLI*a*) with a 10-mole ratio of 2,4-dinitrobenzenesulfonyl chloride under the conditions of (*e*) gave no loss of antibody activity.

(*g*) Kinetic studies showed that increasing the concentration of diazonium reagent (XLIII) increased the rate of reaction with antibody (XLI) clearly less markedly than would be expected for a simple second-order reaction for formation of the labeled antibody (XLV). They demonstrated "that the concentration dependence is entirely accounted for by the unimolecular mechanism" *via* the obligatory formation of a reversible complex (XLIV); that is, the rate of reaction was dependent on the concentration of XLIV.

Wofsy, *et al.* (21), consider that the combining region has been labeled in XLV, again differing, as have Gundlach and Turba (23), with our definition "adjacent to the active site;" some redefinition of "exoalkylation," as discussed later, could clarify these differences in semantics.

Inactivation of Lactic and Glutamic Dehydrogenases.—Evidence for the inactivation of LDH and GDH by 4-(iodoacetamido)salicylic acid (XLVI) by active-site-directed irreversible inhibition has been presented by Baker, *et al.* (19).



XLVI XLVII

(*a*) GDH was about 50% inactivated in 10 minutes at 37° by a 2 m*M* solution of XLVI; iodoacetamide showed no inactivation under the same conditions, thus indicating that a complex between enzyme and inhibitor was necessary for inactivation.

(*b*) GDH was inactivated eight times more rapidly by 2 m*M* 4-(iodoacetamido)salicylic acid (XLVI), then by the same concentration of 4-(iodoacetamido)benzoic acid (XLVII).

This difference in rate was attributed to the difference in enzyme-inhibitor dissociation constants; the K_i for XLVI was smaller, thus giving (at the same inhibitor concentration) a larger amount of reversible enzyme-inhibitor (XLVI) complex; the rate of inactivation is presumably dependent on the concentration of reversible complex.

(*c*) Increasing the concentration of XLVI from 1 m*M* to 2 m*M* increased the rate of inactivation of GDH by 1.23-fold, rather than two-fold; this is considered evidence that the active-site is involved in the irreversible inhibition since the ratio of the rates calculated from K_i is 1.23.

(*d*) Although 4-acetamidosalicylic acid was a reversible inhibitor of GDH as good as XLVI, the former showed no irreversible inhibition of GDH; this experiment was considered evidence that the inactivation by XLVI was not due to a chelation effect of the salicylate structure.

(*e*) The inactivation of GDH by traces of common metallic ions such as silver, iron, copper, chromium, nickel, cobalt, or zinc was considered unlikely since there was no correlation between the trace metal content of XLVI, XLVII, and 4-acetamidosalicylic and their respective rates of inactivation of GDH.

(*f*) That the active site was presumably involved in the inactivation of GDH by XLVI was further indicated by the slower rate of inactivation in the presence of the reversible inhibitor, isophthalate (16)—a protective effect.

The criteria for the inactivation of LDH by XLVI by the exoalkylation mechanism were the same as used for GDH; the same results and con-

clusions were obtained as in the GDH case with two exceptions. The first exception was that the reversible inhibitor, oxamate (30), was used in the protection experiment (*f*) and slowed the rate of inactivation. The second exception was that iodoacetamide as well as XLVI showed irreversible inhibition of LDH, However, 2 m*M* XLVI inactivated LDH at about twice the rate of 4.48 m*M* iodoacetamide. As will be discussed later under the bridge principle, several *N*-substituted iodoacetamides, including 3-(iodoacetamido)oxanilic acid (XLIX), failed to give any measurable irreversible inhibition of LDH, indicating that a properly oriented reversible complex with XLVI is obligatory to inactivation.

ELUSIVE DEFINITION OF AN ACTIVE-SITE

Some difference of opinion exists regarding whether the active-site has been labeled with the specific reagents discussed in the previous section; at this early stage in the science of "mapping the active site," such *a priori* arguments can at best be only differences in philosophy. We have defined alkylation within the active-site stringently as "endoalkylation" (2). Of the work described in the previous section, only that on azaserine (VII), DFP, Shaw's chloromethyl ketone (XXXII), phenylalaninol ditosylate (XXXIV), and diazoacetyl-chymotrypsin (XXXVI) clearly meet these criteria. Those compounds that presumably operate by our definition of "exoalkylation" include Lawson's *N*-bromoacetyl derivative (XXI), Gundlach's *N*-iodoacetyl derivative (XXIX), Singer's hapten-type diazonium salts (XLIII), and Baker's iodoacetamido salicylic acid (XLVI). The argument is dependent upon how large to define the active-site, which obviously cannot yet be done. Two points emerge on which the various involved investigators will most probably agree. (*a*) The "exoalkylation" type definitely forms a covalent bond *adjacent to the reversible combining region of the inhibitor* (region *A* in Fig. 3). The combining region for a substrate or inhibitor is obviously smaller than the total active-site since other groups on the enzyme must be present in the active-site to catalyze the enzymatic reaction. (*b*) Initial complexing with the active-site is an obligatory intermediate to the formation of an irreversible covalent bond; that is, the reaction is controlled by the active-site with its inherent specificity.

The term "exoalkylation" also breaks down in describing adequately the mode of irreversible inhibition caused by 5-(carbophenoxyamino)-salicylic acid (LI), to be discussed in a following section; LI is clearly an acylating agent. Other irreversible inhibitors are neither an alkylating nor an acylating agent, but operate by Michael addition, such as LVI.

At the current level of knowledge concerning the active site, an expression is needed which will cover all of the cases so far discussed. Two expressions emerge which appear to describe the phenomenon adequately without defining the size of the active-site: (*a*) "active-site-directed irreversible inhibition" (31), and (*b*) "affinity-labeling," (21) providing "the remaining question whether the bond is formed with a group actually in the active site or one very close to, but not part of, the site" is left open for future definition when more data become available from mapping studies.

As the title of this review indicates, we plan to use expression (*a*).

BRIDGE PRINCIPLE OF SPECIFICITY

As pointed out in an earlier section, the first two milestones in active-site-directed irreversible inhibition were found by studying the biological mechanism of action of DFP and azaserine (VII). Except for the work in our laboratory, the main concern of subsequent investigators in the period of 1962–1963 was to use the phenomenon for the labeling of active sites of pure enzymes or of specific, though heterogeneous (21*c*), antibodies. Depending upon the goal of the particular investigator, two different criteria for specificity can be envisioned. If the investigator wishes to label the active-site of a pure protein, then he is concerned with finding a reagent that will react specifically with the active-site and have a near zero effect on other parts of the pure protein. In contrast, if the investigator wishes to use "active-site-directed irreversible inhibitors" in chemotherapy, the problem is less concerned with exactly how a given enzyme becomes inactivated and more concerned with the specific inactivation of a single enzyme in an intracellular mixture of enzymes in a functioning cell.

That these two apparently different goals initially converge into one approach becomes apparent when one realizes that the specificity required for inactivation of one enzyme in an intracellular mixture of enzymes must logically reside in the more or less subtle differences in the active-site of enzymes that perform different—but at times mechanistically closely related—enzymic reactions. Once the labeling reagent for an active-site has been found, the experimental approaches to the two goals then diverge dramatically in methodology. The purpose of studying the labeling of the active-site of a pure enzyme is to determine the amino acid sequence surrounding the

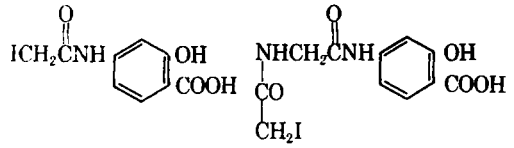
label in order to learn what part of the sequence is at (or close) to the active-site (mapping the active-site). In chemotherapy, the problem is different since the labeling reagent must now be studied to determine how specific it may be in reacting with only one of a number of mechanistically closely related enzymes; it is the latter problem to which we have been devoting our attention, particularly how an active-site-directed irreversible inhibitor may be modified to build in further specificity. This problem has been approached in the following way.

The earlier definition of "exoalkylation" (2) and the experimental support for this phenomenon (19, 21–23, 32) led to the concept (19*b*) of and experimental evidence (31–36) for the bridge principle of specificity; this principle has been restated (31), replacing the word "exoalkylation" by "active-site-directed irreversible inhibition:" "Compared to a reversible inhibitor, the active-site-directed type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly bound inhibitor to bridge to and form a covalent bond with a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being covalently linked."

Nucleophilic Sites.—The bridge principle can be envisioned by inspection of Fig. 3. The group, *B*, of an active-site-directed irreversible inhibitor can form a covalent bond with a nucleophilic group adjacent to the reversible binding region where group *A* is reversibly complexed. In order for this covalent bond to form (shown by the arrow), there must be a proper distance between the *A* and *B* regions of the inhibitor so that bridging can take place; in addition, the electrophilic group, *B*, and the enzymic nucleophilic group must have the ability to interact, since obviously not all electrophilic groups react with all nucleophilic groups. Furthermore, the approach of the electrophilic group to the enzymic nucleophilic group must meet the combined steric and conformational requirements of the active-site, the nucleophilic site, and the environment in between these two sites. These dual site requirements are obviously far more restrictive than the mere additive requirements of each site alone (34). Therefore, the use of the bridge principle should make it possible to obtain highly selective irreversible inhibitors within any group of enzymes that are closely related by the nature of their substrates. Furthermore, it should be noted that an irreversible inhibitor will have a much greater effect on the operation of an enzyme than a reversible inhibitor. The enzyme molecule is still more or less operational in the presence of a reversible inhibitor and substrate, but an irreversibly inhibited enzyme molecule can be completely inoperative. Since theoretically only one irreversible inhibitor molecule is necessary to inactivate one enzyme molecule, and since one active enzyme molecule can convert many substrate molecules to product, an irreversible inhibitor will be far more effective than a reversible inhibitor. Thus, an inhibitor will be more effective on an enzyme that can be first reversibly then irreversibly inhibited than a closely related enzyme that is only reversibly inhibited since enzymes have an estimated concentration of 10^{-10} to 10^{-12} *M*, while substrates have a concentration in the range of 10^{-3} to 10^{-6} *M*.

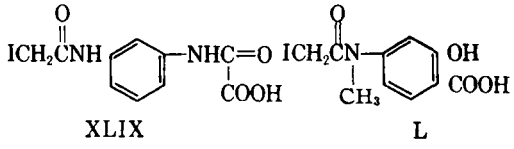
Conformational and Steric Aspects.—For the initial studies on the bridge principle, Baker (33, 34) selected the mechanistically closely related enzymes, GDH and LDH. Earlier, a study of a variety of reversible inhibitors showed that almost all of the compounds that inhibited GDH also inhibited LDH (19*b*, 37); since these two enzymes perform similar reactions, *i.e.*, dehydrogenation at the α-position of an anionic substrate, and are therefore by mechanistic necessity closely related, it is not surprising that the two enzymes are inhibited by similar compounds. In contrast, *the adjacent nucleophilic sites would most likely be dissimilar*, since these nucleophilic sites probably have no function in the mechanistic operation of the enzymes, but are probably part of the protein molecule necessary for the integrity of the protein's secondary-tertiary structure. Thus, it was anticipated that relatively minor changes in inhibitor structure could greatly influence the bridging ability of the inhibitor.

Previously 4-(iodoacetamido) salicylic acid (XLVI) had been shown (19) to be an irreversible inhibitor of both LDH and GDH. A number of closely related compounds were then investigated as irreversible inhibitors of the two enzymes in a search for irreversible specificity (33, 34). When the length of the bridge was increased as in XLVIII, both GDH and skeletal muscle LDH were inhibited both reversibly and irreversibly, thus showing no irreversible specificity (Table I). However, two other compounds showed a crossover in selectivity of irreversible inhibition. Although XLIX was a better reversible inhibitor of skeletal muscle LDH than XLVI, the former showed no irreversible inhibition of skeletal muscle LDH; in contrast, XLIX was an irreversible inhibitor of GDH almost as good as XLVI. A direct antithesis of irreversible inhibition pattern was shown with L; although skeletal muscle



XLVI

XLVIII



XLIX

L

LDH was irreversibly inhibited by L, the latter showed no irreversible inhibition of GDH (Table I).

It was previously noted (1) in a crude model of the active-site of LDH that the oxanilic-LDH complex does not appear to have sufficient space for the complete free rotation of the phenyl group; in addition, it can be demonstrated with molecular models that the iodomethylene group of the oxanilate (XLIX) can approach any point in space that the iodomethylene group of XLVI can approach when the respective enzyme-binding points of the inhibitors are anchored. Therefore, the conformation necessary for the iodomethylene group of XLIX to bridge to the nucleophilic site for reaction within the enzyme-inhibitor complex may not be tolerated due to a restriction of rotation.

The carbonyl carbon of the iodoacetyl group and the *N*-methyl groups of L have a fixed relationship between them; when the iodomethylene group of L attempts to bridge to the nucleophilic site within the GDH-inhibitor reversible complex, there is apparently insufficient space for the counter-balancing *N*-methyl group and bridging for inactivation cannot be completed.

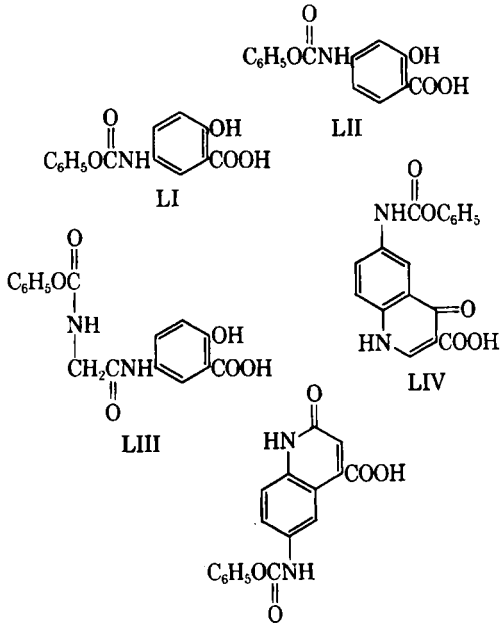
Inactivation via the Carbophenoxy Group.—The second corollary of the bridge principle of specificity is based on the difference in nucleophilicity of the enzymic group being alkylated. It is at this point that the approach for labeling the active-site of a pure enzyme and the approach to chemotherapy become divergent. The α-halogen carbonyl group of an iodoacetamide such as XLVI or a chloroketone such as XXXII have little functional specificity. Of about 15 amino acids in proteins containing a third functional group, a majority of them—such as methionine, histidine, arginine, lysine, cysteine, cystine, tyrosine, aspartic acid, and glutamic acid—have the nucleophilic ability to become alkylated. The α-halogen carbonyl compounds, therefore, have the broadest utility in labeling the active site of a pure enzyme, the selectivity of reaction being controlled by the initial complexing between the inhibitor and the active-site; this very broadness of utility for a pure enzyme is undesirable for the mixture of enzymes in a cell involved in chemotherapy. Ideally, for chemotherapy the greatest selectivity should be obtained by using an attacking group for the enzymic nucleophilic group that is specific for a single amino acid of the group of 15, thus affording a further dimension of specificity. The phenyl ester group such as that of 5-(carbophenoxyamino)salicylic acid (LI) apparently can

TABLE I.—IRREVERSIBLE INHIBITION OF LDH AND GDH BY SELECTED COMPOUNDS

No.	Compd.[a]	Liver GDH $K_i \times 10^4$	Liver GDH Rate of Inactivation[b]	Skeletal Muscle LDH $K_i \times 10^4$	Skeletal Muscle LDH Rate of Inactivation[b]	Heart Muscle LDH $K_i \times 10^4$	Heart Muscle LDH Rate of Inactivation[b]
XLVI	4-ICH_2CONH—SA	8.5	1.0[c]	17	1.0[c]	45	0
XLVIII	4-ICH_2CONH—GSA	18	1.2	4.0	0.87	25	1.0[c,g]
XLIX	3-ICH_2CONH—OA	23	0.75	1.3	0	1.2	0
L	4-$ICH_2CON(CH_3)$—SA	33	0	33	1.0	...	...
LI	5-C_6H_5OCONH—SA	4.0	2.0	15	0	14	0.58[h]
LII	4-C_6H_5OCONH—SA	8.5	0	11	0.98	19	0[g]
LIII	5-C_6H_5OCONH—GSA	1.6	2.0	5.9	0	18	0
LIV	6-C_6H_5OCONH—QA	2.3	0[d]	1.1	0[d]	...	...
LV	6-C_6H_5OCONH—CA	2.4	1.3[e]	2.4	0[f]	...	...
LVI	4-cis-HOOCCH=CH(SA—NHCO)	43	0	18	0.9	30	0[g,i]
LVIII	Maleanilic acid	...	...	83	0	114	0

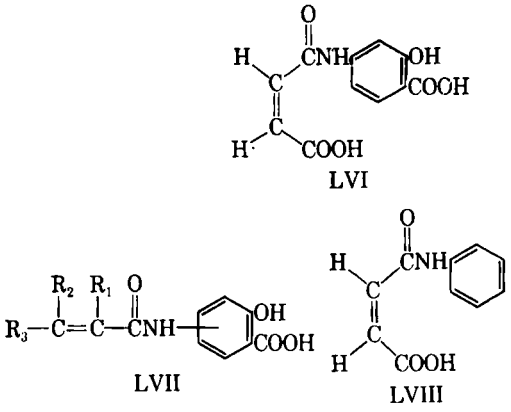
[a] Abbreviations used: SA = salicylic acid; OA = oxanilic acid; GSA = glycylamidosalicylic acid; QA = 4-hydroxyquinoline-3-carboxylic acid; CA = 2-hydroxycinchoninic acid; data taken from References 31–36, 38. [b] Concentration of inhibitor was 2 mM unless otherwise indicated. [c] Arbitrary standard rates that are not the same for the three enzymes. [d] Concentration of inhibitor was 0.13mM, the maximum solubility at pH 7.4 that could be attained in the inactivation procedure [e] Concentration of inhibitor was 0.2 mM. [f] Concentration of inhibitor was 0.4mM. [g] Concentration of inhibitor was 4mM. [h] Concentration of inhibitor was 3mM. [i] Preliminary unpublished data.

only react with a primary amino group, thus having complete functional specificity (35, 36).



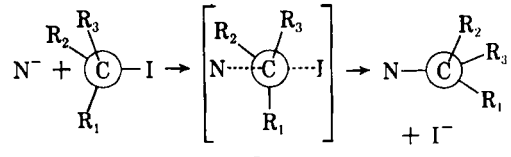
5-(Carbophenoxyamino) salicylic acid (LI) inactivated GDH at twice the rate of the standard, 4-(iodoacetamido) salicylic acid (XLVI); in contrast, LI showed no irreversible inhibition of skeletal muscle LDH (Table I). A cross-over specificity was noted with LII, which inactivated skeletal muscle LDH about the same rate as the standard (XLVI), but showed no inactivation of GDH. The peptide, LIII, with a longer side-chain still inactivated GDH, presumably by folding the chain back to the point in space equivalent to the position of the carbophenoxy group of LI. Although LIII can also fold back to the point in space equivalent to the position of the carbophenoxy group of LII, LIII failed to show the inactivation of LDH that LII showed; this difference is attributed to hindrance at the transition state which is discussed in a later section. In contrast, LIV failed to inactivate either LDH or GDH, showing that the fixed position of the carbophenoxy group in LIV cannot bridge to the available enzymic amino group (32). That a bicyclic compound could inactivate GDH at low concentration was shown with LV; again LDH was not inactivated by this compound (32).

Inactivation via the Maleamyl Group.—The α,β-unsaturated system of LVII should react in general most rapidly with SH groups, much slower with amino groups, and extremely slowly with hydroxyl groups by a Michael addition reaction. For initial studies, 4-(maleamyl)salicylic acid (LVI) was selected for



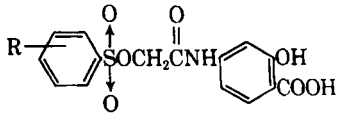
study (38). 4-(Maleamyl)salicylic acid (LVI) inactivated skeletal muscle LDH at about the same rate as the standard compound, XLVI; however, LVI failed to inactivate GDH, thus showing the selectivity of the maleamyl group. That a reversible complex between LVI and skeletal muscle LDH was an obligatory intermediate to inactivation was strongly indicated by the failure of maleanilic acid (LVIII)—which does not have the necessary groups for reversible binding—to inactivate skeletal muscle LDH. Preliminary studies have shown that 4-(acrylamido)salicylic acid (LVII, R=H) irreversibly inhibited both GDH and skeletal muscle LDH (39); further studies on the selectivity that might be obtained by varying the R groups LVII are in process (39).

Hindrance to the Transition State.—In a displacement reaction of the S_N2 type, whether bimolecular or intramolecular, the transition state appears to be planar (LIX) (40). There-



LIX

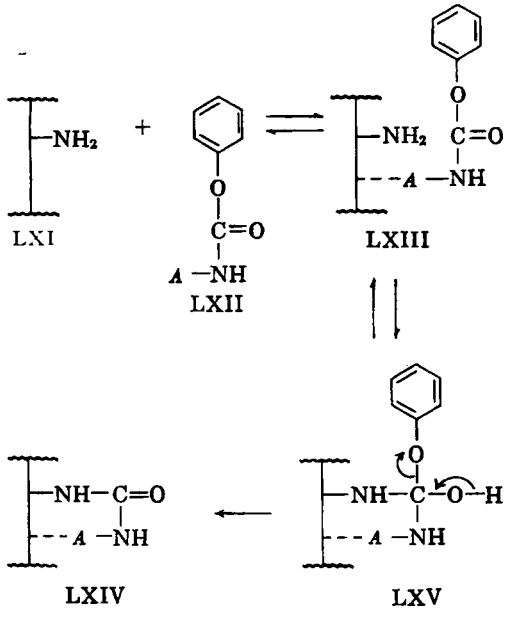
fore the attacking group should not be hindered by a bulky leaving group, since the two groups are on opposite sides of the carbon atom undergoing reaction; that such is the case has been indicated experimentally (41). When such an intramolecular reaction takes place in an enzyme-inhibitor reversible complex, the enzymic nucleophilic group which is attacking in



LX

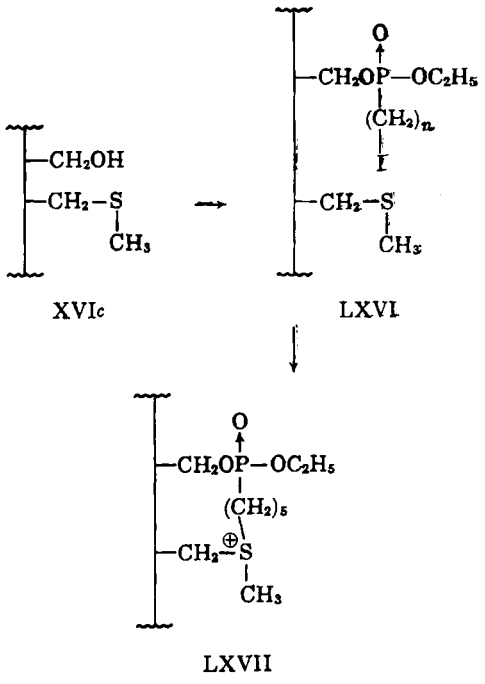
the displacement reaction is part of the extremely large (compared to inhibitor) enzyme molecule; as a result, there may be hindrance to the formation of the transition state by steric interaction of the enzyme and a bulky leaving group. Such a study is underway with the sulfonate leaving group (LX); when R = Me, LX is an irreversible inhibitor of GDH and skeletal muscle LDH (42) and studies with bulky R groups are in process.

Another type of reaction which may be subject to specificity dependent upon the transition state is the inactivation mechanism by phenyl esters such as LI and LIII. The inactivation is



The enzyme (LXI) initially forms a reversible complex with the inhibitor (LXII) through the binding area, *A* (see Fig. 3). In order for an amine on the enzyme surface in the complex (LXIII) to form a covalent bond, it is necessary that the transition state, LXV, be formed; LXV can then lose the phenoxy group resulting in the mixed urea, LXIV, the inactivated enzyme. For this reaction to proceed, there can be no steric interaction between the phenoxy group and the enzyme surface. Stated in another way, in order for the transition state to form, the leaving phenoxy group in the transition state must not be in contact with the enzyme—else the transition state could not form.

5-(Carbophenoxyglycylamido)salicylic acid (LIII) fails to inactivate skeletal muscle LDH, whereas LII can inactivate this enzyme. Since the carbonyl in the carbophenoxy group of LIII can reach any point in space that the same carbonyl group of LII can reach when the reversible binding groups of the salicylic acid structure are



anchored, the carbophenoxy group of LIII most probably cannot approach this critical point for formation of the transition due to a steric interaction between the phenoxy group and the enzyme (31, 36). A useful system for study of possible specificity by the steric interaction of the phenoxy leaving group and the enzyme could be the inactivation of both GDH and heart LDH by LI (Table I) (31). A number of related compounds with substituents on the phenoxy group have been synthesized and are currently being evaluated to determine if such a specificity of the irreversible reaction between these two enzymes can be found (43).

In another approach to find selectivity, some compounds related to 4-(iodoacetylglycylamido)-salicylic acid (XLVIII) having optically active D and L forms of alanine or valine instead of the glycine residue have been synthesized (42): there is a possibility for asymmetric tolerance of the bulky group of the substituted glycine residue in the enzyme-inhibitor reversible complex that may influence whether the bridge can be completed as in Fig. 3. These compounds are being evaluated as irreversible inhibitors, the hope being that only a D-isomer may be an irreversible inhibitor of one enzyme, such as LDH, and only an L-isomer an irreversible inhibitor of a closely related enzyme, such as GDH.

Bridging Phenomenon with Chymotrypsin. —In the earlier discussion on chymotrypsin, it was pointed out that α-haloacetamidoacyl-chymotrypsins (XX*a*) (Fig. 4) could undergo

intramolecular alkylation by attack of a methionine sulfur atom to form XXIII (22). That the proper bridge length is necessary for the reaction to occur is shown by the fact that iodoacetyl-chymotrypsin does not undergo such an intramolecular reaction with methionine or any other amino acid of chymotrypsin (44). In preliminary studies with ethyl 5-iodopentylphosphonofluoridate, Gold (45) has shown the phosphonyl-chymotrypsin (LXVI, $n = 5$) undergoes loss of iodide presumably by an internal alkylation reaction; the ethyl 5-iodopentylphosphonofluoridate does not release bound iodine under the same conditions without enzyme. Furthermore, XLVI with shorter or longer alkyl groups gave zero release of iodide in the presence of enzyme. It is probable that the same methionine attacked intramolecularly in the acylated chymotrypsin of type XX*a* is also attacked in this case to give XLVII, since the bridging distance is nearly the same.

That azaserine (VII) inactivates the appropriate enzyme while diazoacetyl-chymotrypsin (XXXVI) is stable, unless photolyzed, is an interesting comparison. The diazo group of XXXVI certainly has the capacity to react with a carboxyl, phenolic, hydroxyl, or mercapto group; apparently such a group is not within bridgeable distance to the diazomethyl group of XXXVI (Fig. 8).

SELECTIVE INHIBITION OF LACTIC DEHYDROGENASE FROM TWO DIFFERENT TISSUES

The enzymic nucleophilic site that covalently binds an active-site-directed irreversible inhibitor can be, but is not necessarily, adjacent to the active-site in the secondary-tertiary structure of the protein (see Fig. 3). The substrate-identical enzyme from different tissues frequently gives no cross-reaction with specific antisera (46, 47), indicating differences in tertiary structure. Therefore, Baker (34) suggested that it may be possible to obtain highly selective irreversible inhibition of the substrate-identical enzyme from different tissues by use of the bridge principle.

By antisera cross-reaction studies (47, 48) and by amino acid analysis (48), it has been shown that LDHs from heart and skeletal muscle in the same animal are quite different, but that heart LDHs from different species are more similar; also skeletal muscle LDHs from different species are more similar. As a first approximation, a study on selective irreversible inhibition of LDHs from rabbit skeletal muscle and beef heart was undertaken (31, 49).

The standard compound for irreversible inhibition of skeletal muscle LDH, 4-(iodoacetamido)salicylic acid (XLVI), failed to inhibit heart LDH irreversibly (Table I). When the bridge distance was lengthened (as in the peptide, XLVIII), both LDHs were irreversibly inhibited, but at different absolute rates. For further work, XLVIII was used as a standard for heart LDH. Selectivity of action was shown with 5-(carbophenoxyamino)salicylic acid (LI) which could inactivate heart LDH, but failed to inactivate skeletal muscle LDH. A cross-over in specificity was noted with the 4-isomer (LII), which inactivated skeletal muscle LDH, but not neart LDH. It was previously noted that 4-(maleamyl)salicylic acid (LVI) could inactivate skeletal muscle LDH; in contrast, preliminary studies indicate that heart LDH is not inactivated by LVI.

If this irreversible specificity for substrate-identical enzymes from different tissues can be transposed to such critical areas for cell division as (*a*) purine or pyrimidine biosynthesis or (*b*) the folic cofactor area, the benefits that could accrue to chemotherapy would be obvious. Such studies in area (*a*) (50) and area (*b*) (51–56) are continuing in our laboratory.

EXPERIMENTAL DESIGN FOR UNEXPLORED ENZYMES

Lest the medicinal chemist who has had the patience to read this far be tempted to dash into the laboratory and attach diazoketone, fluorophosphate, halomethyl carbonyl, diazonium, maleamyl, carbophenoxy, or other covalent forming groups to the nearest available substrate or inhibitor, he would be wise to be cautious with the suggestions that follow, else he is apt to be disappointed in his laboratory labors.

The studies described to this point have dealt only with irreversible inhibition of isolated enzymes—a far cry from chemotherapy in a host system. It is obvious that there are a variety of other factors—other than selective irreversible inhibition of isolated enzyme systems—that play important roles in whether the inhibitor would be effective in host system. Among these factors are (*a*) transport to and into the desired target cell from the site of administration of the inhibitor must be achieved; (*b*) the inhibitor must be reactive enough to give a reasonable rate of inactivation of the target enzyme, but not so reactive that insufficient inhibitor reaches the desired cell containing the target enzyme; and (*c*) selectivity of inhibition of an enzyme in the target cell such as a cancer cell or an invading organism. This review has been devoted only to one of these important

facets, namely (*c*); the other facets will have to be overcome simultaneously for the inhibitor to be a useful drug. If the candidate inhibitor is only studied for effectiveness in a host system, the jack-pot of (*a*), (*b*), and (*c*) working at the same time will have to be achieved, a remote possibility predicated mostly on extremely good luck.

The design of an effective agent in a host system is still so nebulous that successful design is more likely to be achieved by individual study of the three facets, followed by collective use in order to find eventually chemotherapeutic agents on a less hit or miss basis; that is not to say that synthesis and screening should be discontinued, but the screening approach is a calculated risk of hit or miss that has hit in infectious chemotherapy, but has been so much less successful in cancer chemotherapy to be termed a near miss. In fact, the medicinal chemist will even find it difficult to make a successful guess concerning where to put a covalent-forming group on a substrate or inhibitor to give an active-site-directed irreversible inhibitor for an unexplored enzyme. At this early stage of research in this area, it would appear wise to proceed systematically to determine the binding points of the inhibitor (area *A*, Fig. 3) and the noncontact area of the inhibitor and the enzyme. If the covalent-forming group (*B*) is unfortuitously placed in area *A* (Fig. 3), then an active-site-directed inhibitor will not be obtained since the reversible complex cannot form.

The logical development of where to place the covalent-forming group *B* (Fig. 3) to make an irreversible inhibitor for an unexplored enzyme can be gleaned by considering the previous development of irreversible inhibitors of LDH by reading in order the theoretical background (2), types of inhibitors and points of binding (1, 37), the noncontact areas (1, 37), and finally the bridge principle (31–36, 49); most of this information has been covered in this review, but not necessarily in the same order.

For two enzymes in the folic cofactor area, this approach has been systematically developed to the point where positionings for covalent-forming groups can now be done logically (51–56). An interesting sideline developed by serendipity may well solve the cell wall transport problem for active-site-directed irreversible inhibitors of folic reductase (56).

Having heeded my precautions, and introduced some new ones of his own on how to construct an active-site-directed irreversible inhibitor, and having been successful in synthesizing and enzymically evaluating the inhibitor, the researcher now must face the wall of prejudiced conservatism put up by his peers—the so-called "experts." Most of our work—and perhaps some of yours—may be based on kinetic evaluation of the inhibitors, giving results which may approach being, but do not become, unequivocal. We have criticized our own attempt to demonstrate that a reversible complex between inhibitor and enzyme is an obligatory intermediate to inactivation:

> "If it were possible to obtain *unequivocal* proof for the initial complex formation—and no such experiment for yielding unequivocal proof is apparent to us—solution of this problem would be relatively straight forward. Thus, one is left with the unsatisfying approach of eliminating all other possibilities than the one under consideration; such an approach has the obvious difficulty, as is the case for all kinetic experiments, that a yet unthought of mechanism may be the true one."

Thus, the "expert" may well sum up your work (and mine) by saying you (I) have not yet proved unequivocally your (my) thesis; therefore, the thesis must be wrong. At this point I like to chide the "expert" a little along the following vein, in an attempt to maintain my own sense of humor.

Too many of these "experts" treat the enzyme as a worshipper treats Mecca; they bow to the East and are dazzled by thoughts of the great enzyme temple that there resides. The more adventuresome will travel to Mecca, go inside the enzyme temple, and behold the glory of the active-site. Even the adventuresome worshipper is dazzled by this magnificient workmanship of nature. As past history has shown, some heretic worshipper or some worshipper of a different temple will—beneath his robes—put on his polarized sunglasses, then arise from his kneeling position. With the dazzle now eliminated, he can observe that the temple has been made from bricks, mortar, metal and paint, but of exquisite design; similarly, he may observe that the bricks of the enzyme temple are constructed from the long known elements of carbon, hydrogen, nitrogen, oxygen, and sulfur, then mortared together with peptide and hydrogen bonds and finally decorated with the metal and paint of functional groups—an exquisite and bedazzling design when the enzyme becomes functional for a substrate. Even with this dazzlement, three facts have emerged over the past 50 years that are the basis for active-site-directed irreversible inhibitors and their specificity: (*a*) The macromolecular enzyme has reactive functional groups on its surface; (*b*) this macromolecule can form complexes with simpler molecules such as substrates and inhibitors; (*c*) neighboring group reactions can be accelerated 10,000-fold or more

compared to the same chemical reaction occurring by a bimolecular reaction.

Some "experts" have accepted (*a*) and (*b*), and other "experts" have accepted (*c*). Is it not time for them to accept that the three tenets might be combined into one situation?

The macromolecular enzyme has functional groups on its surface which logically could be attacked selectively in the tremendously accelerated neighboring group reactions capable of taking place within the reversible complex formed between the enzyme and an inhibitor substituted with a properly placed neighboring group.

My own frustrations of researching in this area can be summed up in the following letter which this author sent to Dr. Samuel W. Goldstein, Associate Editor of THIS JOURNAL, in the process of considering another paper in this series for publication.

Thank you for your letter of September 9, 1963, with the referee's comments on our paper "Nonclassical Antimetabolites. XV." I share the quandary expressed by the referee; I too wonder how best to approach experimentally the "receptors of unknown structure." In fact, at times I wonder why I ever got started in this area of research which is so equivocal in its interpretation of data; at other times, I am sufficiently depressed about the reactions of reviewers to this work that I am tempted to drop this line of research.

However, everyone wants to know all about the active-site, but few are ever willing to try experiments to solve this tremendously important problem. Some not willing to experiment in such a difficult area are so conservative they are even unwilling to put their official seal of approval as a referee to such experiments. Although I feel our data are as solid as one can get at this early stage, I certainly agree that the interpretation of the data is equivocal and may even be wrong totally or in part. Nevertheless, I feel the main responsibility for statements in a publication that may ultimately prove to be wrong rests with the authors and not with the referees or editors, although the latter can certainly exercise their rights and reject any manuscript they wish. Actually, if anything turns out to be incorrect it will be my personal reputation that will be on the chopping block; it is this last reason that often tempts me to drop this area of research and stick to straight synthetic chemistry. However, the useful benefits that can potentially accrue to the field of chemotherapy keep me working in this difficult area.

The experimental approach that we have been using would appear to draw on knowledge available from the areas of organic chemistry, physical chemistry, biochemistry, and chemotherapy; I certainly feel my own shortcomings in some of these areas, and I imagine a referee may also feel shortcomings in one or more of these areas. Hence, it is difficult to set up an experimental design, carry out the research, and referee the results.

In conclusion, it is admittedly difficult to get started with a machete through the jungles of the so-called active-site. Until more sophisticated tools can be developed, some paths, including that which I have taken, may lead to naught; nevertheless, this or other paths may ultimately lead to the smooth four-lane highways for the band-wagon boys to jump aboard for the easy ride. Until my approach leads to a blind alley or a four-lane highway, I hope that the referees and editors will take into consideration that the experimental and interpretative difficulties are equivocal, but are being carried on with the highest scientific ideals that I am capable of reaching.

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Research Articles

Phytochemical Investigation of *Carya illinoensis*

By LEON O. WILKEN, JR.†, and FRANK P. COSGROVE

The leaves and petioles of *Carya illinoensis* and a lyophilized aqueous extract of this plant were investigated. Examination of the petroleum ether, ether, and chloroform extracts revealed the presence of unidentified phytosterols and a squalene-like substance in the unsaponified portions, and the presence of capric, lauric, myristic, palmitic, stearic, arachidic, oleic, linoleic, and linolenic acids in the saponified portions. Nonhydrolyzable tannins containing a phloroglucinol and a catechol nucleus were found in the ethanol and methanol extracts. Investigation of the plant extracts revealed the presence of carbohydrates and the absence of discernible amounts of glycosides and alkaloids. A crystalline neutral substance obtained from a neutral lead acetate treated aqueous extract was identified as the *m*-inositol. A crystalline acidic substance isolated from an aqueous extract of the crude drug was identified as 3,4-dihydroxybenzoic acid. Further pharmacologic studies of various extracts are presently in progress.

THIS PHYTOCHEMICAL STUDY was undertaken primarily on the basis of our preliminary screening tests which indicated that certain extracts of the leaves and petioles of *Carya illinoensis* (Wangh) K. Koch (Fam. *Juglandaceae*), common name—pecan, possessed the property of temporarily lowering the blood pressure of test animals on intravenous administration. A search of the available literature revealed little (1, 2) or no scientific information concerning the evaluation of this plant for medicinal properties. The fact that this species has been assigned eight official names (3) throughout its relatively short history may account for the lack of investigation in the *Carya* genus. In 1951, the plant was reclassified under the international code as *Carya illinoensis*.

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Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1963.

EXPERIMENTAL

Source and Preparation of Material.—The plant parts used in this investigation consisted of the air dried leaves and petioles collected in late summer from pecan trees growing near Covington, La. This material, sampled and authenticated,[1] was reduced to a moderately coarse powder with the aid of a W. J. Fitzpatrick, model D, comminuting machine.

Moisture Content and Ash Determination.—The moisture content of the powdered plant material, as determined by the toluene method (4) of the U.S.P.

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† Present address: School of Pharmacy, Auburn University, Auburn, Ala.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1963.

EXPERIMENTAL

Source and Preparation of Material.—The plant parts used in this investigation consisted of the air dried leaves and petioles collected in late summer from pecan trees growing near Covington, La. This material, sampled and authenticated,[1] was reduced to a moderately coarse powder with the aid of a W. J. Fitzpatrick, model D, comminuting machine.

Moisture Content and Ash Determination.—The moisture content of the powdered plant material, as determined by the toluene method (4) of the U.S.P.

[1] The authors are deeply indebted to Dr. B. L. Turner, Professor of Botany and Director of the Herbarium, The University of Texas, and Dr. M. Johnston, Lecturer in Botany, The University of Texas, for authenticating the plant.

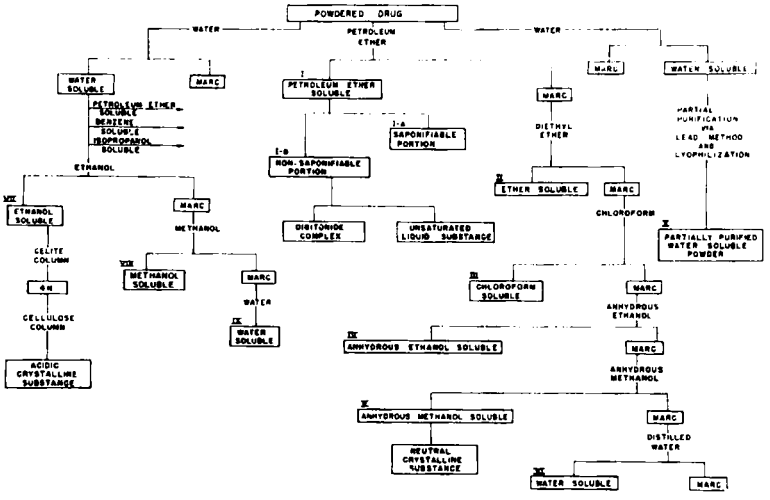


Fig. 1.—A schematic diagram of the preparation of fractions for phytochemical studies.

XVI, was shown to be 9.42%. The determinations for ash content as recommended by the U.S.P. XVI (5) afforded the following results: total ash, 7.03%; acid-insoluble ash, 1.40%; and acid-soluble ash, 5.63%. Further examination of the acid-soluble ash revealed the presence of K, 0.71%; Na, 0.048%; Ca, 0.97%; Mg, 0.34%; and Al, 0.92%. These mineral content values are generally in accordance with the values reported by Robinson and Edgington (6).

Preparation of Extracts for Phytochemical Studies.—Suitable samples of the powdered drug were extracted successively with a series of selective solvents in a soxhlet apparatus according to a modified Rosenthaler procedure (7). The solvents utilized and the per cent of extractives obtained with each solvent were: petroleum ether (b.p. range 30–60°), 2.07%; anhydrous diethyl ether, 2.51%; chloroform U.S.P., 0.79%; anhydrous ethanol, 6.97%; anhydrous methanol, 5.75%; and distilled water (percolation), 7.10%.

A schematic diagram of the preparation of the fractions for the phytochemical studies is shown in Fig. 1.

Fatty Acids.—Accurately weighed samples of the saponifiable portions of the petroleum ether fraction (Fraction I-A in Fig. 1), the diethyl ether fraction (Fraction II-A), and the chloroform fraction (Fraction III-A) were individually esterified according to the method of Fischer, as described by Fieser (8), in order to provide the more volatile methyl esters of the fatty acids. Analyses of these mixtures in an Aerograph gas chromatograph, model A-90-P, equipped with a Brown 1 mv. automatic recorder and comparisons of the resulting chromatograms with a standard chromatogram produced under similar conditions showed the presence of the methyl esters of capric, lauric, myristic, palmitic, stearic,

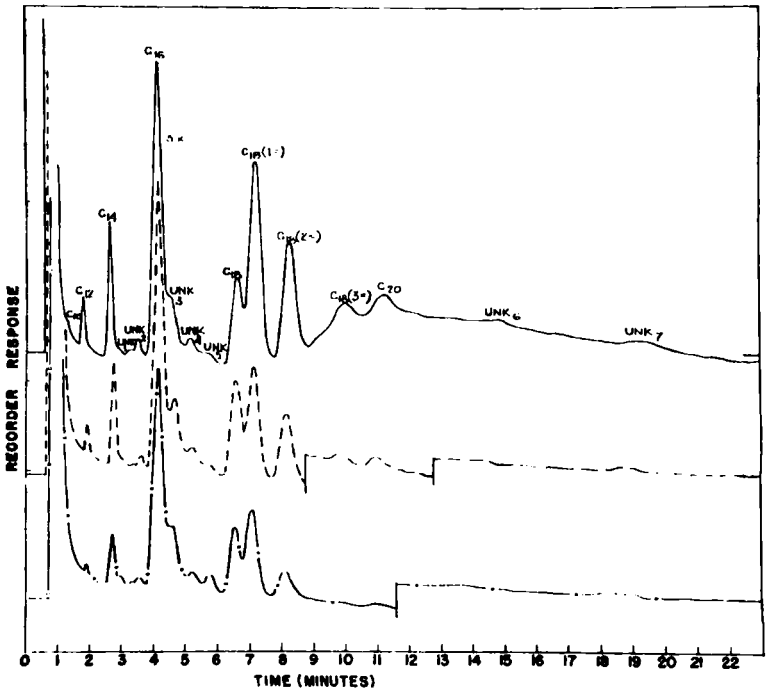


Fig. 2.—Gas chromatograms of the methyl esters of fatty acids found in the petroleum ether extract (——), the diethyl ether extract (- - - - -), and the chloroform extract (—·—·—). Conditions: sample 6.0 μl.; column packing, 20% diethyleneglycol succinate on firebrick; column 62 × 0.25 in.; helium, flow rate, 30 ml./min.; temp., column 223°, collector 183°, detector 240°, injector 260°; current 200 ma.; sensitivity 2.

arachidic, oleic, linoleic, and linolenic acids. The sample for the standard chromatogram contained pure methyl esters of all these named acids. Although seven additional peaks were observed, they were not studied further. The chromatograms obtained are shown in Fig. 2.

Phytosterols.—The unsaponifiable fractions of the petroleum ether extract, (Fraction I-B in Fig. 1), the diethyl ether extract (Fraction II-B), and the chloroform extract (Fraction III-B) were examined for the presence of phytosterols by the digitonin reaction described by Fieser and Fieser (9). Although the quantities of the insoluble digitonides formed in the petroleum ether and the chloroform extracts were too slight for further study, the diethyl ether extract afforded a white digitonide which melted between 205–208°. The presence of phytosterols in all three extracts was verified by the positive results obtained with the reactions of Hesse, Moleschott, and Hirschsohn (10) as well as the Liebermann-Burchard reaction (11).

Squalene-like Substance.—A suitable portion of the unsaponifiable fraction of the petroleum ether extract was extracted with boiling anhydrous methanol. When the alcohol soluble portion was decanted and placed in a freezer at a temperature of −18° overnight, a white flocculant precipitate formed which upon drying resulted in a viscous liquid having solubility and unsaturation characteristics similar to squalene. The isolation, purification, and characterization of this substance will be reported in a separate paper at a later date.

Glycosides.—Since a preliminary qualitative examination of the anhydrous ethanol, anhydrous methanol, and distilled water extracts, (Fractions IV, V, and VI, respectively, in Fig. 1) with the aid of Molisch reagent yielded positive results for carbohydrates and/or glycosides, the conventional method of Stas-Otto (12) was utilized in an attempt to verify these findings.

Powdered plant material (200 Gm.) was refluxed for 12 hours with 600 ml. of a 2% ethanolic solution of tartaric acid and then filtered. Evaporation of the filtrate under reduced pressure, using a Rinco rotatory evaporator with steam as the source of heat, afforded a residue which was extracted with hot distilled water. This aqueous extract was cooled, then extracted in a separator with diethyl ether.

The residue from this ethereal extract was dissolved in ethanol and the resulting solution tested for glycosides and/or carbohydrates with Molisch reagent. The negative results both before and after boiling this alcoholic solution with diluted hydrochloric acid indicated that glycosides in significant amounts were absent.

Alkaloids.—The purification procedures of the proximate assay method of the "National Formulary XI" (13) were applied to the diethyl ether extract and the chloroform extract (Fractions II and III, respectively, in Fig. 1) in order to analyze qualitatively for alkaloids. The resulting extracts gave negative tests with mercuric iodide T.S., gold chloride T.S., mercuric-potassium iodide T.S., and iodine-potassium iodide solution.

The negative tests obtained with these same reagents and the aqueous portion of the Stas-Otto extraction indicate the absence of any significant or discernible amounts of alkaloids in the plant material.

Tannins.—Since the conventional method of Feist and Bestehorn (14) for the extraction of tannins compares favorably with the method of obtaining the Rosenthaler extractives, the ethanol and methanol extracts (Fractions IV and V, respectively, in Fig. 1) from the Rosenthaler process were examined for the presence of tannins. Aliquots of 50% ethanolic solutions of these dried extracts gave positive results with the general tannin test reagents. A green solution was produced by the addition of ferric chloride T.S., and precipitates were obtained upon the addition of bromine T.S., quinine hydrobromide solution (1%), lead acetate T.S., gelatin T.S., and potassium dichromate T.S. to samples of each of the aliquots. These positive reactions are those characteristic of a tannin (15). The presence of a condensed (nonhydrolyzable) tannin was indicated by the formation of green with ferric chloride T.S. and by the development of a brown precipitate with bromine T.S. (16). The presence of a catechol nucleus (two adjacent phenolic groups) was indicated by the green obtained with ferric chloride T.S. and by precipitation with lead acetate T.S. (17). The presence of a phloroglucinol group was revealed by the production of a red coloration upon the addition of vanillin-HCl solution and confirmed by a positive Coward-Harris test (18). The absence of a gallic acid nucleus was indicated not only by the negative results with ammonium molybdate but also by the absence of a blue with ferric chloride T.S.

Isolation, Purification, Classification, and Chemical Characterization of a Neutral Crystalline Substance.—A small portion of the anhydrous methanol extract (Fraction V in Fig. 1) from the Rosenthaler procedure was redissolved in methanol and partly purified by treatment with a slight excess of a 20% solution of neutral lead acetate in methanol. The precipitate which formed was removed by filtration, the excess lead in the filtrate precipitated with hydrogen sulfide, and the mixture filtered. When the resulting filtrate, having been reduced in volume on a steam bath, was allowed to stand over calcium chloride for several days, a small quantity of colorless platelet crystals formed. These were shown to be only slightly soluble in methanol but very soluble in water.

To obtain an ample supply of this crystalline substance, 7.460 Kg. of the powdered plant material was extracted with seven portions ($1^1/_2$ gal. each) of distilled water. The 12 gal. (approx.) of aqueous extract collected was reduced in volume to 2 gallons using steam as a source of heat.

The resulting concentrate, partly purified with lead acetate in the manner previously described for the anhydrous methanol extract, was reduced to a

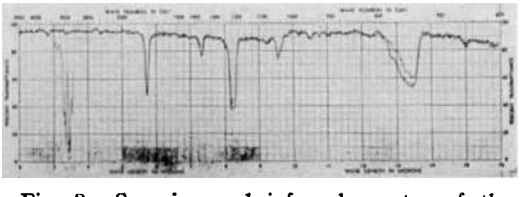


Fig. 3.—Superimposed infrared spectra of the hexa-acetate derivatives of the unknown (dark line) and a known myoinositol (light line) in chloroform.

fine powdery consistency with the aid of a F. S. Stokes lyophilizer, model 200 4LX3. This lyophilized extract was dissolved in a minimum of hot water, methanol added to cloudiness, and placed over phosphorous pentoxide for several days. A large crop of crystals resembling those observed with the previous methanol extract was collected.

Purification.—After six recrystallizations from methanol and water, the colorless needles which were dried *in vacuo* at 80° with potassium hydroxide in an Abderhalden pistol for 12 hours melted sharply at 224°. A yield of 0.08% was obtained by this procedure.

Classification and Chemical Characterization.—The reactions of the crystalline material to some of the chemical classification tests are summarized as follows: sodium fusion test (19) for nitrogen, sulfur, and halides, negative; bromine T.S., negative; potassium permanganate T.S., negative; ferric chloride T.S., negative; ceric nitrate T.S., positive; fuschin-aldehyde test, negative; and Molisch test for carbohydrates, negative. The collected data indicated the presence of a polyhydroxy alcohol, a conclusion which was substantiated by a mixed melting point of the unknown and a known C.P. grade inositol.

Esterification.—A sample of the unknown and the known C.P. inositol were separately acetylated according to the method of the "National Formulary XI" (20). The purified hexa-acetate derivatives melted at 216° both individually and as an intimate mixture.

Infrared Analysis.—To confirm the identity of the inositol, comparative studies of the infrared spectra of the unknown, the known myoinositol, and their hexa-acetate derivatives were made. The nearly identical spectra of the known and unknown samples offer confirmatory evidence of the identity of the two samples. The spectra of the hexa-acetate derivatives are superimposed and shown in Fig. 3.

Chromatographic Analysis.—In an attempt to identify which of the isomeric forms of hexahydroxycyclohexane had been isolated, the method of Angyal, *et al.* (21), utilizing paper chromatography was effectively employed. The unknown and a sample of myoinositol of known purity were spotted alone and together as a mixture with the control, dextrose.

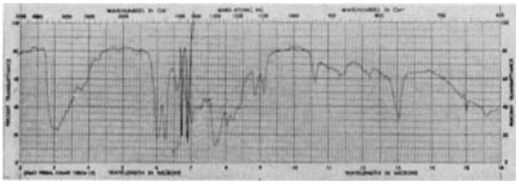


Fig. 4.—Infrared spectrum of the unknown acidic crystalline material in KBr pellet.

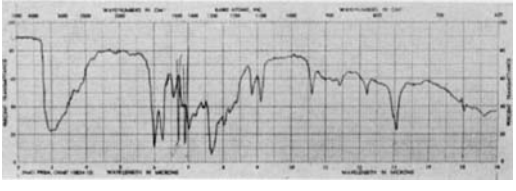


Fig. 5.—Infrared spectrum of a sample of 3,4-dihydroxybenzoic acid in KBr pellet.

The unknown migrated to the same degree as the known sample for four different solvent systems. These results indicate that the unknown is 1,2,3,-5/4,6-hexahydroxycyclohexane or, more commonly, myoinositol. A comparison of an elemental analysis of the unknown with the calculated values for inositol were obtained.

Anal.—Calcd.: C, 40.00; H, 6.71. Found: C, 40.81; H, 6.75.

Isolation of an Acidic Crystalline Substance.—Since preliminary studies of an aqueous extract of the crude material showed that solvent partition techniques were inadequate for the separation of constituents, the combined procedures of selective solvent extraction and column chromatography were employed for this purpose.

Successive extractions of a sample of the lyophilized aqueous extract in a soxhlet extractor with petroleum ether, benzene, isopropanol, ethanol, and methanol yielded significant quantities of extractives only in the ethanol and methanol fractions.

Further separation of the ethanol fraction (Fraction VII in Fig. 1) was accomplished through the use of a Celite 545[2] chromatographic column with elution effected by 1000-ml. portions of isopropanol, isopropanol-ethanol (1:1), ethanol, ethanol-methanol (1:1), and methanol applied in the order of increasing polarity. Since the third, fourth, and fifth fractions (500 ml. each) afforded significant quantities of similar residues when reduced in volume to a viscous syrup, they were combined and labeled 4N.

Separation of this fraction, 4N, was accomplished with the aid of a powdered cellulose chromatographic column utilizing as eluating liquids mixtures (500 ml. each) of solvents—skellysolve C, diethyl ether, ethanol, methanol, and water—applied in the order of increasing polarity. The 18th, 19th, and 20th fractions (100 ml. each) when dried afforded crops of similar brownish crystals and therefore were combined.

Purification.—After five recrystallizations from methanol and water, a crop of grayish-yellow crystals were collected which, after drying for 12 hours *in vacuo* at 80° with potassium hydroxide in an Abderhalden pistol, melted sharply at 199–200°.

Classification and Chemical Characterization.—Some of the qualitative classification tests applied to the crystals are summarized as follows: negative tests with bromine T.S., vanillin-HCl solution; positive tests with potassium permanganate T.S., ferric chloride T.S., lead subacetate T.S., and lead acetate T.S. Blue litmus was reddened and a sodium fusion test (22) for nitrogen, sulfur, and halides proved negative.

The accumulated data indicated the presence of both a phenolic and a carboxylic acid group. Determinations of the molecular weight and neutralization equivalent of the unknown material showed a formula weight of 154 Gm. and a neutralization equivalent of 154. An elemental analysis yielded: C, 54.58%, H, 4.07%.

The collected data compare favorably with the corresponding theoretical values for 3,4-dihydroxybenzoic acid, which are C, 54.55%; H, 3.92%; F.W., 154 Gm.; neutralization equivalent, 154.

A mixed melting point determination of the

[2] The Celite 545 used in this investigation was generously supplied by Johns-Manville, Houston, Tex.

unknown and a 3,4-dihydroxybenzoic acid of known purity showed no lowering of the melting point.

Preparation of the 3,4-Diacetyl Derivative.—The diacetyl derivative of 3,4-dihydroxybenzoic acid was prepared according to the method of Chattaway (23). The crystals obtained melted at 157–158.5°. The reported melting range is 157–159° (24). A mixed melting point determination of this derivative with one similarly prepared from a known 3,4-dihydroxybenzoic acid showed no lowering of the melting point.

Infrared Spectra as Proof of Identity.—The infrared spectra of both the isolated compound and a sample of 3,4-dihydroxybenzoic acid of known purity both in KBr pellets, were determined in an infrared spectrophotometer, model 4-55, Baird Associates, Inc. The tracings of the unknown and the known 3,4-dihydroxybenzoic acids are shown in Figs. 4 and 5, respectively. A comparison of the two spectra clearly shows the high degree of similarity existing between the spectrum of the known and that of the unknown. This identity of spectra, in addition to the other accumulated data, affords conclusive evidence for the identification of the unknown as 3,4-dihydroxybenzoic acid.

SUMMARY

The gas chromatographic analysis of the methylated saponified material of the petroleum ether, the diethyl ether, and the chloroform fractions indicated the presence of capric, lauric, myristic, palmitic, stearic, arachidic, oleic, linoleic, and linolenic acids in these fractions.

The qualitative tests for the presence of phytosterols in unsaponifiable material were positive.

On the basis of preliminary chemical studies, the liquid isolated from the unsaponifiable material of the petroleum ether fraction was partially characterized as an unsaturated squalene-like substance.

The qualitative tests performed for the presence of glycosides and alkaloids were negative.

A series of reactions with chemical reagents were positive for the presence of condensed tannins containing a phloroglucinol nucleus and a catechol nucleus.

From the results of the chemical, infrared, and paper chromatographic studies, the neutral crystalline substance was identified as 1,2,3,5/4,6-hexahydroxycyclohexane (myoinositol).

On the basis of chemical, physical, and infrared studies, the crystalline acidic substance was identified as 3,4-dihydroxybenzoic acid (protocatechuic acid).

Pharmacologic and chemical studies of certain extracts of the leaves and petioles of *Carya illinoensis* are in progress in an attempt to isolate the hypotensive activity and possibly identify the agent responsible.

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Assay Procedure for Pharmaceutical Combinations of Aspirin, Phenacetin, Caffeine, and Itobarbital with Phenothiazine Derivatives I

By PAUL TURI

A procedure is presented for the quantitative determination of combinations of aspirin, phenacetin, caffeine, and itobarbital with salts of phenothiazine derivatives. The powdered mixture is dissolved and passed through a series of partition chromatography columns. The ingredients are separated by specific trap segments, eluted by selective solvents, and assayed by any suitable procedure. The assay technique offers a rapid and accurate determination of the five ingredients.

MIXTURES OF acetylsalicylic acid, acetophenetidin, and caffeine—by themselves or in combination with other drugs—are among the most popular pharmaceutical preparations. To assay quantitatively the components of these mixtures, a number of authors (1–6) applied modified partition chromatography as a very convenient and adequate technique. Most recently, Heuermann and Levine (5) published two procedures: one variation was adapted for assaying APC with barbituric acid derivatives, the other variation for analyzing APC combined with organic bases.

Our objective was to develop a single method which facilitates an assay for APC compounds combined with both barbituric acid derivatives and certain organic bases, particularly with phenothiazine derivatives.

The first and most important problem was to find a stationary phase system providing a selective entrapment for the individual ingredients. The combination column containing a 7.5% tartaric acid–Celite segment and a 10% sulfuric acid–Celite segment which had been described to trap simultaneously caffeine and many organic bases (5), was unsuitable for the phenothiazine derivative containing mixtures for the following reasons: (*a*) phenothiazine derivatives degrade rapidly in presence of mineral acids (such as 10% sulfuric acid) by forming strongly colored products; and (*b*) solubilities of the phenothiazine derivatives and the caffeine are very similar. For this reason, chloroform, which can be used as a selective eluant of caffeine from its mixtures with many stronger bases, cannot be considered for the separation of caffeine-phenothiazine derivative combinations. A part of this study aimed therefore to find a trap segment, which separates the phenothiazines from the other ingredients, particularly from the caffeine.

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In selecting the proper solvent system, chloroform appeared to be superior by its ability to dissolve quantitatively the salts of the phenothiazine derivatives as well as the aspirin, phenacetin, caffeine, and itobarbital. However, in order to achieve transfer of the phenothiazine derivative and the caffeine to the immobile phase in the proper columns, the addition of a more polar solvent (such as diethyl ether) was needed. The distribution coefficients of the solutes are thereby shifted to favor the aqueous phases. The appropriate solvent ratio was investigated.

Once the satisfactory stationary phase and mobile phase had been determined, there was no difficulty in recovering the individual ingredients by the conventionally used eluants.

EXPERIMENTAL

Apparatus

Chromatographic Columns.—Glass tubes ($8 \times {}^{3}/_{4}$ in.) with $3 \times {}^{5}/_{16}$ in. stems were used. The stationary phase solutions were mixed with Celite 545 and packed into the columns in the conventional manner (7). A scheme for the assembly of the chromatography tube series is shown in Fig. 1.

Spectrophotometer.—Assays were performed on a Cary 11 recording spectrophotometer, using 1-cm. cylindrical silica cells.

pH Meter.—Beckman Zeromatic was used.

Reagents

The list of reagents is presented in Table I.

Procedures

Ethyl Ether: Chloroform Solvent Ratio.—Earlier

	Aqueous stationary phase: Composition	ml.	Celite 545: Gm.
I.	Tartaric acid, 1% (w/v)	3	3
II.	Sulfuric acid, 12% (v/v)	3	3
III.	Sodium bicarbonate, 8.4% (w/v)	2	2
IV.	Tripotassium phosphate, 22.1% (w/v)	3	3

Fig. 1.—Schematic assembly of chromatography column series.

TABLE I.—DESCRIPTION OF REAGENTS

Name	% Composition, in water	Grade
Tartaric acid sol.	1 (w/v)	U.S.P. XVI
Sulfuric acid sol.	12 (v/v)	U.S.P. XVI
Sodium bicarbonate sol.	8.4 (w/v)	U.S.P. XVI
Tripotassium phosphate sol.	22.1 (w/v)	U.S.P. XVI
Chloroform		ACS water-washed
Ethyl ether		ACS, redistd., water-washed
Isooctane		U.S.P. XVI

TABLE II.—DISTRIBUTION RATIO OF THIORIDAZINE HYDROCHLORIDE

Phase I	1% Tartaric Acid—		
Phase II	Chloroform	Ether:Chloroform 4:1	Ether:Chloroform 8:1
α^a	0.1	5.6	6.3

[a] α = solute concn. in phase I/solute concn. in phase II.

experimental results indicated that a complete separation of the ingredients can be achieved only by a properly selected solvent system. In this study, the distribution ratio of thioridazine hydrochloride between 1% tartaric acid and several organic solvents has been measured at 25° by shaking extensively 10.0 mg. of this substance with equal volumes of the solvent pair and, after a complete separation, determining the concentrations spectrophotometrically (Table II).

Subsequently, 10.0-mg. amounts of several phenothiazine derivatives had been dissolved in 100.0-ml. portions of chloroform. A 5.0-ml. aliquot of each solution was mixed with 40 ml. of ethyl ether and passed through a chromatography column containing 3 ml. of 1% tartaric acid on 3 Gm. of Celite. The eluates were collected into glass-stoppered Erlenmeyer flasks. To simulate assay conditions, the columns were then washed with consecutive 20, 20, and 10-ml. portions of an ether:chloroform (8:1) solvent, and these washings were also combined in the receiving flasks. The eluates were then evaporated to incipient dryness, the residues dissolved in suitable solvents, and the released amounts were determined spectrophotometrically (Table III).

Separation of Phenothiazine Derivatives from Caffeine.—Thioridazine hydrochloride[1] was used as a model in the following experiments. A mixture of 20.0 mg. of caffeine and 5.0 mg. of thioridazine hydrochloride was dissolved in 50.0 ml. of chloroform. Aliquots of 5.0-ml. of this solution were thoroughly mixed with 40-ml. portions of ethyl ether and passed through separate Celite columns[2] containing aqueous solutions of tartaric acid in different concentrations. (The pH values of these solutions had been measured at 25°.) After collecting the effluents in 100-ml. volumetric flasks, each column was washed with consecutive 20, 20, and 10-ml. portions of the ether:chloroform (8:1) solvent. These eluates were also collected into the same receivers which were finally adjusted to volume with the solvent mixture at 20°. Aliquots of 25.0 ml. of these solutions were then evaporated to incipient dryness, the residues redissolved in 25.0-ml. volumes of chloroform, and the ultraviolet absorption curves were measured (Table IV).

[1] Marketed as Mellaril Hydrochloride by Sandoz Pharmaceuticals.

[2] Before introducing the sample solutions, the columns had been rinsed with 20-ml. portions of ether. The rinsing solvents were then discarded.

TABLE III.—RETENTION OF PHENOTHIAZINE DERIVATIVES

Mobile Phase: Ether:Chloroform (8:1); Stationary Phase: 1% Tartaric Acid–Celite

Compound	% Released	% Retained (calcd.)
Thioridazine HCl	0.24	99.76
Thiethylperazine dimaleate	0.35	99.65
Chlorpromazine HCl	0.32	99.68
Promethazine HCl	0.22	99.78

TABLE IV.—SEPARATION OF THIORIDAZINE AND CAFFEINE

Mobile Phase: Ether:Chloroform (8:1); Stationary Phase: 3 ml. of Tartaric Acid + 3 Gm. of Celite

Concn., % of the tartaric acid sol.	pH	Released caffeine, %	Thioridazine entrapment
7.5	1.35	67.50	complete
3.0	1.70	83.70	complete
1.0	2.07	99.87	complete
0.5	2.25	101.25	complete

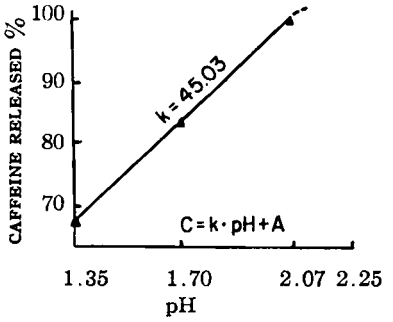


Fig. 2.—pH dependence of the caffeine release.

Quantitative Assay Procedure for the Five Active Ingredients.

Preparation of the Sample.—The average weight of the tablets or capsules was determined. A representative number of tablets was reduced to a fine powder, or a similar number of capsules was emptied and homogenized. In our experiments, the following typical mixture was used: acetylsalicylic acid (200 mg.), caffeine (40 mg.), acetophenetidin (130 mg.), itobarbital[3] (50 mg.), phenothiazine derivative salt (5–20 mg.). In some of the experimental mixtures, inactive ingredients such as stearic acid, talc, sucrose, starch, and lactose were also added; these excipients did not interfere with the assay procedures.

Assay.—A portion of the homogenized powder (equivalent to the weight of a quarter or a half of a dosage unit) was placed into a 50-ml. amber volumetric flask and shaken mechanically with 25 ml. of chloroform for 15 minutes. The solution was then adjusted to volume with chloroform at 20°, mixed

[3] Marketed as Sandoptal by Sandoz Pharmaceuticals.

TABLE V.—ASSAY RESULTS

Sample	% of Label Claim				Phenothiazine Derivative	
	Aspirin	Phenacetin	Caffeine	Itobarbital	Type[a]	% of Label Claim
A1	92.09	104.46	96.94	97.93	T	98.57
A2	92.09	101.92	95.42	100.27	T	99.45
B1	95.10	103.07	99.80	99.66	T	102.20
B2	94.75	103.69	99.80	102.53	T	99.40
C	95.85	100.92	98.16	104.34	T	92.86
D	96.73	99.23	96.84	97.87	TP	108.40
E1	100.70	100.33	97.62	96.85	C	104.12
E2	101.00	100.83	96.35	96.14	C	106.86
F	96.23	99.23	94.82	101.42	P	98.59

[a] Symbols: T = thioridazine hydrochloride, TP = thiethylperazine dimaleate, C = chlorpromazine hydrochloride, P = promethazine hydrochloride.

well, and a 5.0-ml. portion was transferred (without filtration) into a beaker containing 40 ml. of ethyl ether. (Sample solution.)

The assembled column series (Fig. 1) was washed with 35 ml. of ether and the washing discarded. A 250-ml. glass-stoppered Erlenmeyer flask was placed under the column series. The sample solution was quantitatively transferred onto column I, allowed to flow through the column series, and collected in the receiver. After the passage of the first eluate, the column system was eluted with consecutive 20, 20, and 10-ml. portions of the ether:chloroform (8:1) solvent. The eluates were combined in the receiver and evaporated to dryness. The residue which constituted the phenacetin portion of the mixture was redissolved completely in 5.0 ml. of chloroform, diluted with 45.0 ml. of isooctane, and assayed spectrophotometrically.

Immediately after the passage of the last ether:chloroform eluate, the column series was disassembled and column III was eluted first to minimize the time of residence of the aspirin on the column. (A prolonged stay would result in a partial *in situ* degradation of the acetylsalicylic acid.) Elution of the column and the determination of the aspirin were performed in the usual manner (7).

To recover the phenothiazine derivative,[4] the tartaric acid containing column I was eluted with consecutive 20-ml. portions of ammonia-saturated chloroform; the eluates were collected in a 100-ml. volumetric flask and adjusted to volume with the eluant at 20°. An aliquot of the eluate was then evaporated to dryness, redissolved in a suitable solvent, and assayed by the ultraviolet absorption curve.

In the further course, caffeine and itobarbital were eluted from their respective columns and assayed by the technique described in the literature (5, 6).

RESULTS AND DISCUSSION

Martin and Synge (8) in their basic theoretical work on the column partition chromatography developed an equation which expressed the mathematical relation between the movements of the solute and the mobile phase

$$R = \frac{A}{A_m + \alpha A_s} \qquad \text{(Eq. 1)}$$

where R = movement of chromatography band/movement of developing fluid, A = the cross-section area of the column, A_m = the cross-section area of the mobile phase, A_s = the cross-section area of the stationary phase, and α = partition coefficient (solute concentration in the stationary phase/solute concentration in the mobile phase).

Equation 1 indicates that the speed of the solute movement is inversely proportional with the partition coefficient. The distribution ratio of thioridazine hydrochloride between 1% tartaric acid solution and some organic solvents were experimentally determined (Table II).

The ratio with an ether:chloroform (8:1) mixture is more than 60 times higher than that with pure chloroform. Consequently, by the use of this ether:chloroform (8:1) solvent mixture, a low R value and a satisfactory retention of the phenothiazine derivative band were anticipated.

The quantitative entrapment of several phenothiazine derivatives by the selected system is shown in Table III.

The released amounts were found invariably less than 0.35%, indicating a practically complete entrapment of each substance.

The next experimental step was to study the selectivity of the phenothiazine derivative entrapment in the presence of caffeine.

As the results in Table IV demonstrate, by using the ether:chloroform (8:1) mobile phase on tartaric acid–Celite columns of various acid concentrations, the thioridiazine component of the mixture was quantitatively trapped on each column, whereas the release of the caffeine appeared to be a function of the acidity of the stationary phase.

Plotting the data of Table IV in a coordinate system, a graphical interpretation of the pH dependency of the caffeine release was obtained as shown in Fig. 2.

The curve is linear between 1.35 to 2.07 pH values and, by determining the slope of this line, an empirical equation was found

$$C = k \cdot \text{pH} + A \qquad \text{(Eq. 2)}$$

where C is the released percentage of caffeine. (The values of k and A were found to be 45.03 and 6.71, respectively.)

Based on the favorable results of the preliminary model experiments, a number of assays had been carried out with five-component mixtures. Results of these determinations are listed in Table V.

Recoveries of the materials are reported as percentages of the theoretical amounts. Samples with identical letter designation were duplicates. The

[4] The column, receiver, and all glassware containing the phenothiazine derivative must be made of low actinic glass (or protected by a dark cloth) to prevent the photo-catalyzed degradation of the substance.

relatively small deviations between the parallel readings indicate a promising accuracy of the described technique.

The operation of the four-column system imposed no technical difficulty, and the total assay of the five ingredients can be performed by an experienced operator in about 2 hours.

Preliminary experiments for some further simplification of the procedure are now in progress; the results will be reported in a subsequent publication.

SUMMARY

The mechanism of separation of some pharmaceutical derivatives of phenothiazine from mixtures with aspirin, phenacetin, caffeine, and itobarbital has been investigated.

A modified partition chromatography procedure was developed which facilitates a simultaneous quantitative assay of the five active ingredients with satisfactory accuracy.

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Absorption and Incorporation of Methionine-S^{35} into Hair

By A. P. INTOCCIA, J. M. WALSH, and R. L. BOGNER

Administration of methionine-S^{35} to guinea pigs by topical, oral, and intramuscular routes resulted in the incorporation and utilization of the S^{35}-radioactivity by the growing dorsal and ventral hair. Oral or intramuscular administration of methionine-S^{35} led to utilization by the growing hair of between 13 and 21 per cent of the total amount administered. Metabolic utilization by these routes was confirmed by the fact that roughly twice as much nonmethionine-S^{35} as methionine-S^{35} was found. The amount utilized was approximately proportional to the amount administered. A greater amount of S^{35}-radioactivity was detected in the dorsal hair than in the ventral hair even after allowing for the greater rate of hair growth in the dorsal area. When the methionine-S^{35} was applied topically to the dorsal area, approximately 10 per cent of the total amount applied was utilized by the total body hair. At the site of topical application, two-thirds of the S^{35} in the hair in the area appeared to be derived locally and the other one-third systemically.

THE CHEMISTRY and physiology of hair growth has been investigated to gain insight into the factors that control growth and differentiation of hair follicles and formation of keratin. Of special interest is the amino acid composition of and uptake into the proteins and other components of the variety of cells that constitute keratinizing structures and keratin.

This report describes the results of radiotracer investigations initiated to study the influence of various routes of administration on the incorporation of amino acids into hair protein. The amino acid methionine, indispensable for all animals, was chosen for study because it is incorporated into proteins and is converted, after demethylation, into cysteine and cystine which are vital components of keratinized structures.

EXPERIMENTAL

Hartley strain male guinea pigs, adequately adjusted to laboratory conditions and diet, were employed. The diet consisted of pregnant rabbit chow supplemented with fresh lettuce and water *ad libitum*. The animals were housed in individual wire mesh cages that were cleaned daily to prevent the accumulation of any radioactive excreta which could possibly be ingested or adsorbed to the hair of the animal. Prior to the administration of the labeled compound, an area of 2 sq. in. was shaved from the dorsal and ventral surfaces of each animal with an electric clipper. Care was taken in this and all subsequent clippings to prevent abrasions of the skin.

The animals were separated into three groups of six animals. Group *A* received methionine by topical application, group *B* by oral administration, and group *C* by intramuscular injection. Three of the animals in each group received a total dose of 500 mcg. of methionine-S^{35}, and three of the animals received a total dose of 250 mcg.

Radioactive *dl*-methionine-S^{35} was obtained from the Radiochemical Centre, Amersham, England, with a specific activity of 55.9 millicuries per millimole (mc./m*M*). The labeled methionine, shown by paper chromatographic methods to be of radiochemical homogeneity, was diluted with sufficient nonlabeled *dl*-methionine so that the animals at the 250-mcg. level were exposed to a total of 52 μc. and

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TABLE I.—METHIONINE EQUIVALENTS IN HAIR OF GUINEA PIGS (VALUES IN mcg. METHIONINE EQUIVALENTS PER GM. OF HAIR)

Route	Topical				Oral				Intramuscular			
Dosage	250 mcg.		500 mcg.		250 mcg.		500 mcg.		250 mcg.		500 mcg.	
Area	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Wk. 1	2.86	0.19	4.14	0.48	0.95	0.53	1.18	0.83	1.57	0.803	1.22	0.60
2	4.46	1.24	10.20	2.32	3.94	3.16	6.69	5.86	3.83	2.78	5.05	4.28
3	4.91	0.85	5.20	1.47	3.47	2.21	7.23	6.36	4.59	3.05	5.19	4.16
4	3.61	1.96	6.48	2.08	2.52	1.66	7.88	4.30	3.83	2.85	6.16	5.16
5	1.97	1.16	5.28	3.31	2.38	2.24	6.50	2.28	3.32	1.36	8.09	6.07
6	1.24	0.65	2.32	1.53	1.17	1.06	4.37	2.96	1.58	1.69	2.93	2.39
7	0.94	0.75	1.85	2.23	0.49	0.53	1.96	1.33	0.73	1.03	1.30	1.35
8	0.50	0.42	1.15	1.12	0.66	0.85	1.73	1.31	0.79	1.10	1.36	1.16
16	0.24	0.25	0.31	0.22	0.31	0.33	0.69	0.83	0.46	0.512	0.63	0.80

the animals at the 500-mcg. level received 128 μc. during the experiment.

The doses administered by topical application were dissolved in a mixture of 18 amino acids in denatured ethyl alcohol[1] to yield solutions that contained methionine concentrations of 250 and 500 mcg./ml. Fifty microliters of these solutions were applied to the shaved areas on the dorsal surfaces of the animals and massaged into the skin with the operator's forefinger encased in a rubber finger cot. Loss of radioactive material by adsorption to the finger cot did not exceed 10% of the daily administered dose.

The oral doses were prepared by dissolving nonradioactive methionine and methionine-S^{35} in distilled water to give the appropriate concentrations of 12.5 and 25.0 mcg./ml. The animals were administered 1 ml. of these solutions intragastrically by means of a 1-ml. syringe and an 18-gauge oral blunted needle.

The intramuscular doses were prepared to contain 125 and 250 mcg./ml. in distilled water; group *C* animals were injected daily with 0.1 ml. of the appropriate solution.

Radioactive methionine preparations were administered daily for 5 consecutive days of each week for 4 weeks. Hair clippings from both the dorsal and ventral surfaces of all animals were collected on the seventh day of each week during the period of methionine-S^{35} administration and for 12 additional weeks after administration was discontinued.

For radioactivity measurements, it was necessary to free the hair of any adsorbed radioactivity prior to S^{35}-measurements. Preliminary tests showed that over 99% of the adsorbed S^{35}-radioactivity on the dorsal hair from the animals treated topically could be removed by washing the hair with 50% ethanol made 1 *N* with hydrochloric acid. Accordingly, 20-mg. samples of the hair from all animal groups were washed, dried, and dissolved in 3 ml. of 1 *M* hydroxide of hyamine 10X. A toluene phosphor containing 0.4% 2,5-diphenyloxazole (PPO) and 0.1% 1,4-bis-2-(5-phenyloxazole)-benzene (POPOP) was added, and the samples were counted in a liquid scintillation spectrometer. Counting efficiency was determined with internal standards. The disintegrations per minute (d.p.m.) were converted to methionine equivalents by assuming that all of the radioactivity in a sample was present as methionine of the same specific activity as that of the administered methionine-S^{35}.

[1] Supplied by Beecham Products, Inc., Clifton, N. J., as Pure Silvikrin which contained ordinary methionine in a concentration of 100 mcg./ml.

For paper chromatography, approximately 20 mg. of the washed hair samples were acid hydrolyzed; the residue was dissolved in distilled water. Aliquots of these solutions were applied to Whatman No. 1 filter paper for chromatography in a solvent of phenol saturated with 6.3% sodium citrate and 3.7% dibasic potassium phosphate. The developed chromatograms were air-dried and cut into segments for liquid scintillation counting or for counting on flat cards in a thin end-window gas-flow proportional counter. Counting in this manner revealed two peaks of radioactivity in the positions of cysteic acid, cystine, and/or glutathione (R_f 0.24) and methionine (R_f 0.80). The dorsal and ventral hair from one animal in each of the three groups for 5 weeks was subjected to chromatographic analysis in the above manner to determine the relative concentrations of these amino acids in the hair.

RESULTS

The "methionine equivalents" of S^{35}-radioactivity of washed dorsal and ventral hair are presented in Table I; each value represents the average of three animals. The data indicated that the hair from the animals that received 250 mcg. of methionine-S^{35} contained approximately half as much activity at peak levels as those that received 500 mcg. The dorsal hair of the animals receiving methionine-S^{35} by topical application in doses of 250 and 500 mcg. showed the highest average S^{35} levels at the third and second week, respectively. Thereafter, the levels decreased gradually up to the point where administration was discontinued after 4 weeks. The average S^{35} levels of the ventral hair of these animals was considerably lower than those found in the dorsal hair. In general, the S^{35} persisted in the hair at least 1 week after discontinuation of administration before the concentration declined more sharply.

The difference between the dorsal and ventral hair of the topical group was not of the same magnitude in the oral and intramuscular groups. The higher values found in the dorsal hair of these latter two groups suggested preferential incorporation of systemically administered methionine-S^{35} by dorsal hair of the guinea pig.

In addition, the peak levels of radioactivity in both oral and intramuscular groups appeared later than did the peaks in the topical group; the peak value in the intramuscular group receiving 500 mcg. methionine-S^{35} did not appear until 1 week after administration was discontinued.

Examination of the dorsal hair data indicated that all routes of administration were equally efficient.

TABLE II.—METHIONINE EQUIVALENTS (mcg.) OF TOTAL HARVESTED HAIR AND TOTAL BODY HAIR

	250-mcg. Dose			500-mcg. Dose		
	Dorsal Harvested Area	Ventral Harvested Area	Total Body	Dorsal Harvested Area	Ventral Harvested Area	Total Body
Oral	1.65 ± 0.40	0.72 ± 0.31	31.92	4.56 ± 0.34	1.70 ± 0.48	84.68
Intramuscular	2.83 ± 0.69	1.05 ± 0.40	52.50	5.45 ± 1.47	2.09 ± 1.05	101.91
Total Topical	3.77 ± 0.84	0.53 ± 0.12	28.51	6.38 ± 1.25	0.79 ± 0.12	43.15
Local	2.39	...		4.33	...	
Systemic	1.38	0.53		2.05	0.79	

However, it was seen that S^{35}-uptake by the ventral hair of the oral and intramuscular groups was similar, but the level of incorporation into the ventral hair of the topical group was significantly lower.

Table II shows the actual weights of hair harvested from the dorsal and ventral surfaces of the animals multiplied by the radioactivity concentrations to give the actual average "methionine equivalents" laid down in the area of harvest. From the weights of hair harvested, the rate of hair growth in the dorsal area averaged 2.16 times the rate of growth in the ventral areas. On the other hand, the average "methionine equivalents" harvested from the animals of the orally administered and intramuscularly injected groups indicated that 2.6 times more radioactivity was recovered from the dorsal areas than from the ventral areas. Since the radioactivity ratio is greater than the growth rate ratio, it was concluded that a preferential uptake of systemic methionine-S^{35} or its S^{35}-labeled conversion products occurred in the dorsal area over and above that due to the higher rate of hair growth.

The values of the ventral areas of the topically applied groups were multiplied by the factor 2.6, previously found to reflect the directional influence of the systemic distribution of S^{35} between the dorsal and ventral hair of the oral and intramuscular groups. These calculated values represented the apparent systemic utilization or incorporation of the S^{35} in the dorsal hair of the topically treated groups and are included in Table II. Local utilization of the S^{35} in the dorsal hair of the topically treated animals was obtained by difference. No local utilization was assumed in the ventral hair of the topically treated groups and it was also assumed that the actual uptake of S^{35} by the animals in the oral and intramuscular groups reflected true systemic utilization.

Groups receiving the methionine-S^{35} by topical application showed high local dorsal utilization but lower systemic utilization. The conclusion was that the topical application of methionine-S^{35} to the small defined areas of the back yielded higher recoveries of S^{35} in the hair from these areas than the recoveries from the other two routes of administration, since higher recoveries were presumably due to local utilization of the methionine-S^{35} superimposed upon a smaller incorporation from penetration of the skin, systemic distribution, and delivery of the S^{35} to the same site. Approximately two thirds of the radioactivity in this area was due to local utilization and one-third was provided by systemic utilization.

Total utilization of the S^{35} over the entire body (Table II) was calculated by multiplying the quantities for the uptake in the areas of harvest by factors which integrated them in terms of total body hair growth. These factors were obtained by determining the weight of the harvested hair both dorsally and ventrally and the weight of the remaining dorsal and ventral hair of three test animals. It was then possible to arrive at factors relating the S^{35} in the harvested area to the remainder of the hair for both the dorsal and ventral areas of each animal. These factors were 14.17 for dorsal hair and 11.81 for ventral hair. It should be noted that the results of the oral and intramuscular administration were not dissimilar, although the intramuscular route appeared to be the more efficient of the two routes.

TABLE III.—RATIO OF NONMETHIONINE-S^{35} TO METHIONINE-S^{35}

Route	Level, mcg.	Dorsal Area	Ventral Area
Oral	250	2.4	3.3
	500	2.3	3.4
Intramuscular	250	2.5	1.5
	500	2.3	1.6
Topical	250	1.4	1.8
	500	1.5	1.7

From the data for the total "methionine equivalents" in the total body hair, estimations could be made of the percentages of the total dose utilized. It was found that the total utilization by either the oral or intramuscular route was between 13–17 and 20–21%, respectively, of the total S^{35} administered. The topical route led to a total incorporation of approximately 10% of the applied S^{35}, which represented 89 and 54% of that utilized by the more efficient oral and intramuscular routes, respectively, at the 250-mcg. dose and 51 and 42% at the 500-mcg. dose. Table III presents the average ratios of total nonmethionine-S^{35} to total methionine-S^{35} in the dorsal and ventral areas for the first 5 weeks of the experiments. Nonmethionine-S^{35} activity was not resolved chromatographically into cysteic acid, cystine, or glutathione. It is difficult to interpret the increased ventral ratio by the oral route and the increased dorsal ratio by both the oral and intramuscular routes. Since the S^{35}-radioactivity arrived systemically, similar results were expected in each case. The low ratio in the dorsal area by the topical route suggested local utilization of methionine *per se*.

DISCUSSION

Earlier studies (1) demonstrated that oral feeding of methionine-S^{35} to rats resulted in the appearance of cystine-S^{35} in hair and tissues. Similar results were obtained in guinea pigs by topical application, oral feeding, and intramuscular injection of methionine-S^{35} (2). However, no evidence of local utilization of topically applied methionine has been reported prior to the studies described here.

Several investigators have demonstrated the intense localization of sulfur-containing radioactive

amino acids in the keratogenous zone of hair. Belanger (3) noted localization of methionine-S^{35} and cystine-S^{35} after subcutaneous injections into rats. Fleischer, *et al.* (4), injected S^{35}-yeast protein hydrolysate containing cystine-S^{35}, methionine-S^{35}, and methionine sulfoxide-S^{35} intravenously into rats and found a rapid incorporation of S^{35} into hair, particularly over the length of the intensely vascularized part of the hair. Ryder (5) and Harkness and Bern (6) also found a rapid and marked uptake of S^{35} in the keratogenous zone of anagen follicles after injecting cystine-S^{35}; the latter investigators also noted uptake of C^{14} in the bulb and keratogenous zone after injecting C^{14}-labeled algal protein hydrolysate of undefined amino acid composition into mice.

Bern, *et al.* (7), have pointed out that transport of cystine from the circulatory system *via* absorption through the hair bulb is conceivable, but that a lateral transport from the capillaries around the follicle shaft (8) is also possible.

Apparently, S^{35}-amino acids are at least as equally utilized for hair protein synthesis as for plasma or tissue protein synthesis. The S^{35} specific activity of rabbit hair keratin was found (9) similar to that of the S^{35} activity of tissue proteins following intravenous injections of S^{35}-labeled yeast protein hydrolysate. A much higher specific activity of the cystine-S^{35} in wool than in plasma was noted following the intravenous administration of cystine-S^{35} to sheep (10). The latter concluded cystine is incorporated into wool as a free amino acid.

Only traces of cystine and methionine as free amino acids have been found in aqueous extracts of the roots (bulb, prekeratinized fiber portion, and attached inner root sheath) of keratinized structures, although more methionine is present in the protein of the inner root sheath of hair follicles, as detected by administering S^{35}- and C^{14}-labeled methionine to rats (11) and large quantities of cystine occur in the hair protein itself. According to Rogers (12), the pool of free amino acids in the hair roots is presumably available for keratin synthesis and the small concentration of cystine may be related to a high demand for cystine by the growing fiber and to mechanisms for maximum utilization of the sulfur-containing amino acids. Recently, it has been reported that other amino acids, H^3-labeled alanine and leucine and C^{14}-labeled serine, were incorporated into the growing hair of guinea pigs in the same pattern as cystine-S^{35} after intraperitoneal injection (13); however, no other studies of local utilization following topical application of amino acids by growing hair have been reported.

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Degradation of Phenylephrine Hydrochloride in Tablet Formulations Containing Aspirin

By A. E. TROUP and H. MITCHNER†

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PHENYLEPHRINE HYDROCHLORIDE is frequently incorporated in multi-ingredient pharmaceutical preparations. Analytical assay methods for phenylephrine in such mixtures have been directed both to the development of specific molecular procedures and to methodology involving the separation of the phenylephrine followed by its analysis. Assay problems have been further complicated in the endeavor to evaluate degradation during stability testing where the

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amino acids in the keratogenous zone of hair. Belanger (3) noted localization of methionine-S^{35} and cystine-S^{35} after subcutaneous injections into rats. Fleischer, *et al.* (4), injected S^{35}-yeast protein hydrolysate containing cystine-S^{35}, methionine-S^{35}, and methionine sulfoxide-S^{35} intravenously into rats and found a rapid incorporation of S^{35} into hair, particularly over the length of the intensely vascularized part of the hair. Ryder (5) and Harkness and Bern (6) also found a rapid and marked uptake of S^{35} in the keratogenous zone of anagen follicles after injecting cystine-S^{35}; the latter investigators also noted uptake of C^{14} in the bulb and keratogenous zone after injecting C^{14}-labeled algal protein hydrolysate of undefined amino acid composition into mice.

Bern, *et al.* (7), have pointed out that transport of cystine from the circulatory system *via* absorption through the hair bulb is conceivable, but that a lateral transport from the capillaries around the follicle shaft (8) is also possible.

Apparently, S^{35}-amino acids are at least as equally utilized for hair protein synthesis as for plasma or tissue protein synthesis. The S^{35} specific activity of rabbit hair keratin was found (9) similar to that of the S^{35} activity of tissue proteins following intravenous injections of S^{35}-labeled yeast protein hydrolysate. A much higher specific activity of the cystine-S^{35} in wool than in plasma was noted following the intravenous administration of cystine-S^{35} to sheep (10). The latter concluded cystine is incorporated into wool as a free amino acid.

Only traces of cystine and methionine as free amino acids have been found in aqueous extracts of the roots (bulb, prekeratinized fiber portion, and attached inner root sheath) of keratinized structures, although more methionine is present in the protein of the inner root sheath of hair follicles, as detected by administering S^{35}- and C^{14}-labeled methionine to rats (11) and large quantities of cystine occur in the hair protein itself. According to Rogers (12), the pool of free amino acids in the hair roots is presumably available for keratin synthesis and the small concentration of cystine may be related to a high demand for cystine by the growing fiber and to mechanisms for maximum utilization of the sulfur-containing amino acids. Recently, it has been reported that other amino acids, H^3-labeled alanine and leucine and C^{14}-labeled serine, were incorporated into the growing hair of guinea pigs in the same pattern as cystine-S^{35} after intraperitoneal injection (13); however, no other studies of local utilization following topical application of amino acids by growing hair have been reported.

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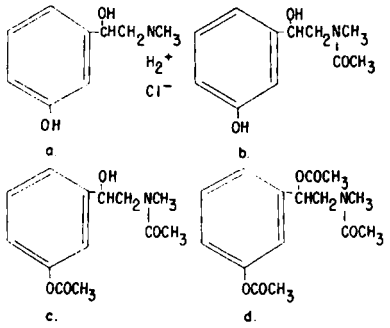


Fig. 1.—Chemical structures of phenylephrine and its synthetic acetyl derivatives: *a*, phenylephrine hydrochloride; *b*, *N*-monoacetylphenylephrine; *c*, *O,N*-diacetylphenylephrine; *d*, triacetylphenylephrine.

breakdown pathway is frequently impossible to predict. With heated solutions of phenylephrine hydrochloride and tetracaine, Schriftman (1) reported the presence of unknown breakdown products which were not found in solid formulations. Paper chromatographic procedures were utilized for the separation of degradation products. Evaluating the available analytical methodology, Schriftman (1) pointed out that phenylephrine assay procedures were based on reactions involving the hydroxyphenol nucleus (2, 3) and failed to detect changes on the side chain of the molecule. To overcome this latter problem, Kelly and Auerback (4) developed an ion exchange procedure which reportedly separated phenylephrine from interferring ingredients and resulted in a higher degree of analytical specificity for phenylephrine stability studies.

In working with stability samples of multiingredient tablet preparations containing phenylephrine hydrochloride, this laboratory found that the secondary amine characteristic of phenylephrine was being destroyed. To facilitate studies concerning the cause of the degradation, analytical procedures were developed which were specific for secondary amine character in the mixtures studied, did not involve complex separations, and were quite rapid. It was found that acetylsalicylic acid (aspirin) was involved in the phenylephrine degradation. Studies were conducted to determine what factors con-

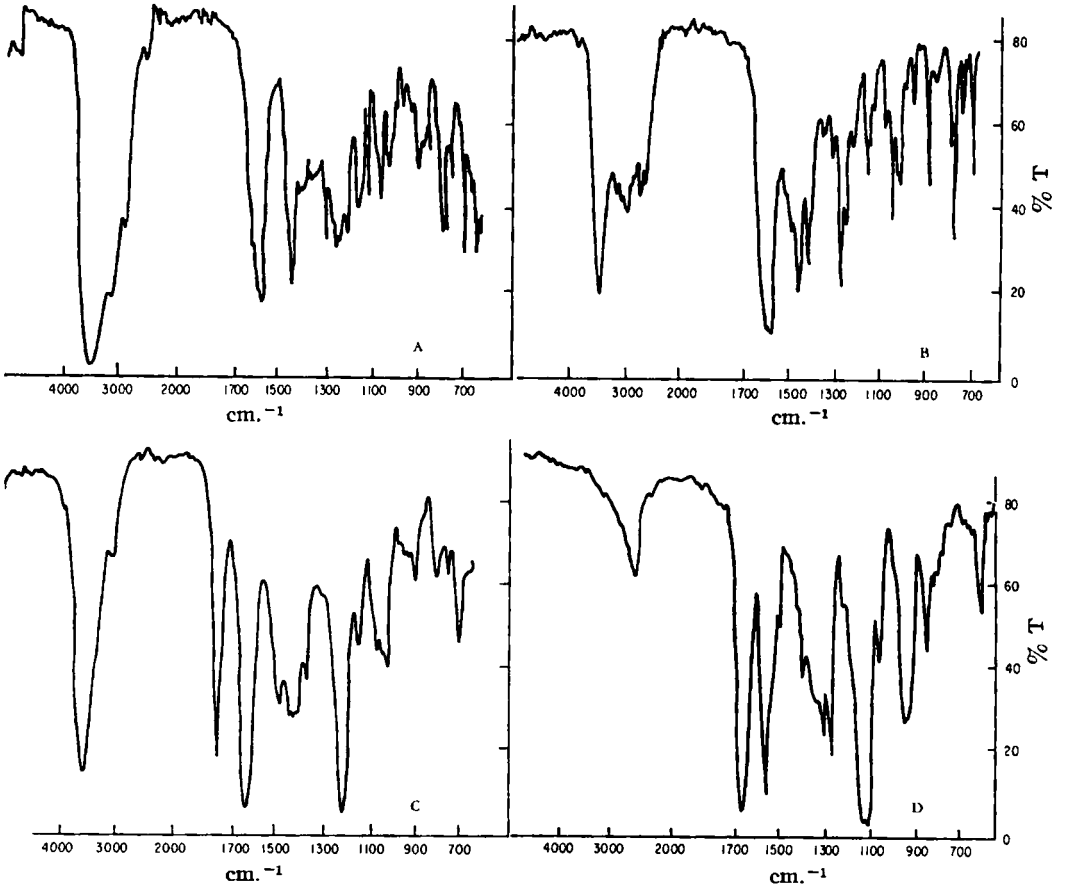


Fig. 2.—Infrared spectra of phenylephrine and its synthetic acetyl derivatives: *a*, phenylephrine hydrochloride; *b*, *N*-monoacetylphenylephrine; *c*, *O,N*-diacetylphenylephrine; *d*, triacetylphenylephrine. Spectra *a*, *b*, and *c* run in KBr disks. Spectrum *d* run in carbon tetrachloride with solvent compensation. All were recorded on a Beckman IR-4. Purity of synthetic derivatives was about 90%.

tributed to the degradation, the mechanism thereof, and the characterization of the breakdown products.

EXPERIMENTAL

Carbon Disulfide Assay for Secondary Amine Function.—All solvents and reagents were of analytical reagent quality and used without additional purification. Carbon disulfide:isopropyl alcohol:benzene mixture was prepared by pipeting 5 ml. of carbon disulfide and 25 ml. of isopropyl alcohol into a 100-ml. volumetric flask and diluting to volume with benzene. Ammoniacal copper solution was prepared according to Stanley, *et al.* (5). Acetic acid solution was a 30% v/v aqueous solution. As a standard stock solution, about 100 mg. of phenylephrine hydrochloride (Winthrop Laboratories) was accurately weighed, transferred to a 200-ml. volumetric flask, and diluted to volume with water. This solution was diluted exactly 1:10 with water as a working standard.

Procedure.—A representative tablet or powder sample equivalent to about 5 mg. phenylephrine hydrochloride was accurately weighed and transferred to a 100-ml. volumetric flask. The flask, to which about 50 ml. of water was added, was shaken 15 minutes. The mixture was diluted to volume with water, then filtered through Whatman No. 42 filter paper. Exactly 4 ml. of sample or working standard was transferred, in duplicate, to 50 ml. glass-stoppered centrifuge tubes. Exactly 10 ml. of carbon disulfide:isopropyl alcohol:benzene reagent and 1 ml. ammoniacal copper reagent was added to each tube. The tubes were shaken exactly 15 seconds on a Fisher clinical shaker; exactly 2 ml. of 30% acetic acid was added to each tube. The tubes were again shaken 15 seconds. The upper layer was filtered through Whatman No. 42 filter paper and the absorbances of the standards and samples read against benzene on a Beckman DU spectrophotometer at 434 mμ.

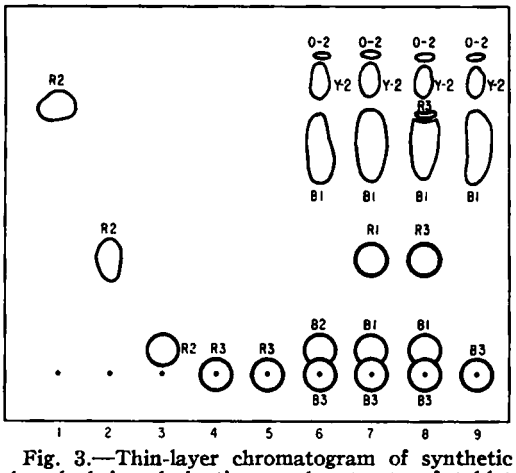


Fig. 3.—Thin-layer chromatogram of synthetic phenylephrine derivatives and extracts of tablet stability samples. Key: *1*, triacetylphenylephrine; *2*, *N*-diacetylphenylephrine; *3*, *N*-monoacetylphenylephrine; *4*, phenylephrine hydrochloride; *5*, phenylephrine base; *6*, tablet stored 6 months at room temperature—carbon disulfide assay method indicated 98% phenylephrine remaining; *7*, tablet stored 34 months at room temperature—carbon disulfide method indicated 14% loss, 4-aminoantipyrine method indicated 100% phenylephrine remaining although chromatogram indicated a trace of diacetylphenylephrine; *8*, tablet stored 2 months at 50°C.—carbon disulfide method indicated 93% loss of phenylephrine; *9*, a placebo containing all tablet ingredients except phenylephrine. Colors: R red, B brown, O orange, Y yellow, 1 faint, 2 moderate, 3 intense.

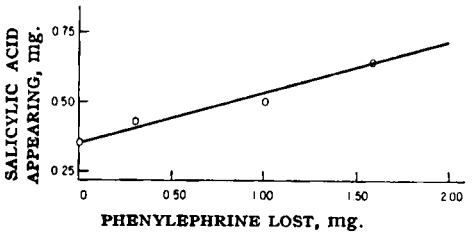


Fig. 4.—Decrease in phenylephrine activity plotted against increase in free salicylic acid for a multi-ingredient tablet containing phenylephrine hydrochloride and acetylsalicylic acid with storage at 70°C. for 4 weeks.

Synthesis of Acetylated Phenylephrine Derivatives.—*N*-Monoacetylphenylephrine (*N*-(*m*,β-dihydroxyphenethyl)-*N*-methylacetamine), Fig. 1*b*, was prepared according to Bretschneider (6).

N-Diacetylphenylephrine (*N*-(*m*-acetoxy-β-hydroxyphenethyl)-*N*-methylacetamide), Fig. 1*c*, was prepared according to Pratt (7).

Triacetylphenylephrine (*N*-(*m*,β-diacetoxyphenethyl)-*N*-methylacetamide), Fig. 1*d*, was prepared by dissolving about 10 Gm. of phenylephrine base in 30 ml. glacial acetic acid and 60 ml. acetic anhydride. The mixture was refluxed 6 hours, then placed in a glass-stoppered flask in a 105° oven for 7 days. The volume was reduced by evaporation at 50° under vacuum. The oily residue was dissolved in water and then extracted with several portions of carbon tetrachloride. The solvent was removed by evaporation on a steam bath. A thin-layer chromatogram of the product showed only one spot.

The identity of the mono and diacetyl derivates were confirmed by melting point. Further characterization of structure was obtained by infrared spectra (Fig. 2) and with thin-layer chromatograms which showed only single ingredient spots (Fig. 3).

Thin-Layer Chromatography.—A Brinkman model "S" applicator was used for all chromatograms. As an adsorbant, a 250-μ thickness of silica gel G (Brinkman) was applied. The best developing solvent was found to be the chloroform layer from an equilibrated mixture of chloroform, glacial acetic acid, and water (5:1:1). Color development was obtained by use of aqueous solutions of 0.3% *p*-nitroaniline dissolved in 1 *N* hydrochloric acid, 5% w/v sodium nitrite, and 20% w/v sodium carbonate.

Procedure.—For a synthetic acetylated compound about 10 mg. was dissolved in a mixture of 2 ml. ethanol and 0.5 ml. acetone. With tablets, two well ground tablets were thoroughly mixed with 2 ml. of ethanol and 0.5 ml. of acetone and the clear supernatant solution used. About 0.02 ml. of each solution was spotted to previously prepared and activated silica gel plates. After development the plates were air dried at room temperature. Detection of spots was accomplished by (*a*) spraying the plates with diazotized *p*-nitroaniline (to 25 ml. of

the acidic 0.3% *p*-nitroaniline, 1.5 ml. of sodium nitrite was added immediately prior to use), (*b*) drying the plates at 105° for about 10 minutes, and (*c*) spraying the plates with the 20% sodium carbonate solution.

RESULTS AND DISCUSSION

Analytical and Stability Factors.—Tablet preparations containing as active ingredients phenylephrine hydrochloride, acetylsalicylic acid, chlorpheniramine maleate, acetophenetidin, and caffeine were prepared and assayed for ingredient stability under accelerated temperature conditions. Only changes in acetylsalicylic acid and phenylephrine could be detected. For any one formulation held at an elevated temperature, the increase in salicylic acid content plotted against the decrease in phenylephrine content gave a linear relationship. Figure 4 shows a typical plot. It appeared, therefore, that there could exist some interrelationship between these degradations. Breakdown of phenylephrine was detected in the mixtures with the ion exchange procedure of Kelly and Auerbach (4) which further suggested that the secondary amine function of phenylephrine was involved.

Because of the involved nature of the Kelly and Auerbach procedure, efforts were directed toward other possible analytical procedures, particularly those that did not involve separation procedures. Of all the active ingredients and tablet excipients only phenylephrine contained a secondary amine function; this function was altered as indicated by the Kelly and Auerbach ion-exchange procedure. Specific secondary amine procedures were adaptable to the complex pharmaceutical mixtures under study. A modification of a procedure by Umbreit (8) worked. A second very rapid procedure with low blank values was developed and utilized for the work reported in this paper. This later method was based on a procedure reported for the assay of dimethylamine in methylamine by Stanley, Baum, and Gove (5), and prior ingredient separations were not needed. Evaluation of changes on the hydroxyphenol nucleus of the phenylephrine molecule was possible with the use of the 4-aminoantipyrine assay procedure of Koshy and Mitchner (9) which could also be applied to the tablet mixtures without prior separations.

Possible tablet ingredient interactions were further explored by submitting a series of powder mixtures to accelerated temperatures. These mixtures contained the active ingredients previously mentioned with the addition of various tableting agents such as binders, diluents, lubricants, and disintegration agents.

Mixtures were: [I] phenylephrine hydrochloride, acetylsalicylic acid (1:20); [II] phenylephrine hydrochloride, acetylsalicylic acid, magnesium stearate (10:200:1); [III] phenylephrine hydrochloride, acetylsalicylic acid, talc (1:20:1); [IV] phenylephrine hydrochloride, starch, magnesium stearate (50:150:1); and [V] phenylephrine hydrochloride, chlorpheniramine maleate, ascorbic acid, acetophenetidin, caffeine, starch (5:10:25:150:30:2). Analysis for secondary amine function with exposure to 70° for extended periods of time gave the results shown in Fig. 5.

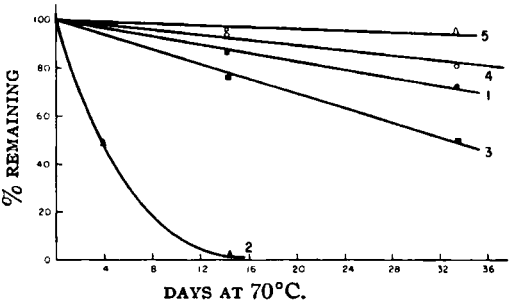


Fig. 5.—Decrease in secondary amine of phenylephrine in powder mixtures with storage at 70°C. Assay by carbon disulfide method. Key: *1*, phenylephrine hydrochloride, acetylsalicylic acid; *2*, phenylephrine hydrochloride, acetylsalicylic acid, magnesium stearate; *3*, phenylephrine hydrochloride, acetylsalicylic acid, talc; *4*, phenylephrine hydrochloride, starch, magnesium stearate; and *5*, phenylephrine hydrochloride, chlorpheniramine maleate, ascorbic acid, acetophenetidin, caffeine, starch.

TABLE I.—PHENYLEPHRINE CONTENT (MG./TABLET) AFTER STORAGE AT 70°C.

	Carbon Disulfide Method	4-Aminoantipyrine Method
Original, days	4.41	4.48
6	4.12	4.30
9	3.46	4.10
12	2.87	3.72
17	2.67	3.61
21	1.81	...
30	1.44	3.01

Combinations of phenylephrine hydrochloride and acetylsalicylic acid showed the greatest rates of phenylephrine degradation. With the addition of magnesium stearate, the breakdown was markedly accelerated. Very little phenylephrine was degraded in Mixture I with only phenylephrine hydrochloride and acetylsalicylic present and very little free salicylic acid was formed. Large amounts of free salicylic acid were formed in stability samples of Mixture II, which differed only from Mixture I by the addition of magnesium stearate. Previously, the effect of magnesium stearate in accelerating the breakdown of acetylsalicylic to salicylic acid and acetic acid in aspirin tablet formulations was shown by Ribeiro, *et al.* (10). It was concluded that breakdown of acetylsalicylic acid was prerequisite to the degradation of phenylephrine. The probable route of phenylephrine degradation was postulated as an acetylation of the secondary amine function with a possible contributory action from magnesium stearate since Castello and Mattocks (11) have shown that magnesium stearate can react with amine salts to release the free base. The free base of phenylephrine would be expected to be readily acetylated.

A multi-ingredient tablet formulation containing phenylephrine hydrochloride, acetylsalicylic acid, and magnesium stearate was stored at 70° and analyzed periodically for its phenylephrine content by both the carbon disulfide assay described in this paper and the 4-aminoantipyrine method (9). The results obtained are shown in Table I.

The reaction of 4-aminoantipyrine with a phenol

such as phenylephrine depends upon the *para* position to the phenyl group being unsubstituted (12). Loss of phenylephrine activity by attack only on the secondary amine portion of the side chain would not, therefore, be picked up by the 4-aminoantipyrine assay. A comparison of the results of the two assay techniques as shown in Table I suggested that both the phenolic portion of the phenylephrine and its secondary amine were being attacked under accelerated conditions but at considerably different rates. The secondary amine was more susceptible to attack. This was confirmed with samples held under less severe conditions. Similar tablet formulations held 2 months at 50° showed a 36% loss of phenylephrine by the 4-aminoantipyrine and 93% loss by the carbon disulfide assay. On samples stored at room temperature, loss of phenolic activity could not be detected after 34 months and secondary amine loss was 14% at that time.

Identification of Phenylephrine Degradation Products.—The identities of the degradation products were established by thin-layer chromatography of stability samples of tablets. The R_f values of spots not present in fresh samples were identical with the synthetic samples of mono, di, and triacetylated phenylephrines. Structures of derivatives are shown in Fig. 1. A thin-layer chromatogram of the three synthetic phenylephrine derivates compared to extracts of stability samples of multi-ingredient tablets containing phenylephrine hydrochloride and acetylsalicylic acid is shown in Fig. 3. From the chromatographic results it was apparent that (*a*) *N*-monoacetylphenylephrine formed under relatively mild conditions and in a short time, (*b*) *O*, *N* diacetylphenylephrine formed in addition to the monoacetyl compound under more severe conditions or extended periods of time at room temperature, and (*c*) all three acetylated compounds formed under severe accelerated stability conditions.

A number of different solvent systems for the thin-layer chromatography of phenylephrine and its degradation products were evaluated with the best system as previously described. Although not all of the systems yielded resolution of all of the degradation products, it was possible to confirm comparatively the identity of the unknown spots that did appear with the synthetic phenylephrine derivatives.

Oxidative Degradation of Phenylephrine.—Both the 4-aminoantipyrine method and the carbon disulfide assay methods were checked regarding applicability for the detection of oxidative degradation. Aqueous solutions of phenylephrine hydrochloride oxidized by boiling or by the addition of either hydrogen peroxide or manganese dioxide showed loss of phenylephrine of the same magnitude by either procedure.

SUMMARY

Formulations containing both phenylephrine hydrochloride and acetylsalicylic acid were found susceptible to loss of phenylephrine activity.

Degradation of phenylephrine resulted from acetylation, with acetylsalicylic acid providing the acetyl function. Breakdown products were identified as the mono, di, and triacetylated phenylephrine derivatives.

Tableting agents such as magnesium stearate which could accelerate the breakdown of acetylsalicylic acid were found to be contributory agents to a rapid loss of phenylephrine.

An assay was developed to detect loss of secondary amine activity in phenylephrine. With complex mixtures, specific functional group analysis could detect degradation on either the intact hydroxyphenol portion of the phenylephrine molecule or on the secondary amine of the side chain.

Oxidative degradation of phenylephrine could be detected by either the 4-aminoantipyrine or carbon disulfide method.

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Measurement of a Color Gamut Obtained from FD&C Colorants

By ALLAN M. RAFF

A method is illustrated to determine what colors may be achieved utilizing the FD&C aluminum lakes as colorants. Lakes were mixed with a tablet excipient, compressed, and the reflectance was measured. A color solid was constructed at fixed values of lightness. Reduction in the color solid caused by limitations imposed by Federal regulations and pharmaceutical requirements is also illustrated. The use of such a technique aids in making color matches and defines the extent of realizable colors.

THE PURPOSE OF this study is threefold. First, it illustrates a method whereby the pharmaceutical formulator can define the gamut of colors he may achieve with a given set of colorants in any pharmaceutical dosage form as long as the color of the dosage form can be measured instrumentally. Second, it establishes the color gamut of the FD&C aluminum lakes. They were chosen as representative of a class of colorants important in taking full advantage of the techniques of direct tablet compression (1, 2). Third, this study further serves to illustrate the need for considering colorants as more than spectrophotometric curves. They also possess varying degrees of physical and chemical stability, tinctorial power, etc. (3, 4).

An introduction and orientation to the basic concepts utilized in this study are contained in an appendix[1] and bibliography of a previous article (5).

Instrumentation.—The instrumental approach to color matching, and more specifically the use of the spectrophotometer for industrial color formulation, is the method chosen in most instances.

Davidson and Hemmendinger (6–8) have developed several instruments to expedite the work involved in spectrophotometric color matches based upon a two-constant theory of pigment mixtures (9–11). Others, such as Billmeyer, *et al.* (12, 13), and later Alderson, *et al.* (14), described the use of large digital computers as an aid in color formulation. Saltzman (15) described the use of a pigment library as an aid in pigment identification and color matching. Duncan (16) investigated the mechanisms of color production and derived a set of formulas for the prediction of color matches.

The making of a spectrophotometric color match can only be achieved when the same colorants that are present in the color sample are used in the preparation of the pharmaceutical dosage form. It is often stated that *color* matching is best attained by *colorant* matching (17).

In the case of color matching in industrial pharmacy we assume that we are definitely going to get a metameric[2] color match. The reason for this assumption is as follows. The pharmacist is shown a color sample on a paper that was achieved with printing inks, or he is given the liquid dosage form of a drug from which it is now desired to make a tablet, or someone just happens to be wearing a tie that has an "appealing" color. In the case of the color samples being made from printing inks in the first example, and textile dyes in the last, it is obvious that these dyes cannot be used in the preparation of pharmaceutical products. In the example of the liquid dosage form, it was made with water-soluble dyes and, while these may be used, they defeat the purpose of direct compression. If we further assume that in the majority of cases we are only trying to match the colors of our own products, and not those of our competitors, then we already know the exact colorant composition of the color in the liquid and do not need to resort to spectrophotometry for color matching. One would also have the problem here of differences in the attributes of the appearance modes (18, 19) of a liquid with a transmitted color *versus* a tablet with a reflected color. The color may not appear the same even with the same colorant formulation, achieved with or without a spectrophotometer. When a nonmetameric match cannot be made it is necessary to resort to colorimetry (6).

In other words, the industrial pharmacist in his role as a colorist faces problems that may be more acute than those of the colorist in the paper coating, printing, textile, ceramic, plastic, or paint industry. At the present time the U. S. Food and Drug Administration (FDA) lists only 11 FD&C coal-tar dyes (20). Others, in the D&C category, have a more tenuous existence and may eventually be decertified. Since the number of colorants is so limited (and if we add further limitations such as the requirement for a dry colorant for direct tablet compression), the primary question in the development of a color for a new product should be "What colors are available to us?" not "Can we make a match of a color standard using different colorants?" It is for this reason that this example of a color gamut is presented.

Color Gamut.—A color gamut, or color solid, is of value because it enables one to tell, with a minimal amount of effort, whether a color standard can be matched.

A color solid may be constructed because color

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[1] Available from the author.

[2] A metameric match is one that does not appear to match under all conditions of illumination.

can be considered as a three-dimensional system. These dimensions may be described in the terminology of the Munsell (21) system or that of the C.I.E. (22, 23) system. The first dimension, "hue" (Munsell), is that attribute which distinguishes a red from a green. In the C.I.E. system this is the dominant wavelength. The second dimension is "value" (Munsell), or "lightness" (C.I.E.), the lightness compared with a gray scale from white to black. It may be thought of as the attribute which allows the differentiation of light from dark colored objects (17). The third dimension is "chroma" (Munsell), "purity" (C.I.E.) or saturation, the "redness" of a red as it goes from a dull brick red to a brilliant scarlet (15).

There are a number of additional attributes which will affect the appearance of a colored object, such as size, shape, sparkle, transparency, glossiness, and luster (19). While these factors cannot be ignored, they were, in a sense, eliminated from this experiment by evaluating all colors in tablets with the appearance modes having the same attributes.

While the number of colors perceived may be considered as infinite, those available to the industrial pharmacist are probably more severely limited than in any other field. Aside from all the natural factors that limit the available colors—such as their toxicity, light fastness, and chemical reactivity—the pharmaceutical industry may use only those colorants approved by the FDA (20). In the case of the FD&C aluminum lakes, only nine FD&C dyes are utilized in their manufacture.

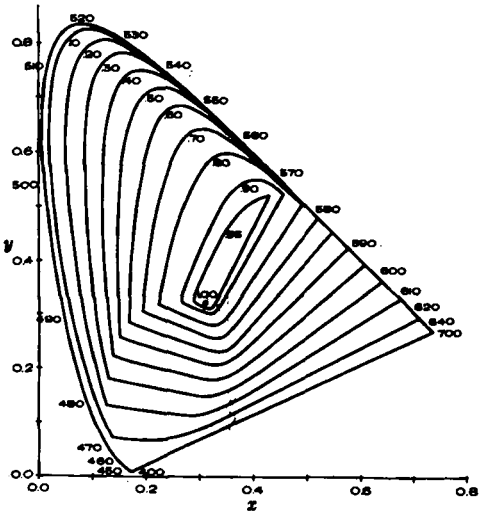


Fig. 1.—The (x, y) chromaticity diagram of the C.I.E. system. The parts of the spectrum locus are identified by wavelength in mμ. The region bounded by this locus and the straightline (purple border) joining its extremes represent all chromaticities producible by actual stimuli. Within the outermost locus are the contours of theoretically realizable surface colors at various levels of lightness. Illuminant "C" assumed. [From MacAdam, D. L., (25).]

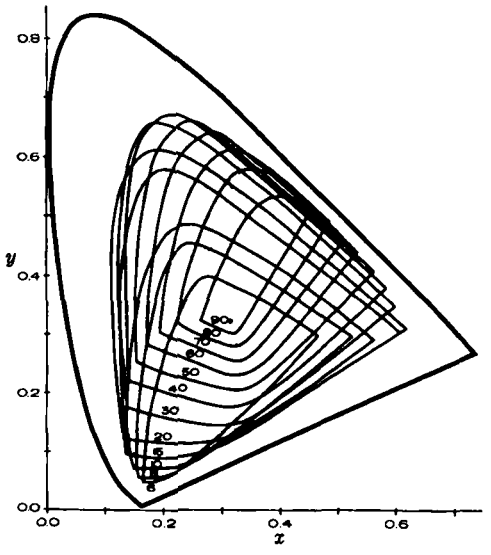


Fig. 2.—MacAdam limits modified by surface reflection factor. [From Atherton, E., and Peters, R. H., (27).]

Limitations.—Before defining the color gamut of these nine colorants, the theoretical limitations should first be examined.

Figure 1 illustrates a chromaticity diagram. All mixtures of the spectrum colors lie within the area enclosed by the solid line. The area contains all the real colors (18, 22, 23).

MacAdam developed the theory of the maximum visual efficiency of colored materials (24) and then subsequently (25) showed what surface colors are theoretically realizable. One of his diagrams is reproduced in Fig. 1. It is obvious that even without the limitation of specific colorants being introduced the color gamut has lessened.

The further limitations to the gamut of surface colors were studied for fabrics by Vickerstaff (26) and for paints by Atherton and Peters (27). The modifications in the range of realizable surface colors for materials such as paints, or compressed tablets with a glossy surface, are a result of the fact that no surface absorbs 100% of the incident light. Figure 2 contains the modifications in the MacAdam diagram when surface reflectance is taken into account.

The studies cited had as their objective the description of the theoretically realizable surface colors in order to determine where best to expend

TABLE I.—DATA OBTAINED ON FIRST SERIES OF TABLETS COLORED WITH FD&C BLUE NO. 2 ALUMINUM LAKE, CONCENTRATED

Concn., % w/w	Tristimulus Value			Chromaticity Coordinates		Lightness (Percentage)	Munsell Value V_T
	X	Y	Z	x	y		
0.00	0.9784	1.0025	1.1320	0.3143	0.3220	100.25	9.91
0.01	0.9020	0.9247	1.1447	0.3066	0.3144	92.47	9.60
0.10	0.7325	0.7506	1.0024	0.2947	0.3020	75.06	8.83
1.00	0.4120	0.4194	0.6920	0.2704	0.2753	41.94	6.92
10.00	0.1420	0.1375	0.3040	0.2434	0.2356	13.75	4.26
100.00	0.0230	0.0185	0.0584	0.2302	0.1852	1.85	1.41

future research efforts. The problem was to determine those areas of color in which new colorants should be made available. All industries have in common the need and desire for colorants that will be light and at the same time saturated or intense. Such studies as these are of great value in this respect.

However, in the pharmaceutical industry the problem is somewhat different since we have little to say about the development of additional colorants. This is solely within the province of the chemist, tempered by economics, and constantly scrutinized by the Federal government. In the sense in which colorants are legally defined, the problem then becomes one of determining the color gamut defined by specific materials under a given set of conditions.

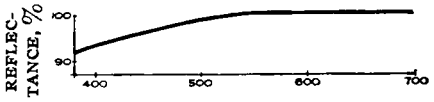


Fig. 3.—Reflectance curve of tablet containing no colorants.

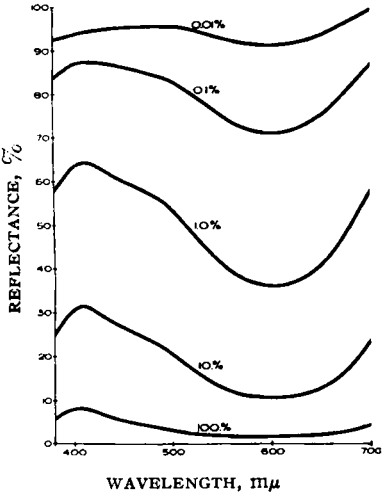


Fig. 4.—Reflectance curves of tablets containing various percentages of FD&C Blue No. 2 aluminum lake, concentrated.

EXPERIMENTAL

Materials.—A spray dried basic tablet granulation (28) having the following formulawas used.

Calcium sulfate, dihydrate	90.585% w/w
Solka Floc, BW-100	4.950
Acacia	3.465
Magnesium stearate	1.000

Two series of National Aniline FD&C aluminum lakes were used. The dye content of one series was nominally 15%; that of the other, designated by the manufacturer as "Concentrated," was nominally 40%. Other manufacturers produce these aluminum lakes in other concentrations; the data of the following experiments are not perfectly valid for any concentrations other than those described. The colorants evaluated were the aluminum lakes of the following FD&C dyes: Violet No. 1, Blue No. 1 and No. 2, Green No. 1, Yellow No. 5 and No. 6, and Red No. 2, No. 3, and No. 4.

Equipment.—A Mikro-Samplmill fitted with a No. .010 HB screen; Carver hydraulic press; Hobart mixer, K4-B; punch and die ($1^1/_2$ in., flat face, bevel edge) for Stokes model F tablet press; and G.E. recording spectrophotometer with Librascope integrator (29) were utilized.

Preparation of Tablets.—Five batches of tablets were prepared: FD&C aluminum lake 0.01%, *A;* 0.10%, *B;* 1.0%, *C;* 10.0%, *D;* 100.0%, *E;* with basic granulation *q.s.* in each.

A series of logarithmic dilutions was chosen to

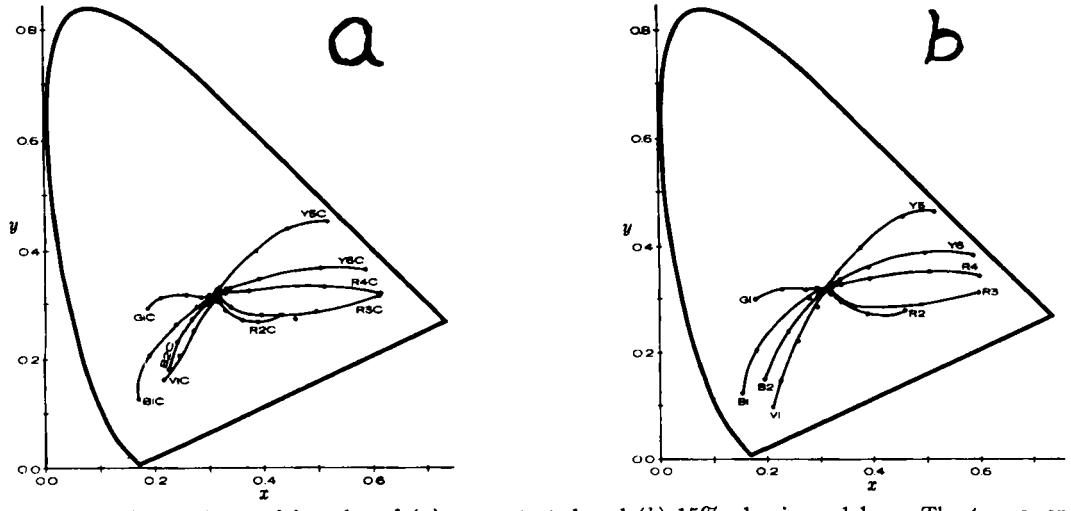
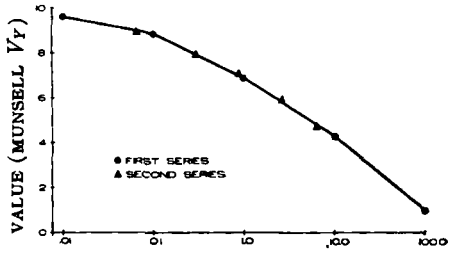


Fig. 5.—C.I.E. chromaticity plot of (*a*) concentrated and (*b*) 15% aluminum lakes. The traces, or curved lines, represent chromaticity loci of a concentration series of the lakes measured individually. Key for *a:* B1C = FD&C Blue No. 1; B2C = FD&C Blue No. 2; V1C = FD&C Violet No. 1; R2C = FD&C Red No. 2; R3C = FD&C Red No. 3; R4C = FD&C Red No. 4; Y6C = FD&C Yellow No. 6; Y5C = FD&C Yellow No. 5; G1C = FD&C Green No. 1; aluminum lakes, concentrated, respectively. Key for *b:* B1 = FD&C Blue No. 1; B2 = FD&C Blue No. 2, V1 = FD&C Violet No. 1; R2 = FD&C Red No. 2; R3 = FD&C Red No. 3; R4 = FD&C Red No. 4; Y6 = FD&C Yellow No. 6; Y5 = FD&C Yellow No. 5; G1 = FD&C Green No. 1; aluminum lakes, 15%, respectively.



PER CENT CONCENTRATION OF FD&C BLUE NO. 2, AL. LAKE, CONCD., IN TABLETS

Fig. 6.—Concentration of FD&C Blue No. 2 aluminum lake, concentrated, in tablet *vs.* Munsell value level.

by Davidson (31) was utilized, and the tristimulus value, Y, which is also a function of lightness, was converted to the Munsell value function V_Y (32). These values were then plotted against their respective concentrations (Fig. 6).

Using the graph of Fig. 6, concentrations were determined which would yield values of V_Y from 9 down to that value that could be obtained using no more than 1% of pure dye content in a tablet. Tablets were then made having those concentrations determined by the use of the graph of Fig. 6. The tristimulus values, chromaticity coordinates, lightness, and values for V_Y, are shown in Table II.

The color solid could have been illustrated at

TABLE II.—DATA OBTAINED ON SECOND SERIES OF TABLETS COLORED WITH FD&C BLUE NO. 2 ALUMINUM LAKE, CONCENTRATED

Concn., % w/w	Tristimulus Value			Chromaticity Coordinates		Lightness (Percentage)	Munsell Value V_Y
	X	Y	Z	x	y		
0.065	0.7443	0.7622	1.0117	0.2956	0.3027	76.22	8.89
0.290	0.5681	0.5815	0.8601	0.2827	0.2893	58.15	7.95
0.900	0.4391	0.4472	0.7178	0.2737	0.2788	44.72	7.11
2.600	0.2787	0.2802	0.5177	0.2589	0.2603	28.02	5.82
6.200	0.1752	0.1723	0.3623	0.2468	0.2427	17.23	4.71

cover effectively the psychological value scale. This scale has an exponential relationship to perceived lightness (30). These dilutions also allowed each series to be plotted on a single graph which permitted a ready comparison of the changes in the curves that resulted from changes in concentrations.

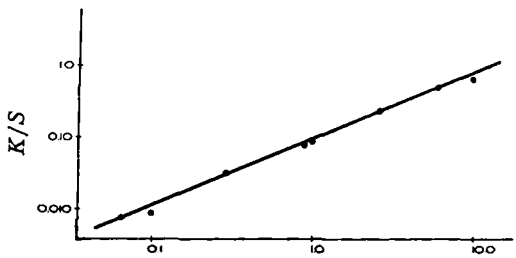
In formulas *A–D* the colorant was mixed with the basic granulation in the Hobart mixer and then passed through the Mikro-Samplmill. The material was then placed in a punch and die assembly in the Carver press and compacted at a pressure of 12,000 p.s.i. For the compaction of the pure aluminum lake it was necessary only to coat the die with a lubricant prior to compaction to effect the release of the tablet.

Each tablet was marked so that the upper surface could be measured in every case. This was necessary because the upper tablet face had a greater gloss than the lower face due to the nature of compaction in the Carver press.

Measurement of Tablets.[3]—The reflectance of the tablets was measured using a G.E. recording spectrophotometer equipped with a Librascope integrator using illuminant C. The specular component was not measured. Magnesium oxide was the white standard. The tristimulus values, chromaticity coordinates, lightness, and values for V_Y (see below) for FD&C Blue No. 2, aluminum lake, concentrated, are shown in Table I.

Figure 3 shows the reflectance curve of a tablet containing no colorant; Fig. 4 shows the reflectance curves of the tablets containing FD&C Blue No. 2, aluminum lake, concentrated. Similar curves were obtained for each colorant. The results of this initial series are plotted in Fig. 5. It can be seen that the color gamut is greatly reduced from that shown in Fig. 2.

Continuing to use the results obtained from FD&C Blue No. 2 aluminum lake, concentrated, as an example, a modification of a method developed



PERCENTAGE CONCENTRATION OF FD&C BLUE NO. 2, AL. LAKE, CONCD. IN TABLETS

Fig. 7.—Concentration of FD&C Blue No. 2 aluminum lake, concentrated in tablet *vs.* absorption coefficient/scattering coefficient (K/S). Reflectance measured at 410 mμ.

various levels of reflectance rather than at Munsell values. In this case the value of K/S[4] would be plotted *versus* concentration as shown in Fig. 7 and the concentrations required to obtain the desired levels of reflectance determined.

The reflectance curves of the second series of tablets containing FD&C Blue No. 2, aluminum lake, concentrated, are shown in Fig. 8.

Figure 9 is composed of a series of contours at Munsell value 9. This is what is possible when there is a limitation concerning the colorants which may be used and a further restriction that the maximum concentration may not exceed 1% of pure dye content. The other systems are shown at the same value level for comparison.

Color Matching.—It is obvious from Fig. 9 that the color gamut at this value level 9 is far less than the theoretical limits. This is equally true at all other value levels. For example, at a value of 4 there is only a single point because no other colorant was this dark at a pure dye concentration of less than 1%. Another restriction was the use of only the aluminum lakes as colorants without using a black, such as carbon black. This meant that the values

[3] The author thanks Messrs. Best, Leary, Seeber, and Croce of National Aniline Division, Allied Chemical Corp., for their assistance in having the spectrophotometric measurements of the tablets made.

[4] $K/S = (1 - R_\infty)^2/2 - R_\infty$, the Kubelka-Munk function (9).

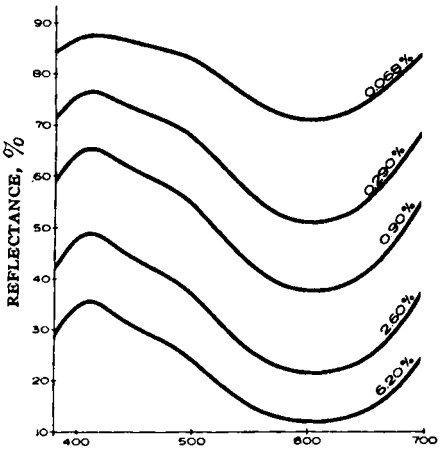


Fig. 8.—Reflectance curves of tablets containing various percentages of FD&C Blue No. 2 aluminum lake, concentrated.

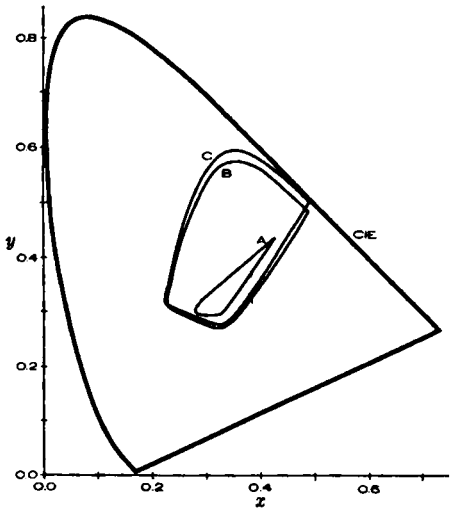


Fig. 9.—Contours at Munsell V_Y = 9. Key: contour *A*, limit of aluminum lakes; contour *B*, limit of Atherton and Peters; contour *C*, limit of MacAdam; contour C.I.E., limit of spectral colors.

were modified solely by the amount of colorant in the tablet formulation. Additional experiments are now in progress that will determine the maximum saturation of each colorant and will also utilize a carbon black to vary the lightness of each colorant. This should serve to enlarge the color gamut. However, the system will have to be evaluated for reproducibility of color from batch to batch as a function of the dispersion of the carbon black.

The principal usefulness of such a color mixture diagram is that it enables one to obtain from measurements, using either spectrophotometers or abridged spectrophotometers, information about the range of hues obtainable without actually making all the necessary physical combination of hues. Duncan (16) showed that the recording of the position of a color on the C.I.E. diagram enabled a determination of whether it would be possible to make a match with the colorants available. "In general, the theory indicates that any color can be reproduced when its chromaticity coordinates fall within an area that is generated by drawing lines connecting the points representing the chromaticity coordinates of the colorants being used. If the color to be reproduced falls outside of such an area, it is sufficient evidence that the color cannot be produced with the colorants available" (33). It must be kept in mind at all times that color is a three-dimensional concept, so it is necessary to consider the lightness of the proposed color as well as the hue and saturation. For this reason, the color gamut described here is shown at definite levels of lightness.

For matching a color, the procedure would then be as follows. The color to be matched would be measured in a spectrophotometer, and the chromaticity coordinates and lightness value determined. This point would be plotted on the correct level of reflectance (or V_Y) to see whether it lies within the area. If it does not lie within the area, there is little likelihood that any match, metameric or nonmetameric, can be achieved. If the point does lie within the area, then it may be achieved by utilizing those colorants that bound it.

DISCUSSION

The color gamut in Fig. 9 shows that in this case there is relatively little opportunity to make a light and a saturated color in a tablet at the same time. If one further restricts the colorant concentrations to the more usually used concentrations of 0.1%, the color gamut then contains only pastel colors.

The curve of the colorant traces (Fig. 5) indicates that, as the concentration of the colorants is increased, both the hue and the lightness value change. Unlike the color additive mixture of lights—hue, value, and chroma for pigments are not independent properties, nor are their changes predictable *a priori*.

More extensive discussions of the usefulness of color grids are given by Evans (34) and Saltzman (33) as well as those cited earlier. The making of color matches having the minimum metamerism possible with a given set of colorants has been aided by the development of a tristimulus difference computer by Davidson and Hemmendinger (35, 36). The instrument permits the application of the relation between the tristimulus values and concentration without any precalibration except that involving the spectral measurement of the component pigments.

SUMMARY

Color matching in industrial pharmacy, as in other fields, is a difficult problem. However, in pharmacy, exact matches, which can only be made with the same colorants as those of the standard, are the exception rather than the rule. Considering this fact and that industrial pharmacists have relatively few colorants—and these cannot be used indiscriminately—then it behooves us to define the gamut of colors available to us for such dosage forms as liquids, compressed and coated tablets, etc. Having this information will then allow a quick determination of whether a color sample can be matched to any extent.

"It is necessary that this problem be recognized so that a great deal of difficulty may be avoided in asking for an exact match where it is evident, from the

difference between the two colorant systems, that different colorants must be used and therefore an exact match is not obtainable" (3).

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Effect of Cortisone on the Minimal Carcinogenic Dose$_{50}$ of Methylcholanthrene in Albino Mice

By RALPH T. MANCINI, RONALD F. GAUTIERI, and DAVID E. MANN, JR.

The administration of 0.4 mg. of cortisone acetate per 30 Gm. of body weight, injected subcutaneously five times a week over a 16-day period (12 injections) during the initial phase of the experiment, caused a slight decrease in the incidence of methylcholanthrene-induced tumors. The CF-1 albino mice used in this experiment also exhibited a definite retardation or even a significant decrease in body weight during the 16-day period of cortisone acetate administration. However, when cortisone acetate was withdrawn, the mice gained weight rapidly. At the termination of the experiment, the groups of mice which had received cortisone (Groups B and C) exhibited a mean weight approximately equal to that of the control groups.

THE ISOLATION of cortisone from the adrenal cortex by Kendall, *et al.* (1), Reichstein (2), and Wintersteiner and Pfiffner (3) in 1936 was followed by the extraction and identification of approximately 45 additional steroids, five of which—besides cortisone—exhibited significant physiological activity: corticosterone, 11-desoxycorticosterone, 11-dehydrocorticosterone, 17-α-hydroxycorticosterone (hydrocortisone), and 17-hydroxy-11-desoxycorticosterone. Of these, cortisone has been subjected to the most thorough clinical evaluation in such diverse conditions as arthritis, leukemia, Addison's disease, allergic states, skin diseases, and ophthalmic disorders. In addition, the influence of the steroid on carcinogenesis has been studied.

One of the earliest experiments to determine the effects of cortisone on the growth of malignant tumors in mice was conducted in 1944 by Heilman and Kendall (4). They showed that the administration of cortisone in drinking water inhibited lymphosarcomas, while withdrawal caused the initiation of carcinogenesis. However, growth was not reversed by the subsequent readministration of cortisone. Other investigations have demonstrated that cortisone exerted a temporary inhibition of ependyomas (5), lymphosarcomas (6), adenocarcinomas (7), and rhabdomyosarcomas (8).

In a study conducted by Baserga and Shubik (9), it was observed that cortisone reduced the

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Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

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By RALPH T. MANCINI, RONALD F. GAUTIERI, and DAVID E. MANN, JR.

The administration of 0.4 mg. of cortisone acetate per 30 Gm. of body weight, injected subcutaneously five times a week over a 16-day period (12 injections) during the initial phase of the experiment, caused a slight decrease in the incidence of methylcholanthrene-induced tumors. The CF-1 albino mice used in this experiment also exhibited a definite retardation or even a significant decrease in body weight during the 16-day period of cortisone acetate administration. However, when cortisone acetate was withdrawn, the mice gained weight rapidly. At the termination of the experiment, the groups of mice which had received cortisone (Groups B and C) exhibited a mean weight approximately equal to that of the control groups.

THE ISOLATION of cortisone from the adrenal cortex by Kendall, *et al.* (1), Reichstein (2), and Wintersteiner and Pfiffner (3) in 1936 was followed by the extraction and identification of approximately 45 additional steroids, five of which—besides cortisone—exhibited significant physiological activity: corticosterone, 11-desoxycorticosterone, 11-dehydrocorticosterone, 17-α-hydroxycorticosterone (hydrocortisone), and 17-hydroxy-11-desoxycorticosterone. Of these, cortisone has been subjected to the most thorough clinical evaluation in such diverse conditions as arthritis, leukemia, Addison's disease, allergic states, skin diseases, and ophthalmic disorders. In addition, the influence of the steroid on carcinogenesis has been studied.

One of the earliest experiments to determine the effects of cortisone on the growth of malignant tumors in mice was conducted in 1944 by Heilman and Kendall (4). They showed that the administration of cortisone in drinking water inhibited lymphosarcomas, while withdrawal caused the initiation of carcinogenesis. However, growth was not reversed by the subsequent readministration of cortisone. Other investigations have demonstrated that cortisone exerted a temporary inhibition of ependyomas (5), lymphosarcomas (6), adenocarcinomas (7), and rhabdomyosarcomas (8).

In a study conducted by Baserga and Shubik (9), it was observed that cortisone reduced the

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TABLE I.—COMPOSITE RESULTS OF PART 1 AND PART 2

Group	Total MCA Admin., mcg.[a]	Effective Total Mice/Group	Tumorous Mice/Group	Tumor Incidence, %	Male	Female	Incidence of Multiple Tumors	Male	Female
A	336–504	58	29	50.0	17	12	11	9	2
B	312–504	55	26	47.3	14	12	5	3	2
C	288–504	53	18	33.9	9	9	3	2	1
D	360–504	59	26	44.0	12	14	9	5	4

[a] The first column of numbers refers to the smallest quantity of methylcholanthrene (expressed in micrograms) needed to induce the formation of a tumor in at least one CF-1 albino mouse in that group. The second column of numbers represents the total amount of methylcholanthrene (expressed in micrograms) applied to all animals during the 10.4 week period of the experiment. MCA-methylcholanthrene.

TABLE II.—PART 1.—QUANTITY (MCG.) OF METHYLCHOLANTHRENE APPLIED (AS A 0.12% ACETONE SOLUTION) UNTIL ONSET OF TUMORS IN MICE

Groups	Sex	Tumors, No.	Applications, No. Range	Applications, No. Av.	Total Admin., mcg. Range	Total Admin., mcg. Av.	Induction Time, Days Range	Induction Time, Days Av.
A	M	7	16–21	19	384–504	456	56–71	66
	F	4	14–21	19	336–504	462	49–72	66
B	M	8	15–21	18	360–504	426	54–72	62
	F	7	17–21	20	408–504	483	60–72	70
C	M	4	13–21	17	312–504	408	46–72	59
	F	3	15–21	17	312–504	416	53–72	60
D	M	8	15–21	18	360–504	441	53–72	65
	F	8	15–21	19	360–504	450	53–72	65

tumor incidence in mice receiving topical applications of methylcholanthrene.

Sugiura, *et al.* (10), noted, however, that cortisone was inconsistent in temporarily inhibiting different types of tumors. Using cortisone acetate, they observed a marked inhibition of rat sarcoma R-39, mouse lymphosarcoma, and mouse osteogenic sarcomas, while the hormone only slightly inhibited Lewis sarcoma T-241 and exerted no effect on sarcoma 180 or mammary adenocarcinoma E-0771.

Contrary to the results obtained in the foregoing experiments, which indicated that cortisone inhibited neoplastic growths to varying degrees, other investigators have compiled evidence that cortisone treatment caused a definite increase in the tumor incidence (11–14).

Possibly conflicting results have occurred because investigators have failed to employ a valid dose/response relationship for the carcinogen used. With the establishment of the minimal carcinogenic dose$_{50}$ (MCD$_{50}$) of methylcholanthrene (15), the elucidation of tumor enhancement or inhibition by an agent can be determined. The purpose of this investigation was to discern the effects of cortisone on methylcholanthrene-induced skin carcinogenesis in mice.

MATERIALS AND METHODS

In this study the experiment was performed twice to verify and confirm the MCD$_{50}$ of methylcholanthrene described in a previous paper (15).

A total of 240 CF-1 albino mice approximately 10 to 11-weeks-old—four groups of 60 mice each (30 males and 30 females)—were used in this experiment. The cages employed, diet, methylcholanthrene solution, hair trimming procedure, weighing, manipulation of the mice, recording of data, preparation of tumors for pathological study, and the method of application of the carcinogen are described in a previous paper (15).

The cortisone acetate[1] used in this experiment was a saline suspension containing 25 mg. of cortisone acetate per milliliter. Two milliliters (50 mg.) of cortisone acetate suspension was diluted in a 25-ml. volumetric flask with normal saline solution. The diluted suspension, containing 2 mg. of cortisone acetate per milliliter, was used for the subcutaneous administration of cortisone acetate to CF-1 mice according to body weight (0.4 mg. per 30 Gm. of body weight).

In this experiment the four groups of mice were *Group A* (control group), received 504 mcg. through 21 bi-weekly applications of 0.02 ml. of a 0.12% solution of methylcholanthrene in acetone. This is the MCD$_{50}$ of methylcholanthrene. *Group B* (cortisone group) received the MCD$_{50}$ of methylcholanthrene with the concomitant subcutaneous administration of cortisone acetate suspension three times a week (Monday, Wednesday, and Friday) alternately in the left and right inguinal area over a 16-day period from the day of the initial application of methylcholanthrene. *Group C*, (cortisone group) was the same as *Group B*, except that the cortisone acetate was administered five times a week (Monday through Friday). *Group D* (normal saline control group) received the MCD$_{50}$ of methylcholanthrene with the concomitant subcutaneous administration of normal saline solution five times a week, equivalent in volume to the injections of cortisone acetate suspension (based on body weight).

EXPERIMENTAL

The hands were protected by heavy neoprene gloves when handling the mice or the apparatus used for the application of the carcinogen. The mice were grasped with the index finger and the thumb at the base of the tail during the applications. When the mice were held in this manner, the solution of

[1] Marketed as Cortone Acetate by Merck Sharp and Dohme, Philadelphia, Pa.

TABLE III.—PART *2*.—QUANTITY (MCG.) OF METHYLCHOLANTHRENE APPLIED (AS A 0.12% ACETONE SOLUTION) UNTIL ONSET OF TUMORS IN MICE

Groups	Sex	Tumors, No.	Applications, No. Range	Av.	Total Admin., mcg. Range	Av.	Induction Time, Days Range	Av.
A	M	10	14–21	17	336–504	413	48–71	59
	F	8	18–21	19	432–504	459	62–72	66
B	M	6	14–21	20	336–504	432	48–72	62
	F	5	13–21	17	312–504	403	44–71	57
C	M	5	12–21	17	288–504	418	41–72	60
	F	6	18–21	20	432–504	476	62–72	68
D	M	4	15–21	19	360–504	450	51–72	64
	F	6	18–21	20	432–504	476	63–72	68

TABLE IV.—MEAN WEIGHT AND STANDARD DEVIATION IN WEIGHT OF EACH GROUP AT ONSET OF EXPERIMENT, AT THE END OF CORTISONE ADMINISTRATION, AND AT THE TERMINATION OF THE EXPERIMENT

Group (30 Mice/ Group)	Part *1* Mean Wt., Gm.	Part *1* S.D.	Part *2* Mean Wt., Gm.	Part *2* S.D.
A[a]	20.25	±1.81	25.49	±2.73
A[b]	24.09	±2.06	26.33	±2.93
A[c]	30.05	±3.16	30.40	±2.87
B[a]	20.22	±1.60	23.26	±3.57
B[b]	20.80	±2.18	22.25	±2.99
B[c]	30.32	±2.85	29.00	±3.74
C[a]	20.54	±1.85	23.18	±2.53
C[b]	19.79	±1.77	21.21	±2.24
C[c]	29.41	±2.99	29.57	±3.30
D[a]	20.54	±1.95	23.92	±3.25
D[b]	23.94	±1.92	25.64	±3.36
D[c]	31.41	±2.45	29.98	±3.15

[a] Initial weight. [b] Weight at end of cortisone therapy. [c] Termination weight.

methylcholanthrene in acetone was applied by placing the interscapular area beneath the tip of the micropipet. A 2-cm. square area of hair in the interscapular region was trimmed with a pair of nickel-plated scissors to enable the solution of methylcholanthrene in acetone to spread evenly by capillarity to all parts of the area of application.

The recording of tumors was initiated at the appearance of the first macroscopically observed tumor. After the appearance of the initial growth, the interscapular area was examined at least three times a week with a Stanley 701-A magnifying glass. The mice that died before the recording of the first tumor were not included in the effective total.

The surviving mice were sacrificed with chloroform at the termination of the experiment. Tumors were randomly excised from each of the four groups of animals and placed in a 10% solution of formaldehyde for histopathological study.

All data obtained from Parts *1* and *2* of this study were compiled in four tables. Only those mice exhibiting tumors measuring 1 mm. or more in one dimension (width *versus* height) were recorded. Table I shows the composite results of Parts *1* and *2* obtained after 21 bi-weekly applications of 0.02 ml. of a 0.12% solution of methylcholanthrene in acetone to the control mice (Groups *A* and *D*) and to the cortisone groups (Groups *B* and *C*). Tables II and III exhibit the quantity in micrograms of methylcholanthrene applied until the onset of tumors in each part of the experiment. The induction time for the occurrence of at least one epidermal tumor in each group is also indicated. Table IV exhibits the mean weight and standard deviation in the weight of each group of mice at the onset of the experiment, at the end of cortisone administration, and at the termination of the experiment.

DISCUSSION

The results of this investigation indicated that the subcutaneous administration of cortisone acetate five times weekly (Group C) decreased the incidence of methylcholanthrene-induced tumors. The control mice (Group A) exhibited a confirming MCD_{50} for methylcholanthrene, as previously shown by Gautieri and Mann (15) in CF-1 albino mice.

These results are in agreement with those obtained by Baserga and Shubik (9), who demonstrated that methylcholanthrene-induced carcinogenesis was inhibited by the subcutaneous administration of 0.5 mg. of cortisone acetate, daily, 6 days a week for 19 weeks.

Table II (Part *1*) and Table III (Part *2*) show that in both phases of the experiment the first tumor was observed at approximately the same time. In Part *1*, the first tumor appeared 46 days after the initial application of methylcholanthrene; in Part *2* the first tumor occurred 41 days after the first methylcholanthrene application. These data also agree with previous studies (15).

Epilation was initiated 5 to 7 days after the first application of a 0.12% solution of methylcholanthrene in acetone. The epidermis of the interscapular area of the control mice (Groups A and D) appeared hyperemic with the application of the methylcholanthrene solution. However, similar applications of methylcholanthrene to the skin of the interscapular area of mice treated with cortisone acetate suspension did not cause a comparable degree of hyperemia. The number of epidermal lesions appearing in the interscapular area of the control mice (Groups A and D) seemed to increase as the experiment progressed. Approximately 1 week after the termination of cortisone therapy, a mild vasodilation became apparent in the mice of the cortisone groups (Groups B and C). Skin lesions appeared in the interscapular area in some of the cortisone-treated mice toward the end of the experiment.

A resurgence of hair growth in the control mice (Groups A and D) occurred 16 to 20 days after the beginning of the application of methylcholanthrene. This same resurgence of hair growth in the mice receiving cortisone acetate suspension (Groups B and C) did not commence until 33 to 36 days after the initial application of methylcholanthrene. The resurgent hair did not have the glossy appearance and natural conformation to the body contours that was characteristic of normal hair; it was stiff and difficult to cut with a pair of scissors.

Table IV shows that the cortisone-treated mice (Groups B and C) in *Parts 1 and 2* of this study had a definite retardation or even a significant decrease in mean body weight at the termination of the cortisone injections, while the control mice (Groups A and D) exhibited a definite increase in mean body weight during the same period.

SUMMARY

The administration of 0.4 mg. of cortisone acetate per 30 Gm. of body weight, injected subcutaneously five times a week over a 16-day period (12 injections) during the early phase of the experiment, caused a slight decrease in the incidence of methylcholanthrene-induced tumors.

When administered subcutaneously, cortisone acetate suspension decreased the skin irritation and subcutaneous vasodilation caused by the application of a 0.12% solution of methylcholanthrene in acetone to the interscapular region. However, this effect was noticeable only during the interval of cortisone therapy and for a short period thereafter.

The CF-1 albino mice used in this experiment also showed definite retardation or even a significant decrease in body weight during the 16-day period of cortisone acetate administration. However, when cortisone acetate was withdrawn, the mice gained weight rapidly. At the termination of the experiment, the groups of mice which had received cortisone (Groups B and C) exhibited a mean weight nearly equal to the mean weight of the control groups (Groups A and D).

The administration of cortisone acetate to the mice of Groups B and C delayed the resurgence of hair growth approximately 2 weeks beyond the resurgence of hair growth in the control groups.

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The bi-weekly, intraperitoneal administration of sodium cobaltinitrite in doses of 50, 60, and 70 mg./Kg. in mice subjected to the MCD$_{50}$ of methylcholanthrene resulted in a reduction of tumor incidence to 31, 31, and 25 per cent, respectively, compared to 47 per cent for the controls.

FROM THE MANY diverse factors that have been shown to modify experimentally induced carcinogenesis, an intriguing concept has arisen which stresses the need for a deeper understanding of the role played by oxygen in the inception and regulation of the cancerization process. According to Warburg (1), a normal cell becomes cancerous because of irreversible damage to its respiratory mechanism. Cells which are unable to compensate immediately to the abrupt change in intracellular respiration die, while others survive only by adjusting to a fermentation type of respiration, thus becoming undifferentiated and cancerous. In short, the cancer cell derives energy from fermentation which fulfills its metabolic demands as adequately as intracellular respiration meets the requirements of the normal cell. On the other hand, Weinhouse (2) presented experimental evidence that the respiration of tumor slices is approximately equal to the oxidative metabolic activity of normal cells. In further support of these findings, isotope studies performed in 1949 (3) have shown that tumor cells can oxidize glucose to carbon dioxide at rates comparable to those of normal cells. Therefore, it

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appears unlikely that an impairment of oxygen consumption or utilization is an aberration common to all neoplasms.

The present investigation was undertaken to observe the effect that an *in vivo* oxygen deficiency might produce on the growth of chemically induced tumors in mice—specifically on squamous cell carcinomas resulting from the topical application of methylcholanthrene. The basis for this study was an established method which has elicited a consistent tumor response. Accordingly, the bi-weekly application of 0.02 ml. of a 0.12% methylcholanthrene solution to the shaved interscapular area for 10.4 weeks has repeatedly resulted in tumor formation in approximately 50% of the treated animals. This dose/response relationship has been termed the minimal carcinogenic dose$_{50}$ (MCD_{50}) of methylcholanthrene (4) and has provided a satisfactory procedure for the evaluation of various factors, such as the effects of gonadectomy and/or estrogen (5), and cortisone (6), on controlled tumor production.

The present investigation was undertaken to observe the tumor incidence in normal and hypoxic mice subjected to the MCD_{50} of methylcholanthrene. A temporary hypoxic state was produced through the formation of methemoglobinemia, a condition which results in a decreased oxygen-carrying capability of the blood. Several groups of agents are known to induce methemoglobinemia, including nitrites and *p*-amino phenol derivatives. Sodium cobaltinitrite, $Na_3Co(NO_2)_6$, was chosen because it is superior to sodium nitrite as an antidote for cyanide poisoning in mice, presumably from its ability to convert hemoglobin to methemoglobin (7).

EXPERIMENTAL

The experiment was divided into three separately performed parts, designated as trials *1*, *2*, and *3*. In each trial, 300 or 360 mice (CF-1) were divided into groups of 60 mice, each with 30 males and 30 females. Table I lists these groups and the treatment which each received. Cages employed, diet, handling, shaving, and method of application of the carcinogen were described in a previous paper (4). Ampuls containing 10 ml. of 0.12% methylcholanthrene (Eastman-Kodak) in acetone were prepared and refrigerated until used. The carcinogen was applied twice weekly (Tuesday and Thursday); freshly prepared aqueous solutions of sodium cobaltinitrite were injected intraperitoneally on Mondays and Fridays. This schedule of injections was begun on the Friday of week 1 of each trial; therefore, the mice had been exposed to two previous applications of methylcholanthrene. Concentrations of the cobaltinitrite ranged from 0.5 to 1.6%, depending on the dose to be administered, so that the volume of solution injected would not exceed 0.3 ml.

The intraperitoneal LD_{50} of sodium cobaltinitrite, determined by the method of Litchfield and Wilcoxon (8), was 159 (149 to 170)mg./Kg. Since the animals were to receive a total of 20 injections over a period of 72 days, it was necessary to choose dosage levels which, while producing a definite methemoglobinemia, would not elicit toxic symptoms. After a single intraperitoneal injection of 50 to 100 mg./Kg., mice exhibited ataxia, reduced respiratory activity, and a moderate degree of cyanosis within 5 minutes. However, noticeable toxic manifestations were not apparent 1 or 2 hours later; the animals appeared normal in all respects. When doses greater than 100 mg./Kg. were administered, marked cyanosis accompanied by gasping and occasional convulsions

TABLE I.—GROUPING AND TREATMENT OF MICE IN TRIALS *1*, *2*, AND *3*

Group[a]	Treatment
Trial *1:* Mar. 20 to May 31, 1962	
A-1	Methylcholanthrene (controls)
B-1	Methylcholanthrene + sodium cobaltinitrite,[b] 30
C-1	Methylcholanthrene + sodium cobaltinitrite, 40
D-1	Methylcholanthrene + sodium cobaltinitrite, 50
E-1	Methylcholanthrene + sodium cobaltinitrite, 60
Trial *2:* June 7 to Aug. 18, 1962	
A-2	Methylcholanthrene (controls)
D-2	Methylcholanthrene + sodium cobaltinitrite, 50
E-2	Methylcholanthrene + sodium cobaltinitrite, 60
F-2	Methylcholanthrene + sodium cobaltinitrite, 70
G-2	Methylcholanthrene + sodium cobaltinitrite, 80
Trial *3:* Sept. 25 to Dec. 6, 1962	
A-3	Methylcholanthrene (controls)
B-3	Methylcholanthrene + sodium cobaltinitrite, 30
C-3	Methylcholanthrene + sodium cobaltinitrite, 40
D-3	Methylcholanthrene + sodium cobaltinitrite, 50
E-3	Methylcholanthrene + sodium cobaltinitrite, 60
F-3	Methylcholanthrene + sodium cobaltinitrite, 70

[a] Each group consists of 60 mice, equally divided regarding sex. [b] All doses (mg./Kg.) of sodium cobaltinitrite are by intraperitoneal injection.

TABLE II.—FORMATION AND DURATION OF METHEMOGLOBINEMIA IN MICE AFTER INTRAPERITONEAL INJECTIONS OF SODIUM COBALTINITRITE (80 mg./Kg.) OR SODIUM NITRITE (80 mg./Kg.)

After Injection, min.	% Methemoglobin[a] Sodium Cobaltinitrite	Sodium Nitrite
10	15 ± 3	30 ± 2
20	32 ± 4	39 ± 2
40	39 ± 6	34 ± 3
60	27 ± 5	30 ± 2
90	16 ± 7	16 ± 4
120	11 ± 3	4 ± 3
180	3 ± 2	...

[a] Expressed as per cent of total hemoglobin converted to methemoglobin. Each value listed is the mean of six different determinations ± standard error.

TABLE III.—EFFECT OF INTRAPERITONEAL INJECTIONS OF SODIUM COBALTINITRITE ON TUMOR INCIDENCE IN MICE RECEIVING THE MCD_{50} OF METHYLCHOLANTHRENE

Treatment (mg./Kg.) and Group No.	Trial 1 Tumor Incidence[a]	Trial 1 Tumors, %	Trial 2 Tumor Incidence	Trial 2 Tumors, %	Trial 3 Tumor Incidence	Trial 3 Tumors, %	Total Trials Tumor Incidence	Total Trials Tumors, %
Methylcholanthrene controls (A-1, A-2, A-3)	30/59	51	24/58	41	28/59	48	82/176	47
Na Cobaltinitrite, 30[c] (B-1, B-3)	27/59	46	...	..	20/58	35	47/117	40
Na Cobaltinitrite, 40 (C-1, C-3)	26/60	43	...	..	20/59	34	46/119	39
Na Cobaltinitrite, 50 (D-1, D-2, D-3)	20/58	35	13/60	22	21/59	36	54/177	31[b]
Na Cobaltinitrite, 60 (E-1, E-2, E-3)	16/58	28	24/57	42	14/59	24	54/174	31[b]
Na Cobaltinitrite, 70 (F-2, F-3)	...	..	16/59	27	14/59	24	30/118	25[b]
Na Cobaltinitrite, 80 (G-2)	...	..	18/59	31	...	..	...	..

[a] Ratio expressed as: $\frac{\text{No. of mice bearing one or more tumors}}{\text{Effective total of mice in group}}$. [b] Significant reduction of tumor incidence compared with control value. (Chi² test, $P < 0.01$). [c] Dose is mg./Kg.

occurred. Death usually ensued within 2 hours. On the basis of these preliminary observations, it was decided not to exceed the 80-mg./Kg. dose level.

A blood study was conducted on a separate group of mice to determine the extent and duration of the methemoglobinemia produced by sodium cobaltinitrite (80 mg./Kg.) and by sodium nitrite at a comparable dose in terms of nitrite present. The colorimetric method of Evelyn and Malloy (9) was employed; the results of this experiment are listed in Table II. The data indicated that a peak methemoglobin concentration was achieved within 60 minutes after the time of injection and decreased until negligible concentrations remained after 3 hours. This peak value represented the conversion of approximately 40% of the total hemoglobin to methemoglobin. It should be kept in mind that 80 mg./Kg. was the highest dose of sodium cobaltinitrite utilized, and that the methemoglobinemic response was, naturally, less at the lower dosage schedules. Several determinations at a dose of 60 mg./Kg. gave results indicating peak values of approximately 25% methemoglobinemia, which diminished appreciably by the end of 2 hours.

In comparing the cobaltinitrite response with that produced by sodium nitrite, a similar overall picture was observed. Peak levels were practically identical and occurred at approximately the same time. However, 2 hours after injection, the 80-mg./Kg. cobaltinitrite-treated mice exhibited higher methemoglobin values than the corresponding nitrite group. Thus, the one difference attributed to sodium cobaltinitrite—compared with sodium nitrite—was a slight prolongation in duration of methemoglobinemia.

It is unnecessary to describe in detail the macroscopic changes which occurred in the mice as they were exposed to repeated methylcholanthrene applications, since these observations have been published (4). It is sufficient to mention that the epidermal reactions were similar and consisted of an initial period of epilation which lasted for about 3 weeks. This was followed by another period of renewed hair growth, at which time the animals were left with longer hair in the interscapular region than in other areas of their bodies.

The appearance of tumors was first noted on a few mice during the sixth week of each trial. Both

TABLE IV.—INITIAL AND FINAL WEIGHTS OF MICE

Group	Initial Wt. (Gm. ± S.D.)	Final Wt. (Gm. ± S.D.)
Trial *1*		
A-1	25 ± 3	33 ± 4
B-1	25 ± 5	31 ± 3
C-1	26 ± 3	33 ± 3
D-1	25 ± 2	32 ± 3
E-1	25 ± 2	31 ± 6
Trial *2*		
A-2	20 ± 2	30 ± 4
D-2	21 ± 2	29 ± 3
E-2	21 ± 2	29 ± 3
F-2	20 ± 2	28 ± 3
G-2	22 ± 3	30 ± 3
Trial *3*		
A-3	25 ± 4	30 ± 3
B-3	24 ± 3	30 ± 2
C-3	25 ± 3	29 ± 3
D-3	26 ± 3	30 ± 3
E-3	26 ± 3	29 ± 3
F-3	25 ± 2	29 ± 3

control and cobaltinitrite groups responded at the same time; *i.e.*, no outstanding delay in tumor onset could be attributed to the methemoglobinemia, although at a given time there were usually less tumors in the test groups than in the controls. It was not our purpose to study the chronological progression of tumor growth, for it was apparent that a close daily examination of 360 mice would be extremely impractical. Rather, the experiment was designed to use the established method of producing tumors in a 72-day period, and to compare the tumorous mice in the test groups with the number of tumorous mice in the controls at the end of this time.

At the termination of each trial, all growths which measured at least one dimension (width *versus* height) of 1 mm. or greater were drawn and recorded on individual data sheets. All mice were chloroformed and the tumors excised and immersed in Bouin's fixative. Histological examination revealed that these growths were true cancers of the type that are produced on the epidermis by methylcholanthrene, *viz.*, squamous cell carcinomas.

DISCUSSION

Table III contains the complete data with respect

to tumor incidence in the separate groups at the termination of each trial and lists the composite results of the three trials. These totals were obtained by combining the corresponding individual groups of each trial, thus giving a single "per cent tumor" value for the control mice and for each of the five cobaltinitrite groups. The effective total of mice in each group was, in most cases, less than the original number of 60. Obviously, this was due to deaths which occurred during the trial period. Of the 960 mice employed in all three phases of the experiment, only 28 died. Mice which died before tumors were noted in the group were not counted in the effective total. When a death occurred after growths had appeared, the animal was examined for the presence of a tumor, and the data obtained were incorporated into the final results. No evidence of cumulative toxicity was ever apparent throughout the experiment; the animals seemed normal in all respects. Table IV, which shows the initial and final weights of the various groups, fails to reveal any differences that might indicate some toxic manifestation.

Table III shows that of 176 control mice (those which received only the methylcholanthrene), 82 developed tumors of 1 mm. or greater in one dimension. This corresponds to a 47% tumor incidence, a result which is very close to the expected value of 50% and which reconfirms the MCD_{50} of methylcholanthrene. The mice which had received the two lower doses (30 and 40 mg./Kg.), responded to the MCD_{50} with a slight but insignificant decrease in tumors. However, at the 50-mg./Kg. dose level and higher, a significant reduction of tumor incidence was seen. Thus, bi-weekly, intraperitoneal injections of 50, 60, and 70 mg./Kg. of sodium cobaltinitrite were capable of reducing the per cent of tumor bearing mice to 31, 31, and 25, respectively, compared to the 47% control value.

It is difficult to accept that the reduction in tumor incidence is the result of the transient hypoxia produced by sodium cobaltinitrite. Periods of methemoglobinemia which lasted no longer than 3 hours and were elicited twice weekly certainly should not be unquestionably concluded as being the sole cause of this tumor inhibition. To reiterate the initial statements, the role of oxygen in the inception of the cancerization process is obscure, as is the basic mechanism of methylcholanthrene-induced carcinogenesis. It is quite possible that some metabolic effect other than hypoxia is responsible for or contributes to the decreased tumor incidence observed.

As a consequence of the theory that sulfhydryl enzymes may be involved in the induction of chemically-induced carcinogenesis by a fixation of the carcinogen to cellular proteins through sulfur linkages (10) an interesting concept arises. Lusky, *et al.* (11), obtained significant tumor reduction by applying dimercaprol (BAL) to mice during painting with 3,4-benzpyrene and reasoned that the additional SH-groups might compete with cellular SH for the carcinogen. Coupling this theory to the report by Vollmer and Carey (12)—that methemoglobinemia produced by sodium nitrite and *p*-amino propiophenone induces an increase in blood SH-enzyme levels—leads to the similar possibility that the increased SH-groups in the blood might also compete with the SH-groups of epidermal cell proteins, thereby affecting the neoplastic changes.

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Nonketol Reduction of Tetrazolium Salts in Pharmaceutical Analysis

By EDWARD F. SALIM†, PETER E. MANNI‡, and JOSEPH E. SINSHEIMER

Nonketol compounds of pharmaceutical interest have been tested for reduction of tetrazolium salts; the quantitative applications of these reactions have been evaluated.

IN CONTRAST TO most organic compounds, the reduction of tetrazolium salts yields highly colored compounds. Based essentially upon this property, the production of these formazans by various reducing systems has led to a diverse range of biological and chemical applications. Lakon (1) has used triphenyltetrazolium chloride to detect viability of seeds, while Straus, Cheronis, and Straus (2) have applied tetrazolium salts in the study of carcinomatous tissue.

Chemical applications of tetrazolium salts include the quantitative analysis of reducing sugars (3), ketol steroids (4), and ascorbic acid (5). Rosenkrantz (6) has compared the effect of struc-

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Nonketol compounds of pharmaceutical interest have been tested for reduction of tetrazolium salts; the quantitative applications of these reactions have been evaluated.

IN CONTRAST TO most organic compounds, the reduction of tetrazolium salts yields highly colored compounds. Based essentially upon this property, the production of these formazans by various reducing systems has led to a diverse range of biological and chemical applications. Lakon (1) has used triphenyltetrazolium chloride to detect viability of seeds, while Straus, Cheronis, and Straus (2) have applied tetrazolium salts in the study of carcinomatous tissue.

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tural changes on the reactivity of catecholamines with blue tetrazolium. Polyhydroxy phenols and readily oxidizable thiols have been determined qualitatively as a result of their ability to reduce tetrazolium salts (7).

To date, quantitative measurements involving tetrazolium salts have dealt primarily with the analysis of α-ketols. The objectives of this study were to determine the extent of nonketol reduction of tetrazolium salts and to evaluate quantitative applications in pharmaceutical analysis. Included in this investigation are polyhydroxy phenols, thiols, active hydrogen compounds, and other pharmaceutical reducing agents; that is, other compounds easily oxidized in the presence of air and light.

EXPERIMENTAL

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer equipped with Bakelite thermospacers. Melting points are corrected.

Reagents.—*Blue Tetrazolium Reagent Solution.*—The 3,3'-(3,3'-dimethoxy-4,4'-biphenylene) bis[2,5-diphenyl-2H-tetrazolium chloride] (Dajac Laboratories) was purified by recrystallization from ethanol–anhydrous ether (8) until a portion of the salt in absolute ethanol showed constant absorbance at 254 mμ. Twenty-five milligrams of the purified salt was dissolved in 10 ml. of U.S.P. ethanol.

Red Tetrazolium Reagent Solution.—Twenty-five milligrams of 2,3,5-triphenyl-2H-tetrazolium chloride (Nutritional Biochemicals) was dissolved in 10 ml. of U.S.P. ethanol. (These solutions should be freshly prepared and protected from light.)

Tetramethylammonium Hydroxide Reagent Solution.—Ten milliliters of 10% aqueous solution was diluted to 100 ml. with absolute ethanol.

Test Compounds.—The compounds listed in Tables I–IV met the manufacturer's requirement for pharmaceutical use and were of official purity where applicable.

Screening Procedure.—Two milliliters of blue tetrazolium reagent solution followed by 2 ml. of tetramethylammonium hydroxide reagent solution were added to 10 mg. of test compound dissolved in 10 ml. of absolute ethanol. The reaction was allowed to proceed for 60 minutes at room temperature and quenched by the addition of 1 ml. of glacial acetic acid. The absorbance at 530 mμ was measured against a reagent blank.

Rate of Color Development.—From 10 to 400 mcg. of test compound was dissolved in 10 ml. of absolute ethanol and allowed to equilibrate in a constant temperature bath at 30.5 ± 0.1°. Two milliliters of blue tetrazolium reagent solution was added, followed by 2 ml. of tetramethylammonium hydroxide solution. The absorbance at specified time intervals compared to a reagent blank was measured at 530 mμ. Solutions were held in the thermostated (30.0 ± 0.1°) cell compartment of the spectrophotometer.

Assay Procedures.—An epinephrine standard of 10 mcg. in 10 ml. of absolute ethanol was prepared; a dilution of epinephrine solution (B.P.) of comparable strength in absolute ethanol was compared to this standard using blue tetrazolium reagent solution. The reaction was quenched after 40 minutes with glacial acetic acid.

About 80 mg., accurately weighed, of powdered 25-mg. diethylpropion hydrochloride tablets was suspended in 2 ml. of water and diluted to 250 ml. with absolute ethanol. A 10-ml. aliquot of filtered sample was compared to a diethylpropion hydrochloride standard of 200 mcg. in 10 ml. of absolute ethanol using blue tetrazolium reagent solution. The reaction was quenched as described above.

Table I.—Reactivity of Blue Tetrazolium with Polyhydroxy Phenols

	M.p., °C.	Absorptivity at Time, min. 10	30	60
Apomorphine HCl	[a]	2480	2490	2490
Epinephrine	210 dec.	5250	5240	5200
Hexylresorcinol[b]	64–65	328	416	438
Isoproterenol sulfate	119.5 to 122.5	3360	3380	3330
Levarterenol bitartrate[c]	97–101	2880	2950	2950
Protokylol HCl[d]	172–173	2460	2490	2450
Pyrogallol	133 to 134.5	3920	4140	4180
Resorcinol	109–110	133	378	661
Rutin[e]	206 to 209.5	340	617	830

[a] Oxidizes in air. Compounds generously supplied by: [b] Merck Sharp & Dohme. [c] Winthrop Laboratories. [d] Lakeside Laboratories. [e] Parke, Davis & Co.

Table II.—Reactivity of Blue Tetrazolium with Thiols

	M.p., °C. or (n^{25}_D)	Absorptivity at Time, min 10	30	60
l-Cysteine	224 dec.	1150	1330	1440
Dimercaprol	(1.5765)	1790	1960	2080
6-Mercaptopurine[a]	307–309 dec.	...	...	0.9
Methimazole[b]	144–145	...	...	7.8
dl-Penicillamine	196.5 dec.	612	634	643
Thiamylal[c]	131–133	...	...	0.2

Compounds generously supplied by: [a] Burroughs Wellcome & Co. [b] Eli Lilly & Co. [c] Parke, Davis & Co.

Table III.—Reactivity of Blue Tetrazolium with Other Pharmaceutical Reducing Agents

	M.p., °C. or (n^{25}_D)	Absorptivity at Time, min. 10	30	60
Anthralin[a]	173–176	783	1010	1100
Menadione	103.5 to 104.5	1440	1490	1500
Phytonadione[b]	(1.5254)	35.0	193	371
Thiamine HCl	247–249 dec.	7.6	28.0	68.6
d-α-Tocopheryl acetate[c]	(1.4924)	...	...	16.1

Compounds generously supplied by: [a] Abbott Laboratories. [b] Merck Sharp & Dohme. [c] R. P. Sherer.

RESULTS AND DISCUSSION

Nonketol pharmaceutical compounds were screened with blue tetrazolium for 60 minutes at room temperature. Only compounds which produced an absorptivity value over 20, based on the

TABLE IV.—REACTIVITY OF BLUE TETRAZOLIUM WITH ACTIVE HYDROGEN COMPOUNDS

	M.p., °C.	Absorptivity at Time, min. 10	30	60
Adiphenine HCl[c]	113–114	270	549	644
Bishydroxycoumarin[d]	285–287	...	...	0.7
Bromindione[e]	142–144	...	...	0.2
Cycloserine[f]	147 dec.	36.1	37.2	36.8
Diethylpropion HCl[g]	169–174 dec.	75.6	224	405
Dihyprylone[h]	101–103	...	...	1.0
Dimethindene maleate[c]	154 to 156.5 dec.	123	351	560
Diphenadione[i]	145–145.5	...	...	0.04
Ethotoin[d]	91–92	1230	1230	1210
Furazolidone[j]	268 dec.	665	1450	2000
Glutethimide[e]	81–85	...	...	0.01
Methantheline Br[k]	171–173	139	98.4	61.6
Methylphenidate HCl[e]	204–206 dec.	...	...	0.04[a]
Methyprylon[h]	74 to 76.5	...	...	18.6
Nitrofurantoin[j]	256–257 dec.	10.5	43.4	85.4
Nitrofurazone[j]	234.5 to 236.5 dec.	33.6	75.6	126
Phensuximide[l]	69–70	1180	1180	1170
Piperidolate HCl[m]	193–195	119	266	351
Potassium penicillin G[i]	211–215 dec.	...	...	10.4
Propantheline Br[k]	160 to 161.5	138	103	68.6
Santonin	172–174	...	...	0.1
Thiphenamil HCl[n]	125–127	462	477	470
Tolazoline HCl[e]	174–176	...	...	0.7
Valethamate Br[o]	122.5 to 124.5	...	...	0.02
Warfarin sodium[p]	160–162[b]	...	...	0.4

[a] First neutralized with tetramethylammonium hydroxide. [b] Melting point of Warfarin. Compounds generously supplied by: [c] Ciba Pharmaceutical Products, Inc. [d] Abbott Laboratories. [e] U. S. Vitamin & Pharmaceutical Corp. [f] Eli Lilly & Co. [g] Wm. S. Merrell Co. [h] Roche Laboratories. [i] Upjohn Co. [j] Eaton Laboratories, Inc. [k] G. D. Searle & Co. [l] Parke, Davis & Co. [m] Lakeside Laboratories, Inc. [n] Wm. P. Poythress & Co. [o] Ayerst Laboratories, Inc. [p] Endo Laboratories, Inc.

concentration of reducing agent, were considered sufficiently reactive to be of quantitative interest. Those compounds with an absorptivity over 20 were examined for rate of color development. Rates of reduction of blue tetrazolium by polyhydroxy phenols, thiols, and other easily oxidized compounds are summarized in Tables I–III by the absorptivity values at 10, 30, and 60 minutes. Also included are results for those compounds of low reactivity by listing the absorptivity at 60 minutes obtained from the screening procedure.

The reaction of triphenyltetrazolium chloride with alicyclic ketones (9) led to an extensive screening of

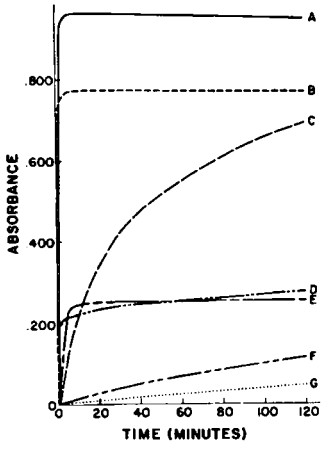


Fig. 1.—Rate of color development for 1×10^{-5} M solutions with blue tetrazolium. Key: A, epinephrine; B, apomorphine hydrochloride; C, rutin; D, dimercaprol; E, menadione; F, resorcinol; and G, thiamine hydrochloride.

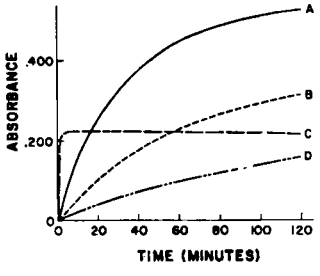


Fig. 2.—Rate of color development for 1×10^{-5} M solutions of active hydrogen compounds with blue tetrazolium. Key: A, furazolidone; B, dimethindene maleate; C, phensuximide; and D, diethylpropion hydrochloride.

active hydrogen compounds. Table IV summarizes the results of this study. Excluded from this table were 30 compounds in which the only activation of the hydrogen resulted from one or more aromatic rings. None of these compounds produced an absorptivity greater than 1.0 at 60 minutes.

Figures 1 and 2 are absorbance *versus* time graphs obtained for representative nonketol pharmaceutical compounds tested for rate of color development with blue tetrazolium. Figure 3 presents a comparison of the reduction of blue and red tetrazolium salts by ketol steroids determined under the same conditions as nonketols. The reaction of tetrazolium salts with steroids, exemplifying an established quantitative procedure, serves as a direct comparison to the compounds in Figs. 1 and 2.

Favorable comparison to the steroids concerning sensitivity of reaction and stability of color presents a strong indication of a successful assay for many compounds in Figs. 1 and 2 and others in Tables

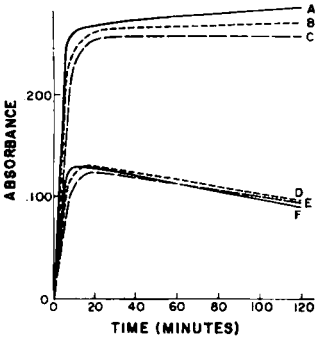


Fig. 3.—Rate of color development for 1×10^{-5} *M* solutions of ketol steroids with: I. blue tetrazolium—A, cortisone acetate; B, hydrocortisone; C, prednisolone acetate; and II. red tetrazolium—D, hydrocortisone; E, prednisolone acetate; F, cortisone acetate.

I–IV. For example, epinephrine (Fig. 1) exhibits greater sensitivity than steroids. The quantitative determination of epinephrine solution (B.P.) was highly satisfactory—ten analyses gave a recovery value of 97.54 ± 0.64% of labeled amount of epinephrine.

Diethylpropion hydrochloride (Fig. 2), in contrast to ketol steroids, represents a compound of lower sensitivity and does not plateau within 2 hours. Although this compound represents a less ideal example than epinephrine, ten analyses of 25-mg. diethylpropion hydrochloride tablets resulted in a satisfactory recovery of 99.24 ± 0.93% of labeled amount of diethylpropion hydrochloride.

A limited study was conducted using more vigorous reaction conditions to increase absorptivity values of compounds which were moderately reactive to blue tetrazolium. Rates of color development for methyprylon, *d*-α-tocopheryl acetate, and cycloserine were determined at 40°, other reaction conditions remaining unchanged. Methyprylon exhibited an absorptivity increase from 18.6 to 52.8 at 60 minutes, while *d*-α-tocopheryl acetate gave only a slight increase from 16.1 to 21.3. Cycloserine, which at 30° showed good stability of color but only moderate sensitivity, did not increase in sensitivity at 40°—and, in fact, produced a noticeable decrease in stability at this temperature. Although an increase in reaction temperature can have analytical significance in some cases, in limited examples it was of only minor importance and in the case of cycloserine was actually detrimental.

The reactivity of nonketol pharmaceutical compounds had been based exclusively on the reduction of blue tetrazolium. However, official monographs employing tetrazolium salts specify triphenyltetrazolium chloride. Rates of color development of several ketol and nonketol steroids were determined using red tetrazolium. Data for steroids are represented in Fig. 3, a comparison of the blue and red tetrazolium curves indicates greater color stability with blue tetrazolium. This superiority as well as the greater sensitivity of blue tetrazolium (4) was also exhibited with the nonketol reductions.

SUMMARY

Nonketol compounds have been tested for reduction of tetrazolium salts. Many compounds were reactive in low concentrations, indicating applicability to quantitative analysis in pharmaceutical formulations.

Specific procedures have been developed for epinephrine solution (B.P.) and diethylpropion hydrochloride tablets.

Nonketol compounds should be recognized as possible sources of interference to tetrazolium assays.

Comparison of blue and red tetrazolium salts using ketol and nonketol reductions indicates blue tetrazolium as a superior reagent.

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Evaluation of Polymeric Materials I

Screening of Selected Polymers as Film Coating Agents

By BILL J. MUNDEN, H. GEORGE DEKAY, and GILBERT S. BANKER

A procedure has been developed for the systematic evaluation of mechanical, thermal, and stability properties of polymeric materials through the study of unsupported films. Films of selected vinyl, acrylic, and cellulosic polymers were prepared by the knife edge applicator method and the mercury substrate technique. The mercury substrate technique afforded superior control of film thickness and facilitated film removal. Films that were very permeable to water vapor were rather impermeable to oxygen and vice versa. This relationship is discussed as it relates to a combination of the hydrophilic-lipophilic and pore theories of film structure. The unsupported vinyl films possessed superior mechanical properties of those tested. Moisture absorption by the polymer films was negligible. There was no discernible decomposition according to infrared analysis of the films, due to thermal, photo-oxidation, and/or aging effects.

PHARMACEUTICAL PREPARATIONS are required by law and ethics to contain the ingredients advertised and the potency specified by the manufacturer. The first condition is easily fulfilled, but drug potency often presents a problem. The manufacturer also encounters the difficulty of overcoming unpleasant drug tastes and odors.

The decomposition of most drugs can be classified either as hydrolysis or oxidation. Both of these degradation reactions are catalyzed by atmospheric effects. This has led to the use of dehydrated products sealed in ampuls and protective coatings for solids. Sugar and gelatin were among the first and most popular protective coatings for solids, but had the disadvantage of merely delaying degradation of the product. Pharmaceutical preparations such as sugar coated tablets have a pleasant taste and odor but do not necessarily contain the original potency specified.

This disadvantage of the sugar coating has led to the investigation of other types of protective coatings, such as the film coat. Film coating of tablets on an industrial scale first occurred in 1954 when Abbott Laboratories introduced their "Filmtab." Since then a more general and concentrated interest in this technique has existed.

Film coatings may be defined as thin pliable layers of a physiologically acceptable coating composition. Additives in a film coat may include plasticizers, which make the plastic film former more flexible, and colorants, flavors, and surfactants which facilitate spreading. Modifier materials, such as soluble waxes, are added to affect film solubility or pliability (1–3).

Due to the great increase in the number of polymeric materials developed in recent years, an examination of these new polymers and their properties for application in pharmaceutical coating is appropriate. An improved method of evaluation is proposed in this work. Films of the polymers were cast and subjected to evaluation for water vapor transmission, oxygen permeability, water absorption, durability, mechanical, and thermal and photo-oxidation effects.

Some of the possible advantages of the experimental method proposed by this study are: (*a*) the polymers are evaluated for their pharmaceutical coating properties without interference from modifying additives; (*b*) a more rapid and comprehensive screening of new polymers is provided; and (*c*) the results can provide a logical basis for the selection of the modifying additives in the coating formulation.

EXPERIMENTAL

Initial Screening Procedure.—Various polymeric materials currently being used in the plastics industry were screened for their film forming characteristics and were classified according to chemical type. A second screening was performed on the basis of oral toxicity and method of drying of the film (air dry, forced dry, or baked), and the following groups of polymers were selected for further study on this basis (4): acrylate, alkyd, cellulose acetate butyrate, cellulose nitrate, chlorinated paraffin, chlorinated rubber, cyclized rubber, hydrocarbon, maleic, oleoresin, polyamide, polyester, silicone, silicone alkyd, polystyrene, styrenated alkyd, vinyl acetate, vinyl alcohol, vinyl butyral, and vinyl chloride acetate.

The following solvent system was selected after analyzing solvent systems suggested by various manufacturers and after studying the Antonides, Fennell, and Peirano investigations (5–7), butyl alcohol, 25%; butyl acetate, 25%; and acetone, 50%. Formulations were prepared containing the particular polymer and the proposed solvent system to yield a 10% solution by weight of the polymer. Clean microscope slides were dipped into the poly-

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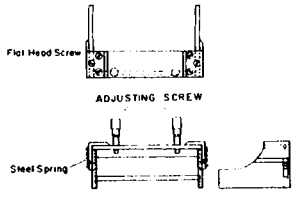


Fig. 1.—Knife edge applicator.

mer solutions to a depth of approximately 3 cm. The coated slides were then observed at 25° for noticeable film formation.

Film test strips to evaluate the polymers further were prepared with a brass film casting knife which was designed according to specifications suggested by the Hercules Powder Co. (See Fig. 1.) The casting knife permitted rapid preparation of films of fairly uniform thickness. The apparatus consisted basically of a knife edge supported by a frame mounted on two runners. A precise gap could be set between the knife edge and the casting surface by using screw adjustments. A smooth plane such as a chromium surface was suggested as ideal for the casting surface (8). The knife, sliding on the support runners, was drawn over the pool of film solution, leaving a wet film of fairly uniform thickness. The films were air dried.

An alternate method was investigated using Petri dishes and casting the films on a mercury surface (mercury substrate technique). This method produced films with a controlled constant thickness. A known quantity of the 10% polymer solution was diluted with acetone to approximately 25 ml. to facilitate spreading. The film thickness could be varied by varying the volume of the 10% polymer solution used in the 25 ml. dilution.

Mechanical Properties.—The stress-strain relationship of solid materials is comparable to the viscosity property of liquids. The stress-strain relationship is an expression of resistance of a solid to dimensional change.

Tensile stress (9) is defined as the load per unit area of original cross section at any given moment. It is usually expressed in force per unit area. The maximum tensile stress during the test is called the tensile strength. Gauge length is the initial length of the test specimen, while elongation is the increase in sample length produced by a tensile load and is usually measured at the moment of film rupture. Elongation is expressed in a linear dimension (inches); percentage elongation is the per cent conversion. Strain is the change in length per unit of original length.

With a graph, Lever and Rhys (10) explained the general pattern of the elastic modulus, which can be calculated from the slope of the curve where stress and strain are proportional and is an indication of the stiffness of the test material. (See Fig. 2.) In Fig. 2 the yield point A is a measure of the material's strength. It is also a measure of the resistance of the material under test to permanent deformation.

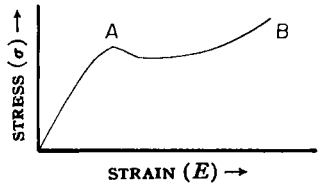


Fig. 2.—Stress-strain relationship.

Elastic deformation then is calculated from the beginning portion of the curve and denotes the ability of a film to return to its original dimensions after being extended. The portion of the curve from A to B (where the sample breaks) is a measure of plastic deformation. Here the strain is not immediately recoverable. A rough measure of toughness can be found by measuring the area under the curve. This represents the work required to break the test material (10).

Lever and Rhys (10) classify materials as (*a*) soft, weak—show low modulus of elasticity, yield point, and low elongation at break; (*b*) hard, brittle—high modulus of elasticity, no well defined yield point, and low strain at break; (*c*) soft, tough—low modulus of elasticity, low yield point, and high strain at break; (*d*) hard, strong—high modulus of elasticity, yield point, and moderate stress and strain at break; and (*e*) hard, tough—high modulus of elasticity, yield point, stress and strain at break. Kinney illustrates some of these physical stress-strain relationships as shown in Fig. 3 (11).

Film samples cast on the mercury pool in a Petri dish were cut to a uniform size of 1 × 5 in. using a razor knife. This size test sample allows a 1-in. width and a 3.5-in. gauge length. Stress-strain curves were determined using an Instron TM strain

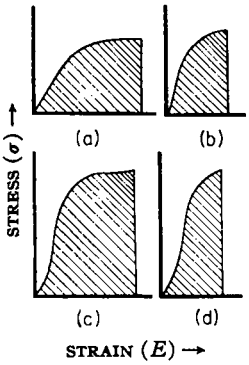


Fig. 3.—Material classification according to stress - strain relationships. Key: (a) soft and hard; (b) weak and brittle; (c) strong and tough; (d) hard and strong.

tester[1] with the CT crosshead, which has a range of 1 to 200 lb. The crosshead speed was 0.2 in. per minute.

"For solids, Hooke's law states that the tension is proportional to the extension or 'stress is proportional to strain,' where stress is the force per unit area (F/A) and strain is the ratio of the extension produced to the original length (l/L). Hence (F × L)/(A × l) = Constant generally denoted by E and is called Elastic Modulus (12)." The elastic modulus (elasticity) was calculated using this formula. Since the Instron strain tester reads in the English system of weights, E was calculated in this system, then converted to the metric system using the conversion factor of 68947.

Linear expansion of the films was measured by the ASTM test No. D 1042-54. Film strips were cut 1 in. wide and 6 in. long and inscribed as prescribed by the American Society for Testing and Materials. The inscribing distance was 100 mm. Holes were punched in one end of the film strips, and the strips were suspended from cross rods. The marked test samples were then subjected to 93% relative humidity and 40° for 24 hours. A saturated solution of ammonium phosphate was used to produce the

[1] Instron Engineering Corp., Canton, Mass.

relative humidity for the test. After 24 hours, the films were again inscribed and the distance measured. Any change in length was observed and measured with a calibrated microscope at ×10 power. The same test samples were then subjected to 60° for 24 hours and again measured under the microscope.

Film test samples in a thickness range of 5 to 10 mm. were cut using a 1 by 3 in. microscope slide as a template. They were then immersed in artificial gastric and intestinal fluids to determine solubility and/or enteric properties. A sample was placed in a vertical position in each chamber of the U.S.P. tablet disintegrator and completely submerged in artificial gastric fluid at 37°. The U.S.P. disintegration apparatus disks were not used since the film strips tended to adhere to them. The end point was complete solution of the film. After 3 hours, the gastric fluid was removed and replaced with artificial intestinal fluid. To confirm the results, three test samples were run for each polymer.

Thermal Properties.—Test samples were cut and were mounted on sample holder cards. These cards are the same dimensions as the calibration card for the Perkin Elmer model 21 spectrophotometer. The infrared spectrum was initially recorded for each polymer and after a 2-month interval to note any changes produced by temperature and aging.

Stability Properties.—*Water Vapor Permeability.*—A modification of ASTM:E96-53T was developed to measure water vapor transmission. Through the use of ASTM designed cups[2] and controlled humidities on both sides of the film, the water vapor transmission was determined. The test was conducted with 0% relative humidity inside the cup (maintained by Drierite)[3] and 93% relative humidity outside at room temperature. The 93% relative humidity was maintained with a saturated solution of ammonium phosphate in a paper chromatography jar, 10 in. in diameter and 18 in. deep. The cups were weighed initially and then every 2 hours over a 24-hour time period, the water vapor transmission was calculated from the straight line portion of the plot of moisture gain *versus* time. Water vapor transmission is usually expressed as the number of grams of moisture gain per 24 hours per square meter. Preliminary data indicated that weighing the films every 4 hours over a 12-hour period was adequate, and this procedure was subsequently followed. Utsumi (13) has suggested the following water vapor transmission formula: $Q = (WL)/(S)$ where W = Gm. of water/24 hours, L = film thickness in cm., S = surface area in cm.^{2}, and Q = weight of water in Gm.cm. thickness/cm.^{2} area 24 hours.

The American Society for Testing Materials suggests the following formula for calculating the water vapor transmission: $WVT = (g \times 24)/(t \times a)$ where g = weight gain or loss, in grams, a = exposed area in square cm., t = time, in hours, during which g was observed, 24 = to convert the actual weighing time, t, to the time basis of 24 hours.

Since Utsumi's formula took the thickness of the film sample into consideration, it was used in this investigation.

Oxygen Permeability.—The method of determining oxygen permeability of the films involved the complexation of oxygen by pyrogallic acid in an alkaline solution (14). The following reagent was used in this study: pyrogallic acid, 4 Gm.; sodium hydroxide, 10 Gm.; and distilled water *q.s. ad.* 1000 ml. Test tubes were filled to a volume of 35 ml. with the reagent solution and sealed with the films under oxygen free conditions as provided by high purity nitrogen. The quantity of oxygen passing through the films was determined by the Beckman model B spectrophotometer at room temperature. The wavelength of 425 mμ was used as suggested by Williams, *et al.* (14). The presence of oxygen was also noted visually by the change of a nearly colorless solution to varying shades of brown and then to black. The quantity of oxygen present was measured at intervals of 30 minutes until a constant rate of gain was reached. The oxygen permeability was calculated according to the first equation.

TABLE I.—POLYMERS USED IN THIS STUDY

Chemical Designation	Trade Name and Manufacturer[a]	Film Formation (Free Film)
Acrylate and methacrylate	Lucite 42 (*1*)	Yes
	Lucite 44 (*1*)	Yes
	Lucite 46 (*1*)	Yes
Alkyd	Sytex H-3 (*2*)	No
Cellulose acetate butyrate	½ Second Butyrate (*3*)	Yes
Chlorinated paraffin	Clorowax 70 (*4*)	No
Chlorinated rubber	Parlon (*5*)	No
Coumarone	Nevidene (*6*)	No
	Neville R16-A (*6*)	No
	Picco 450-L (*7*)	No
Oleoresin modified	Carbasol 6 (*8*)	No
	Carbasol 30 (*8*)	No
	Staybrite Ester 10 (*5*)	No
	Cellolyn 102 (*5*)	No
	Pentalyn A (*5*)	No
Phenol modified	Pentalyn 802A (*5*)	No
	Nevillac Hard (*6*)	No
	F.C.D. 101 (*9*)	No
Vinyl acetate	Gelva V-15 (*10*)	Yes
	Gelva C-3,V-10 (*10*)	Yes
	Vinac E1-52 (*11*)	No
	Bakelite AYAF (*12*)	Yes
	Lemac 541-20 (*13*)	Yes
Vinyl butyral	Butvar B-90 (*10*)	Yes
Vinyl acetate/ stearate	Flexbond B-142 (*11*)	No
Vinyl chloride copolymer	Exon 470 (*15*)	Yes
	Exon 471 (*15*)	Yes
	Exon 497 (*15*)	Yes
Polyvinyl ethyl ether	Bakelite EDBM (*12*)	No
Styrene	Lytron 810 (*15*)	No
	Lytron 820 (*15*)	No
Silicone	S.R. 83 (*16*)	No
	F.C.D. 16 (*9*)	No
	R. 62 (*12*)	No
	Neville 118–123 (*6*)	No
	Bakelite VMCH (*12*)	No
	Piccoflex 100 (*7*)	No
	Bakelite VAGH (*12*)	Yes

[a] The manufacturer of the polymer is designated by the number in parenthesis: *1*, E. I. du Pont de Nemours & Co.; *2*, Jones-Dabney Co.; *3*, Eastman Chemical Products, Inc.; *4*, Diamond Alkali Co.; *5*, Hercules Powder Co.; *6*, Neville Chemical Co.; *7*, Pennsylvania Industrial Chemical Corp.; *8*, C. J. Osborn Co.; *9*, France, Campbell, and Darling; *10*, Shawingan Resins Corp.; *11*, Colton Chemical Co.; *12*, Union Carbide Corp.; *13*, The Borden Chemical Co.; *14*, Firestone Plastics Co.; *15*, Monsanto Chemical Co.; and *16*, Schenectady Resins Div.

[2] Thwing Albert Instrument Co., Philadelphia, Pa.
[3] W. A. Hammond, Drierite Co., Xenia, Ohio.

TABLE II.—EVALUATION OF STRESS-STRAIN RELATIONSHIPS AND LINEAR EXPANSION

Polymer	Stress-Strain Relationships: Film Thickness × 10^{-3} cm.	Elasticity × 10^{9} dyne/cm.^{2}	Tensile Strength × 10^{6} dyne/cm.^{2}	% Elongation	Linear Expansion (% Elongation): Film Thickness × 10^{-3} cm.	At 40°C. + 93% R.H.	At 60°C.
Lucite 44	2.54	2.484	0.436	68.5	5.59	2.19	5.00
Lucite 46	2.00	4.388	1.630	1.7	5.08	−1.00	3.30
Gelva V-15	1.83	5.614	2.642	4.5	4.57	3.00	...
Gelva C-3, V-10	0.970	4.117	2.429	8.9	...	...	...
Bakelite AYAF	0.710	5.506	1.642	3.7	3.05	2.10	0.60
Lemac 541-20	0.990	3.861	2.455	4.2	4.06	−2.70	...
Butvar B-90	1.65	1.735	5.390	27.1	4.32	0.27	−0.92
Exon 471	2.21	0.705	2.375	3.1	5.59	1.80	−8.00
Exon 497	0.380	7.023	1.587	2.1	5.84	−0.30	−2.90
Bakelite VAGH	0.990	5.984	2.153	2.4	...	...	...

Photo-Oxidation.—The procedure of determining photo-oxidation was the same as that used to measure the effect of temperature upon the polymers. A sample card was prepared as previously described for each polymer and an initial spectrum recorded. The sample cards were then exposed to indirect sunlight passing through a north window. Infrared spectra were again recorded after a 2-month interval had elapsed.

Moisture Absorption.—Test samples were prepared as previously described, placed in relative humidity chambers, and weighed at definite time intervals. The per cent moisture absorbed was calculated on a weight basis, and the time required for maximum absorption was determined. Relative humidity chambers were constructed using large battery jars covered with glass plates. Holes were punched in one end of the film strips, and the strips were suspended from cross rods. The film strips were subjected to 10, 52, and 93% relative humidity using saturated solutions, respectively, of zinc chloride, magnesium nitrate, ammonium phosphate, and were weighed at weekly intervals. The Serdex[4] relative humidity meter was used to check the relative humidity.

Coating of Tablets.—In order to evaluate each polymer for coating efficiency on a moving tablet bed, film hardness, and effect upon disintegration time, selected film coatings were applied to placebo tablets. The following formula was selected for the placebo tablets: calcium sulfate, 40%; lactose, 40%; starch, 20%, granulating agent: starch paste, 10%; and lubricant: magnesium stearate, 1%.

Tablet disintegration and friability were used to measure durability, ease of handling, and tablet firmness. To check these properties, tablets were coated, and disintegration and friability tests were conducted. The tablets were coated with a 10% solution of the polymer using the solvent system previously mentioned. One per cent sorbitan monooleate,[5] was added to facilitate spreading on the tablets. A 0.1% concentration of FD&C yellow No. 4 was added to permit visualization of tablet coating. A standard 12-in. coating pan revolving at 36 r.p.m. was used, and the coating solution was ladled on the moving tablet bed.

The disintegration tests were conducted according to the manner prescribed by the U.S.P. XVI for uncoated tablets using both artificial gastric and intestinal fluids. A blank was determined using uncoated tablets.

The friability test was conducted according to the method suggested by Perlman (15). This test utilizes a metal can ($5^1/_4$ in. in diameter and 8 in. high) containing three 1-in. metal baffles. The tablets were placed in the can, and the can was sealed with plastic electrical tape. The tablets were rotated on a jar mill rolling machine[6] for 5 minutes at 90 r.p.m.

RESULTS AND DISCUSSION

Experimental work was conducted to evaluate selected polymeric substances for use as pharmaceutical film coating agents. After preliminary screening the polymers listed in Table I were selected for further evaluation.

Further screening using the film casting knife indicated that 13 polymers formed free unplasticized films. A free unplasticized film was defined as an unsupported sheet of film containing no additives such as plasticizers or alloying agents. These results are also shown in Table I.

At the end of the preliminary screening the 13 polymers forming free films (Table I) were selected for further evaluation of properties of interest in pharmaceutical film coating.

Evaluation of Mechanical Properties of Free Films.—Experimental work was conducted to obtain a comparison of the flexibility and toughness of the polymers evaluated. The polymers were evaluated for modulus of elasticity, tensile strength, and per cent elongation; the results are shown in Table II.

Lever and Rhys (10) indicated that tension tests gave reasonably accurate information when employed under definite test conditions. The tests cannot be considered significant when the application conditions differ widely. It appears from this that these properties are best evaluated when one is modifying an original formulation and desires to know the effect of such modifications. Polymers could also be compared if the same test conditions were used.

For use in film coating, the per cent elongation and tensile stress should both be high in relative value. A polymer having these properties would produce a strong and tough film, which is especially desirable in tablet coating. The value of the elastic modulus could be either high or low, depending upon the intended application of the polymer. A higher value denotes a harder film (*e.g.*, tablet coating),

[4] Serdex, Inc., Boston, Mass.
[5] Marketed as Arlacel 80 by the Atlas Powder Co., Wilmington, Del.
[6] Paul Abbe, Little Falls, N. J.

TABLE III.—EFFECT OF SOLVENT UPON STRESS-STRAIN RELATIONSHIPS

Polymer	Age	Film Thickness $\times 10^{-3}$ cm.[2]	Elasticity $\times 10^{9}$ dyne/cm.[2]	Tensile Strength $\times 10^{8}$ dyne/cm.[2]	% Elongation
Exon 471	4 mo.	2.21	0.705	2.375	3.1
	2 days	1.55	0.884	0.294	74.5
Butvar B-90	4 mo.	1.65	1.735	5.390	27.1
	2 days	1.47	2.378	...	over 125

while a lower value indicates a soft and more flexible film (*e.g.*, spray bandage).

Table II shows that most of the elastic moduli were 10^9 dyne/cm.[2] with 2 to 10% elongation. It appeared from the data presented that nearly all of the polymers would have application as film coating agents. Lucite 44 has a low tensile strength when compared to the other polymers and might be unsuitable. The data in Table II resulted from samples that had been aged approximately 4 months. Table III indicates that residual solvent can have an effect upon the mechanical properties of a polymer and indicates that these tests should be conducted on aged films.

The tensile strength for Butvar B-90 is unavailable because the film did not break under the test conditions. The extension limit of the testing procedure was exceeded. This also accounts for the approximate value for the per cent elongation. As was expected, the film samples became more flexible but less tough due to the residual solvent being present (16). This could account for the fact that commercial film coated pharmaceuticals often chip and crack easily upon aging, while freshly coated samples may have been chip resistant and resilient in the laboratory.

When the results from Tables II and III were compared to those of Utsumi (13), it was found that his synthesized polymers exhibited a somewhat greater elastic modulus (the higher the elastic modulus, the stiffer the film). Some of the difference may be attributed to the different methods used as well as the different polymers employed.

The linear expansion of the film test samples was in the order of ±3% as shown in Table II. Lucite 46, Exon 497, and Lemac 541-20 probably would be unsuitable for film coating where both high temperature (40°) and high relative humidity (93%) are involved. Any shrinkage in a film would be likely to cause a film coating on a pharmaceutical product to fissure and become unacceptable. It is interesting to note that the linear dimensions of Butvar B90 and Exon 471 test samples increased slightly upon exposure to high relative humidities, but decreased when subsequently exposed to high temperature (60°). The only difficulty in evaluating polymers using this method is that some samples are not able to be measured accurately due to

TABLE IV.—PERMEABILITY OF FREE FILMS TO WATER AND OXYGEN

(Gm.cm./cm.[2] 24 HOURS)

Polymer	Sample Thickness $\times 10^{-3}$ cm.	Water Permeability $\times 10^{-6}$	Av. $\times 10^{-6}$	Sample Thickness $\times 10^{-3}$ cm.	Oxygen Permeability $\times 10^{-4}$	Av. $\times 10^{-4}$
Exon 497	0.432	1.11				
	0.406	1.48	1.29	3.10	1.33	1.33
Exon 470	0.950	1.95		0.915	4.25	
	0.910	1.85		0.915	4.97	4.61
	0.900	1.51	1.77			
Butvar B-90	0.432	2.32		1.80	23.8	
	0.381	2.05	2.18	1.65	19.0	21.4
Lucite 44	0.457	4.91		2.70	14.9	
	0.482	4.04	4.47	2.30	17.2	16.0
Bakelite VAGH	0.127	8.18		0.990	6.41	
	0.102	7.64	7.91	0.970	5.70	6.06
Lucite 46	1.75	6.01		2.10	13.9	
	1.96	6.31	6.16	1.96	15.6	14.8
Lemac 541-20	0.356	8.40		1.10	8.48	
	0.331	8.60		0.990	7.52	8.00
	1.02	7.90	8.10			
Lucite 42	1.47	7.89		1.27	9.85	9.85
	1.44	8.96				
	1.55	8.32	8.39			
Gelva V-15	1.02	10.9		1.32	5.86	
	0.810	10.4		2.39	3.34	4.60
	0.483	8.90	10.1			
Bakelite AYAF	0.787	9.63		0.740	5.80	5.80
	0.711	8.25	8.94			
Exon 471	2.34	13.8		2.20	1.27	
	2.03	14.8	14.3	2.30	1.60	1.16
Gelva C-3, V-10	2.59	16.7		2.70	9.48	
	0.991	19.2		2.20	8.09	8.78
	2.54	16.3	17.4			
1/2 Second Butyrate	1.57	74.0		0.330	2.27	
	1.27	56.8		0.380	1.79	2.03
	1.52	74.4	68.4			

curling and/or melting of the film at the higher temperature (60°). Bakelite AYAF and Exon 471 exhibited the least dimensional distortion at an elevated temperature or at an elevated temperature and humidity. It would appear that these polymers would be suitable as pharmaceutical coating agents.

Film test strips were cut as previously described and placed in artificial gastric and intestinal fluids to determine solubility and/or enteric properties. All of the polymers were insoluble in both artificial gastric and intestinal fluids, except Bakelite AYAF and Gelva C-3,V-10 which exhibited possible enteric properties. From this it would appear that those polymers which are insoluble would not be suitable for film coatings unless modified with other agents. However, it is possible that all coated products do not disintegrate by dissolution of the coating, but some release the medicament according to the water permeability of the coating. Experimental data that support this hypothesis will be shown in a later section.

Evaluation of Thermal Properties of Free Films.—Test samples of the polymers were prepared as previously described and evaluated for their temperature stability at 25, 40, and 60° for a period of 2 months. Visual examination of the films disclosed no physical changes during that period of time. Infrared spectra were also recorded for each of the polymers initially and after storage. Comparison of the spectra disclosed no differences and/or formation of new peaks. It was concluded that there was no decomposition of the films.

Evaluation of Stability Properties of Free Films.—*Water Vapor Transmission and Oxygen Permeability.*—The water vapor transmission and oxygen permeability of the polymers have been determined by the procedure outlined in the *Experimental* section. The magnitude was in the order of 10^{-3} to 10^{-5} Gm.cm./cm.^{2} 24 hours as shown in Table IV.

The values for water permeability all fall within a range which indicates that they would be suitable film coating agents (13) according to the value for shellac of 8.813×10^{-4} Gm.cm./cm.^{2} 24 hours, which has been suggested as a control for maximum permissible water vapor transmission.

One might assume that these films would be as effective against oxygen as they are against water vapor transmission, although no experimental work was found in the literature to indicate what range of oxygen permeability was satisfactory for the prevention of auto-oxidation of film coated drugs. Future work along these lines will be undertaken.

It appears from Table IV that there was an inverse relationship between the water vapor transmission and oxygen permeability. The noticeable exceptions were the values for Exon 497 and 470 which were low in both cases and Gelva C-3,V-10 which was high. The following hypothesis is offered to explain this relationship. The water vapor permeability of a free film has been shown to be dependent upon the number of polar groups that the polymer contains (17).

Polymers that contain hydrophilic groups attract the water molecule and cause it to pentrate more readily than those with lyophilic groups. The "Pore Theory" could be applicable to the method of oxygen permeability. Since the oxygen molecule is smaller than the water molecule, it can penetrate molecular holes in the film which are too small to permit passage of the water molecule. Films which are lipophilic and contain very small molecular holes would have a small rate of both water and oxygen permeability, such as Exon 497 and 470. This would be a desirable property of a film coating agent. Conversely, a hydrophilic film with large pores would be highly permeable to both water and oxygen.

Moisture Absorption.—Experimental work was conducted to evaluate the effect of relative humidity upon the film properties of the polymers. The film samples were weighed at 2, 4, 8, and 12-week intervals, and the average weight change was determined for each sample. The polymers were neither affected by changes in relative humidity, nor did they absorb moisture during the test period. The standard deviations of the weight changes were also determined. The statistical *t*-test values for the mean differences were determined at the 99% confidence level according to the procedure outlined by Davies, *et al.* (18).

No statistically significant increases in moisture absorption were determined for film samples exposed to any of the three relative humidities. Exon 471 when exposed to 10% relative humidity exhibited a 1.5% average increase in weight over the 12-week period. This was the greatest increase in moisture content of any of the polymers studied at the three relative humidities.

Some of the polymers decreased in average weight over the test period. This can occur due to the gradual evaporation of any remaining residual solvent. The greatest decrease was .81% average weight loss by Gelva V-15 when exposed to 93% relative humidity. There were no visual differences in the samples. This magnitude of moisture absorption correlates with the results obtained by Goodman (8) for ethyl cellulose, representative of a typical water insoluble polymer.

Evaluation as Tablet Coating.—Coating solutions were prepared as previously described. Initially, some preliminary work was conducted using a small wire basket, and the tablets were dipped into the coating solution. This method in its cursory evaluation was unsatisfactory. No dusting powder was used so that the tablets could be evaluated for the apparent effectiveness of the polymer coating alone. Tackiness was present with some of the coatings and was appreciable in the films of Butvar B-90.

TABLE V.—PHYSICAL PROPERTIES OF FILM COATED TABLETS

Polymer	Friability Coat Loss	Disintegration Rate Artificial Gastric Fluid	Disintegration Rate Artificial Intestinal Fluid
Uncoated tablets	...	30–45 Sec.	40 Sec.
Gelva C-3, V-10	0.07%	480	150
1/2 Second Butyrate	0.37	70	60
Bakelite VAGH	0.56	180	60
Exon 471	0.78	120	90
Exon 470	1.13	135	180
Lucite 44	1.20	420	300
Gelva V-15	1.42	240	270
Lucite 42	1.43	300	240
Exon 497	1.46	225	90
Lemac 541-20	2.23	210	300
Lucite 46	2.47	180	180
Butvar B-90 (5%)	6.67	90	540
Bakelite AYAF	12.85	210	240
Butvar B-90 (10%)	20.20	...	...

It was necessary to dilute the solution of Butvar B-90 to a 5% concentration to facilitate tablet coating. To evaluate the durability, ease of handling, and coating friability, disintegration and coating friability tests were conducted as described in the *Experimental* section. The results are shown in Table V.

The disintegration tests were conducted using a different sample of tablets for each of the artificial fluids since the tablets quickly disintegrated. It is believed that the film coated tablets disintegrated by means of water vapor transmission through the film with the transmitted water, reacting with the starch that was present in the tablets.

A 10% coating solution of Butvar B-90 and Bakelite AYAF produced a coating that was rather friable. All of the other tablets lost only a small percentage of their coating.

Correlation of Screening Procedure with a Formulated Product.—A coating formulation was developed from information obtained by the above screening procedure. The mercury substrate technique was employed using various concentrations of diethyl phthalate and sorbitan monooleate[6] from 8 to 30% of each by weight of the polymer. Exon 470 was selected as the film former. Visual examination of the cast films indicated that the following formula was superior to any of the others for use with Exon 470: Exon 470, 5%; diethyl phthalate, 20% by weight of the polymer; Arlacel 80, 2%; FD&C red No. 3, 0.05%; isopropyl alcohol, 25%; butyl acetate, 40%; and acetone, *q.s. ad.*, 100%.

The coating formulation was applied to placebo tablets in a 12-in. coating pan using a Binks airless spray system.[7] A nozzle tip with a 40° dispersion and a 0.009-in. opening was used, and the solution was applied under a pressure of 30 p.s.i. The following formula was selected for the placebo tablets: calcium sulfate, 90%; starch, 10%; granulating agent: methylcellulose 1500, 3% solution; and lubricant: talc, 3%.

The disintegration and friability tests were conducted according to the procedure outlined in the *Experimental* section. The placebo tablets disintegrated in approximately 55 minutes in either artificial gastric or intestinal fluid, while the coated tablets disintegrated in approximately 2 hours and 15 minutes and 2 hours and 50 minutes, in the respective fluids. There was 1.98% coat loss. The tablets had a smooth glossy appearance and retained their original design and shape.

SUMMARY AND CONCLUSIONS

1. This investigation has led to the development of a screening procedure to evaluate unsupported polymeric films for their mechanical, thermal, and stability properties.

2. The results can provide a logical basis for the selection of the modifying additives in the coating formulation.

3. Unsupported film test samples of each polymer were prepared by two different methods—knife edge applicator and mercury substrate technique. The mercury substrate technique was superior because control of film thickness and film removal was easier.

4. The 13 polymers remaining after the primary screening were evaluated according to the physical and mechanical properties of: elastic modulus, tensile strength, linear expansion, and solution rate. The following polymers appeared to be superior of the 13 tested: (*a*) 1/2 Second Butyrate, (*b*) Bakelite AYAF, (*c*) Lucite 44, (*d*) Gelva V-15, and (*e*) Exon 470.

5. There was no discernible decomposition of any of the 13 polymers due to thermal, photo-oxidation, and/or aging effects according to infrared analysis.

6. Evidence that an inverse relationship may exist between the water permeability and oxygen permeability of the free films could be explained by a combination of the hydrophilic-lipophilic and pore theories.

7. Moisture absorption by the polymers was negligible.

8. Nearly all of the films appeared to be applicable as film coating agents based upon friability and disintegration studies. Butvar B-90 and Bakelite AYAF produced a highly friable coating and would not be suitable.

[7] Binks Manufacturing Co., Chicago, Ill.

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Kinetic Study on Stability of Fluprednisolone Acetate in Aqueous Solution

By E. H. JENSEN and D. J. LAMB

The shelf-life of a potential formulation of fluprednisolone acetate was predicted from kinetic measurements. Subsequent assay of samples stored at ambient temperatures confirmed the predicted stability. The degradation of the steroid ester to products devoid of the 17-dihydroxyacetone moiety followed a two-step sequence: ester → alcohol → degradation products. The kinetics of this pathway were interpreted on the basis of consecutive first-order reactions. Quantitative thin-layer chromatography was used to determine the two major components in solution.

ACCELERATED STUDIES on the stability of drugs in solution have been used extensively in attempts to predict the approximate shelf-life of potential pharmaceutical products. Several investigators have reported on the stability of steroids in solution (1–5). It has been shown that although the steroidal A-ring is subject to degradation, it is essentially a photolytic process and may be ignored in stability studies and in finished products, provided adequate protection from light is afforded. The rate of degradation of the 17-dihydroxyacetone moiety of cortical steroids in solution, however, is affected by its immediate environment, *i.e.*, of buffers, oxygen tension, pH of the solution, type of steroid, concentration, etc. Ideally, the effect of each component on the rate of decomposition may be determined independently and subsequently combined to yield an expression capable of predicting the shelf-life of a finished formulation. However, such an approach is quite time consuming. In product development it is often preferred to select what may appear to be a superior formulation based upon certain established criteria, then determine the stability of the active ingredient in this preparation.

Fluprednisolone acetate[1] has been found to possess strong antiinflammatory activity, both systemically and topically. This property made it a suitable candidate for incorporation into ophthalmic preparations. A kinetic study on the stability of fluprednisolone acetate in solution was undertaken to determine whether the steroid was sufficiently stable for consideration in ophthalmic solutions.

Previous reports on steroid esters have suggested that the side chain degradation of these compounds may involve both hydrolysis of the ester linkage and degradation of the dihydroxyacetone side chain (6, 7).

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[1] Fluprednisolone acetate is 6α-fluoroprednisolone acetate.

It was assumed that partially degraded solutions of fluprednisolone acetate would contain fluprednisolone acetate, fluprednisolone, and some unknown steroid degradation products. The presence of the side chains in fluprednisolone acetate and fluprednisolone may be determined by the tetrazolium assay. Degradation products without the dihydroxyacetone side chain do not react to this test. Thus, the tetrazolium procedure may be used for determination of the total amount of fluprednisolone (acetate + alcohol). However, to meet the objectives of studying both the hydrolysis and the side-chain degradation, it was necessary to determine the relative amounts of each of the steroids. This was done by utilizing thin-layer chromatography in combination with the tetrazolium assay.

EXPERIMENTAL

Preparation of Solution

The solution under study was fluprednisolone acetate, 0.5 mg./ml.; polysorbate 80, 60 mg./ml.; sodium chloride U.S.P., 6.5 mg./ml.; phenylmercuric nitrate N.F., 0.025 mg./ml.; neomycin sulfate, 5.75 mg./ml.; water for injection U.S.P. *q.s. ad.* 1.00 ml. The pH of the solution was 7.20. The solution was filled into 5-ml. ampuls which were sealed and placed in constant temperature oil baths at temperatures ranging from 40 to 70°.

At requisite time intervals, ampuls were removed from the baths and assayed.

Assays

Total Fluprednisolone.—The total fluprednisolone (acetate + alcohol) content was determined by the blue tetrazolium procedure (8).

Reagents.—BLUE TETRAZOLIUM SOLUTION.—Blue tetrazolium (Fisher certified reagent) dissolved in 3A alcohol in a concentration of 5 mg./ml.

TETRAMETHYLAMMONIUM HYDROXIDE SOLUTION.—A dilution of 10% aqueous tetramethylammonium hydroxide with 3A alcohol (1:10).

REFERENCE STANDARD SOLUTIONS.—Solutions of fluprednisolone (reference standard) and fluprednisolone acetate (reference standard) in 3A alcohol in concentrations of 10 mcg./ml.

Procedure.—A 2.00-ml. aliquot of the ophthalmic solution was diluted with 2 ml. of water and extracted

with three 25-ml. portions of chloroform. The chloroform layers were separated, filtered through cotton, and collected in a 100-ml. volumetric flask. The volume was made up to 100.0 ml. with chloroform. A 10.0-ml. aliquot of the chloroform solution was evaporated to dryness *in vacuo*. The dry residue was dissolved in 10.0 ml. of 3A alcohol. Exactly 1.00 ml. of the blue tetrazolium solution was added, followed by 1.00 ml. of tetramethylammonium hydroxide solution. Exactly 45 minutes after the addition of the former solution, 1.00 ml. of glacial acetic acid was added to quench further color formation and to stabilize the color already formed. The absorbance of the solution was measured at 525 $m\mu$ in a Beckman DU spectrophotometer with a reagent blank in the reference cell. Parallel runs on standard solutions of fluprednisolone and fluprednisolone acetate were carried out for each sample assayed.

Separation of Fluprednisolone and Fluprednisolone Acetate by Thin-Layer Chromatography.—Four milliliters of the ophthalmic solution were extracted with three 25-ml. portions of chloroform. The chloroform extracts were combined, evaporated to dryness, and redissolved in ethylene dichloride–methanol (1:1) to a total volume of 1.00 ml. Exactly 25 μl. of this solution was spotted on a thin-layer chromatography plate coated with silica gel G in a thickness of 250 μ. The plate was developed in a mobile phase of chloroform-methanol (9:1 v/v) for 80 minutes and dried at 40° for 5 minutes. (Using this procedure, the approximate R_f values for fluprednisolone and fluprednisolone acetate are 0.12 and 0.50, respectively.) The positions of fluprednisolone and fluprednisolone acetate on the plate were located by viewing the plate under a Mineralight, model SL 2537. The silica gel areas containing the steroids were scraped off the plate separately with a razor blade and collected in small Büchner sintered-glass funnels. The silica gel was then eluted with 3A alcohol and the solution filtered through the glass funnel. The volume of the filtrate was made up to 10.0 ml., and the steroid content was determined by the blue tetrazolium procedure described above.

The absorbance readings for fluprednisolone and fluprednisolone acetate, respectively, indicated the relative proportions of the two steroids in the original sample. By applying this proportionality figure to the total fluprednisolone assay, the absolute amounts of fluprednisolone and fluprednisolone acetate in the sample were calculated. Absorptivity values for the calculations were determined from parallel assays of standard solutions of fluprednisolone and fluprednisolone acetate.

Verification of Assay Procedures.—The validity of the assays was checked on freshly prepared solutions of fluprednisolone acetate and fluprednisolone of known concentrations. The solutions were mixed in various proportions and then assayed by the procedures outlined above. The results are shown in Table I.

TABLE I.—RECOVERY OF FLUPREDNISOLONE AND FLUPREDNISOLONE ACETATE FROM SOLUTIONS OF THE TWO STEROIDS OF KNOWN CONCENTRATION

Steroid in Soln., mmoles/L.		Steroid Recovered, mmoles/L.	
Fluprednisolone	Fluprednisolone Acetate	Fluprednisolone	Fluprednisolone Acetate
0	1.226	0.012	1.218
0.632	0.920	0.642	0.917
1.262	0.613	1.244	0.630
1.893	0.307	1.928	0.287
2.524	0	2.537	0

The results show that the separation of fluprednisolone and fluprednisolone acetate in the assay is quite complete. The accuracy of the determination of the major component is within ±2% of theory.

RESULTS AND DISCUSSION

It has been reported in the literature that the hydrolysis of steroid esters is pH dependent (6, 7). Therefore, in the present study it was necessary to stabilize the pH of the solution in order to eliminate the effect of this variable. Neomycin sulfate provided sufficient buffer effect to maintain the pH within 0.6 unit of the initial pH of 7.20.

The assay procedures described above were applied to the individual samples to determine at each temperature the concentrations of fluprednisolone and fluprednisolone acetate in solution as a function of time. A semilog plot of the concentration of fluprednisolone acetate *versus* time is shown in Fig. 1. The linear relationships indicate that the disappearance of fluprednisolone acetate is first order with respect to drug concentration. The corresponding rate constants, k_1, were calculated and are listed in Table II.

The main objective of the study was to determine the suitability of the fluprednisolone acetate solution as a potential product. For this reason, it was of interest to estimate the rate constant, k_1, at 25°. This was done by extrapolation from the higher temperature data while the study was in progress, and k_1 was determined to be 0.0081 days^{-1}. This estimate was later verified by actual experimentation at 25°, and the identical value was obtained. Using this value for k_1, it was calculated that at room temperature 10% of the fluprednisolone acetate would degrade in 13 days ($t_{0.90}$ = 13 days), a period too short to consider the ophthalmic solution a potential product.

Examination of the assay results showed that the decrease in the concentration of fluprednisolone acetate was accompanied by an increase in the concentration of fluprednisolone which reached a maximum and then decreased (Fig. 2). This pattern suggested that consecutive first-order reactions were involved and that fluprednisolone acetate was hydrolyzed to fluprednisolone which then subsequently decom-

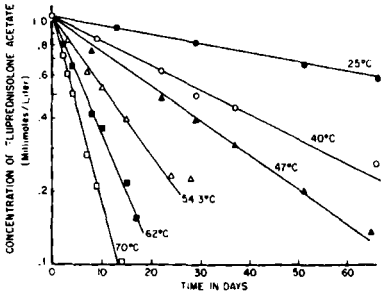


Fig. 1.—First-order plot of the disappearance of fluprednisolone acetate from aqueous solution at various temperatures.

TABLE II.—RATE CONSTANTS[a] FOR DEGRADATION OF FLUPREDNISOLONE ACETATE IN AQUEOUS SOLUTION

Temp., °C.	k_1, days^{-1}	$\beta_{max.}$	K	k_2, days^{-1}
70	0.186	0.494	0.513	0.0952
62	0.113	0.501	0.497	0.0560
54.3	0.0678	0.493	0.516	0.0350
47	0.0339	0.505	0.495	0.0168
40	0.0250	No maximum	...	...
25	0.0081	No maximum	...	...

[a] These are pseudo first-order rate constants since both reactions are pH dependent.

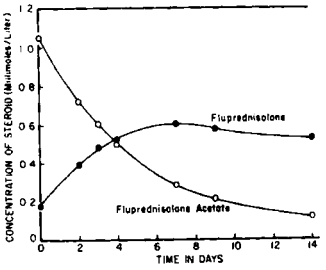


Fig. 2.—Typical plot showing the changes in concentrations of fluprednisolone and fluprednisolone acetate in solution as a function of time (temperature 70°C.).

posed to further degradation products. This is shown schematically in

$$\text{Fluprednisolone acetate} \xrightarrow[(A)]{} \underset{k_1}{} \text{fluprednisolone} \xrightarrow[(B)]{k_2} \text{products} \quad (C)$$

where k_1 and k_2 are first-order rate constants.

The rate of change in the system may then be expressed by the differential equations

$$\frac{dA}{dt} = -k_1A \qquad \text{(Eq. 1)}$$

$$\frac{dB}{dt} = k_1A - k_2B \qquad \text{(Eq. 2)}$$

$$\frac{dC}{dt} = k_2B \qquad \text{(Eq. 3)}$$

Equation 1 may be integrated to give

$$A = A_oe^{-k_1t} \qquad \text{(Eq. 4)}$$

or

$$\log A = -\frac{k_1t}{2.303} + \log A_o$$

where A_o = initial concentration of A.

Thus, k_1 may be obtained by plotting log A *versus* time as shown in Fig. 1.

Equation 2 may be integrated to give

$$B = \frac{A_ok_1}{k_2 - k_1}(e^{-k_1t} - e^{-k_2t}) \qquad \text{(Eq. 5)}$$

To obtain rate constant, k_2, Eqs. 4 and 5 are transformed using dimensionless parameters and variables as described by Frost and Pearson (9). Let $\alpha = A/A_o$; $\beta = B/A_o$; $q = k_1t$; and $K = k_2/k_1$ then Eqs. 4 and 5 become

$$\alpha = e^{-q} \qquad \text{(Eq. 6)}$$

$$\beta = \frac{1}{K-1}(e^{-q} - e^{-Kq}) \qquad \text{(Eq. 7)}$$

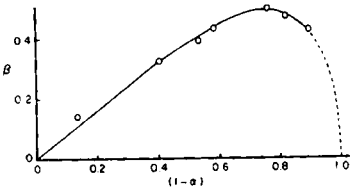


Fig. 3.—Typical plot of β *vs.* (1-α) (temperature 70°C.).

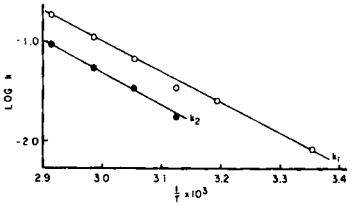


Fig. 4.—Arrhenius plot of the rate constant data ($Ea.k_1$ = 14.2 Kcal./mole; $Ea.k_2$ = 15.2 Kcal./mole).

When β is plotted *versus* (1 − α), a curve is obtained which goes through a maximum, the position of which depends on K. By using Eq. 7 and setting $d\beta/dq = 0$, it is found that $q_{max.} = lnK/(K-1)$.

The value of β at the maximum is

$$\beta_{max.} = K^{K/(1-K)} \qquad \text{(Eq. 8)}$$

Determination of $\beta_{max.}$ is carried out graphically by plotting β *versus* (1 − α) and reading the value of β at the maximum off the graph. A typical plot of β *versus* (1 − α) is shown in Fig. 3. The value for $\beta_{max.}$ is used for the determination of K from Eq. 8.[2] The rate constant, k_1, was determined from Eq. 4. Thus, it is possible to determine rate constant, k_2, from $k_2 = K \times k_1$.

Values of k_2 and corresponding values of $\beta_{max.}$ and K are listed in Table II. An Arrhenius plot of the rate constant data is shown in Fig. 4.

The fact that the pathway of degradation of fluprednisolone acetate follows a consecutive first-order reaction sequence implies that the 17-dihydroxyacetone side chain is, in effect, protected by substitution at the 21 position. However, in the case of fluprednisolone the acetate ester does not afford adequate protection to assure stability in aqueous, neutral solution.

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[2] Equation 8 may be solved conveniently with an electronic computer. In the absence of a computer, K may be determined graphically. A curve is constructed from Eq. 8 using K as the abscissa and $\beta_{max.}$ as the ordinate, assigning different values for K, and calculating the corresponding values for $\beta_{max.}$ K may then be determined from the graph as the abscissa value which corresponds to the ordinate value of $\beta_{max.}$ as determined from Fig. 3.

Rate of Solute Transport out of Emulsion Droplets in Micron Size Range

By WILLIAM I. HIGUCHI

An experimental verification of the theory for rate of external-phase-diffusion-controlled solute transport from emulsion droplets in the micron size range has been carried out. The Coulter counter was employed to follow the change in size distribution in aqueous sodium chloride solutions of dibutyl phthalate droplets with time. The rate was proportional to particle radius according to theory, and the diffusion coefficient of dibutyl phthalate calculated from data and theory agreed well with the Stokes-Einstein law predictions. The effect of the presence of a water immiscible component in the dibutyl phthalate droplet was also studied.

As PART OF the program on the behavior of dispersed systems in these laboratories the transport of solute out of emulsion droplets is under study. This problem has bearing on a number of important pharmaceutical situations. In many instances the understanding of the release rate of a drug from orally and parenterally administered emulsions may be critically dependent on a consideration of factors associated with this phenomenon. It is also important in the understanding of the physical degradation process in emulsions taking place *via* molecular diffusion (1).

This report gives the results of an experimental examination of the theory for the diffusion-controlled dissolution from small droplets. The method of study is simple and unique. It should be useful in similar studies of more complex systems.

THEORY

Recently (2, 3), the theory for the rate of diffusion-controlled dissolution and growth of small spherical particles was examined. Although the equations were aimed at and applied to dissolution and growth from solid particles, the same model may be employed here. The expression for the steady state rate, G, may be written

$$G = 4\pi a D\,\Delta C \qquad \text{(Eq. 1)}$$

Here D is the diffusion coefficient of the solute in the external phase, a is the droplet radius, and ΔC is the difference between the solute concentration at the surface but on the external phase side[1] and the solute concentration in the external phase but far away from any droplet. According to theory, Eq. 1 is in reality a limiting equation valid when hydrodynamic effects on the solute diffusion near the droplets become negligible. Thus, for large particles ($\gtrsim 50\ \mu$) (because even small shearing in the external phase may alter the concentration gradients in a region within a few particle diameters of a droplet and because sedimentation or creaming effects would be more important) Eq. 1 would not be expected to be always in quantitative agreement with experiments. However, for smaller particles ($\lesssim 1\ \mu$) Eq. 1 would be expected to be obeyed even under moderate amounts of agitation and shear due to gravitational forces.[2]

If the droplet is substantially one component, we may equate G to the rate of change in mass of the droplet, *i.e.*

$$G = \frac{dm}{dt} = 4\pi a^2 \rho \frac{da}{dt} \qquad \text{(Eq. 2)}$$

where m is the mass of the droplet, $^4/_3\pi a^3\rho$, ρ is the density of the droplet, and t is time. Combining Eqs. 1 and 2 and integrating we obtain as before (2, 3)

$$a^2 = a_o^2 + \frac{2D\Delta C}{\rho} t \qquad \text{(Eq. 3)}$$

where ΔC is positive for growth and negative for dissolution.

It should be mentioned that Eq. 3 was recently (3, 4) incorporated into the theory for the dissolution rate of a finely divided powder. Comparison of this theory with data on micronized methylprednisolone was satisfactory. However, some deviations were observed. This suggested that a much better test of Eq. 3 would be provided by suitably selected emulsion droplets.

EXPERIMENTAL

General Considerations.—The primary aim of this work was to test Eq. 3 with emulsion droplets. It was decided to employ the Coulter counter[3] to follow the time change in the number and size distribution of an emulsion of a slightly miscible liquid in aqueous media. The instrument is uniquely able to count and size particles conveniently and rapidly in the size range of $\sim 0.5\ \mu$ and above.

Dibutyl phthalate was selected as the oil phase because the water solubility (miscibility) of this material in water appeared to be small enough so

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[1] For external phase diffusion-controlled processes, this surface concentration would be in equilibrium with the droplet and therefore would be the solubility. This means, furthermore, that no concentration gradients in the droplet and no interfacial barriers are present.

[2] It is assumed that interfacial turbulence, spontaneous emulsification, and gross droplet distortion effects are absent. Also it is assumed that no reactive agents are present in the external phase other than the main external phase component itself.

[3] Coulter Industrial Sales, Chicago, Ill.

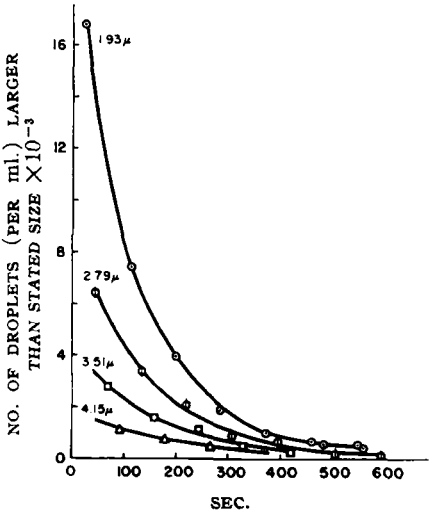


Fig. 1.—Coulter counter data showing changes in cumulative counts with time at various threshold settings when 5 μl. of the dibutyl phthalate emulsion was pipeted into 100 ml. of saline.

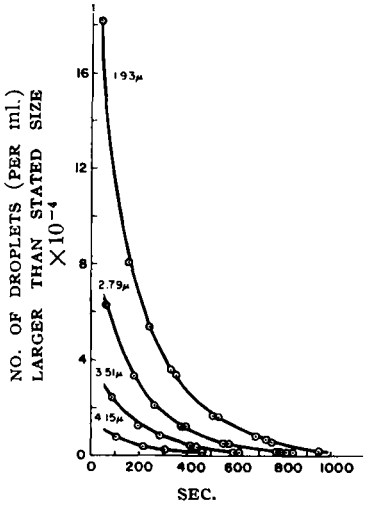


Fig. 2.—Coulter counter data showing changes in cumulative counts with time at various threshold settings when 50 μl. of the dibutyl phthalate emulsion was pipeted into 100 ml. of saline.

that dissolution would not occur too rapidly according to Eq. 3, yet large enough so that at a convenient droplet concentration the water would not become saturated (*i.e.*, $\Delta C \rightarrow o$) before an appreciable change in sizes occurred.

Procedure.—Fisher reagent grade dibutyl phthalate was distilled at 115° under reduced pressure. Approximately 1% by weight of this material in 0.10% aqueous sodium lauryl sulfate[4] was emulsified by about 10 minutes of ultrasonic irradiation generated by the Acoustica cleaning unit (model DR-125AH, 40 kc).[5] Dibutyl phthalate containing various amounts of hexadecane was also emulsified in this manner. These latter experiments were conducted to see the effects of an immiscible second component in the oil phase.

The Coulter counter was calibrated with monodispersed polystyrene latex particles[6] as in the previous work (5, 6). The 50-μ aperture tube was used in the present studies.

From 5 to 100 μl. of the emulsion was pipeted into 100 ml. of the Abbott[7] 0.90% sodium chloride solution for injection contained in a 150-ml. beaker ready in counting position. Temperature was maintained at 25.0 ± 0.5°. Slow (~50 r.p.m.) to moderate (~300 r.p.m.) stirring of the saline

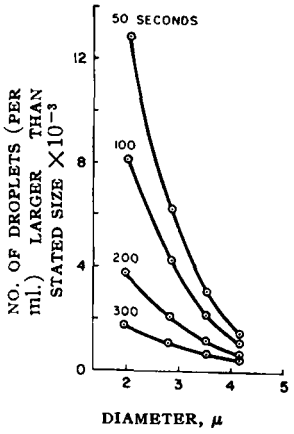


Fig. 3.—Cumulative size distribution curves constructed from the data in Fig. 1. These illustrate the dissolution of the dibutyl phthalate droplets with time.

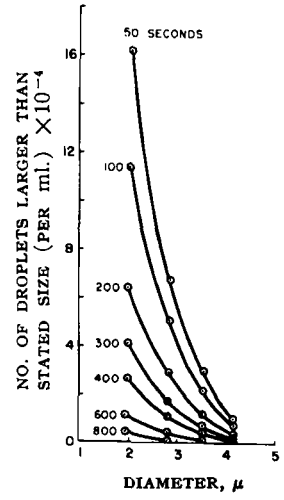


Fig. 4.—Cumulative size distribution curves constructed from data in Fig. 2 showing dissolution of the dibutyl phthalate droplets with time.

solution was provided during and after the addition of the aliquot of emulsion. This was achieved by a conventional glass stirrer (blade length 3 cm., width 0.8 cm., and at maximum pitch) positioned at a slight angle and driven from above the beaker. A timer was initiated after the addition, and both the counts per sample volume and the times were

[4] A highly purified sample kindly supplied to us by Professor K. J. Mysels.
[5] Acoustica Associates, Inc., Mineola, N. Y.
[6] Kindly supplied to us by Dr. J. W. Vanderhoff, Dow Chemical Co., Midland, Mich.
[7] Abbott Laboratories, North Chicago, Ill.

recorded at various settings of the threshold dial. The time period for counting the usual sample volume of 0.05 ml. was about 5 seconds; the time between counts was about 25 seconds. Count rates were always sufficiently low so that coincidence errors were negligible.

The solubility (miscibility) of dibutyl phthalate in saline was determined by pipeting 0.10, 0.20, and 0.50 ml. of dibutyl phthalate into 100 ml. of the saline and shaking vigorously for periods up to 48 hours at 25.0 ± 0.5°. Samples were removed from time to time, centrifuged, diluted 1:1 with

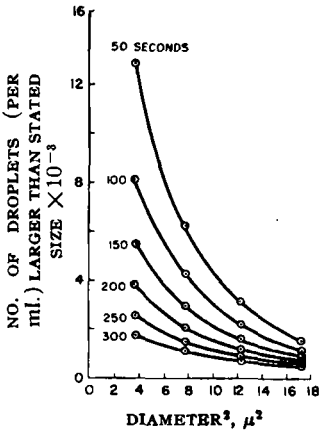


Fig. 5.—Plots of cumulative counts *vs.* diameter squared showing fit of data to the diffusion controlled theory for small droplets; 5-μl. run, obeys $a_i^2 = a_{i0}^2 - 1.2 \times 10^{-10} t$.

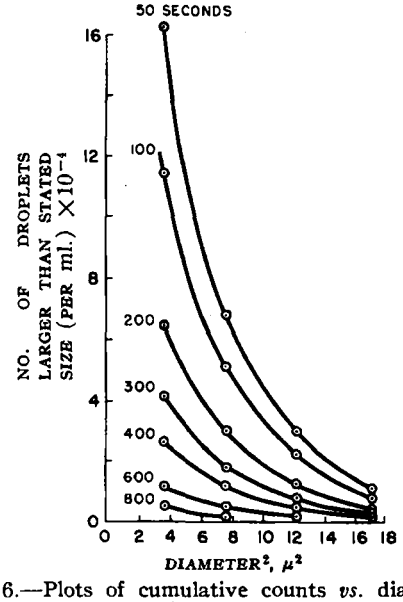


Fig. 6.—Plots of cumulative counts *vs.* diameter squared showing fit of data to theory; 50-μl. case.

ethanol and assayed at 266.5 mμ with a Beckman DU spectrophotometer. A constant value of 1.20 (± 0.10) × 10^{-6} Gm. per ml. was obtained for all three mixtures at sufficiently long times.

Dissolution rate runs were also carried out on dibutyl phthalate emulsions prepared in 0.10% polysorbate 20[8] solutions. These were run in both

[8] Marketed as Tween 20 by the Atlas Powder Co., Wilmington, Del.

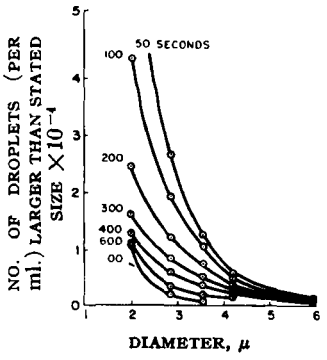


Fig. 7.—Cumulative count curves showing dibutyl phthalate release from droplets initially containing 12.5% hexadecane.

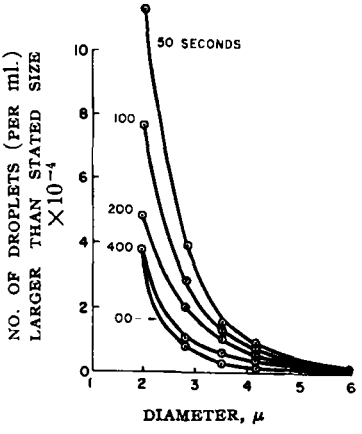


Fig. 8.—Cumulative count curves showing dibutyl phthalate release from droplets initially containing 25% hexadecane.

plain saline and in saline containing 0.1% polysorbate 20.

RESULTS AND DISCUSSION

Pure Dibutyl Phthalate Case.—Figures 1 and 2 give the Coulter counter data obtained when 5 and 50 μl., respectively, of the 1% dibutyl phthalate emulsion (0.10% sodium lauryl sulfate) were pipeted into 100 ml. of saline solution. These results were quite reproducible and independent of the stirring rate over a range of more than a factor of two.

Interpolated values from the smoothed curves in Figs. 1 and 2 were used to construct the constant time plots of the cumulative particle size distribution curves shown in Figs. 3 and 4. The background saline blank counts of 200 counts per ml. for the 1.93 μ setting, 30 counts per ml. for the 2.79 μ setting, and zero blank for the other settings were subtracted from the Figs. 1 and 2 data prior to making the Figs. 3 and 4 plots. Numerical differentiation of the $t = 50$ seconds curves in Figs. 3 and 4 shows that only about one-half of the total dibutyl phthalate can be accounted for in the sizes greater than 1.93 μ.

If Eq. 3 is applicable to the present data, it is apparent that plots of cumulative count (ordinate) *versus* the square of particle size (abscissa) should give curves that are displaced along the abscissa with time but without changes in shape. Further-

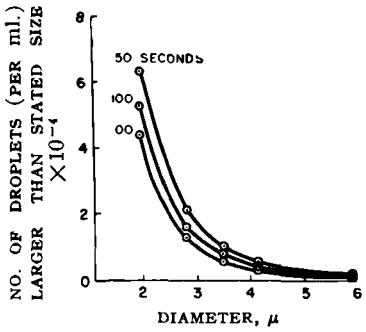


Fig. 9.—Cumulative count curves showing dibutyl phthalate release from droplets initially containing 50% hexadecane.

more, such displacements should be constant with time if ΔC is constant. In Figs. 5 and 6 the same data are plotted in this manner. In accordance with Eq. 3 it can be seen that any two points on a given curve move horizontally at the same rate. In the 5 μl. results (Fig. 5) the displacements with time are constant and obey

$$a_i^2 = a_{io}^2 - 1.2 \times 10^{-10} t \qquad \text{(Eq. 4)}$$

where a_{io} and a_i are the radii of droplet i at time $t = 0$ and $t = t$, respectively. The constancy of the time coefficient was not unexpected in this instance because ΔC was not expected to decrease by more than 5%, even when all of the droplets were dissolved.

By comparing Eq. 4 with Eq. 3 we obtain $D = (0.60 \times 10^{-10} \rho)/\Delta C$. Since $\rho = 1.04$ for dibutyl phthalate and $\Delta C = 1.20 \times 10^{-5}$ Gm. per ml., we obtain $D = 5.2 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ Now from the Stokes-Einstein relation we have

$$D = \frac{kT}{6\pi vs} \qquad \text{(Eq. 5)}$$

where k is the Boltzmann constant, T is absolute temperature, v is the viscosity, and s is the hydrodynamic radius of the dibutyl phthalate molecule. For the present case $T = 298.2°K$, $k = 1.38 \times 10^{-16}$ ergs deg.$^{-1}$ molecule^{-1}, $v = 0.0091$ poise, and $s = 4.7 \times 10^{-8}$ cm. The value for s was estimated from the equation $^4/_3\pi s^3\rho = M/N$, where M is the molecular weight of dibutyl phthalate and N is Avogadro's number. Thus we obtained from Eq. 5 $D = 5.1 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ which agrees well with the above value calculated from the rate experiment and Eq. 3.

The 50-μl. runs (Fig. 6) did not show a constant dissolution rate. Comparison of Figs. 5 and 6 show that between 50 and 100 seconds the rate of dissolution in the 50-μl. run was about 70% of the 5-μl. run value. Then the rate steadily decreased to about 50% of the 5-μl. run value. It is noteworthy that even though ΔC varied in the 50-μl. run, the dependence on a was consistent with Eq. 3.

Essentially identical results were obtained with emulsions of dibutyl phthalate prepared using 0.10% polysorbate 20 as the emulsifying agent. This was expected as the amount of surfactant in either case was very small in the saline dissolution media, *i.e.*, about 5×10^{-5}%. Solubility determinations indicated no increases in solubility with this amount of surfactant in the saline.

A few runs were made with 0.10% polysorbate 20 in the saline dissolution media. Dissolution rates were greater by about a factor of about two than those rates found in pure saline. Attempts were made to measure the solubility of dibutyl phthalate in these polysorbate solutions, but constant results were not obtained. Solution concentrations generally continued to increase slowly with time. Concentrations as great as 25 to 50 times greater than the solubility in pure saline were observed, yet the dissolution rates were only a factor of two or so greater. This would be consistent with Eq. 3 only if it can be assumed that the diffusion coefficients involved are much smaller than the above values for pure saline. This is reasonable (7) if the diffusing species are primarily large swollen micelles of dibutyl phthalate in polysorbate 20. After the centrifugation step in the solubility measurements of dibutyl phthalate in 0.10% polysorbate 20 it was observed that the supernatant liquid possessed a bluish tint when viewed in indirect light, suggesting the presence of colloidal species. More definitive work is planned on the effect of colloids on transport rates.

Other Cases.—Results of runs with dibutyl phthalate containing various amounts of hexadecane are given in Figs. 7–9. In all of these cases dissolution begins at about the same rate as the pure dibutyl phthalate emulsion droplets. However, the rates slow up and stop when nearly all of the dibutyl phthalate is released from the droplets, leaving behind mostly pure hexadecane. It can be seen (particularly from Figs. 7 and 8) that the smaller emulsion droplets tend to reach the final state more rapidly than the larger droplets. In experiments where 100 μl. or more of the emulsion was added to the saline solution, this situation resulted in a "rebound" phenomenon, *i.e.*, the small particles gave up more than their equilibrium share at early times, but regained their overlosses at later times. This was apparently not significant in the experiments with 50 μl. of emulsion because the solution buildup of dibutyl phthalate was not very great.

To explain these results quantitatively with dibutyl phthalate containing hexadecane it would be necessary to know (*a*) the distribution coefficient of dibutyl phthalate for the hexadecane-water pair and (*b*) the partial molal (or specific) volumes for dibutyl phthalate in dibutyl phthalate-hexadecane solutions. This information may be incorporated into Eqs. 1 and 2 to give an expression analogous to Eq. 3.

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Kinetics of Hydrolytic Degradation in Unbuffered Solutions

Theoretical Analysis of Autocatalytic Processes Controlled Primarily by Specific Acid Catalysis

By NICHOLAS G. LORDI and STEPHEN F. BARON

The kinetics of degradation of neutral and acidic molecules which form acidic products in unbuffered solutions has been considered. The validity of the analytic solutions for autocatalytic cases involving specific acid catalysis in competition with specific base catalysis or spontaneous degradation has been verified through analog simulation of the reactions. Since the time required for 10 per cent degradation of a drug is of pharmaceutical interest, the effect on this parameter of variation in initial concentration of drug, rate constants, and dissociation constants of reactants and products was investigated. The equations presented may be applied to the estimation of drug stability under the conditions described, if the relevant kinetic parameters are known.

THE PROBLEMS involved in treating stability in pharmaceutical systems in which the products influence the course of degradation have not been considered in detail. In his comprehensive review of pharmaceutical stability, Garrett (1) suggests that "... autocatalytic degradation may need special treatment..." to apply the principles of stability prediction which have been elaborated. Kinetic mechanisms involving autocatalysis have been described for certain autoxidations (2), biochemical processes (3), and thermal degradations (4, 5). The degradation of amyl nitrite (6) has been shown to be autocatalytic. It is also well known that the "spontaneous" hydrolysis of esters in aqueous solution is autocatalytic, the catalyst being the acid formed upon hydrolysis. In this respect, Zawidzki (7) has reported studies of the autocatalytic hydrolysis of alkyl sulfates and Dawson and Lowson (8, 9) that of ethyl acetate. General theoretical treatments of autocatalytic kinetics have been presented by Zawidzki (10) and Reed and Berkson (11). The latter have discussed the application of the "logistic" function to autocatalytic processes.

This study considers the kinetics of homogeneous degradation of drugs in unbuffered systems which are subject to specific acid and/or base catalysis and form acids and/or bases as products. Sound kinetic technique dictates that stability analysis of such drugs be carried out in buffered solutions to simplify interpretation of experimental data and to extract the maximum information from the study concerning mechanism of reaction and pH of optimum stability. However, there are instances where it may prove undesirable to prepare buffered pharmaceutical systems containing these drugs. General acid and/or base catalysis may prove sufficiently severe so that buffering actually results in decreased stability when compared to the unbuffered system. Buffers may be incompatible with the drug or with other components of the preparation. In other instances, the pH of maximum stability may be too high (>10) or too low (<2) to permit effective buffering for optimum stability. Drugs of a high order of stability are often formulated in unbuffered systems. Drugs of a low order of stability may be used in unbuffered solution forms which are prepared prior to use and may often have been stored for periods of several weeks. It would seem desirable to have a valid means for predicting the possible extent of degradation in such instances as well as for kinetically analyzing data obtained in the absence of pH control. Although Zawidzki and Dawson have developed theoretical treatments for some specific cases of the type under consideration, no prior *systematic* quantitative treatment of cases of this sort has otherwise been reported.

The specific cases dealt with in this report concern the hydrolytic degradation of neutral and acidic molecules which form acidic products. Examples of the model reactions included in this report are listed in Table I. Although the theory has been developed assuming only hydrolytic degradation, the proposed models may apply to other kinds of degradative processes as well.

Differential equations descriptive of the models have been derived and solved analytically. The complexity of some of the solutions obtained prohibit their ready application to practical

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TABLE I.—MODEL REACTIONS TREATED

Case	Reactant	pH-Determining Product	Examples	Ref.
I	Neutral[a] molecule	Strong acid	Ethyl trifluoroacetate	(12)
	$N \rightarrow H^+ + A^-$			
II	Neutral[a] molecule	Weak acid	Choline esters	(13)
	$N \rightarrow HA_2 \rightleftharpoons H^+ + A_2^-$			(14)
			Alkyl sulfates	(15)
III	Weak acid	Weak acid	Acylsalicylates	(16)
	$HA_1 \rightarrow HA_2$		Alkyl-*dl*-α-(2-piperidyl)-phenylacetates	(17)
	$H^+ + A_1^- \quad H^+ + A_2^-$			(18)

[a] Neutral in the sense of acidity, not charge.

problems, necessitating some degree of approximation. An analog computer was used to simulate the models and thus generate the experimental data required to test the validity and useful range of the computational techniques developed which may have specific application to stability prediction in pharmaceutical systems.

THEORY

A generalized differential equation describing the rate of irreversible hydrolytic reactions in unbuffered solutions in the absence of general acid and base catalysis may be written (1)

$$-\frac{d[X]}{dt} = k_H[H^+][X] + k_0[X] + k_{OH}[OH^-][X] \quad \text{(Eq. 1a)}$$

where k_H is the bimolecular rate constant characterizing the specific acid-catalyzed degradation, k_{OH} is the bimolecular rate constant characterizing the specific base-catalyzed degradation, and k_0 is a first-order rate constant which may characterize the spontaneous reaction of a drug with the solvent or an intramolecular catalysis. $[X]$ may be considered to be a neutral, acidic, or basic species of reacting drug. It will prove more convenient to write this rate expression in the form

$$-\frac{d[X]}{dt} = k_H[H^+][X] + ak_H[X] + \frac{bk_H[X]}{[H^+]} \quad \text{(Eq. 1b)}$$

where $a = k_0/k_H$ and $b = k_{OH}Kw/k_H$.

The problem reduces to defining hydrogen ion concentration $[H^+]$ in terms of reactant concentrations and appropriate constants to arrive at differential equations which are amenable to integration and computer simulation. Degradations of the type discussed in this report may be followed in practice by measuring changes in pH or reactant concentration. Equations describing the degradation rate may be written as functions of either or both these parameters. In general, equations will be expressed here in terms of concentration of undegraded drug.

The development which follows is based on the premise that the bulk of the observed degradation in the systems studied will be the result of specific acid catalysis, at least in systems which are initially neutral or weakly acidic. The existence of acids initially in the system, specific base catalysis, and/or first-order degradative processes in this view serve primarily to initiate degradation and thus determine the length of the induction period observed before degradation significantly begins to accelerate. Systems which are perturbed by the presence of bases are not specifically considered in this report. In these instances, specific base catalysis and first-order processes may account for significant degradation.

Case I. Homogeneous Degradation of Neutral Molecules Which Form Strong Acids.—In this case[1] the hydrogen ion formed in the degradation will be reduced by the hydroxyl ion present in solution. In pure water

$$[H^+] = [N_0] - [N] + [OH^-] = [N_0] - [N] + \frac{Kw}{[H^+]}$$

or

$$[H^+]^2 - \{[N_0] - [N]\}[H^+] - Kw = 0 \quad \text{(Eq. 2a)}$$

where $[N]$ is the concentration of undegraded drug at any time t and $[N_0]$ its concentration at $t = 0$. The positive root of Eq. 2a is

$$[H^+] = \frac{[N_0] - [N]}{2} + \frac{1}{2}\sqrt{\{[N_0] - [N]\}^2 + 4Kw} \quad \text{(Eq. 2b)}$$

Since it will prove generally true that $[N_0] \gg Kw$,

$$[H^+] \sim [N_0] - [N] \quad \text{(Eq. 2c)}$$

during the bulk of the degradation; at $t = 0$

$$[H^+] = [H_0] = Kw^{1/2} \quad \text{(Eq. 2d)}$$

in pure aqueous solution. Equation 2c is substituted in Eq. 1b which is then integrated[2] to give

$$\ln \frac{\sqrt{\{[N_0] - [N]\}^2 + a\{[N_0] - [N]\} + b}}{[N]} - \left(\frac{b}{[N_0]} + \frac{a}{2}\right)\int \frac{d\{[N_0] - [N]\}}{\{[N_0] - [N]\}^2 + a\{[N_0] - [N]\} + b} = \{[N_0]^2 + a[N_0] + b\}k_H t + \text{constant} \quad \text{(Eq. 3a)}$$

[1] All derivations have been made in terms of concentrations rather than activities, thus presuming constancy of ionic strength during the degradation.

[2] The integration was carried out using integral No. 99 as listed in the Table of Integrals, "Handbook of Chemistry and Physics," 43rd ed., The Chemical Rubber Publishing Co., Cleveland, Ohio, 1961, pp. 254–280. Equations 3b and 3c were obtained by substitution in integrals No. 87 and 88.

TABLE II.—PARTICULAR SOLUTIONS OF EQ. 1*b* (HOMOGENEOUS DEGRADATION OF NEUTRAL MOLECULES WHICH FORM STRONG ACIDS)

$\ln X - Y = \ln Z + C\,k_H t$

Condition	X	Y	Z	C	Equation
$a = b = 0$	$\frac{[N_0] - [N]}{[N]}$	0	$\frac{[H_0]}{[N_0]}$	$[N_0]$	4[a]
$b = 0$	$\frac{[N_0] - [N] + a}{[N]}$	0	$\frac{[H_0] + a}{[N_0]}$	$[N_0] + a$	5
$a = 0$	$\frac{\sqrt{([N_0] - [N])^2 + b}}{[N]}$	$\frac{\sqrt{b}}{[N_0]}\left\{\tan^{-1}\frac{[N_0] - [N]}{\sqrt{b}} - \tan^{-1}\frac{[H_0]}{\sqrt{b}}\right\}$	$\frac{\sqrt{[H_0]^2 + b}}{[N_0]}$	$\frac{[N_0] + b}{[N_0]}$	6

[a] Equation characteristic of second-order autocatalytic processes (11, 19).

TABLE III.—PARTICULAR SOLUTIONS OF EQ. 1*b* (HOMOGENEOUS DEGRADATION OF NEUTRAL MOLECULES WHICH FORM WEAK ACIDS)

$$A \ln \frac{\sqrt{\beta} + \sqrt{\beta - [N]}}{\sqrt{\beta} + \sqrt{\beta - [N_0]}} - B \ln \frac{\sqrt{\beta} - \sqrt{\beta - [N]}}{\sqrt{\beta} - \sqrt{\beta - [N_0]}} + (B - A) \ln Y + Z = Ka_2^{1/2} k_H t$$

Condition	A	B	Y	Z	Equation
$a = b = 0$	$\frac{1}{\sqrt{\beta} + \frac{Ka_2^{1/2}}{2}}$	$\frac{1}{\sqrt{\beta} - \frac{Ka_2^{1/2}}{2}}$	$\frac{[H^+]^a}{[H_0]^b}$	0	13[c]
$b = 0$	$\frac{1}{\sqrt{\beta} + \frac{Ka_2^{1/2}}{2} - \frac{a}{Ka_1^{1/2}}}$	$\frac{1}{\sqrt{\beta} - \frac{Ka_2^{1/2}}{2} + \frac{a}{Ka_2^{1/2}}}$	$\frac{[H^+] + a}{[H_0] + a}$	0	14
$a = 0$	$\frac{\sqrt{\beta} + \frac{Ka_2^{1/2}}{2}}{\left(\sqrt{\beta} + \frac{Ka_2^{1/2}}{2}\right)^2 + \frac{b}{Ka_1}}$	$\frac{\sqrt{\beta} - \frac{Ka_2^{1/2}}{2}}{\left(\sqrt{\beta} - \frac{Ka_2^{1/2}}{2}\right)^2 + \frac{b}{Ka_1}}$	$\sqrt{\frac{[H^+]^2 + b}{[H_0]^2 + b}}$	$D^d\left\{\tan^{-1}\frac{[H^+]}{\sqrt{b}} - \tan^{-1}\frac{[H_0]}{\sqrt{b}}\right\}$	15

[a] Given by Eq. 10*a*. [b] Given by Eq. 11 or 2*d*. [c] Dawson and Lowson (9) had derived Eq. 13 expressed in terms of product rather than reactant concentrations. [d] $D = (A - B) - (A + B)\left\{\left(\frac{(Ka_2/4 + (b/Ka_2)}{\sqrt{\beta}}\right)\right\}\frac{Ka_2^{1/2}}{\sqrt{b}}$.

If $a^2 > 4b$ the integral will have the value

$$-\frac{2}{\sqrt{a^2 - 4b}} \tanh^{-1} \frac{2\{[N_0] - [N]\} + a}{\sqrt{a^2 - 4b}} \quad \text{(Eq. 3b)}$$

If $4b > a^2$ the integral will be

$$\frac{2}{\sqrt{4b - a^2}} \tan^{-1} \frac{2\{[N_0] - [N]\} + a}{\sqrt{4b - a^2}} \quad \text{(Eq. 3c)}$$

Particular solutions of Eq. 3*a* that apply to situations in which only specific acid catalysis, specific acid catalysis and first-order degradation, or specific acid and specific base catalysis are involved result when $a = b = 0$, $b = 0$, or $a = 0$, respectively. These are listed in Table II.

In systems free of acid prior to degradation $[H_0]$ is given by Eq. 2*d*. If strong acid is initially present, $[H_0]$ becomes equal to its concentration, and $[N_0]$ is increased by the amount of acid present. On the other hand, if a weak acid HA_0 with a dissociation constant[3] Ka_0 is present in solution at a fixed concentration C_0, then $[H^+] = [OH^-] + [A_0^-] + [N_0] - [N]$. Therefore

$$[H^+] = \frac{[N_0] - [N]}{2} + \frac{1}{2}\sqrt{\{[N_0] - [N]\}^2 + 4\{Ka_0C_0 + Kw\}} \quad \text{(Eq. 7a)}$$

assuming $C_0 \sim [HA_0]$. At $t = 0$,

$$[H^+] = [H_0] = \sqrt{Ka_0C_0 + Kw} \quad \text{(Eq. 7b)}$$

The contribution of the weak acid to the rate of degradation will be significant only during the early stages of the reaction if $[H_0] \ll N_0$; otherwise $[H_0]$ must be added to $[N_0]$ wherever it appears in Eqs. 4–6.[4]

When only specific base-catalyzed degradation is considered, Eq. 1*a* reduces to $-(dN/dt) = k_{OH}Kw\text{-}[N]/\{[N_0] - [N]\}$ integration of which leads to

$$\ln \frac{[N_0]}{[N]} - \left(1 - \frac{[N]}{[N_0]}\right) = \frac{k_{OH}Kw}{[N_0]}\,t \quad \text{(Eq. 8)}$$

This equation may be modified in the manner discussed when applied to systems containing acid prior to the initiation of degradation.

Case II. Homogeneous Degradation of Neutral Molecules Which Form Weak Acids.—Assuming that a weak acid HA_0 other than the product HA_2 of

[3] Dissociation constants are assumed to be classical in form so that all $[H^+]$ functions may be expressed in terms of stoichiometric concentrations. If apparent dissociation constants Ka′ are used, then the substitution $Ka = Ka'/f_H$ must be made. Also note that the thermodynamic ion product of water $Kw = f_H f_{OH} \cdot Kw$. f_H and f_{OH} are the activity coefficients of hydrogen and hydroxyl ion.

[4] This constitutes an approximation of Eq. 7*a* which is necessary, since one cannot readily arrive at an exact solution to Eq. 1*b* employing Eq. 7*a*.

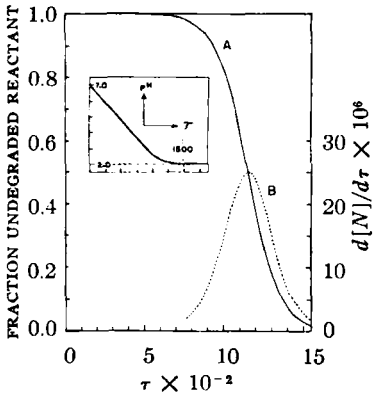


Fig. 1.—Computer simulated degradation (according to Eq. 4) of a neutral compound subject only to specific acid catalysis in unbuffered solution. Degradation product is a strong acid; $[N_0] = 0.01$ M. Fraction of undegraded reactant (curve A), $[N]/[N_0]$, and rate of degradation (curve B), $d(N)/d\tau$, are shown as a function of the time parameter $\tau = k_H t$. Inset shows pH variation with τ for this system.

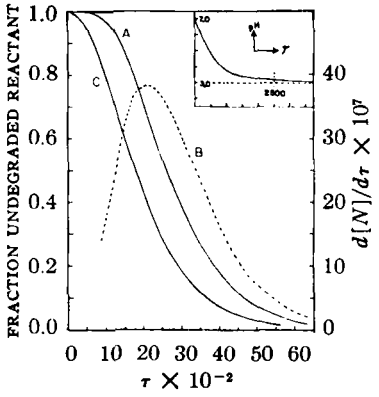


Fig. 2.—Computer simulated degradation (according to Eq. 13) of a neutral compound subject only to specific acid catalysis in unbuffered solution. Degradation product is a weak acid, $pK_{a_2} = 4.0$; $[N_0] = 0.01$ M. Fraction of undegraded reactant (curve A), $[N]/[N_0]$, and rate of degradation (curve B), $d[N]/d\tau$, are shown as a function of the time parameter $k_H t$. Curve C represents the theoretical course of degradation according to Eq. 16. Inset shows pH variation with τ for this system.

the degradation is also present in solution at a fixed concentration $C_0 = [HA_0] + [A_0^-]$, the $[H^+]$ function characteristic of this system may be derived in the following manner. Since $[H^+] = [A_2^-] + [A_0^-] + [OH^-]$ or

$$[H^+] = \frac{Ka_2[HA_2]}{[H^+]} + \frac{Ka_0[HA_0]}{[H^+]} + \frac{Kw}{[H^+]} \quad \text{(Eq. 9}a\text{)}$$

and $[N_0] - [N] = [HA_1] + [A_1^-]$ therefore,

$$[H^+]^2 - Ka_2\{[N_0] - [N] - [A_2^-]\} - Ka_0\{C_0 - [A_0^-]\} - Kw = 0 \quad \text{(Eq. 9}b\text{)}$$

where $[N_0]$ is the initial concentration of reactant, and Ka_2 and Ka_0 are the dissociation constants of the product and other acid present in the system. It may be further assumed that during the course of the degradation $[A_2^-] > 20\ [A_0^-]$ and $[A_2^-] \gg [OH^-]$, so that $[A_2^-] \sim [H^+]$. The resulting quadratic expression can then be solved for $[H^+]$, the positive root of which is

$$[H^+] = Ka_2^{1/2}\{\sqrt{\beta - [N]} - Ka_2^{1/2}/2\} \quad \text{(Eq. 10}a\text{)}$$

where $\beta = \{(Ka_2/4) + (Kw/Ka_2) + (Ka_0/Ka_2) \times C_0 + [N_0]\}$. If $Ka_2 \ll [N_0]$, then

$$[H^+] = Ka_2^{1/2}\sqrt{\beta - [N]} \quad \text{(Eq. 10}b\text{)}$$

At $t = 0$

$$[H^+] = [H_0] = \sqrt{\frac{Ka_0^2}{4} + Ka_0C_0 + Kw} - \frac{Ka_0}{2} \quad \text{(Eq. 11)}$$

in the presence of weak acid, or $[H^+]$ is defined by Eq. 2d in its absence.

Substitution of Eq. 10a in Eq. 1b results in a differential equation which adequately represents all significant features of the model. Its solution[5] takes the form

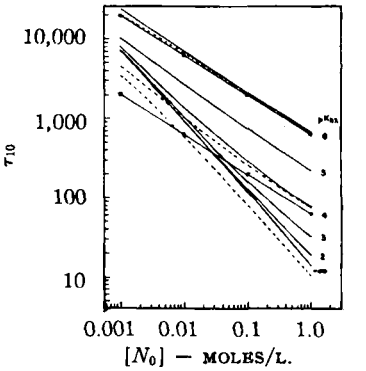


Fig. 3.—Dependence of the time required for 10% degradation of neutral compounds in unbuffered solution on the initial reactant concentration ($[N_0]$) and product acidity (pKa_2). Key: ———, τ_{10}-values computed from Eqs. 4 ($pKa_2 - \infty$) and 13 (pKa_2 2–6) assuming $[H_0] = 10^{-4}$ M; – – – – τ_{10}-values computed from Eqs. 4 ($pKa_2 - \infty$) and 13 (pKa_2 4 and 6) assuming $[H_0] = 10^{-5.5}$ M; and –●—●–, τ_{10}-values computed from Eq. 16 (pKa_2, 4 and 6) assuming $[H_0] = 10^{-7}$ M.

$$A \ln(\sqrt{\beta} + \sqrt{\beta - [N]}) - B \ln(\sqrt{\beta} - \sqrt{\beta - [N]}) + (B - A) \ln \sqrt{\{\beta - [N]\} + \lambda\sqrt{\beta - [N]} + \gamma} + \left\{A\left(1 - \frac{\gamma}{\sqrt{\beta}}\right) - B\left(1 + \frac{\gamma}{\sqrt{\beta}}\right)\right\} \int \frac{d(\sqrt{\beta - [N]})}{\{\beta - [N]\} + \lambda\sqrt{\beta - [N]} + \gamma} = Ka_2^{1/2}k_H t + \text{constant} \quad \text{(Eq. 12)}$$

where the constants A, B, λ, and γ are complex functions of Ka_2, β, a, and b. Table III lists particular solutions of Eq. 12 for cases where $a = b = 0$, $b = 0$, and $a = 0$, Eqs. 13–15, respectively.

The differential equation[6] which results when Eq.

[5] Equation 12 was derived by the method of partial fractions and subsequent application of integral No. 29 and 93 (see *Footnote 2*).

[6] This equation is basically the one reported by Zawidzki (7) to characterize the rate of hydrolysis of potassium methane sulfate.

TABLE IV.—EXAMPLES OF KINETIC PARAMETERS RELEVANT TO AUTOCATALYTIC HYDROLYSIS OF SOME COMPOUNDS

Reactant	Ethyl Acetate (9)	Acetyl β-Methyl Choline (14)		Sodium Ethyl Sulfate (15)	Acetylsalicylic Acid (16)	Methyl DL-α-Phenyl-2-piperidyl Acetate HCl (17)		Phosphoramidic Acid (20)
Temp., °C.	25	25	60	90	25	25	80	36.8
pKa$_1$	...	...	...	...	3.69	8.8	[a]	3.0[b]
pH-Determining product	Acetic acid	Acetic acid	...	Sodium bisulfate	Salicylic acid	[a]	[a]	Ammonium biphosphate
pKa$_2$	...	...	...	...	2.97	[a]	[a]	7.2[b]
e	4.75	4.75	4.8	2.9	0.81	(1.0)[c]	(1.0)[c]	1.0
f	1.0	1.0	1.0	1.0	(0.91)[d]	...	...	0.555
k_H[e]	...	...	...	...	1.50 × 10^{-3}	7.02 × 10^{-6}	2.87 × 10^{-3}	7.17 × 10^{-3}
k_0[e]	6.50 × 10^{-6}	3.59 × 10^{-3}	1.91 × 10^{-2}	3.30 × 10^{-3}	[g]	[f]	[f]	
k_{OH}[e]	[f]	[f]	[f]	7.92 × 10^{-6}	[g]	7.59 × 10^{2}	1.48 × 10^{4}	
a	6.5	66.7	385.2	...	...	...	...	1.29 × 10^{-2}
b	...	...	...	2.4 × 10^{-3}	...	...	...	
pH$_0$	1.0 × 10^{-11}	1.85 × 10^{-11}	1.93 × 10^{-9}	...	...	1.08 × 10^{-7}	1.33 × 10^{-6}	
$t_{10\%}$ (min.)[h,i]	7.0	7.0	6.51	6.23	2.86[h]	5.4[h]	...	2.57[h]
Pseudo-1st order	1.6 × 10^{5}	1.56 × 10^{4}	8.74 × 10^{2}	1.31 × 10^{4}	5.02 × 10^{4}	5.46 × 10^{4}	...	4.1
Autocatalytic	3.59 × 10^{5}	6.32 × 10^{5}	1.17 × 10^{5}	5.66 × 10^{3}	5.0 × 10^{4}	(1.95 × 10^{5})[j]	...	11.9

[a] Values not available. [b] Values listed are for 25°, not the temperature specified. [c] Estimated values. [d] Value for the second degradation product, acetic acid, indicates that it is not a pH-determining product. [e] L. mole^{-1} sec.$^{-1}$. [f] Values are too small to have a significant effect or be measurable. [g] Values are not relevant to autocatalytic degradation. [h] Computed assuming that initial reactant concentration was 0.01 M. [i] Computed assuming that pKa$_2$ = 3.0. [j] Computed neglecting ionic strength effects.

10b is substituted in Eq. 1b leads to a solution of the same form as Eq. 12, but with less complex coefficients. For the particular case where $a = b = 0$, the solution may be written as

$$\tanh^{-1}\sqrt{\frac{\beta - [N]}{\beta}} = \tanh^{-1}\sqrt{\frac{\beta - [N_0]}{\beta}} + \frac{k_H\beta^{1/2}Ka_2^{1/2}}{2}t \quad \text{(Eq. 16)}$$

If only specific base catalyzed-degradation is considered, *i.e.*, $k_H = k_0 = 0$, then integration of the reduced form of Eq. 1a substituted by Eq. 10a results in

$$\tanh^{-1}\sqrt{\frac{\beta - [N]}{\beta}} - \sqrt{\frac{\beta - [N]}{\beta}} + \frac{Ka_2^{1/2}}{4\beta^{1/2}}\ln\frac{[N]}{[N_0]} = \tanh^{-1}\sqrt{\frac{\beta - [N_0]}{\beta}} - \sqrt{\frac{\beta - [N_0]}{\beta}} + \frac{k_{OH}Kw}{2\beta^{1/2}Ka_2^{1/2}}t \quad \text{(Eq. 17)}$$

Equation 17 can be reduced to a first-order expression, if $\beta \sim [N_0]$, which applies during the very early stages of degradation, since $\tanh^{-1}\sqrt{x} \sim \sqrt{x}$ when $x < 0.07$.

Case III. Homogeneous Degradation of Weak Acids Which Form Weak Acids.—The pH of a solution of a weak acid HA_1 with dissociation constant Ka_1 prior to degradation and in the absence of other acids of comparable or greater strength will be determined by the concentration of that acid. If upon degradation another weak acid HA_2 with dissociation constant Ka_2 is formed, the pH of the system will shift in proportion to the relative values of the respective dissociation constants as well as to the extent of degradation. Let $[A_1] = [HA_1] + [A_1^-]$ be the concentration of reactant and $[A_2] = [HA_2] + [A_2^-]$ the concentration of product at any time t. During the course of degradation, the $[H^+]$ function may be represented by $[H^+] = [A_1^-] + [A_2^-]$. Therefore

$$[H^+]^2 - Ka_1[HA_1] - Ka_2[HA_2] = 0 \quad \text{(Eq. 18}a\text{)}$$

Since $[A_0] - [A_1] = [HA_2] + [A_2^-]$ where $[A_0]$ is the reactant concentration at $t = 0$

$$[H^+]^2 - Ka_1\{[A_1] - [A_1^-]\} - Ka_2\{[A_0] - [A_1] - (A_2^-]\} = 0 \quad \text{(Eq. 18}b\text{)}$$

To simplify Eq. 18b one of two limiting assumptions may be made. If we assume that $Ka_2 > Ka_1$, the pH of the system will be determined largely by the dissociation of the product, except during the early stages of degradation. Furthermore, since $[HA_1] \gg [A_1^-]$, except during the latter stages of degradation, Eq. 18b can be revised to

$$[H^+]^2 + Ka_2[H^+] - Ka_1[A_1] - Ka_2\{[A_0] - [A_1]\} = 0 \quad \text{(Eq. 18}c\text{)}$$

whose root is

$$[H^+] = \frac{1}{2}\sqrt{Ka_2^2 + 4Ka_2[A_0] - 4(Ka_2 - Ka_1)[A_1]} - Ka_2/2 \quad \text{(Eq. 19}a\text{)}$$

or

$$[H^+] = Ka_2^{1/2}\left\{\sqrt{\beta - e[A_1]} - Ka_2^{1/2}/2\right\} \quad \text{(Eq. 19}b\text{)}$$

where $\beta = \{Ka_2/4 + [A_0]\}$ and $e = 1 - Ka_1/Ka_2$.

The properties of Eqs. 19*a* and 19*b* can readily be ascertained by considering two limiting conditions. If $Ka_1 = Ka_2$, then $e = 0$ and Eq. 19*a* reduces to

$$[H^+] = [H_0] = \frac{1}{2}\sqrt{Ka_1^2 + 4Ka_1[A_0]} - Ka_1/2 \quad \text{(Eq. 20}a\text{)}$$

which gives the $[H^+]$ of a solution of reactant prior to degradation. The pH of the solution will be constant and the degradation will be first order. On the other hand, if $Ka_2 \gg 100Ka_1$, then $e \sim 1$ and Eq. 19*b* reduces to

$$[H^+] = Ka_2^{1/2}\{\sqrt{\beta - [A_1]} - Ka_2^{1/2}/2\} \quad \text{(Eq. 20}b\text{)}$$

which is of the same form as Eq. 10*a*. Comparison of Eqs. 19*b* and 10*a* indicates that Eqs. 13–15 also apply to the degradation of weak acids in homogeneous systems when appropriate changes in symbols are made, *e.g.*, $e \cdot [A_1]$ replaces $[N]$, etc.

If the degradation product were a weaker acid than the reactant, *i.e.*, $Ka_1 > Ka_2$, the pH of the system would be largely determined by the reactant concentration, except during the latter stages of degradation. It follows from Eq. 18*b* that in this instance

$$[H^+] = Ka_1^{1/2}\{\sqrt{\beta + f[A_1]} - Ka_1^{1/2}/2\} \quad \text{(Eq. 21}a\text{)}$$

where $\beta = \{Ka_1/4 + [A_0]Ka_2/Ka_1\}$ and $f = 1 - Ka_2/Ka_1$. If $Ka_1 \gg 100Ka_2$, then $f \sim 1$ and

$$[H^+] = Ka_1^{1/2}\{\sqrt{\beta + [A_1]} - Ka_1^{1/2}/2\} \quad \text{(Eq. 21}b\text{)}$$

Solutions of Eq. 1*b* substituted by Eq. 21*a* similar in form to Eqs. 13–15 may be derived.

More than one weak acid may be formed in a degradation. The pH of the system will be controlled by the strongest acid present as long as sufficient differences in dissociation constants exist. If HA_1 on degradation forms HA_2 and HA_3, assuming that $Ka_2 > Ka_3$ and Ka_1, a $[H^+]$ function can be derived so that

$$[H^+] = Ka_2^{1/2}\{\sqrt{Ka_2/4 + (1 + Ka_3/Ka_2)[A_0] - (1 + Ka_3/Ka_2 - Ka_1/Ka_2)[A_1]} - Ka_2^{1/2}/2\} \quad \text{(Eq. 22)}$$

This function should adequately describe the pH shift in homogeneous systems, except during the early stages of degradation. It reduces to Eq. 19*b* if $Ka_2 \gg 100\ Ka_3$.

EXPERIMENTAL

Equation 1*b* with appropriate $[H^+]$ functions substituted was programmed and solved on an analog computer. Programs were designed so that changes in reactant concentration, hydrogen-ion concentration, or overall rate of degradation could be followed with respect to time. The effect of variation in the following parameters on the kinetics of degradation was investigated: (*a*) dissociation constants of reactants and products, (*b*) initial concentration of reactants, (*c*) spontaneous or hydroxyl ion–catalyzed reactions in parallel with hydrogen ion–catalyzed degradation, and (*d*) concentration of weak acid other than reactant or product. Computer solutions and results computed from the analytical solutions generally agreed to within better than ±2% (the range of error anticipated with the particular computer and components employed) when crosschecked, thus supporting their validity.

RESULTS AND DISCUSSION

Solvolysis Rates, Acidity, and Concentration.—The essential features of the specific acid-catalyzed hydrolysis of a neutral molecule in solution uncomplicated by the existence of competitive spontaneous or specific base-catalyzed reactions are expressed by Eq. 4 if the degradation product is a strong acid and Eq. 13 if a weak acid.[7] Figures 1 and 2 show the *S*-shaped curves characteristic of autocatalytic processes as they would be observed in reactions of the type described in the absence of acid other than that derived from the degradation. In these instances the reaction is initiated by $[H^+]$ resulting from the dissociation of water. These curves may be viewed as limiting cases indicative of the slowest

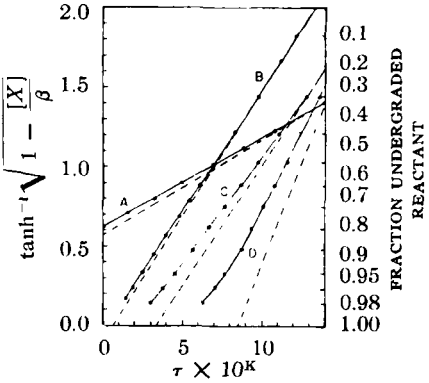


Fig. 4.—Analysis, using Eq. 16, of computer simulated degradation of neutral compounds which form weak acids as products and are subject only to specific acid catalysis in unbuffered solution: curve *A*, $pKa_2 = pKa_0 = 4.0$, $[N_0] = 0.01\ M$, $C_0 = 0.0044\ M$, $\beta = 0.0144\ M$, $K = -2.0$; curve *B*, $pKa_2 = 4.0$, $\beta = [N_0] = 0.1 \cdot M$, $K = -2.0$; curve *C*, $pKa_2 = 4.0$, $\beta = [N_0] = 0.01\ M$, $K = -2.477$; curve *D*, $pKa_2 = 2.0$, $\beta = [N_0] = 0.1\ M$, $K = -1.204$. Key: O represent values computed from Eq. 16; dashed lines represent the theoretical slope for each case $= \sqrt{\beta K a_2}/2$.

degradation rate observable for the particular conditions they represent. Data are plotted as a function of a time parameter $\tau = k_H t$. The time scale is defined so that it is independent of the actual value of the rate constant k_H.

The most important feature of an autocatalytic process is its induction period. Reactions characterized by Eqs. 4 and 13 significantly begin to accelerate only when the hydrogen-ion concentration becomes a significant fraction (*ca.* 1%) of the reactant concentration. As shown by the insets in Figs. 1 and 2, the bulk of the observed pH shift takes place during the induction period. For cases represented by Eq. 4, the time course of degradation is symmetrical, the rate of degradation being a maximum at $[N] = {}^1/_2[N_0]$ (curve *B*, Fig. 1). Degradations better represented by Eq. 13 are unsymmetrical, the rate being a maximum at $[N] = {}^2/_3[N_0]$ (curve *B*, Fig. 2).[8]

[7] Dawson and Lowson have established the validity of Eq. 13 by application to the autocatalyzed hydrolysis of ethyl acetate (9).

[8] One can readily demonstrate that $d[N]/dt$ will be a maximum at $[N] \sim {}^2/_3\ \beta$ by differentiation of the equation $-(d[N]/dt) = k_H[H^+][N]$, where $[H^+]$ is given by Eq. 10*b*. In the example described by Fig. 2, $\beta = [N_0]$.

A convenient measure of the induction period which is also a pharmaceutically significant parameter is the time required for 10% degradation, *i.e.*, τ_{10} in terms of the time parameter. Data comparing the magnitude of decreases in τ_{10} produced by increases in product acidity and reactant concentration are shown in Fig. 3. If $[N_0] - [N] < 10\ Ka_2$, Eqs. 13 and 4 give substantially the same result. The acid can be considered to be effectively 100% dissociated, thus behaving as a strong acid.

If acid other than that owing to the degradation is initially present in the system, it can be seen from the definition of β (Eq. 10*a*) that its effect would be to increase $[N_0]$ by the amount Ka_0C_0/Ka_2 as well as decrease the initial pH (Eq. 11), thus acting to reduce the induction period. The weak acid normally present in aqueous solution will be carbonic acid (pK_1 6.35 at 25°) which results from solution of atmospheric carbon dioxide. A good grade of distilled water in equilibrium with a normal atmosphere has a pH $\sim$ 5.5. For this system the product Ka_0C_0 will be $\sim 10^{-11}$, a value too small to change β, yet significant enough to reduce τ_{10} because of its effect on the initial pH of the system. The extent of reduction in τ_{10} produced by carbonic acid contaminated water compared to carbon dioxide–free water is also shown in Fig. 3. The magnitude of this effect is an inverse function of $[N_0]$ and a direct function of Ka_2.

When a weak acid decomposes to form another stronger weak acid, the pH of the solution is initially fixed at a value determined by the concentration and acidity of the reactant (Eq. 20*a*). τ_{10} will be shortened in proportion to the relative values of the two dissociation constants involved. No actual induction period will exist if the initial pH of the system is low (<4) or if $Ka_1/Ka_2 > 0.01$ ($e < 1$). Equation 19*b* was derived assuming that the dissociation of reactant was negligible. This assumption fails if the reactant concentration is small ($\sim$0.01 *M*). Equation 19*b* predicts that the $[H^+]$ during the early stages of degradation can be smaller than the $[H_0]$ calculated for the system (Eq. 20*a*), particularly if $e < 1$. The initial small increase in pH which should be observed is indicative of the shifting in control of acidity of the system from reactant to product. A good example is acetylsalicylic acid which is cited in Table IV. This table lists relevant kinetic parameters for several compounds of the types considered in this report and compares predicted $t_{10\%}$-values for autocatalytic and pseudo-first-order (at constant $[H^+] = [H_0]$) processes. If 10% degradation has taken place in an 0.01 *M* solution of aspirin, the calculated $[H^+]$ of the system is 1.25×10^{-3} *M*, whereas $[H_0]$ is 1.37×10^{-3} *M*. The effect is small enough so that pseudo-first order degradation can be assumed for purposes of analysis. If 50% degradation has taken place, $[H^+]$ is 2.05×10^{-3} *M*, indicating that the reaction rate is now controlled by the acidity of the salicylic acid. An equation of the form of Eq. 13 then more accurately describes the process.

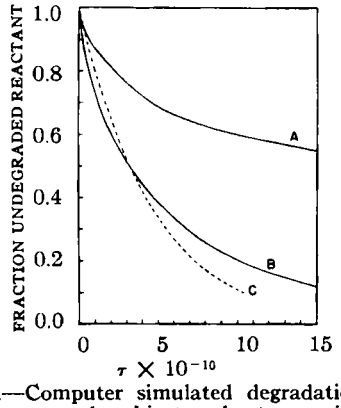


Fig. 5.—Computer simulated degradation of a neutral compound subject only to specific base catalysis in unbuffered solution: curve *A*, degradation product is a strong acid (defined by Eq. 8), $[N_0] = 0.01\ M$; curve *B*, degradation product is a weak acid $pKa_2 = 4.0$ (defined by Eq. 17), $[N_0] = 0.01\ M$. Curve *C* represents a first-order degradation shown for purposes of comparison.

If the functional group being hydrolyzed is not acidic, one would generally expect that at least one of the degradation products of a weak acid would be stronger acid or one comparable in strength to the reactant. If an acidic group is degraded, acids weaker than the reactant may only be formed. One example of this type is phosphoramidic acid (see Table IV) which would exhibit a negative autocatalytic effect upon degradation in unbuffered solution since the $[H^+]$ continually decreases in proportion to the degree of reaction.

Equation 13 is cumbersome; computations made with it are involved. Unfortunately, the less complex Eq. 16 will not give a valid prediction of τ_{10}, since the assumption upon which it is based (in effect that the dissociation of the product is negligible[9]) fails in the early stages of degradation. The degree of error observed in calculating τ_{10} using Eq. 16 is shown in Fig. 3; it decreases in proportion to product pKa and reactant concentration. A comparison of the form of solutions obtained for Eqs. 13 and 16 is shown in Fig. 2 (curves *A* and *C*, respectively). These curves illustrate the failure of Eq. 16 to account for the induction period accurately, because it makes no provision for the initial state of the system. However, Eq. 16 may have application to the analysis of stability data obtained in the absence of pH control when autocatalytic processes of the type described are involved. It predicts that a straight line with slope equal to $k_H\beta^{\frac{1}{2}}Ka_2^{\frac{1}{2}}/2$ will be obtained if $\tanh^{-1}\{1 - [X]/\beta\}$ is plotted as a function of time. Plots of this type are shown in Fig. 4 which illustrates the limitation of the technique: reactant concentration must be large (1 − 0.1 *M*), and only data corresponding to the latter stages of degradation should be weighted. These limitations may be overcome in part if the acid degradation product is initially added to the system so that $\beta = [X_0] + C_0$ (the concentration of added acid). Dawson and Lowson (8) have derived an equivalent form of Eq. 16 which they applied to a study of the acetic acid catalyzed hydrolysis of ethyl acetate.

Competitive First-Order and Hydroxyl Ion–Catalyzed Processes.—Specific base-catalyzed or spontaneous degradation also result in reduction of the autocatalytic induction period. Figure 5 shows the course of degradation if it is only specific base-catalyzed. These curves are characterized by a rapid initial rate of degradation which decreases in proportion to the acidity of the product. When

[9] This assumption is implicit in the derivation of equations generally used for estimating pH (21).

both specific base-catalyzed and acid-catalyzed processes are in competition, the rate of degradation passes through a minimum (Fig. 6) which corresponds to that point in time where the reaction changes from one controlled by the pOH of the system to one controlled by the pH. At this point $[H^+] = \sqrt{b}$ (the catenary of the pH-rate profile), since the rates of specific acid and base catalysis are equal. The $\tan^{-1}$ terms which appear in Eqs. 6 and 15 significantly influence computations only in the vicinity of the minimum.[10]

The relative importance of specific base catalysis in influencing the overall degradative rate is measured by the b-value, a few examples of which are listed in Table IV to show the range in values which may be observed. Figure 7 shows the variation in τ_{10} as a function of b at different levels of product acidity and reactant concentration. Comparison is made with τ_{10}-values computed assuming pseudo-first order kinetics at constant $[H^+] = [H_0]$. If b has a relatively large value, the system will be more stable in unbuffered solution than when maintained at constant pH_0; if it has a small value, the system will be less stable in unbuffered solution.

Where spontaneous hydrolysis is important in initiating degradation, the initial rate will be first

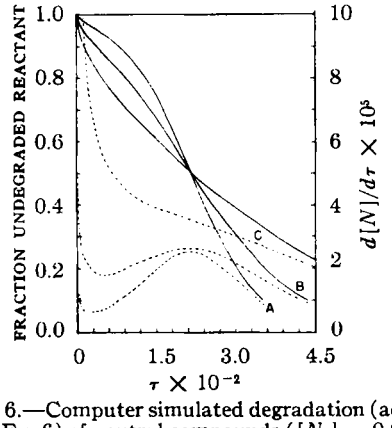


Fig. 6.—Computer simulated degradation (according to Eq. 6) of neutral compounds ($[N_0] = 0.01\ M$) which form strong acids as products and are subject to both specific acid and base catalysis: curve A, $b = 10^{-7}$; curve B, $b = 10^{-6}$; and curve C, $b = 10^{-5}$. For purposes of comparison, k_H was adjusted so that τ_{50}-values were equal. Key: ———, represents plots of fraction of undegraded reactant as a function of τ; – – – –, represents plots of rate of degradation, $d[N]/d\tau$, as a function of τ.

order. Its relative importance in the overall degradation is measured by the a-value (see Table IV). Figure 8 shows the variation in τ_{10} as a function of a at different levels of reactant concentration and product acidity. It gives an indication of the effective range of application of Eqs. 5 and 14. It should be noted that phosphoramidic acid would show transition from autocatalytic to first-order control as its degradation proceeded, in contrast to the data presented in Fig. 8.

[10] Dawson and Lowson (9) divided their analysis of the autocatalytic degradation of ethyl acetate into two stages. In the first stage they evaluated the combined catalytic action of hydrogen-ion and hydroxyl-ion by numerical solution of the differential equation. They employed a form of Eq. 13 in their analysis of the second stage which dealt with only hydrogen-ion catalysis.

Temperature and Solvent Effects.—The effect of temperature on the autocatalytic rate process will depend upon the degree of thermal variation of the dissociation constants of all acid species present (including the solvent) and on the energies of activation of the reactions involved. The dissociation constant may increase or decrease with temperature depending upon the nature of the acid and the temperature range (22). However, the effect is sufficiently small in the case of carboxylic acids to be neglected. The significant increase in acidity of water observed at elevated temperatures will be reflected as an increase in value of b (Table IV). The relative dependency of autocatalytic degradation on hydroxyl-ion catalysis is thereby increased.

The solvent effects which can be analyzed are those owing to moderate changes in dielectric constant and ionic strength. Their influence on the rate constant has been detailed by Garrett (1). It

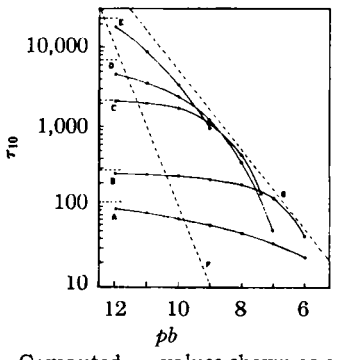


Fig. 7.—Computed τ_{10}-values shown as a function of $b = k_{OH}k_w/k_H$: curves A ($[N_0] = 0.1\ M$) and D ($[N_0] = 0.001\ M$), degradation product is a strong acid, defined by Eq. 6; curve B ($[N_0] = 0.1\ M$), degradation product is a weak acid ($pKa_2 = 4.0$), defined by Eq. 15; curves C ($[N_0] = 0.1\ M$) and E ($[N_0] = 0.001\ M$), degradation product is a weak acid ($pKa_2 = 6.0$), defined by Eq. 15. Curve F represents τ_{10}-values computed assuming pseudo-first order degradation at constant $pH = pH_0 = 7.0$. Curve G represents values computed assuming pseudo-first order degradation at constant $pH = p\sqrt{b}$.

should be noted that increases in ionic strength tend to increase the apparent dissociation constants of weak acids; decreases in dielectric constant tend to decrease them (22). Since the present theory has been developed in terms of the classical dissociation constant, the exact analysis of these effects requires a knowledge of f_H and f_{OH} for the specific system being studied (see *Footnote 3*). In most practical work activity coefficients can be assumed to be unity. The effect of changing ionic strength as a consequence of degradation cannot readily be quantified. Observed deviations from results predicted for autocatalysis, owing to changes in ionic strength, will be especially significant if the degradation product is a strong acid and the reactant concentration is large.

CONCLUSIONS

The validity of the equations which have been presented to describe autocatalytic degradation in systems of the type considered in this report depend on the validity assigned to the $[H^+]$ functions derived. For purposes of estimating stability (as

measured by $t_{10\%}$) in unbuffered aqueous pharmaceutical systems, the exact expressions (*e.g.*, Eqs. 13–15) must be employed. The examples of computed $t_{10\%}$-values shown in Table IV indicate that significant errors can be made in estimates of stability if pseudo-first order degradation is assumed.

The models discussed fail to take into account general acid and base catalysis or reversible reactions such as those which might be observed in simple ester hydrolysis. However, these effects certainly can be neglected for estimation of $t_{10\%}$.

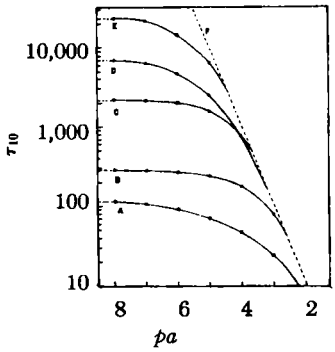


Fig. 8.—Computed τ_{10}-values shown as a function of $a = k_0/k_H$: curves A ($[N_0] = 0.1\ M$) and D ($[N_0] = 0.001\ M$), degradation product is a strong acid, defined by Eq. 5; curve B ($[N_0] = 0.1\ M$), degradation product is a weak acid ($pK_{a_2} = 4.0$), defined by Eq. 14; curves C ($[N_0] = 0.1\ M$) and E ($[N_0] = 0.001\ M$), degradation product is a weak acid ($pK_{a_2} = 6.0$), defined by Eq. 14. Curve F represents τ_{10}-values computed assuming pseudo-first order degradation at constant $pH = pH_0 = 7.0$.

Other cases can be considered, such as degradation of neutral molecules or weak bases which form weak bases as products and are subject primarily to specific base catalysis in unbuffered solution. Treatment of these cases leads to solutions of the same form as those described for *Cases II* and *III*.

The power of the analog computer as a tool in analyzing cases of this sort can readily be ascertained by noting the complexity of the solutions obtained to the differential equations. The computer will prove especially useful in analyzing systems where analytical solutions cannot readily be arrived at—for example, cases complicated by reversible reactions.

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Several known antiemetics and other compounds were evaluated for their ability to prevent protoveratrine induced emesis in dogs and found to be ineffective.

Protoveratrine produces a potent reflex vasodepressor effect in dogs and humans (1). However, the range between the therapeutic and the toxic dose is quite narrow (2), and it is often difficult to obtain good clinical results without some side effects; the major side effects are nausea and vomiting. Alterations in the protoveratrine molecule to increase the margin between effectiveness and nausea have been attempted (3). A second approach has been directed at the prevention of nausea and vomiting by the administration of antiemetic agents.

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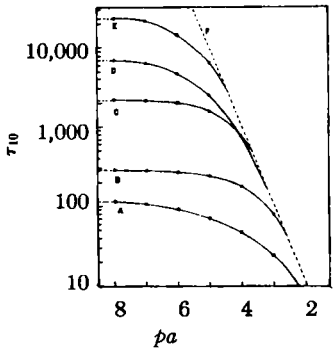


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TABLE I.—RELATIVE EMETIC POTENCIES OF VERATRUM ALKALOIDS IN DOGS FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION

	Emetic Dose 50					
	Intravenous			Oral		
Drug	No. Dogs	ED_{50}, mcg./Kg.	Slope	No. Dogs	ED_{50}, mcg./Kg.	Slope
Protoveratrine A	29[a]	3.24 (2.63 to 3.99)[b]	1.6 (0.9 to 3.0)	24	3.4 (2.9 to 4.0)	1.5 (1.3 to 1.7)
Protoveratrine B	40	3.63 (3.03 to 4.36)	1.4 (1.1 to 1.8)	20	20.0 (15.9 to 25.2)	1.3 (0.9 to 2.1)
PAB	37[a]	3.82 (3.38 to 4.32)	1.3 (1.1 to 1.5)	...	...	...

[a] From Abreu, Richards, Alexander, and Weaver (1954). [b] 95% confidence limits.

This report explores the feasibility of the latter procedure.

METHODS

Adult mongrel dogs, unselected as to sex, were used in all experiments. They were maintained on an adequate diet consisting of canned horse meat and dry pellets with water given *ad libitum*, except during the period of testing.

Stock solutions of protoveratrine A and protoveratrine B were prepared by dissolving a sufficient amount of each in its stochiometric equivalent of glacial acetic acid, then adding sufficient distilled water to make a 0.1% concentration of the base. These stock solutions were refrigerated and dilutions freshly prepared from them prior to each experiment. A commercially available solution of the proveratrines A and B (PAB)[1] was used.

The emetic doses for 50% of animals (ED_{50}) and their slopes were calculated by the method of Litchfield and Wilcoxon (4).

An emetic dose of protoveratrine A was injected at different rates and in different volumes of solvent to determine the importance of these factors on the occurrence of the side effects which usually accompany its administration.

In some experiments the intravenous (i.v.) protoveratrine A challenge (6 mcg./Kg.) was repeated at 15, 30, 40, 120, or 135-minute time intervals to determine whether there was a development of tolerance to certain responses, particularly the convulsive (collapse and leg extension) and emetic effects, upon repeated administration.

Several drugs with known and unknown antiemetic properties were tested for their ability to prevent protoveratrine A emesis in dogs. All animals were observed for 60 minutes following the injection of protoveratrine A (5.6 mcg./Kg., i.v.). The procedure for the preliminary evaluation of 4'-fluoro-4(1-[4-hydroxy-4-(4'-chloro)-phenylpiperidino])-butyrophenone (haloperidol) can best be followed by referring to Table IV. A subsequent experiment was performed in which three dogs each received orally 1, 2, 5, 10, and 20 mg./Kg./day for 4 days each and then were challenged 4 hours after final dose with protoveratrine A.

Dogs being used to establish dosage levels of piperacetazine[2] for chronic toxicity studies were tested to determine the effects of piperacetazine on protoveratrine A emesis. One dog each received orally 0.1, 0.25, 0.5, or 1.0 mg./Kg./day for 7 days, respectively, then 0.5, 1.25, 2.5, or 5.0 mg./Kg./day for 14 days, followed by 7 days without drug, and finally 2.5, 5.0, 2.5, or 5.0 mg./Kg./day for 7 days. They were challenged with protoveratrine A 2 hours after the last dose. Two additional dogs received 5 mg./Kg./day of piperacetazine for 21 or 44 days. The first dog was challenged after 6 and 21 days; the other dog was challenged after 44 days.

PAB (6.3 mcg./Kg.) was employed as the emetic agent in the final experiment of this series. The challenging dose was administered to six dogs after chlorphenoxamine[3] (10 mg./Kg., i.v.) and simultaneously with reserpine (32 mcg./Kg., i.v.) in another six dogs.

TABLE II.—EFFECT OF ALTERING CONCENTRATION AND/OR INJECTION RATE ON UNTOWARD RESPONSES TO PROTOVERATRINE ADMINISTRATION

	Proto. A, 6 mcg./Kg., i.v.[a]				
Dog	Vol., ml.	Rate, sec.	Collapse and/or Convulsions	Salivation Diarrhea and/or Urination	Onset Emesis, min.
♀	0.42	2	+	+	11
7.0	5.0	30	0	+	3
Kg.	5.0	30	0	+	4
♂	0.46	2	0	+	5
7.7	5.0	30	+	+	4
Kg.	5.0	30	+	+	3
♀	0.39	2	+	+	15
6.5	5.0	30	0	+	5
Kg.	5.0	30	+	+	4
♂	0.64	2	+	+	6
10.6	5.0	30	+	+	2
Kg.	5.0[b]	30	0	+	60

[a] Time between protoveratrine A injections in the same dog was 48 hours or more. [b] A portion of the drug went subcutaneously.

RESULTS

The emetic potencies of the veratrum alkaloids in dogs following intravenous and oral administration are shown in Table I. It is readily apparent that there was no significant difference among the emetic potencies of the three preparations following intravenous administration or between the two routes of administration following protoveratrine A. On the other hand, protoveratrine B was considerably less active following oral administration.

Neither the rate of injection nor volume of solvent administered consistently altered the side effects usually seen after the intravenous injection of an emetic dose of protoveratrine A. The side effects usually observed in the customary order of their appearance were: collapse, tonic extension of all legs,

[1] Veralba, Pitman-Moore Co. brand of protoveratrines A and B, containing 53% A and 47 B standardized chemically, both for total alkaloids and for A and B content.

[2] Available from the Pitman-Moore Co. for experimental use under the trademark, Quide.

[3] Available from Pitman-Moore Co. under the trademark, Phenoxene.

TABLE III.—EFFECT OF DRUGS ON PROTOVERATRINE A INDUCED EMESIS IN DOGS

Drug	Dose, mg./Kg.	Route	Interval,[a] min.	No. Emesis/ No. Dogs
		Antiemetics		
Perphenazine	0.1	i.v.	15	1/1
	0.1	i.v.	35	2/2
	0.1	i.v.	60	1/1
Piperacetazine	1.5[b]	i.g.	60	4/4
Pipamazine	0.002	i.v.	30	3/3
Prochlorperazine	0.1	i.v.	15	1/2
	0.25	i.v.	20	2/2
Triflupromazine	5.0	s.c.	5	2/3
Trimethobenzamide hydrochloride	25.0	i.g.	60	1/2
	50.0	i.g.	60	2/2
	100.0	i.g.	40	2/2
Meclizine	5.0	i.v.	10	3/3
		Miscellaneous		
Atropine sulfate	1.0	i.v.	45	3/3
	1.0	i.v.	60	3/3
	2.0	i.v.	15	3/3
Nicotinic Acid	10.0	i.v.	10	2/4
	10.0	i.v.	20	2/3
P-275[c]	250.0[b]	i.g.	60	4/4

[a] Between drug administration and protoveratrine A (5.6 mcg./Kg., i.v.) challenge. [b] Dose repeated three times—challenge 60 minutes after last dose. [c] *N,N*-Diisopropyl-*N'*-*n*-butyl-*N'*-diethylaminoethylurea.

central nervous system depressant side effects from haloperidol appeared during the 5 mg./Kg./day dosage and continued throughout the remainder of the experiment.

Complete protection against protoveratrine A induced emesis was obtained in the four dogs receiving increasing dosage of piperacetazine but not by the other two dogs. Subsequent attempts to control protoveratrine emesis have not been successful; the conclusion is that chronic administration is also ineffective.

Chlorphenoxamine and reserpine were each successful 50% of the time in preventing PAB-induced emesis.

DISCUSSION

Protoveratrines A and B are very potent hypotensive and emetic agents in the dog. Protoveratrine A is equally potent as an emetic by the intravenous and oral routes. On the other hand, protoveratrine B is only one-fifth as potent as an emetic by the oral as by the intravenous route. Similar findings have been reported for protoveratrine B in the human (5).

It is interesting that neither the rate of injection nor the volume of solution containing the emetic dose of protoveratrine A were critical within the limits of this test. Furthermore, successive doses at various intervals produced emesis but not convulsive type activity in six of seven cases. These results do not conflict with earlier work of a similar nature (6).

TABLE IV.—EFFECT OF HALOPERIDOL ON PROTOVERATRINE A INDUCED EMESIS IN DOGS

Day	Dog 1			Dog 2			Dog 3			Dog 4			Dog 5		
	H[a]	P[b]	E[c]	H	P	E	H	P	E	H	P	E	H	P	E
1	0	+	+	100	+	0	100	+	+	200	+	+	200	+	0
2	0	0	0	0	0	0	100	0	0	100	0	0	0	0	0
3	0	0	0	0	0	0	100	0	0	200	0	0	0	0	0
4	200	+	+	0	+	+	100	+	0	200	+	+	0	+	+
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	200	+	+	100	+	+	180	+	0	200	+	+	200	+	+

[a] Haloperidol dose in mcg./Kg., i.v.—challenge 60 minutes after drug. [b] Protoveratrine A: +, 5.6 mcg./Kg., i.v.; 0, no drug. [c] Emesis: +, present; 0, absent.

some convulsive-like activity, urination, diarrhea, salivation, and emesis. Often these effects occurred in such a rapid sequence that it was difficult to determine which came first. The results are presented in Table II.

When the protoveratrine A challenge (6 mcg./Kg., i.v.) was repeated after an interval of 15, 30, 40, 120, or 135 minutes, all dogs vomited regardless of the time interval. Of the seven dogs used in this experiment, six vomited after the first injection of protoveratrine A. Only the dog which failed to vomit after the first dose showed the convulsive movements after the second protoveratrine A dose.

None of the compounds tested proved to alter significantly the emetic effects of protoveratrine A (Table III) under the conditions of the experiment. For the most part, none of the other side effects were altered by these compounds. As expected, atropine prevented the salivation usually observed.

Haloperidol was inconsistent in preventing protoveratrine A induced emesis (Table IV) in the exploratory investigation and uniformly unsuccessful in preventing emesis and convulsive activity in the three dogs used in the subsequent study. Toxic

The investigator should not be misled by the constancy of emesis observed in data reported in Table II. Although most dogs showed emesis following a dose of about 6 mcg./Kg., we have found dogs who failed to respond or did so inconsistently. Thus, *protection* as first obtained with chronic administration of piperacetazine must be viewed with suspicion until several dogs are used.

That many agents have been reported to reduce the susceptibility of the dog to the emetic effect of apomorphine is well documented. On the other hand, no compound has been found successful in preventing emesis induced by protoveratrine A in the dog. A decade ago chlorpromazine was shown to be effective against apomorphine (7–9) but not veratrum (10). More recently, chlorpromazine, triflupromazine, perphenazine, and fluphenazine were also found effective against apomorphine induced but not veratrum induced emesis (11). We confirmed this work for triflupromazine and perphenazine. Furthermore, three additional phenothiazines (pipamazine, piperacetazine, and prochlorperazine) were shown to be ineffective against protoveratrine induced emesis.

Compounds other than phenothiazines were also investigated for their ability to block protoveratrine induced emesis. Three potent antiemetic agents, haloperidol, meclizine, and trimethobenzamide (12, 13) were found to be without effect. Similarly, atropine, nicotinic acid, and P-275, which have not been reported to be antiemetic, were ineffective. Furthermore, chlorphenoxamine and reserpine did not consistently block PAB emesis. Other workers (6, 14) have reported that parasympatholytics (atropine, scopolamine, and methantheline), a ganglionic blocker (tetraethylammonium), a sympathomimetic (ephedrine), an antihistaminic (dimenhydrinate) and reserpine failed to prevent veratrum emesis in dogs.

Until more data are forthcoming on the mechanism of the emetic action of protoveratrine A, the inability of potent antiemetics to prevent the effect will remain obscure.

SUMMARY

Protoveratrine A is equally potent as an emetic agent by the intravenous and oral routes. On the other hand, protoveratrine B is only one-fifth as potent orally as by the i.v. route.

Neither the rate of injection nor the volume of solution containing the emetic dose of protoveratrine A is important with respect to modifying the side effects which usually accompany the intravenous injection of the drug.

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None of the drugs tested in this study were effective in preventing protoveratrine induced emesis in dogs.

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Gas Chromatography of Esters of Plant Acids and Their Identification in Plant Materials

By HARMON M. KELLOGG, E. BROCHMANN-HANSSEN, and A. BAERHEIM SVENDSEN*

The gas chromatographic retention data of methyl and ethyl esters of a number of common plant acids are reported. Special emphasis is placed on the acids of the Krebs cycle. Of four liquid phases used, Apiezon L appears to be most generally applicable and the only one which permits satisfactory separation of fumarate and succinate. The esters of citric acid and isocitric acid (lactone) are readily separated on all four columns. The method has been used for identification of the acids in various plant materials. It is sensitive and selective and should be valuable for biochemical studies concerning the production and function of organic acids in plants.

PLANTS CONTAIN a large number of organic acids, some of which may be characteristic of certain plant families. Others, such as a group of di and tricarboxylic acids, are found more widely distributed throughout the vegetable kingdom and are usually referred to simply as the plant acids.

In 1937, Krebs and Johnson (1) showed that these acids play a central role in cellular respiration, and the series of reactions which are involved is known as the Krebs cycle. Some of the acids of the Krebs cycle, such as succinic, fumaric, malic, and citric acids, are found in appreciable concentrations in practically every type of plant material. Most of the others are present only in small amounts and require sensitive and specific methods for their detection. The keto acids, particularly oxaloacetic acid, are unstable and may easily be lost during the isolation procedures commonly used for plant acids and may thus escape detection. The enzymes

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Compounds other than phenothiazines were also investigated for their ability to block protoveratrine induced emesis. Three potent antiemetic agents, haloperidol, meclizine, and trimethobenzamide (12, 13) were found to be without effect. Similarly, atropine, nicotinic acid, and P-275, which have not been reported to be antiemetic, were ineffective. Furthermore, chlorphenoxamine and reserpine did not consistently block PAB emesis. Other workers (6, 14) have reported that parasympatholytics (atropine, scopolamine, and methantheline), a ganglionic blocker (tetraethylammonium), a sympathomimetic (ephedrine), an antihistaminic (dimenhydrinate) and reserpine failed to prevent veratrum emesis in dogs.

Until more data are forthcoming on the mechanism of the emetic action of protoveratrine A, the inability of potent antiemetics to prevent the effect will remain obscure.

SUMMARY

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In 1937, Krebs and Johnson (1) showed that these acids play a central role in cellular respiration, and the series of reactions which are involved is known as the Krebs cycle. Some of the acids of the Krebs cycle, such as succinic, fumaric, malic, and citric acids, are found in appreciable concentrations in practically every type of plant material. Most of the others are present only in small amounts and require sensitive and specific methods for their detection. The keto acids, particularly oxaloacetic acid, are unstable and may easily be lost during the isolation procedures commonly used for plant acids and may thus escape detection. The enzymes

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required for the various steps of the Krebs cycle have also been identified in higher plants (2).

Certain plant acids belonging to the Krebs cycle tend to accumulate in special plant families. Isocitric acid, for example, is found in large concentration in succulents of the *Crassulaceae* family; before its chemistry and biological significance was known, it was commonly referred to as crassulacean malic acid (3). Aconitic acid has been found in many plants, but is present in large amounts in *Aconitum septentrionale* L. (4). Such an accumulation of individual plant acids indicates that there is a partial block of a particular step in the cycle due perhaps to a low enzyme concentration or the presence of an enzyme inhibitor (2).

TABLE I.—RELATIVE RETENTION TIMES OF METHYL ESTERS OF PLANT ACIDS

	Stationary Liquid		
Ester	SE-30[a]	Apiezon L	Craig Ester[b]
Oxalate	0.09	0.14	0.19
Malonate	0.20	0.22	0.23
Succinate	0.39	0.43	0.29
Fumarate	0.36	0.48	0.28
Oxaloacetate	0.66	1.04[c]	...
Malate	1.00	1.00	1.00
α-Ketoglutarate	1.85	1.60	1.12
Tartrate	3.15	2.55	2.80
Aconitate	10.42	4.56	2.06
Citrate	15.73	6.08	4.16
Lactoisocitrate	21.20	10.33	11.50
Temp., °C.	55	122	182
Inlet pressure p.s.i.	20	30	25
Outlet flow rate, ml./min.	74	...	...
Carrier gas	Argon	Nitrogen	Nitrogen
Support, mesh	60–80	60–80	60–80
Liquid phase, % w/w	4	15	10
Malate time, min.	6.1	5.9	6.5

[a] Methyl silicone polymer, General Electric Co. [b] Butanediol succinate. [c] The sample appeared to decompose on the column; no well defined peak was obtained.

Three dicarboxylic acids commonly found in plants are not components of the Krebs cycle—oxalic, malonic, and tartaric acid. It has been postulated that oxalic acid may be formed from glycolic acid *via* glyoxylic acid (2). One might also speculate concerning the function of malonic acid which is a highly specific inhibitor of succinic dehydrogenase.

For biochemical studies of the plant acids, it is imperative to have analytical methods which are suitable for qualitative and quantitative determination of micro quantities. The most common method for isolation of plant acids is based on precipitation with lead acetate or lead subacetate, followed by liberation of the free acids by hydrogen sulfide. The individual acids may then be identified and determined by fractional precipitation or by fractional distillation of their esters. However, neither of these methods produces complete separation nor permits determination of an acid present in small quantities. The ester-hydrazide method of Franzen, *et al.* (5, 6), represents a considerable advancement. More recent work (7) has made use of fairly specific micro methods based on chemical and enzymatic reactions. Ion-exchange resins and paper partition chromatography have in many instances given excellent results (8, 9). However, the separation of individual acids is often not sufficient to identify and determine small amounts of certain acids in the presence of large amounts of others.

It would seem that gas-liquid chromatography might offer considerable advantages over the methods presently used. It is extremely rapid and sensitive, highly selective, and lends itself equally well to qualitative and quantitative analysis. Furthermore, it permits determination of trace components of a mixture. Gas chromatography was developed by Martin and James

TABLE II.—RELATIVE RETENTION TIMES OF ETHYL ESTERS OF PLANT ACIDS

	Stationary Liquid			
Ester	SE-30	Apiezon L	Igepal[a]	Craig Ester
Oxalate	0.18	0.21	0.13	0.19
Malonate	0.26	0.30	0.20	0.26
Maleate	0.49	0.54	0.35	0.39
Succinate	0.54	0.57	0.26	0.33
Fumarate	0.56	0.67	0.26	0.32
Malate	1.00	1.00	1.00	1.00
α-Ketoglutarate	1.85[b]	1.42[b]	...	1.22[b]
Tartrate	2.03	1.86	...	2.90
Aconitate	10.61	5.46	...	2.15
Citrate	12.72	6.35	...	4.25
Lactoisocitrate	17.08	7.07	...	10.17
Temp., °C.	80	150	130	182
Inlet pressure, p.s.i.	20	30	20	25
Outlet flow rate, ml./min.	67	...	100	...
Carrier gas	Argon	Nitrogen	Argon	Nitrogen
Support, mesh	60–80	60–80	60–80	60–80
Liquid phase, % w/w	4	15	5	10
Malate, min.	3.6	4.3	6.8	4.7

[a] Nonylphenoxypolyethyleneoxy—ethanol. [b] Major peak.

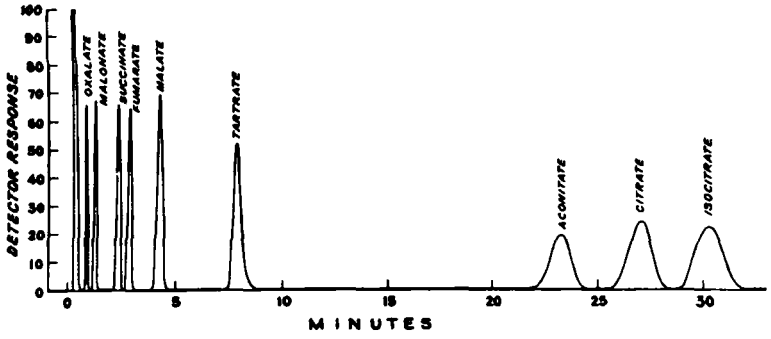


Fig. 1.—Gas chromatogram of the ethyl esters of nine plant acids on Apiezon L. The experimental conditions are shown in Table I.

(10) in 1952 as a tool for studying fatty acids. Since that time, it has become so widely used that it has practically displaced all other methods for routine analysis of fats and oils and for biochemical research concerning fatty acids. Much less work has been done with gas chromatography of di and tricarboxylic acids. James, *et al.* (11), determined the structure of unsaturated fatty acids by oxidative degradation followed by esterification and gas chromatography of the dicarboxylic acids produced. Nowakowska, *et al.* (12), and Miwa, *et al.* (13), used gas chromatography to separate and identify the dicarboxylic acids formed during oxidation of fats and oils. The dicarboxylic acids present in cigarette smoke were identified and determined quantitatively by Quin and Hobbs (14). In his book on gas chromatography, Bayer (15) refers to unpublished work dealing with gas chromatographic separation of methyl esters of certain di and tricarboxylic acids belonging to the Krebs cycle.

EXPERIMENTAL

Esterification of Plant Acids.—Ethyl esters were produced by refluxing the dried acids with anhydrous ethanol containing 5% of hydrogen chloride. With small samples of acids (10 to 20 mg.), the esterification appeared to be complete in about 2 hours. The reaction mixture was evaporated almost to dryness in a rotating vacuum evaporator. The residue was transferred to a small separator, 5 ml. of water was added, and the ester was extracted twice with 10 ml. of ether. The combined ether extracts were washed with a little sodium bicarbonate solution, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure at room temperature.

The methyl esters were synthesized by three different methods, all of which gave satisfactory results with most acids. The method which appeared to be most generally applicable was esterification with diazomethane. The microscale procedure described by Schlenk and Gellerman (16) produced methyl esters in good yield from most of the acids. Depending on the nature of the acid, the esterification was complete in from 10 to 30 minutes. Side reactions may occur with special acids unless the reagent concentration is carefully controlled. Keto acids gave rise to by-products by reaction between

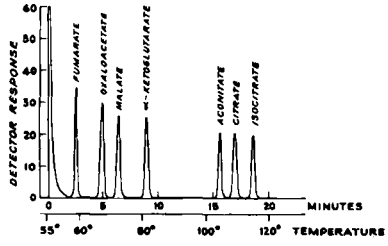


Fig. 2.—Temperature programmed gas chromatogram of methyl esters of plant acids on silicone rubber SE-30, 4%.

diazomethane and the keto group. These acids were best esterfied with methanol catalyzed by boron trifluoride (17). The acid (10 to 100 mg.) was dissolved in 3 ml. of BF_3-methanol reagent in a test tube, and the solution was heated gently on a steam bath for 2 minutes, then cooled under running water and transferred to a small separator with the aid of about 5 ml. of water. The ester was extracted twice with 10 ml. of ether. The combined ether extracts were dried over a little anhydrous sodium sulfate, filtered, and evaporated to dryness at room temperature under reduced pressure. This method appeared to be effective with most of the acids studied, but gave low yield with tartaric acid.

The third method used for production of the methyl esters was similar to the procedure described for the ethyl esters. The keto acids showed considerable decomposition by this method, evidenced by the appearance of several peaks on the gas chromatogram. However, the other acids gave methyl esters in good yield.

Isocitric acid is not known as the free tricarboxylic acid in a crystalline state. When evaporated and dried, the acid loses water and is converted to the lactone (3). Isocitric acid lactone and its barium salt are commercially available. On esterification, both compounds gave esters which had the same retention times as the corresponding authentic diethyl and dimethyl esters of lactoisocitric acid.[1]

Isolation of Acids from Plant Materials.—Ten grams of finely ground plant materials was extracted twice with 100 ml. of warm ethanol each time. The combined extracts were evaporated to dryness under reduced pressure, the residue was dissolved in about 25 ml. of water, and filtered. The filtrate was heated to about 60°, and a saturated solution of lead acetate was added until no further precipitation took place. Lead subacetate solution was then

[1] We are indebted to Professor A. Nordal of Oslo University for supplying us with these authentic esters.

added in the same way. The total lead precipitate was filtered off, washed with water, suspended in 50 ml. of 50% ethanol, and the free acids were regenerated with hydrogen sulfide. The lead sulfide was removed by filtration, and the filtrate evaporated to dryness in a rotating vacuum evaporator at a low temperature. The last traces of moisture were removed by addition of absolute ethanol and evaporation to dryness. This procedure was repeated twice. The acid residue was esterified as described above.

Gas Chromatography of Esters of Plant Acids.— Two gas chromatographs were used for the work reported here. One was a Barber-Colman model 15 gas chromatograph equipped with an argon ionization detector (50 μc. of Ra-226). The columns for this instrument were glass U-tubes, 4 ft. long and 4 mm. in inside diameter. The other instrument was a Wilkins HY-FI gas chromatograph having a hydrogen flame ionization detector and stainless steel columns, 6 ft. long and ⅛ in. in outside diameter. Gas-Chrom P was used as the solid support. It was washed with concentrated hydrochloric acid, methanolic potassium hydroxide, dried, and treated with hexamethyldisilazane (18). The dried and siliconized support was coated with the liquid phase as described by Horning, *et al.* (19). When the stationary phase was nonpolar, such as Apiezon or silicone rubber SE-30, a coating of 0.1% of polyethylene glycol 9000 was applied prior to the main liquid phase (18, 20). This did not seem to alter the retention data, but markedly reduced tailing of the polar hydroxy esters. The ester sample was injected into the flash heater, maintained at 175°, with a Hamilton microsyringe. The usual sample was 0.5 μl. of an acetone solution containing 2 to 10 mcg. of each ester. If the flash heater temperature was much above 175°, certain decomposition reactions could sometimes be observed. Malic acid esters, for example, under such conditions produced three peaks, due to partial dehydration to maleate and fumarate.

RESULTS AND DISCUSSION

The gas chromatographic retention data of the esters of 12 di and tricarboxylic acids are given in Tables I and II. Maleate is included because of its structural relationship to fumarate, although the acid has never been isolated from plant materials. A typical gas chromatogram of esterified plant acids is illustrated in Fig. 1. Temperature programing is useful in speeding up the elution of aconitate, citrate, and isocitrate and producing sharper peaks. Figure 2 represents a temperature programed gas chromatogram on silicone rubber SE-30.

It is apparent from Tables I and II that the greatest number of esters could be separated on Apiezon L. This stationary phase was, furthermore, the only one that produced satisfactory separation of succinate and fumarate. The geometric isomers, maleate and fumarate, were separated as the ethyl esters on all four columns. Maleate and succinate, however, could only be separated on polar columns. Citrate and lactoisocitrate showed great differences in retention times.

The acetone solutions of the esters are unstable and should be gas chromatographed without much delay. The esters of the keto acids and the hydroxy-acids are particularly subject to decomposition in solution. After a day or two, several gas chromatographic peaks were produced indicating substances which were not present in the original solution.

The plant acids were isolated from *Celtis occidentalis, Urticaceae*, as described above. The following acids were identified: oxalic, malonic, fumaric, succinic, malic, citric, and isocitric.

The dried roots of *Angelica archangelica* L. subsp. *norvegica, Umbelliferae*, contained oxalic, malonic, fumaric, succinic, malic, aconitic, and citric acid. This corresponds to the acids identified in this plant by fractional distillation of the esters, followed by hydrolysis and paper chromatography of the liberated acids (21).

A fraction of ethyl esters from *Sempervivum tectorum* L., *Crassulaceae*,[2] showed the presence of succinic, malic, citric, and isocitric acids.

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[2] The ethyl ester fraction was kindly supplied by Professor A. Nordal.

Effect of Temperature on Absorption Rates of Drug Implants

By BERTON E. BALLARD, SUZANNE S. BALLARD, and EINO NELSON

The absorption rates of drug pellet implants have been shown to be temperature dependent in frogs. For neutral compounds the rate equation tested was $(\bar{R}/\bar{A})r = Kr \cdot Dr \cdot (Ss)r/\delta r$, where $(\bar{R}/\bar{A})r$ is the mean absorption rate per mean area at a given temperature relative to that at 0°C., Kr is a constant, Dr is the relative diffusion coefficient, $(Ss)r$ is the relative solubility of drug, and δr is the relative diffusion layer thickness. To minimize body movement, the frog, *Rana pipiens*, was paralyzed with 10 mg./Kg. of *d*-tubocurarine chloride. After temperature equilibrium was attained in a water or ice bath, a small incision into the dorsal lymph sac was made, and two preweighed pellets of sulfanilamide or acetanilid were introduced. The pellets were removed, dried, and reweighed. A plot of $(\bar{R}/\bar{A})r$ *versus* $Dr \cdot (Ss)r/\delta r$ yielded a straight line with a slope 1.09 for the two compounds.

THE ABSORPTION rates of subcutaneously implanted drug pellets have been shown to be rate limited by the dissolution process at the site of implantation. Although considerable literature has appeared where the pellet implantation technique has been used, apparently no quantitative work has appeared on what influence the animal's body temperature has on the absorption rates of solid implants (1).

The purpose of this study was to determine what the relationship is between the absorption rates of two neutral drugs subcutaneously implanted and the influence of body temperature on the physical and chemical properties of drug and the fluid medium surrounding it.

THEORETICAL

Equations relating absorption rates of implanted drug pellets and drug's physicochemical properties have been derived under conditions of a constant body temperature (2). The following derivations were based, in part, on the assumptions previously made.

The absorption rate of implanted solid drug was assumed to be a diffusion-controlled process where diffusion of drug molecules took place normal to a nearly constant solid surface area. Fick's law in one dimension is

$$da/dt = DA\,dc/dx \qquad \text{(Eq. 1)}$$

where da/dt is the rate of flow of dissolved drug through a plane of area, A, normal to the direction of diffusion, and is (in this case) the absorption rate of the drug implant. The diffusion coefficient is D. The area, A, under these conditions was assumed to be the area of the solid drug pellet at any time. The concentration gradient across the region through which diffusion takes place is dc/dx. If $\bar{R}$, which equals da/dt, is the mean absorption rate, and is the mean pellet area, then rearranging Eq. 1 gives

$$\bar{R}/\bar{A} = D\,dc/dx \qquad \text{(Eq. 2)}$$

where $\bar{R}/\bar{A}$ equals the mean absorption rate per mean pellet area. Under conditions of steady state diffusion from solid, the concentration gradient may be approximately represented

$$\bar{R}/\bar{A} = D\frac{(Cs - C)}{\delta} \qquad \text{(Eq. 3)}$$

where Cs is the concentration of a nearly saturated solution of drug in body fluids in the diffusion layer (3) at a point adjacent to the pellet surface, and C is the drug concentration in the bulk of the body fluids. The boundary or diffusion layer thickness is δ. For most substances which are foreign to the animal, like most drugs, the term C can be assumed to be small compared to Cs and will be neglected. Thus

$$\bar{R}/\bar{A} = DCs/\delta = kDSs/\delta \qquad \text{(Eq. 4)}$$

where Cs equals kSs. For the purposes here Ss is the saturation solubility of a neutral drug in body fluids, and k is a proportionality constant. When the animal's body temperature is varied, the terms D, Ss, and δ all should vary independently with temperature. Equation 4 can be written as

$$(\bar{R}/\bar{A})r = \frac{(\bar{R}/\bar{A})}{(\bar{R}/\bar{A})^\circ} = \frac{(k)}{(k)^\circ}\cdot\frac{(D)}{(D)^\circ}\cdot\frac{(Ss)}{(Ss)^\circ}\cdot\frac{\delta^\circ}{\delta} = Kr \cdot Dr \cdot (Ss)r \cdot (1/\delta_r) \qquad \text{(Eq. 5)}$$

where $(\bar{R}/\bar{A})r$ is the relative absorption rate or the mean absorption rate per mean area, $\bar{R}/\bar{A}$, at an absolute temperature T divided by the mean absorption rate per mean area, $(\bar{R}/\bar{A})^\circ$, at 273.2° A or T°, and the superscript zero associated with other terms in Eq. 5 means that the term also must be evaluated at 273.2° A, Kr is a constant whose value should be unity, Dr is the relative diffusion coefficient, $(Ss)r$ is the relative solubility, and δ_r is the relative diffusion layer thickness.

Relative Diffusion Coefficient, *Dr*.—According to the Einstein-Stokes formula (4), the diffusion coefficient of a large molecule in water may be approximated by

$$D = k'T/6\pi\eta r \qquad \text{(Eq. 6)}$$

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where k' is Boltzmann's constant, T is the absolute temperature, r is the Stokes' radius of the diffusing molecule, and η is the viscosity of the medium. Equation 6 may be rewritten for the two temperatures as

$$Dr = \frac{D}{D^\circ} = \frac{T}{T^\circ}\cdot\frac{\eta^\circ}{\eta} \qquad \text{(Eq. 7)}$$

where it is assumed that the Stokes radius r equals r°, and the other terms have the same meaning as before.

For water it can be shown that

$$\left(\frac{\eta^\circ}{\eta}\right)_{H_2O} = \left(\frac{\eta r^\circ}{\eta r}\right)_{H_2O} \qquad \text{(Eq. 8)}$$

where ηr and ηr° are the relative viscosities of water at temperatures T and T°, respectively. Since the viscosities of frog body fluids containing the drugs used in this study have not been measured as a function of temperature, it is convenient to make use of Hess' rule, which states for a biological medium having a fixed protein concentration that

$$\left(\frac{\eta r^\circ}{\eta r}\right)_{H_2O} \cong \left(\frac{\eta r^\circ}{\eta r}\right)_f \qquad \text{(Eq. 9)}$$

where $(\eta r^\circ/\eta r)_f$ is the ratio of the relative viscosities of body fluids, like blood, serum, or plasma, at temperatures T° and T (5, 6).

Relative Solubility (Ss)r.—If it can be assumed that the ratio of drug solubility in body fluids to that in water is equal to a constant that is independent of temperature, then one can write

$$\left[\frac{(Ss')}{(Ss')^\circ}\right]_{H_2O} = \left[\frac{(Ss)}{(Ss)^\circ}\right]_f \qquad \text{(Eq. 10)}$$

where $[(Ss/(Ss)^\circ]_f$ is the ratio of the solubilities of drug in the fluids at the implantation site at the temperature T and T°.

Relative Diffusion Layer Thickness, δr.—The thickness of the diffusion layer at a given temperature may be estimated from the equation discussed by Jost (7)

$$\delta \cong \sqrt{\eta L/v\rho} \qquad \text{(Eq. 11)}$$

where η is viscosity in poise, L is the linear dimension of surface of solid across which the medium flows in cm., v is the velocity of flow of the medium as a result of stirring in cm. sec.$^{-1}$, and ρ is the density of the medium in Gm. cm.$^{-3}$ Equation 11 may be written as

$$\delta r = \frac{\delta}{\delta^\circ} = \left(\frac{\eta L/v\rho}{\eta^\circ L^\circ/v^\circ\rho^\circ}\right)^{1/2} \qquad \text{(Eq. 12)}$$

where the use of the superscript zero has the same meaning as before. Under conditions of this experiment, it can be assumed with little error that $L = L^\circ$, since only a small weight was absorbed from each pellet, and that $\rho \cong \rho^\circ$, since water density is not a sensitive function of temperature in the 0–30° C. range. The terms v and v° are not necessarily equal, since normal body movement (or stirring velocity) is often temperature dependent (see *Discussion*). In this experiment the influence of body movement was minimized so that $v \cong v^\circ$. The only terms greatly influenced by temperature changes are η and η°. Equation 12 may be simplified to give

$$\delta r \cong (\eta/\eta^\circ)^{1/2} \qquad \text{(Eq. 13)}$$

By making use of the arguments previously proposed, Eqs. 8 or 9 may be substituted into Eq. 13 to give

$$\delta_r = (\eta r/\eta r^\circ)_{H_2O}{}^{1/2} = (\eta r/\eta r^\circ)_f{}^{1/2} \qquad \text{(Eq. 14)}$$

Substitution of Eqs. 7–10, 12, and 14 into 5 gives for an aqueous *in vitro* system that is slowly stirred

$$(\bar{R}/\bar{A})_r = Kr(T/T^\circ)\,(\eta r^\circ/\eta r)_{H_2O}{}^{1.5}[(Ss')/(Ss')^\circ]_{H_2O} \qquad \text{(Eq. 15)}$$

and for an *in vivo* system

$$(\bar{R}/\bar{A})_r = Kr(T/T^\circ)(\eta r^\circ/\eta r)_f{}^{1.5}[(Ss/(Ss)^\circ]_f \qquad \text{(Eq. 16)}$$

Thus, if $(\bar{R}/\bar{A})_r$ is plotted against all the righthand terms in Eqs. 15 or 16 (except Kr), a linear plot should result having a slope of Kr.

MATERIALS AND METHODS

Implants.—Thin, cylindrical disks of acetanilid and sulfanilamide having weights in the range of 51.0 to 83.0 mg. and of 63.1 to 100.7 mg., respectively, were compressed in a similar manner to that described previously (2), except that the drug powders were first moistened with distilled water and punched wet. The pellets were then air dried for 48 or more hours before weighing. Pellets formed by this technique were hard and more suitable for implantation than those prepared by the dry method.

Drug grade materials were used without further purification. The melting points of acetanilid and sulfanilamide were 114° C. and 163° C. (corrected), respectively. No binders, excipients, dilutents, or lubricants were used.

TABLE I.—ACETANILID ABSORPTION DATA AND PROPERTIES OF DRUG AND MEDIUM AT VARIOUS TEMPERATURES

t, °C.	No. of Animals	$\bar{R}/\bar{A}$[a] Gm./hr./cm.^{2} × 10^4	$(\bar{R}/\bar{A})_r$	Solubility Gm./100 ml. H_2O	$(Ss)_r$	$\frac{Dr}{\delta r}$[b]	$\frac{(Ss)_r Dr}{\delta r}$
0	8	3.57 (±0.033)	1.00	0.361	1.00	1.00	1.00
9	6	7.59 (±0.68)	2.13	0.432	1.20	1.59	1.91
15	6	13.69 (±0.15)	3.83	0.497	1.38	2.08	2.87
20	7	16.60 (±0.27)	4.65	0.563	1.56	2.55	3.98
25	6	22.50 (±0.31)	6.30	0.641	1.78	3.10	5.52
29	6	27.36 (±1.00)	7.66	0.712	1.97	3.59	7.07

[a] Mean absorption rate per mean area, followed by the 95% confidence interval in parentheses. [b] Values in this column can be calculated by either of two methods: Substitution into the empirical equation which holds for the temperature range 0 to 33° C. $[Dr/\delta r = 1.00 + 5.5713 \times 10^{-2} t + 1.0572 \times 10^{-3} t^2 + 3.65 \times 10^{-6} t^3]$ where t is in degrees C. $Dr/\delta r$ at 15° C. = 2.08. Substitution into Eqs. 7, 8, and 14. At 15° C. the ratio equals $Dr/\delta r = T/T^\circ(\eta r^\circ/\eta r)_{H_2O}{}^{1.5} = (288.2°/273.2°)^{1.5}(1.000/0.6363)^{1.5} = 2.08$.

TABLE II.—SULFANILAMIDE ABSORPTION DATA AND PROPERTIES OF DRUG AND MEDIUM AT VARIOUS TEMPERATURES

t, °C.	No. of Animals	$\overline{R}/\overline{A}$[a] Gm./hr./cm.^{2} × 10^4	$(\overline{R}/\overline{A})_r$	Solubility Gm./100 ml. H_2O	$(Ss)_r$	D_r/δ_r	$(Ss)_r D_r/\delta_r$
0	6	0.0907 (±0.026)	1.00	0.158	1.00	1.00	1.00
10	7	0.3096 (±0.037)	3.41	0.302	1.91	1.66	3.18
15	6	0.6149 (±0.193)	6.78	0.400	2.53	2.08	5.27
20	11	0.8201 (±0.085)	9.04	0.530	3.35	2.55	8.54
25	6	1.4033 (±0.13)	15.47	0.728	4.61	3.10	14.29
29	6	2.1155 (±0.429)	23.32	0.931	5.89	3.59	21.15

[a] Mean absorption rate per mean area, followed by the 95% confidence interval in parentheses.

Temperature Bath.—One to two 2000-ml. beakers, each containing layer of about 1 cm. of tap water, were immersed to a depth of about two-thirds their height into a constant temperature bath regulated to ±0.5°. A cardboard cover with a small hole was placed over each beaker opening to minimize the formation of air convection currents. A thermometer was inserted through the cover, and the bulb was rested in the water layer. A short piece of laboratory rubber tubing about 30 cm. long was placed on the bottom of the beaker with the coil touching the edge.

Animals.—Frogs of each sex (*Rana pipiens*) having a weight range of about 30 to 70 Gm. were injected in the ventral lymph sac with a solution of *d*-tubocurarine chloride (Upjohn). The dose of this drug needed to cause complete paralysis has been previously reported (8–11), although the dosage reported by King (9) seems to be low by a factor of 10. The dose used in this work was 10 mg./Kg., which usually gave a paralysis of sufficient duration to complete the experimental run. If any voluntary movement was observed during the course of an experiment, the animal received another smaller maintenance dose.

For temperatures above 0° C., three to four paralyzed frogs were partially immersed in the water layer of each beaker with their pharyngeal regions resting on the rubber tubing. The frogs were kept there for 15 minutes or longer until temperature equilibrium between them and the bath was attained. For the 0° C. point, all the paralyzed frogs were placed in an insulated chest containing chopped ice (12). Most of their bodies were covered with a thin layer of ice.

After temperature equilibrium was attained, the frogs were removed briefly one at a time from the temperature controlled environment, and an incision of about 0.75 cm. was made through the dorsal skin. Two preweighed pellets were inserted into the dorsal lymph sac. The frogs were then rapidly returned to their previous environment for the duration of the experimental run. At that time, the pellets were removed from the animals, placed on filter paper, and gently moved about to remove traces of biological fluid from their surfaces. The pellets were air dried for 48 hours or more. The time of implantation was adjusted so that pellets would lose from 5 to 10 mg. during the experiment. Data obtained from frogs which did not have an observable heart beat after the experiment were discarded. The mean absorption rates per mean areas of the pellets were calculated by a graphic method (13), except that a "ghost" correction was not made, since the duration of implantation was short.

Solubilities and pK Values.—Numerous reports on the water solubility of acetanilid as a function of temperature have appeared (16–19). The data given by Logan (18, 19) were used in this study because they seemed most reliable. The solubilities were recalculated to give solubility of drug in grams per 100 ml. of water. For purposes of interpolation the data were fitted to a third degree polynomial in temperature to give [solubility in H_2O (Gm./100 ml.) = $0.3611 + 6.3034 \times 10^{-3} t + 1.7064 \times 10^{-4} t^2 + 9.9729 \times 10^{-7} t^3$] where t is in degrees centigrade and includes the range from 0–30° (see Table I, column 5). Literature values for sulfanilamide water solubilities at various temperatures were converted to solubility in grams per 100 ml. water (20–22). A plot of log solubility *versus* $1/T$ was made over the temperature range 0–38°. A smooth curve was fitted by eye, and only points falling near or on this line were used in estimating the water solubility. Sulfanilamide solubilities were fitted to [solubility in H_2O (Gm./100 ml.) = $0.158 + 1.4101 \times 10^{-2} t - 1.8289 \times 10^{-4} t^2 + 2.1225 \times 10^{-6} t^3$] where t is in degrees centigrade (see Table II, column 5). Salt effects on the solubilities of the two drugs were considered small in biological fluids and were ignored.

The influence of drug pK on its solubility can be ignored at physiological pH's. The pK's for acetanilid (23–25) and sulfanilamide (20, 26–28) indicate that the compounds behave as neutral species under conditions of this experiment. Drug solubility should, therefore, be independent of diffusion layer pH (2).

RESULTS

Tables I and II summarize experimental and literature values needed to solve Eq. 16 for acetanilid

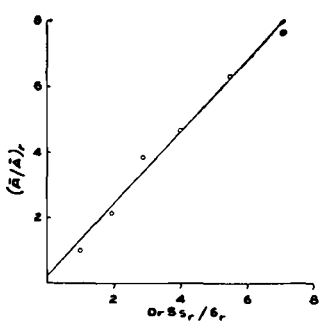


Fig. 1.—A plot of $(\overline{R}/\overline{A})_r$ *vs.* $Dr\ (Ss)r/\delta r$ for acetanilid. The line represents a plot of the regression equation, $Y = 0.203 + 1.09\ X$. The standard error of the estimate, S.E., is 0.271.

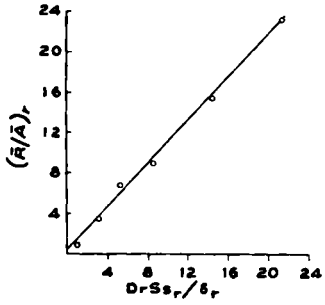


Fig. 2.—A plot of $(\bar{R}/\bar{A})r$ *vs.* $Dr(Ss)/\delta r$ for sulfanilamide. The line represents a plot of the regression equation, $Y = 0.130 + 1.09\ X$. The standard error of the estimate, S.E., is 0.450.

and sulfanilamide absorption. Figures 1 and 2 show plots of $(\bar{R}/\bar{A})r$ *versus* $Dr(Ss)r\ (1/\delta r)$ for the two compounds. Examination of Figs. 1 and 2 indicates that the slope, Kr, for the two compounds is approximately unity. For acetanilid and sulfanilamide, the equations for the straight line by the least squares method were $Y = 0.203 + 1.09\ X$ with a standard error of the estimate, S.E., of 0.271, and $Y = 0.130 + 1.09\ X$, with a standard error of the estimate of 0.450, respectively. Thus, there is a reasonable agreement between the theoretically predicted slope of unity and the experimental slopes.

DISCUSSION

Absorption Kinetics.—The linear relationship shown in Figs. 1 and 2 gives additional support to our view that the rate-limiting step in absorption after implant of the neutral drugs used was the dissolution process at the absorption site. The two drugs were largely dissimilar with respect to their chemical, physical, and pharmacological properties; yet, when suitable approximations were made for the temperature dependency of some of the terms in Eq. 16, the relative absorption rate could be described in terms of the physicochemical properties of drug and the fluids at the absorption site.

Variables in Absorption Equations.—In Eq. 1 the diffusion coefficient term, D, should be considered as being an apparent one. When neutral drugs are dissolved into biologic media at the dissolution site, drug-protein interactions would be expected to occur. If M refers to a drug molecule and P refers to one of many different protein molecules present which could potentially interact with the drug, then a large number of drug-protein species could be postulated having formulas of the form, $M_x \cdot P_y$, where x and y could have values of one or greater. Each species could be a diffusable one, or be a carrier for the drug, and would have a diffusion coefficient of its own. Under steady state conditions at any temperature, the apparent diffusion coefficient for the overall process equals the diffusion coefficient for a particular drug containing species times the fraction of the total diffusing drug represented by this species summed over all possible drug containing species. If it can be assumed that the extent of protein binding for each species is relatively independent of temperature, then the mathematical treatment shown in Eqs. 3 and 5 should be approximately correct. In this connection, Klotz and Urquhart (32) have shown with certain organic anions that the heat of protein binding is quite small.

In Eqs. 6 to 9 it was assumed that the relative diffusion coefficient of drug in water and biological fluids as a function of temperature could be estimated satisfactorily by the Einstein-Stokes equation (4), assuming that the Stokes law radius of the diffusing species in solution was temperature independent. A consideration of Longsworth's data (15) for dextrose, a neutral molecule having a molecular weight of the same order of magnitude as acetanilid and sulfanilamide, allows one to make an *in vitro* test of this assumption. Table III shows the experimental diffusion coefficients for dextrose at the four temperatures 1, 13, 25, and 37°, the observed relative diffusion coefficient, which equals the diffusion coefficient at any temperature divided by the value at 1° C., and the calculated relative diffusion coefficient using the relationship

$$Dr \text{ calcd.} = \frac{T}{T^{1°}} \cdot \left(\frac{\eta r^{1°}}{\eta r}\right)_{H_2O} \quad \text{(Eq. 17)}$$

When Eq. 8 is substituted into Eq. 7, an expression similar to Eq. 17 is found, except that the reference temperature for Eq. 17 is 1° C. instead of 0° C. The percentage error made by Eq. 17 did not exceed 2.7% over the 36° C. temperature range. Thus, the temperature dependency of the Dr term used in Eqs. 15 and 16 could be satisfactorily accounted for by the application of the Einstein-Stokes and the Einstein-Stokes-Hess equations.

In Eq. 10 it was assumed that the ratio of a neutral drug's solubility at two temperatures in water equalled the ratio of the drug's solubility at the same two temperatures in biological fluids. In the case of sulfanilamide this statement seems to hold. The van't Hoff equation is

$$\log Ss = \frac{-\Delta H}{2.303\ RT} + \text{constant} \quad \text{(Eq. 18)}$$

where Ss is the drug solubility in biological fluids, ΔH is the heat of solution in Kcal. per mole, T is the absolute temperature, and R is the gas constant. An analogous expression could be written for the solubility, Ss', of drug in water. If Eq. 10 is valid, then the heats of solution of drug in biological fluids and water should be nearly identical. Clark, *et al.* (29), found the solubilities of sulfanilamide in human serum to be 0.981 and 1.970 Gm. per 100 Gm. solvent at 25 and 37° C., respectively. The calculated value for ΔH from their data was 10.7 Kcal./mole. This value agrees favorably with the heat of solution of 10.9 Kcal. per mole reported by Kienle and Sayward (30) for the drug in water over the same temperature range. Clark, *et al.* (29), also gave water solubility values for sulfanilamide at these two temperatures, but their value at 25° C. seems high, while the value at 37° C. is in close agreement with that given by Kienle and Sayward (30).

TABLE III.—DIFFUSION COEFFICIENTS (15) FOR DEXTROSE

t, °C.	1	13	25	37
$D \times 10^6$ cm.^{2} sec.$^{-1}$	3.137	4.736	6.728	9.088
Dr observed	1.000	1.510	2.145	2.897
Dr calcd.	1.000	1.502[a]	2.107	2.820

[a] Dr calculated (Eq. 17) = [(286.2)/(274.2)·(1.0000)/(0.6947)] = 1.502.

Of the four independent variables in Eq. 11 for the thickness of the diffusion layer, only two are highly sensitive to changes in temperature. They are the viscosity of the medium, η, which was corrected for by a modification of Hess' rule, and the velocity of flow of the biological medium, v, as a result of body movement or stirring. In biological systems body movement is often temperature dependent. Crozier and Stier (31) have shown that the pharyngeal respiratory rhythm of frogs (*Rana pipiens*) was temperature dependent and followed the Arrhenius equation

$$k = A \exp[-E/RT] \qquad \text{(Eq. 19)}$$

where k is the reaction rate, A is a constant, E is the activation energy, and R and T have the same meanings as before. Crozier and Stier (31) found that the experimental activation energy for the rate of frog pharyngeal movement was 8.8 Kcal. In this experiment, it was important that animal body movement should be controlled so that the influence of temperature on drug absorption rate could be readily estimated. The method chosen here was that of paralyzing the frog with large doses of *d*-tubocurarine chloride, so that the velocity of stirring or movement of body fluids was biologically nearly zero. Body movement never can be reduced to zero, since heart function must be maintained. The isolated frog (*Rana temporaria*) heart rate has been shown to be temperature dependent (14). Little could be done to control this variable in body movement, except to note that *d*-tubocurarine in the frog was reported to cause slowing of heart rate in addition to its other effects (11).

The fact that the slopes of the lines in Figs. 1 and 2 are slightly greater than unity indicates that the term Kr might not be completely temperature independent. If experimental errors are ignored, there are at least three reasons why the slope should be greater than unity. First, the value for Dr as calculated from Eq. 17 is always slightly smaller than Dr observed, excepting the 1° C. value. Second, the density of water (and presumably body fluids saturated with drug) decreases at temperatures below and above 4° C. In Eq. 12 at temperatures a little greater than 8° C., ρ° for water is slightly greater than ρ. And third, heart rate, a source of body movement even in curarized animals, is probably greater at higher temperatures.

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ERRATUM

In the paper titled "Production of Pyridoxine and Niacin by *Chlorella vulgaris* and *C. pyrenoidosa*" (1), Table III at page 152 should be clarified to read:

Table III.—Value of *C. vulgaris* Relative to *C. pyrenoidosa* as a Source of Vitamin B_6 and Niacin at Different Harvest Times

Vitamin	mmcg. per mg. Dry Wt. 2 wks.	mmcg. per mg. Dry Wt. 3 wks.	mmcg. per ml. Culture 2 wks.	mmcg. per ml. Culture 3 wks.
	1	*2*	*3*	*4*
B_6	0.66	0.90	0.57	0.75
Niacin	1.06	1.18	0.93	1.07

(1) Pratt, R., and Johnson, E., This Journal, **53**, 151 (1964).

Of the four independent variables in Eq. 11 for the thickness of the diffusion layer, only two are highly sensitive to changes in temperature. They are the viscosity of the medium, η, which was corrected for by a modification of Hess' rule, and the velocity of flow of the biological medium, v, as a result of body movement or stirring. In biological systems body movement is often temperature dependent. Crozier and Stier (31) have shown that the pharyngeal respiratory rhythm of frogs (*Rana pipiens*) was temperature dependent and followed the Arrhenius equation

$$k = A \exp[-E/RT] \qquad \text{(Eq. 19)}$$

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The fact that the slopes of the lines in Figs. 1 and 2 are slightly greater than unity indicates that the term Kr might not be completely temperature independent. If experimental errors are ignored, there are at least three reasons why the slope should be greater than unity. First, the value for Dr as calculated from Eq. 17 is always slightly smaller than Dr observed, excepting the 1° C. value. Second, the density of water (and presumably body fluids saturated with drug) decreases at temperatures below and above 4° C. In Eq. 12 at temperatures a little greater than 8° C., ρ° for water is slightly greater than ρ. And third, heart rate, a source of body movement even in curarized animals, is probably greater at higher temperatures.

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ERRATUM

In the paper titled "Production of Pyridoxine and Niacin by *Chlorella vulgaris* and *C. pyrenoidosa*" (1), Table III at page 152 should be clarified to read:

Table III.—Value of *C. vulgaris* Relative to *C. pyrenoidosa* as a Source of Vitamin B_6 and Niacin at Different Harvest Times

Vitamin	mmcg. per mg. Dry Wt. 2 wks.	mmcg. per mg. Dry Wt. 3 wks.	mmcg. per ml. Culture 2 wks.	mmcg. per ml. Culture 3 wks.
	1	*2*	*3*	*4*
B_6	0.66	0.90	0.57	0.75
Niacin	1.06	1.18	0.93	1.07

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Effects of Selected Variables on the Extractability of Oils from Coacervate Capsules

By LOUIS A. LUZZI and ROBERT J. GERRAUGHTY

The hypothesis that certain pharmaceuticals may be stabilized by coacervation led to the investigation of encapsulation requirements for pharmaceutical oils. Saponification values, acid values, and surfactant properties were imparted to light liquid petrolatum by the introduction of oils with high saponification values, organic acids, and surfactants. The effects of these substances on the degree of protection offered by coacervation were determined by submitting the coacervates to extraction with ethyl ether for periods up to 1 hour, then comparing the extracts spectrophotometrically to known absorption spectra. Results showed that saponification values had little effect on encapsulation and that acid values and surfactants decreased the degree of encapsulation.

THE TERM "coacervation" has recently been used to describe the salting out of a lyophilic solid into liquid droplets rather than solid aggregates (1). The term was introduced into colloidal chemistry by Kruyt and Bungernberg de Jong (2) to describe the flocculation or separation of liquids from solution, where at least one of the liquids contained a colloidal solute.

Coacervation has been subdivided into simple coacervation and complex coacervation. Briefly, simple coacervation usually deals with systems containing only one colloidal solute, while complex coacervation usually deals with systems containing more than one colloid (3).

Simple coacervation is a process involving the addition of a strongly hydrophilic substance to a solution of a colloid. This added substance causes two phases to be formed—one phase rich in colloidal droplets and the other poor in such droplets. This process is dependent primarily on the degree of hydration produced, and this is more difficult to control.

Complex coacervation, however, has been found to be primarily dependent on pH. It has been reported that in a gum arabic–gelatin system, complex coacervation into microcapsules occurred at pH values below the isoelectric point of the gelatin and would not occur above this pH regardless of how other factors were changed (4). The same was true of other systems containing two dispersed colloids of opposite electrical charge.

The optimum conditions for complex coacervation were achieved by adjusting the pH to a point at which equivalents of oppositely charged molecules of the two colloids were present, since the greatest number of salt bonds were formed at that point (4). In such a system, the pH must be adjusted so that the gelatin particles are positively charged (below the isoelectric point), since gum arabic particles are always negative in colloidal dispersions (5). Therefore, the principal condition necessary for successful complex coacervation is pH. Since this factor is more easily and precisely adjusted and controlled than the degree of hydration, it was decided that the process of complex coacervation would be more practical to the purposes of this study.

Green and Schleicher made practical use of the process of coacervation in various patents (6–10). Two of the patents (7, 10) indicated that various oils, some of which contained dissolved dyes, had been entrapped in gelatin-acacia microcapsules. No quantitative method to evaluate the strength of the coacervate or the degree of coacervation was given.

It appeared that encapsulation through coacervation has great potential application in pharmacy. Some properties that might be especially useful include (*a*) prevention of vaporization of volatile substances (*e.g.*, volatile oils), (*b*) protection of moisture-sensitive or light sensitive substances (*e.g.*, vitamins A and K), (*c*) separation of incompatible substances within a single system, and (*d*) dispersion of water insoluble substances in an aqueous medium more readily.

Before such applications could be explored, it was apparent that some methods should be available to predict necessary conditions to achieve good encapsulation. Since the properties of oils used in pharmacy differ considerably, it was decided to study the effects of varying saponification value, acid value, and surface activity to ascertain their effects, if any, on the encapsulation process. A second objective was to attempt to develop a method to determine the permeability of the coacervate shell.

EXPERIMENTAL

Preparation of Coacervates.—The method of Green and Schleicher (7, 10) for preparation of

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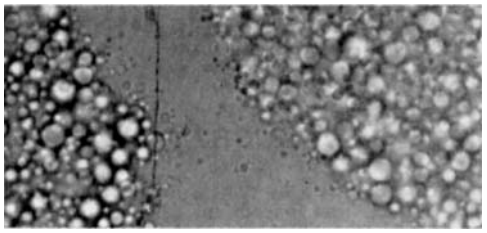


Fig. 1.—Photograph of the coacervation process at pH 4.5 and at 10°.

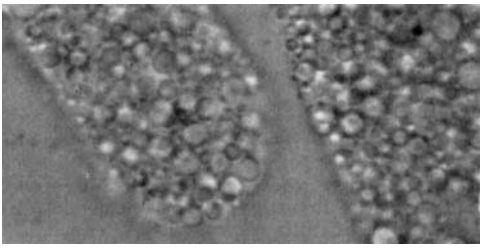


Fig. 2.—Photograph of the coacervation process after the addition of formaldehyde at pH 4.5 and at 10°.

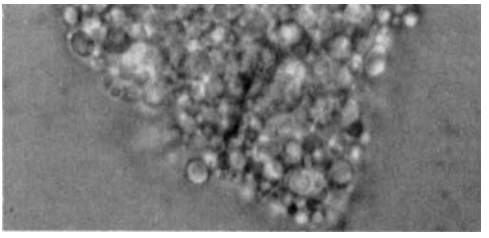


Fig. 3.—Photograph of the coacervation process at pH 9.

complex coacervates was modified. The primary coacervating solutions were made by dissolving 3.0 Gm. of acacia and 3.0 Gm. of gelatin separately in 100 ml. of purified water at 55°. The acacia used was powdered acacia U.S.P. and the gelatin used was 300 bloom, pigskin gelatin[1] with an isoelectric point at pH 8.0.

A 20% sodium hydroxide solution was used to adjust the pH of the solutions to 6.5. An emulsion was formed by adding 12 ml. of the selected oil to the acacia solution and passing the mixture through a hand homogenizer. The gelatin solution was then added with gentle stirring. At no time was the temperature of the mixture allowed to fall below 50°.

The pH of the mixture was adjusted gradually to 4.5 by the dropwise addition of diluted hydrochloric acid U.S.P. while stirring. At this pH the gelatin particles were positively charged and were attracted to the negatively charged acacia particles. The combined droplets are assumed to have coalesced about the oil droplets. In any event, the oil droplets were isolated from the rest of the system.

Then 10 ml. of formaldehyde solution U.S.P. was added, and the mixture was cooled to 10° in an ice bath. The pH was adjusted to 9.0 with 20% sodium hydroxide solution. The addition of formaldehyde denatured the gelatin-acacia complex and entrapped the oil more permanently. Chilling and adjusting the pH merely enhanced this effect.

The coacervation mixture was diluted to 400 ml. and filtered. After filtration, the wet coacervate was wrapped in blotting paper to remove some of the excess moisture. The resulting soft rubbery mass was passed through a 20-mesh screen and dried at 50° for 24 hours. The coacervate was then coarsely granular.

Method to Evaluate Permeability of Coacervate Shell.—The method used to evaluate the strength of the shell was based on the ability of the shell to prevent or allow extraction of the entrapped oil by ethyl ether (analytical reagent). To measure the degree of extraction, the oils were colored with dye[2] which was present in the oils in the same ratio both before and after extraction. The oils were then encapsulated through coacervation and dried by the method described.

Colored oils were scanned for wavelength of maximum absorption using a Bausch and Lomb spectronic 505 recording spectrophotometer. A broad peak with a maximum at 500 mμ was seen. Beer's law plots were obtained using a Bausch and Lomb spectronic 20 at this wavelength. This facilitated assay of the ether extracts of the various coacervates containing the colored oils.

Choice of Oils for Coacervation Properties Study.—To provide a series of oils with a controlled range of saponification values, light liquid petrolatum N.F., which has no saponification value, was mixed with coconut oil[3] which had a saponification of 250 but no acid value.

A controlled acid value series was prepared by mixing oleic acid U.S.P., which had an acid value of 190 and no saponification value, with light liquid petrolatum N.F. Series using lauric acid and benzoic acid were also prepared.

The effect of surfactants on the coacervation was studied by preparing a series using trioleate 85[4] as an additive to the mineral oil before coacervation was carried out. Sodium oleate, polysorbate 20,[5] and sodium acetate were added to the aqueous phase in separate qualitative studies to see if they exerted an effect on the encapsulation process.

Microphotography of the Coacervation Process.—Photomicrographs of the process were taken at various steps in the procedure. Figure 1 shows the agglomeration of oil droplets that occurred when the pH was lowered to 4.5 and the temperature reduced to 10°. At this stage in the process a matrix apparently had not completely formed. Figure 2 was photographed after the addition of the formaldehyde solution with the pH still at 4.5. The outline of the oil droplets is clearly delineated and, in some areas, shows what appears to be a matrix.

Figure 3 was taken after the pH was raised to 9.0. Although the coacervate consisted of a

[1] Obtained from the American Agricultural Chemical Co., Detroit, Mich.

[2] Sudan III. Marketed by General Dyestuff Corp., New York, N. Y.

[3] Marketed as Coconut Oil 76° by Welch, Holme, and Clark Co., New York, N. Y.

[4] Marketed as Arlacel 85 by the Atlas Powder Co., Wilmington, Del.

[5] Marketed as Tween 20 by the Atlas Powder Co., Wilmington, Del.

TABLE I.—COCONUT OIL SERIES

Coconut Oil, %	Sapon. Value	Oil Extracted in 60 Min., ml.	Oil Extracted as % Total Wt. of Sample
1	2.5	0.006	0.45
2	5.0	0.005	0.40
3	7.5	0.005	0.40
4	10.0	0.006	0.45
5	12.5	0.005	0.40

TABLE II.—OLEIC ACID SERIES

Acid in Oil, %	Acid Value of Oil	Oil Extracted in 60 Min., ml.	Oil Extracted as Total Wt. of Sample, %
0	0	0.012	1.02
1	2.15	0.012	1.02
2	4.22	0.061	5.18
3	6.08	0.091	7.70
4	8.24	0.248	21.08
5	10.47	0.320	27.20

rubbery mass at this point, it was not apparent in the photograph.

RESULTS

Effects of Saponification Value.—The series of oils with saponification values ranging from 2.5 to 12.5 were studied after coacervation. Table I shows that the permeability of the shell was constant since the amount of oil extracted by ether in 1 hour was about the same throughout the series.

Effects of Acid Value.—Results of the coacervation and extraction of the oleic acid series are shown in Table II. The amount of oil extracted increased directly as the acid value increased. Oil samples having higher acid values gave results that were somewhat erratic and difficult to measure quantitatively.

A series using lauric acid followed a similar pattern. However, lauric acid was soluble in mineral oil only to the extent of 2%, thereby limiting the usefulness of this series.

Benzoic acid was used as an additive, since it contributed acid value without surfactant properties (both the oleic acid and lauric acid exhibit some surfactant properties). However, the benzoic acid lowered the pH to 4.0 very abruptly, and the condition of gradual approach to a pH of 4.5 could not be achieved. No acid could be found which was soluble in mineral oil, insoluble in water, and which had no surfactant properties.

Effect of Surfactants.—Since this study involved a water soluble phase and an oil soluble phase it was desirable to include surfactants in first one phase and then the other.

Trioleate 85, an oil soluble surfactant, was used as an additive. The results shown in Table III indicate that this surfactant interfered with the encapsulation process, although the results were somewhat more erratic than those in the coconut oil or oleic acid series.

Polysorbate 20, a water soluble emulsifier, was added to the aqueous phase (acacia solution) prior to coacervation. No quantitative data were obtained, since none of the colored oil was retained by the coacervate after dilution and filtration.

TABLE III.—TRIOLEATE-85 SERIES

Trioleate in Oil, %	Acid Value	Oil Extracted in 60 Min., ml.	Oil Extracted as Total of Sample, %
0	0	0.012	1.02
1	0.14	0.02	1.70
2	0.24	0.14	11.45
3	0.33	0.10	8.50
4	0.55	0.35	30.09
5	0.78	0.13	11.05

Sodium oleate was also tried; the results were the same and indicated that surfactants in the aqueous phase seemed to prevent proper encapsulation.

DISCUSSION

Encapsulation of oils occurred when colloidal droplets of oppositely charged colloids agglomerated about the oil droplets to form a shell which probably consists of several layers of coacervate droplets. Denaturization of the colloidal film or shell gave it a degree of permanence.

Surfactants exhibited interference with the encapsulation process and the strength of the protective shell. It is theoretically possible this effect was because surfactants may compete with the gelatin-acacia complex at the oil-water interface If this reasoning is acceptable, then the shell would not be continuous and leakage could occur through the incomplete shell. It would then follow that the more surfactant present, the greater the number of spaces possible in the shell.

The interference of the oleic acid in the coacervation process might have been due to the surfactant action of this fatty acid, or it might have been due to the acidity. In the saponification value series the esters added did not exhibit surfactant properties, nor were the conditions severe enough to cause hydrolysis to the more surfactant fatty acids.

SUMMARY

A spectrophotometric method was found to evaluate the permeability of coacervate shells.

Oleic acid and lauric acid interfere with encapsulation as the concentration of these acids in the oil was increased.

Surfactants incorporated in either the oil phase or water phase interfered greatly with the encapsulation process, and a possible reason for this behavior is discussed.

Increasing the saponification value of the light mineral oil with coconut oil showed no appreciable effect on the permeability of the coacervate shell.

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Determination of Some Mercurials *via* Acetolysis and Nonaqueous Acidimetric Titration

By KENNETH A. CONNORS and DAVID R. SWANSON

An analytical method for certain mercurials is based upon their solvolysis in acetic acid to yield either mercuric acetate or a monosubstituted mercuric acetate. When an excess of methylamine hydrochloride is added, an amount of strong base is released which is equivalent to the mercury-bound acetate. The solution is titrated with standard acetous perchloric acid to complete the analysis. The method is applicable to many mercurials containing a mercury-nitrogen, mercury-oxygen, or mercury-sulfur bond.

MOST ANALYTICAL procedures for mercurials involve release of the mercury by drastic oxidative or reductive treatment and ultimate conversion to mercuric ion, which is then determined by classical methods. The analysis of mercurials has recently been reviewed by Medwick (1).

Two general disadvantages may be associated with these conventional analytical methods; they may be laborious and time consuming, and they lack specificity. This paper reports a simple and rapid titration procedure suitable for the analysis of many mercurials of pharmaceutical interest.

Principle of Method.—The mercurials used in pharmacy can in most cases be represented by the general structure R—Hg—R′, in which one but not both of the bonds may be a carbon-mercury bond. These compounds undergo solvolysis in acetic acid according to Eqs. 1 and 2. The reaction conditions and the nature of the R group determine whether the reaction is essentially stopped with the formation of the monosubstituted mercuric acetate (Eq. 1) or proceeds to yield mercuric acetate (Eq. 2). If an excess of a halide salt of a base is added to the solvolysis reaction mixture, a quantity of strong base will be released which is equivalent to the acetate bound to mercury (Eqs. 3 and 4).

$$R—Hg—R' + HOAc \rightleftarrows R—Hg—OAc + R'H \quad \text{(Eq. 1)}$$

$$R—Hg—OAc + HOAc \rightleftarrows Hg(OAc)_2 + RH \quad \text{(Eq. 2)}$$

$$R—Hg—OAc + BX \rightarrow R—Hg—X + BOAc \quad \text{(Eq. 3)}$$

$$Hg(OAc)_2 + 2BX \rightarrow HgX_2 + 2BOAc \quad \text{(Eq. 4)}$$

The base which is released can be accurately titrated with standard acetous perchloric acid to give a measure of the original mercurial compound. This procedure is seen to utilize a reversal of the Pifer-Wollish method for the titration of amine salts, in which an excess of mercuric acetate is added and the base which is freed may be titrated with perchloric acid (2). It cannot be expected to be suitable for the analysis of mercuric halides, for their acetolysis is essentially the reverse of the reaction upon which the titration is based.

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EXPERIMENTAL

Materials.—Methylamine hydrochloride (Eastman Kodak) was recrystallized from absolute ethanol; *p*-naphtholbenzein and quinaldine red were used directly; glacial acetic acid (reagent grade) was used directly; mercurial samples were dried under reduced pressure before use.

Reagents.—Perchloric acid (0.1 *N*) was prepared by adding 11.0 ml. of 60% perchloric acid and 36.0 ml. of acetic anhydride to several hundred milliliters of glacial acetic acid and diluting to 1 L. with acetic acid; it was standardized against potassium biphthalate using *p*-naphtholbenzein as a visual indicator; 0.1 *M* methylamine hydrochloride was prepared by dissolving about 675 mg. of recrystallized methylamine hydrochloride in enough glacial acetic acid to make 100 ml.; 0.1 *N* aqueous ammonium thiocyanate was standardized against silver nitrate using ferric ammonium sulfate as an indicator. *p*-Naphtholbenzein (0.4%) in glacial acetic acid and a saturated solution of quinaldine red in glacial acetic acid were also employed.

Potentiometric Titration.—A Beckman model H-2 meter was employed for potentiometric titrations. A general purpose glass electrode served as the indicator electrode, and a fiber-type calomel electrode, modified by replacing the aqueous potassium chloride solution with 0.1 *M* lithium perchlorate in glacial acetic acid, was the reference.

Analytical Procedures.—Some variation in the basic procedure is necessary for certain compounds. The procedures recommended are described, and their applicability will be discussed in the following sections.

Procedure A.—A sample corresponding to 0.5 to 0.8 meq. of mercurial is accurately weighed into a flask and is dissolved in glacial acetic acid. Several drops of indicator solution are added, and the solution is titrated to the end point with standard 0.1 *N* perchloric acid; a 10-ml. buret is used. A small

TABLE I.—COMPARISON OF ASSAY RESULTS BY ACETOLYSIS AND THIOCYANATE PROCEDURES

Mercurial	Acetolysis n[a]	Acetolysis Recovery, %	Acetolysis S.D.[b]	Thiocyanate n[a]	Thiocyanate Recovery, %	Thiocyanate S.D.[b]
Mercuric acetate	6	98.8	0.1	4	99.9	0.3
Red mercuric oxide	6	100.5	0.4	7	100.2	0.5
Yellow mercuric oxide	5	99.9	0.5	4	100.0	0.2
Mercuric succinimide	5	98.3	0.3	6	98.6	0.1
Nitromersol	7	97.9	0.3	3	99.1	0.8

[a] Number of determinations. [b] Standard deviation = $\sqrt{\Sigma(x_i - \bar{X})^2/n - 1}$.

(1–2 ml.) excess of 0.1 *M* methylamine hydrochloride solution is added, whereupon the indicator will revert to its basic color. The titration is continued to the end point. The volume of acid consumed between the two end points is equivalent to the mercurial in the sample. If a smaller buret is used, the sample may be reduced to 0.1 meq.

Procedure B.—The weighed sample is dissolved in glacial acetic acid. Indicator and a small excess of methylamine hydrochloride solution are added, and the solution is titrated to the end point with 0.1 *N* perchloric acid. The volume of acid consumed is equivalent to the mercurial plus any strongly basic component in the sample.

Some compounds do not yield satisfactory visual end points and must be titrated potentiometrically (see *Discussion*).

RESULTS

To assess the validity of the proposed method, several mercurials, both inorganic and organic, were analyzed by the acetolysis procedure and by conventional thiocyanate titration (3–5). A comparison of the results, shown in Table I, indicates that the acetolysis method gives assay values which are, within the reproducibilities of the two independent procedures, essentially identical with those of the thiocyanate method. (In certain instances the methods may be expected to yield different results; see below.) Table II lists all of the titration results obtained by the acetolysis procedure, the molecular and equivalent weights for the mercurials, and the procedure employed in each case.

DISCUSSION

Analytical Procedure.—The selection of *Procedure A* or *B* depends upon the structure of the mercurial. It will be noted, from a comparison of the molecular and equivalent weights listed in Table II, that if either or both of the bonds to mercury is a mercury-oxygen or mercury-nitrogen bond that *Procedure A* is usually satisfactory. Such bonds are easily cleaved at room temperature according to Eqs. 1 and 2. *Procedure A* is the more desirable one, for the preliminary titration (before addition of the methylamine hydrochloride) corrects for any basic groups present in the mercurial or in another component of the sample and renders the method quite specific for the mercurial. Figure 1, for example, shows the analysis of both components of a mixture of sodium and mercuric acetates.

Procedure B is necessary for samples which yield a sharp final end point, representing all strongly basic functions in the sample, but would not permit accurate location of the first end point. Thimerosal is such a substance. The acetolysis of this compound gives a thiol (sodium thiosalicylate) and ethyl mercuric acetate. If *Procedure A* is used to analyze this compound, the first end point (representing the titration of the carboxylate group) is obscured by a further consumption of acid for some unknown reason but perhaps is associated with the presence of the thiol group. *Procedure B* must also be employed if the sample contains a weakly basic component, such as theophylline. *Procedure A* in this case would result in two poorly defined end points, each actually representing the titration of the weak base. [In the case of theophylline the first end point is somewhat sharper than the second because mercuric acetate tends to increase the apparent base strength of theophylline (6).] By using *Procedure B* one is able to titrate the strong base component of the mixture, which is a measure of the mercurial present plus any other strong base in the sample.

If the organomercurial contains a carbon-mercury bond which must be cleaved (as in an alkyl or aryl mercuric chloride) neither of these procedures is suitable. More drastic reaction conditions are necessary to solvolyze such a compound. Experiments using perchloric acid as a catalyst under reflux conditions led to nearly quantitative recovery of 0.6 meq. samples of *o*-hydroxyphenyl mercuric chloride in most trials. It was found that an excess of methylamine hydrochloride was required during the reflux, perhaps to shift the equilibrium toward the solvolysis products. The results were observed to be sensitive to the concentration of perchloric acid and to the time of reflux in an irregular manner, and it is believed that side reactions may occur which may lead to nonquantitative recoveries. The acetolysis procedure under reflux conditions is not recommended.

TABLE II.—ANALYSIS OF MERCURIALS BY ACETOLYSIS PROCEDURE

Mercurial	Procedure	Recovery, %	S.D.	Molecular Wt.	Equivalent Wt.	Bond type
Red mercuric oxide	A	100.5	0.4	216.6	108.3	Hg═O
Yellow mercuric oxide	A	99.9	0.5	216.6	108.3	Hg═O
Mercuric succinimide	A	98.3	0.3	396.8	198.4	2 Hg—N
Nitromersol	A	97.9	0.3	351.7	351.7	Hg—O, Hg—C
Phenyl mercuric acetate	A	100.3	0.2	336.8	336.8	Hg—O, Hg—C
Thimerosal	B	100.1	0.2	404.8	202.4	Hg—S, Hg—C
Mercuric nitrate	A[a]	100.5	0.2	324.6	162.3	2 Hg—O
Phenyl mercuric nitrate	A[a]	99.3	0.8	653	327[b]	Hg—O, Hg—C
Meralluride	B[a]	104.9	1.5	611.0	611.0	Hg—N, Hg—C
Mercuric acetate				318.7	159.4	2 Hg—O
(Without theophylline)	A	101.0	0.2			
(With theophylline)	B[a]	100.8	0.5			

[a] Potentiometric titration. [b] *Phenyl mercuric nitrate* is a mixture of phenyl mercuric nitrate and phenyl mercuric hydroxide.

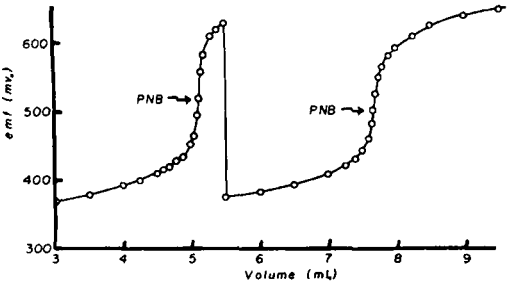


Fig. 1.—Analysis of a mixture of sodium acetate and mercuric acetate by *Procedure A*. Excess methylamine hydrochloride was added at 5.50 ml. The *p*-naphtholbenzein visual end points are indicated.

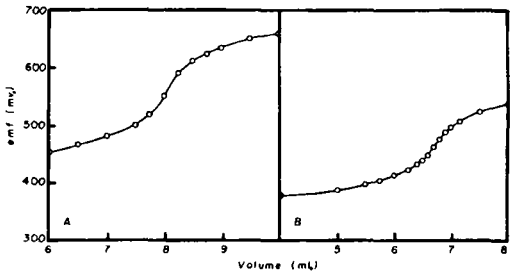


Fig. 2.—(*A*) Potentiometric titration of phenylmercuric nitrate. (*B*) Potentiometric titration of mercuric acetate in the presence of theophylline.

End Point Detection.—Most of the titrations were carried out with visual detection of the end point, using *p*-naphtholbenzein as the indicator. The color change is from yellow (in the basic form) to green (acid form) and is quite sharp. If a large excess of of methylamine hydrochloride is added, the color change becomes rather diffuse due to consumption of perchloric acid by the excess amine salt—hence the direction (see *Experimental*) to keep the excess small. This effect of a large excess of the amine salt is not so noticeable when using quinaldine red as the indicator, probably because quinaldine red is a stronger base than is *p*-naphtholbenzein in the acetic acid system (7, 8).

Mercuric nitrates do not yield accurate visual end points, probably because nitric acid is strong enough in the acetic acid system to compete with perchloric acid. These substances can be successfully analyzed by potentiometric titration (Fig. 2*A*). Potentiometric location of the end point is also necessary if the sample contains theophylline or another very weak base. To demonstrate that the presence of theophylline does not affect determination of the end point, a mixture of mercuric acetate and theophylline was assayed by *Procedure B* with potentiometric titration (Fig. 2*B*); the recovery was essentially identical with that observed in the absence of theophylline (see Table II). Since meralluride contains theophylline, this procedure was employed in its analysis.

Choice of Halide.—Several salts were used in the titration before methylamine hydrochloride was selected as the most suitable one. The quaternary compounds, tetraethylammonium bromide and tetramethylammonium chloride may be employed, but their use always leads to the formation of a precipitate which tends to obscure the indicator color change. Ammonium chloride and potassium chloride are useful salts, but they are relatively insoluble in acetic acid; they were added in the crystalline form. Lithium chloride is soluble in acetic acid, but it appears to be sufficiently ionized in this solvent to be useless for the present purpose. Methylamine hydrochloride is quite satisfactory. It is soluble in acetic acid and is readily purified by recrystallization. If a modest excess is used, sharp end points are observed. It should be recrystallized before use, for its ability to consume a small amount of perchloric acid precludes preliminary neutralization of any free base that may be present as an impurity.

Applicability of Method.—It has been observed with some samples that the proposed acetolysis procedure may give lower results than the thiocyanate titration. This is probably an advantage of the method, for this difference is the result of decomposition of the mercurial, which is not detected by thiocyanate titration. Thus, when a sample of red mercuric oxide was exposed to light and periodically analyzed by both methods, the acetolysis procedure showed a progressive decrease in purity, while the thiocyanate method did not detect this degradation.

Several mercurials cannot be analyzed by the acetolysis method. Mercuric halides and alkyl and aryl mercuric halides have already been discussed. Merbromin, mercuric sulfide, and mercuric sulfate are not soluble in acetic acid. In the analysis of merethoxylline fading end points were noted, though very slow titration eventually led to quantitative recovery, with the equivalent weight equal to one-half the molecular weight. It may be that a slow solvolysis of the amide bond occurs, with consumption of the titrant acid by the product amine.

Differences in solubilities and solvolytic reactivities may permit the analysis of certain mixtures of mercurials by this method.

The acetolysis procedure described in this paper is not of general applicability to the analysis of mercurials. Consideration of the structures of the mercurials listed in Table II will indicate the types of compounds which can be successfully determined. The method possesses the advantages of speed and simplicity for such compounds.

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Soluble Steroids II

Amino Acid Derivatives

By WINTHROP E. LANGE and MARTIN E. STEIN*

A series of amino acid derivatives of cholesterol, 9α-fluorohydrocortisone, 6α-fluoro-16α-hydroxycortisone-16,17-acetonide, and 2α-methyl-5α-dihydrotestosterone have been synthesized in a further attempt to provide compounds with increased aqueous solubility. The acylable hydroxy group of the steroid was converted to the chloroformate and was then condensed with the appropriate amino acid. The potassium salts of the various derivatives which have been prepared possess 100–600 times the aqueous solubility of the parent compounds. Infrared characteristics of the several compounds have been determined. Pharmacological studies have been undertaken and show, in general, reduced activity when the derivatives are administered subcutaneously.

THERE ARE two general methods by which the aqueous solubility of steroids has been increased. The first method is the addition of an adjunct; the second method is the preparation of derivatives having greater water solubility than the parent steroid. Of the many modifications that have been reported, the preparation of carbamates have been mentioned in only a few cases. In the first paper in this series (1) glucamine, glucosamine, and *N*-methylglucamine carbamates of various steroids were prepared. These derivatives possessed eight to 13 times the aqueous solubility of the parent compounds. Thus, it was felt that a series of steroids containing amino acid moieties, capable of salt formation, should give even greater water solubility. The slow hydrolysis of carbamates should give depot solutions of the various steroids.

The references to the use of amino acids to solubilize steroids are found primarily in the patent literature. A British and a Dutch patent describe the preparation of various steroid esters of amino acids and their treatment with hydrohalides to give compounds which are readily soluble in water (2, 3). Another patent covers salts of amino acid esters as well as diethylamine, piperidine, morpholine, and piperazine carbamates of hydrocortisone (4).

Amino acids have been used to increase the water solubility of antibiotics (5). When methionine, glutamic acid, or their *N*-acyl derivatives were allowed to react with streptomycin, dihydrostreptomycin, neomycin, viomycin, or other basic antibiotics, the toxicity of the antibiotics was reduced while the water solubility increased.

The effect of the presence of carbamate or carbonate moieties on the pharmacological activity of steroids has been reported by Brown and coworkers (6). They reported that *N*-alkyl 21-carbamates of corticosteroids had local but no systemic activity, while the 21-ethylcarbonate of hydrocortisone had systemic activity comparable to the free alcohol.

The present investigation is thus intended to make available for pharmacological testing various steroids containing amino acid moieties, such as glycine, phenylalanine, and glutamine.

DISCUSSION

Cholesterol was used as the model steroid for preliminary investigation because of its cost and ease of conversion to its chloroformate. The chloroformate was prepared according to the method described by Wieland, Honold, and Pascual-Vila (7). The chloroformate was then refluxed with an excess of glycine in a dioxane-water mixture. The reaction was completed in 4 hours, and the product, 3β-(5-cholestenyl)*N*-glycylcarbamate, was isolated. Carbon-hydrogen analyses on recrystallized samples of this compound indicated that the desired derivative had been obtained. The glycine derivative was also successfully prepared by refluxing an acetone-water mixture of the starting materials for 4 hours. However, with pyridine as the solvent, only 3β-(5-cholestenyl)formate was obtained.

3β-(5-Cholestenyl) chloroformate was also coupled with phenylalanine, glutamine, and the ethyl ester of β-alanine. However, no product was isolated when the chloroformate was treated with methionine, cysteine, glutamic acid, or serine under identical conditions. Further attempts to prepare these derivatives of cholesterol by either changing the solvent system, refluxing time, or concentration ratio were all unsuccessful.

The potassium salts of the phenylalanyl and glycyl carbamates of cholesterol were prepared by dissolving the carbamates in *n*-butanol and adding, in portions, a 40% solution of potassium 2-ethylhexanoate in *n*-butanol until no further precipita-

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* Fellow of the American Foundation for Pharmaceutical Education, 1961–1962.

TABLE I.—CHLOROFORMATE AND AMINO ACID DERIVATIVES OF SELECTED STEROIDS

Compd.	M.p., °C[a]	Yield, %	Formula	Analyses, %[b] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
Cholesterol-GC[c]	220–222	95	$C_{30}H_{49}NO_4$	73.92	74.16	10.12	9.67
Cholesterol-PC[c]	164–166	60	$C_{37}H_{55}NO_4 \cdot H_2O$	74.59	75.15	9.64	9.65
Cholesterol-GlC[c]	138–140	60	$C_{33}H_{54}N_2O_5$	70.92	70.36	9.76	9.81
Cholesterol-GC, K salt	240–242	85	$C_{30}H_{48}NO_4K$	68.53	67.81	9.19	9.75
Cholesterol-PC, K salt	195–197	70	$C_{37}H_{54}NO_4K$	72.15	71.83	8.83	9.02
Cholesterol-GlC, K salt	212–215	80	$C_{33}H_{53}N_2O_5K$	66.43	65.98	8.95	8.61
Cholesterol-AC[c]	100–102	50	$C_{32}H_{55}NO_4$	74.81	75.56	10.46	10.22
9α-Fluorohydrocortisone-GC	165–166	80	$C_{24}H_{32}FNO_8$	59.87	60.70	6.69	7.32
9α-Fluorohydrocortisone-PC	165–167	75	$C_{31}H_{38}FNO_8$	65.13	65.21	6.70	7.41
9α-Fluorohydrocortisone-GlC	170–172	60	$C_{27}H_{43}FNO_9$	58.65	60.01	6.75	7.45
Fluandrenalone chloroformate	175–177	90	$C_{25}H_{32}ClFO_7$	60.20	60.85	6.27	6.62
Fluandrenalone-GC	164–166	40	$C_{27}H_{36}FNO_9$	60.10	60.14	7.15	6.97
Fluandrenalone-GC, K salt	>300	75	$C_{27}H_{35}FNO_9K$	56.33	55.93	6.13	6.41
2-Methyl-dihydrotestosterone chloroformate	153–156	60	$C_{21}H_{31}ClO_3$	68.85	69.50	8.54	8.15
2-Methyldihydrotestosterone-GC	209–211	25	$C_{23}H_{35}NO_5$	68.20	68.69	8.69	8.44
2-Methyldihydrotestosterone-GC, K salt	223–226[d]	80	$C_{23}H_{34}NO_5K$	62.67	62.45	7.72	8.00

[a] Melting points are uncorrected. [b] Analyses by Weiler and Strauss, Oxford, England. [c] GC is *N*-glycyl carbamate, PC is *N*-phenylalanyl carbamate, GlC is *N*-glutamine carbamate, AC is *N*-(β-alanyl ethyl ester) carbamate. [d] With decomposition.

tion was noticed. The potassium salt of the glycyl carbamate was hygroscopic.

The product obtained after the treatment of 9α-fluorohydrocortisone with phosgene, containing only one chloroformyl group, has been previously described (1). Similarly, it was found that only a monochloroformate of fluandrenalone (6α-fluoro-16α,17α-isopropylidenedioxy-Δ⁴-pregnen-11β,21-dihydroxy-3,20-dione) could be prepared. Treatment of 9α-fluorohydrocortisone and fluandrenalone with glycine, phenylalanine, and glucamine gave the desired carbamates.

An attempt was also made to prepare various amino acid derivatives of 2α-methylandrostan-3-one-17β-ol(2-methyl-dihydrotestosterone). The chloroformate was obtained in a 70% yield, but only the glycine derivative was obtained in a pure form. The incompleteness of the reaction and the difficulty in finding a good purification system affected the preparation of the other amino acid derivatives. The physical properties, analyses, and yields of the various chloroformate and amino acid derivatives are given in Table I.

Infrared Data.—The significant changes which could be observed in the infrared spectra between 2.5 and 7.0 μ of the amino acid derivatives can be attributed to the particular product obtained.

The carbonyl band of the chloroformyl radical exhibits a maximum between 5.62 and 5.63 μ for all of the steroids. The carbonyl band for the substituted carbamates exhibits a maximum between 5.80 and 5.95 μ. The amino acid derivatives also show a new band between 6.5 and 6.6 μ which is attributed to the amide II band of secondary amides or secondary carbamates. The carboxyl group of the amino acids appears as a maximum at 5.70 to 5.82. When the potassium salt was prepared, the carboxyl band at 5.70 to 5.82 μ shifts to 6.20 to 6.30 μ (8). The band at 5.94 to 6.05 μ in some steroids is attributed to the 3-keto function.

Thus, the presence of the characteristic carbamate band, the carboxyl band, the ability of the amino acid compounds to form pure potassium salts, and the preparation of a carbamate with the ethyl ester of β-alanine furnishes evidence that the derivatives are carbamates and not carbonates. The characteristic maxima of the compounds are listed in Table II.

Solubility Studies.—Since the primary objective of this project was a further attempt to prepare a series of steroid derivatives which would have an increased solubility in water over the parent steroids, a solubility study was undertaken. Utilizing the gravimetric procedure previously described (1), it was found that the amino acid moiety had very little effect on the water solubility of the parent steroid. However, when the potassium salt of the glycyl derivative was tested, it increased the solubility of cholesterol 600 times, fluandrenalone 100 times, and 2α-methyl-dihydrotestosterone 200 times. The potassium salt of the glutamine derivative was as effective in increasing the solubility of cholesterol, but the potassium phenylalanyl derivative was only one-half as effective.

Preliminary Pharmacological Results.—At the present time, the potassium salt of the glycyl derivatives of 2-methyl-dihydrotestosterone and fluandrenalone have been tested in the pharmacology laboratories of Eli Lilly and Co.

The fluandrenalone-glycyl carbamate when administered subcutaneously to mice or rats at a dose level of 0.25 to 1.0 mg./Kg. was inactive in either anti-inflammatory or antiestrogenic studies. The 2-methyl-dihydrotestosterone compound, when tested for myotrophic-androgenic activity by subcutaneous injection in rats, was about one-third to one-fourth as active as Dromostanolone propionate (myotrophic-androgenic ratio of 1.58). At present, the studies of the same compounds given by oral administration have not been completed.

EXPERIMENTAL

Where a number of compounds were synthesized by a similar method, a typical preparation is given.

6α - Fluoro - 16α,17α - isopropylidenedioxy - Δ⁴-pregnen-11β-hydroxy-3,20-dione-21-chloroformate.—A solution of 2 Gm. (0.0044 mole) of fluandrenalone (Cordran, Eli Lilly and Co.) in 50 ml. of tetrahydrofuran was saturated with phosgene. The solution was allowed to stand for 3 hours, and the

TABLE II.—INFRARED ABSORPTION CHARACTERISTICS

Compd.	Absorption Maxima, μ[a]						
Cholesterol	2.80	...	...	...	...	...	...
" chloroformate	...	5.62	...	...	...	...	...
" GC[b]	2.90	...	5.70	5.95	...	...	6.35
" GC, K salt	2.95	...	...	5.90	...	6.20	6.55
" PC[b]	2.80	...	5.73	5.89	...	...	6.55
" GlC[b]	2.85	...	5.73	5.89	6.00	6.30	6.45
" GlC, K salt	2.80	...	...	5.89	6.00	6.20	6.50
" AC[b]	2.80	...	5.71, 5.78	5.82	...	...	6.60
9α-Fluorohydrocortisone	2.90	...	...	5.80	6.01	...	...
" chloroformate	2.85	5.60	...	5.90	6.10	...	...
" GC	2.80	...	5.79	...	6.00	...	6.50
" PC	2.85	...	5.75	...	6.05	...	6.60
" GlC	2.86	...	5.77	...	6.00	...	6.50
Fluandrenalone	2.83	...	...	5.83	5.94	...	...
" chloroformate	2.84	5.66	...	5.78	6.00	...	6.60
" GC	2.83	...	5.75	5.80	6.00	...	6.50
" GC, K salt	2.85	...	...	5.80	6.00	6.25	6.50
2α-Methyldihydrotestosterone	2.82	...	...	5.80	5.95	...	...
" chloroformate	...	5.62	...	5.80	6.00	...	...
" GC, K salt	2.82	...	5.75	5.80	5.95	...	6.50
" GC, K salt	2.88	...	...	5.80	5.92	6.25	...

[a] All determinations were made on a Perkin-Elmer model 137 Infracord spectrophotometer. Samples were mounted as Nujol mulls. [b] GC is *N*-glycyl carbamate, PC is *N*-phenylalanyl carbamate, GlC is *N*-glutamine carbamate, and AC is *N*-(β-alanyl ethyl ester) carbamate.

solvent was removed *in vacuo*. The residue was dissolved in hot acetone, the solution was treated with charcoal, and was filtered. The solvent was allowed to evaporate spontaneously; the product which remained melted at 175–177°. The compound darkened on exposure to moisture and light.

21-(6α-Fluoro-16α,17α-isopropylidenedioxy-Δ^4-pregnen-11β-hydroxy-3,20-dione)-N-(carboxymethyl)carbamate Potassium Salt.—To 1.5 Gm. (0.003 mole) of 6α-fluoro-16α,17α-isopropylidenedioxy-Δ^4-pregnen-11β-hydroxy-3,20-dione-21-chloroformate in 30 ml. of acetone was added 0.75 Gm. (0.01 mole) glycine (Merck and Co., Inc.) in 30 ml. of water. The mixture was refluxed for 4 hours, filtered hot, and the resulting filtrate was concentrated by spontaneous evaporation. The resulting precipitate was collected on a filter. Purification of the product was accomplished by treating an acetone solution of the compound with charcoal and recrystallization from ethanol. The tan product, after drying, melted at 164–166°.

The potassium salt was prepared by dissolving 0.35 Gm. of the compound in 5 ml. *n*-butanol and adding 0.5 ml. of a 40% solution of potassium 2-ethylhexanoate in *n*-butanol. The resulting precipitate was collected on a filter and dried. An 80% yield of the water-soluble product, m.p. 212–215°, was obtained.

17β-(2α-Methylandrostan-3-one)N-(carboxymethyl)carbamate Potassium Salt.—To 1.84 Gm. (0.005 mole) of 2α-methylandrostan-3-one-17β-yl-chloroformate (obtained in the usual manner from 2α-methyl-5A-dihydrotestosterone, Eli Lilly and Co.) in 50 ml. of dioxane was added 0.75 Gm. (0.01 mole) glycine in 25 ml. of water. The mixture was refluxed for 2 hours and was allowed to stand overnight. It was again refluxed for 2 hours, cooled, and the solvent evaporated *in vacuo*. The resulting product was recrystallized from acetone. A 25% yield of white platelets, m.p. 205–208°, was obtained.

The potassium salt, prepared in the previously described manner, was obtained in an 80% yield and melted with decomposition at 221–224°.

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New Instrument for Measuring Structure in Fluids

By DUNCAN E. McVEAN† and JERE E. GOYAN‡

A new instrument was developed to study the structural properties of fluids. The instrument consists of a steel sphere suspended from a strain gauge in a fluid sample contained in the core of an electromagnet. Static yield values can be obtained by variation of the electromagnetic field. The behavior of structural fluids in this instrument appears to be in agreement with Bingham's theory on fluid structure.

PREVIOUS EXPERIMENTAL methods designed to evaluate the yield values of suspending agents have involved flow of the sample. Once a flow curve of the sample was obtained, the linear portion of the curve was extrapolated to the stress axis and this intercept taken as the classical the Bingham yield value (1). Figure 1 presents an actual rate of shear *versus* stress curve obtained from the M-2 viscometer (2) for a 0.30% Carbopol-934 mucilage which illustrates the three yield values described by Fischer (3). They are the Bingham yield value, f_B, the value most commonly used in pharmacy; f_L, a lower value which is fixed at the beginning of shear (this is not necessarily laminar flow); and f_m, the maximum yield value which is established presumably by the beginning of laminar flow.

Bingham (4) postulated that a certain portion of the shearing stress was used in overcoming the "internal friction" of the material. He stated that if the material were perfectly elastic, it would undergo elastic deformation at stresses below the yield value. The initial restoring force is zero before stress is applied, then gradually increases to a maximum as the shear force is increased. If the applied force increases beyond the maximum value of the restoring force, flow begins.

In a static system forces must still be acting on the suspended particle even though no flow is occurring. If the forces on the particle can be controlled, then the particle itself can be used to study the physical properties of the suspending agent. One method of controlling forces acting on a particle is through the use of a magnetically susceptible particle. If such a particle is placed in a magnetic field, the downward force exerted by the particle can be controlled by varying the intensity of the field.

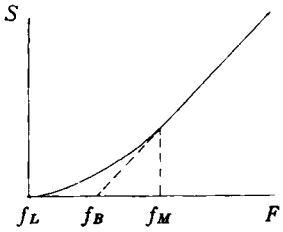


Fig. 1.—Flow curve for a 0.30% Carbopol-934 mucilage illustrates the three classical yield values.

ELECTROMAGNETOYIELDOMETER (EMY)

Figure 2 presents a schematic diagram of the instrument. It consists essentially of a steel sphere attached through a spring to a strain gauge. The sphere is placed in the sample which is contained in the core of an electromagnet. The position of the sphere within the sample can be controlled by adjusting the support of the strain gauge and/or the support of the electromagnet. As voltage is applied to the electromagnet, the sphere is pulled through the sample. The force on the sphere is detected by the strain gauge.

The sphere will descend a lesser distance in a sample of a structural fluid than in a sample of a nonstructural fluid for the same applied voltage, since the structural fluid exerts an additional upward force on the sphere. In a nonstructural fluid there is no additional upward force, and the sphere descends to the point where it is in equilibrium with the upward force of the strain gauge. Thus, the difference between the equilibrium force for a nonstructural fluid and that for a structural fluid is related to the yield value of the structural fluid. This can best be seen by analyzing the forces acting on the sphere.

The force acting downward, $f\downarrow$, is

$$f\downarrow = V(\rho_2)\,g + M \qquad \text{(Eq. 1)}$$

where V is the volume and ρ_2 the density of the sphere, g is the gravitational acceleration, and M is the force due to the magnetic field. For a nonstructural fluid the force acting upward, $f\uparrow$, is

$$f\uparrow = V(\rho_1)g + F_S \qquad \text{(Eq. 2)}$$

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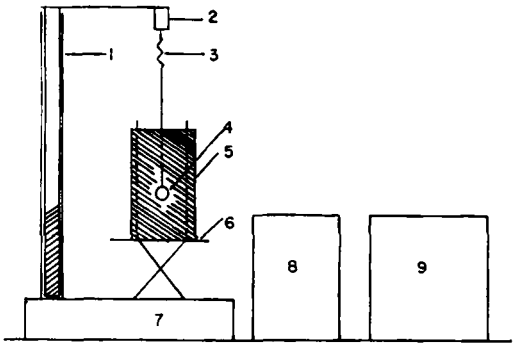


Fig. 2.—The electromagnetoyieldometer. 1, Strain gauge support; 2, Statham transducer, GL-1.5-350, Statham Instruments, Hato Rey, Puerto Rico; 3, spring; 4, steel sphere, diameters 0.635, 0.794, and 1.111 cm.; 5, magnetic wire wound on hollow glass cylinder; 6, laboratory jack; 7, vibradamp, Fischer Scientific Co.; 8, powerstats. type 116, The Superior Electric Co., Bristol, Conn.; 9, Varian recorder, G-10, Varian Associates, Instrument Division, Palo Alto, Calif.

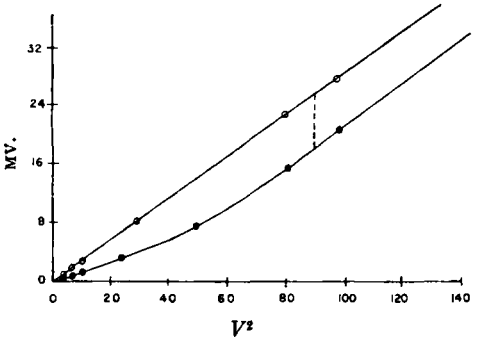


Fig. 3.—A nonstructural fluid, water, (O) compared with a structural fluid, (●) tragacanth supreme mucilage, from data obtained from the electromagnetoyieldometer at high voltages.

where ρ_1 is the density of the sample, and F_S is the upward force of the spring. At equilibrium these forces are equal and

$$F_S = V(\rho_2 - \rho_1)g + M \qquad \text{(Eq. 3)}$$

In the case of a structural fluid the force acting downward is the same as Eq. 1, but the upward force becomes

$$f\uparrow = V(\rho_1)g + F_S + Y \qquad \text{(Eq. 4)}$$

where Y is the resisting elastic force of the fluid. Note that Y will depend on the distance the sphere has moved reaching a maximum just before flow begins. Again, at equilibrium these two forces are equal and

$$F_S = V(\rho_2 - \rho_1)g + M - Y \qquad \text{(Eq. 5)}$$

Therefore, the value for F_S in Eq. 3 is less than the value for F_S in Eq. 5 by the value of Y.

RESULTS OBTAINED WITH THE EMY

The magnetic field intensity is proportional to the square of the applied voltage (5). Therefore, a plot of the force on the sphere (recorded as millivolts) *versus* the square of the applied voltage is linear. Such a plot was designated an EMY curve.

Experiments showed that the sphere could be varied +1 mm. in position with no measurable change in magnetic force. The sphere was allowed to descend no further than 100 μ.

Experimentation was carried out to show that the decrease in force on the sphere in structural liquids was actually due to a structural resistance and not due to a difference in magnetic susceptibility of the liquid or to viscosity. A 0.31% aqueous solution of sodium nitrate, possessing the same viscosity as water but higher ionic strength than the Carbopol-934 solutions, was tested. The EMY curve for this experiment coincided with the EMY curve for water, showing that neither ionic interference or magnetic susceptibility were factors. A sample of 97% glycerin in water whose viscosity was 100 times greater than that of water was studied under the same conditions. The data again showed no difference in the EMY curves.

Experiments did show that the results were very sensitive to temperature changes. As the applied voltage was increased, heating and expansion of the sample occurred. The sample expanded upwards, carrying the sphere with it in the case of structural liquids. This upward movement was detected as a decrease in the strength of the signal from the strain gauge. This heating effect caused resulting EMY curves to bend toward the force axis.

The coefficient of thermal expansion for water at 25° is 64×10^{-6} (degrees^{-1}). Assuming that the temperature is uniform across the core of the electromagnet, a 0.2° temperature change corresponds to a volume change of 1.52×10^{-3} cm.^{3} This would be equivalent to a change in height of the sample in the electromagnet of 15.8 μ. At 3 v. applied potential the sphere moved 50 μ, the error introduced by such a change in height would be approximately 32%. The heating effect was circumvented by applying potentials to the electromagnet rapidly in increments of 2 v. Experimentation ceased when 0.01° change in temperature was noted in a thermistor bridge monitor.

Water was used as the standard nonstructural liquid. Once the linear EMY curve was obtained for water, a structural fluid such as 0.15% Carbopol-934 mucilage was tested under the same conditions. The resulting EMY curve was at first linear with a slope much less than the slope of the curve for water; then it curved upward until it appeared to be parallel to the curve for water. The point where curvature begins can be defined as the elastic limit of the material in analogy to the stress-strain curve of metals. Figure 3 is representative of the EMY curves obtained with structural and nonstructural fluids. The fluids studied were the tragacanth super initial and supreme mucilages and the muci-

TABLE I.—YIELD VALUES OF SELECTED MUCILAGES EXPRESSED IN DYNES/CM.[2]

Mucilage	1.111 cm. Diam. Sphere	0.794 cm. Diam. Sphere
Carbopol-934		
0.23%	175.6	161.2
0.15%	34.5	...
Tragacanth		
Supreme	113.0	...
Super initial	124.8	...

TABLE II.—COMPARISON OF YIELD VALUES OBTAINED FROM THE EMY WITH THOSE OBTAINED FROM THE RISING CYLINDER RHEOMETER[a]

Method	Yield Value			
	Carbopol-934		Tragacanth	
	0.15%	0.23%	Supreme	Super Initial
EMY at 25° C.	34.5	175.6	113.0	124.8
Rising cylinder at 30° C.	16.8	131.7	123.4	156.4

[a] Values are expressed in dynes/cm.²

TABLE III.—EFFECT OF TEMPERATURE ON YIELD VALUE[a]

Mucilage	Temp.,° C.	
	30°	26°
Carbopol-934 0.23%	134.5	175.6
Tragacanth super initial	56.5	78.3

[a] The 1.111-cm. diam. sphere was used in the EMY. Values are expressed in dynes/cm.²

lages of Carbopol-934 prepared in the manner previously described (6).

The values of the forces acting on the different sized spheres in the same sample appeared to be approximately in the ratio of the squares of the diameters of the spheres. Because a suspended particle is being acted upon by forces on all surfaces, it was decided to use the surface area when evaluating the force per unit area.

Figure 3 illustrates the method used to evaluate the yield value of the mucilages. The important section of the figure is the points where the curves for water and the structural liquid are parallel. The distances between these parallel lines are taken as the yield force of the mucilagenous structure. The values of these forces in terms of surface area for the samples tested are listed in Table I. The single value for the 0.794-cm. sphere is presented for comparison of the value obtained from a different sized sphere.

DISCUSSION

The behavior of the EMY curves for structural fluids agrees with Bingham's theory of an opposing elastic force which resists the downward movement of the sphere. This resisting elastic force increases as the sphere moves downward. The curve initially is linear, followed by a nonlinear section where the force is not directly proportional to displacement. Finally, the downward force is large enough to rupture the structure of the fluid. The structure is instantaneously recoverable, so the downward force upon the sphere is continually resisted by the elastic forces. This is the region of the parallel EMY curves. The distance between the curves at the same voltage is the amount of force required to exceed the yield force. If a fluid possesses a structure, it can be detected and evaluated in this manner. When flow curves of fluids possessing structure are evaluated, it should be recognized that a resisting elastic force is always present in addition to the resisting viscosity forces.

Table II presents a comparison of the Bingham yield values obtained from data collected from the Rising cylinder rheometer (6) with the yield values obtained from EMY curves. The correlation of the yield values obtained by extrapolating low shear data with the EMY yield values supports the surface area dependency of the forces acting on a suspended particle.

The data presented in Table II indicate that the method of Bingham is not applicable to the pseudoplastic tragacanth system and that even though the extrapolation to the stress axis was made from an apparently linear portion of the flow curve, curvature was still present. The discrepancy in the data with the Carbopol-934 mucilages is due to the effect of temperature. The data from the EMY were collected at room temperature (approximately 26°), while those from the Rising cylinder rheometer were collected at 30°

To study the effect of temperature upon the yield value, Carbopol-934 mucilage 0.23% and tragacanth super initial mucilage were studied again in the EMY at 30°(Table III). As was expected, the yield values of the mucilages were less at 30° than at 26°. The yield value of the Carbopol-934 mucilage appeared to agree favorably with the yield value obtained with the Rising cylinder. However, the value for the tragacanth mucilage did not. This mucilage was studied again at 26°, and its yield value at this temperature did not agree with the previous determination. It was noted that the previous determination had been made on freshly prepared mucilage, while the mucilage currently being studied was 1 month old. This indicated that the tragacanth mucilage lost considerable suspending ability in 1 month.

Bingham yield values were obtained for other tragacanth mucilages utilizing data collected from the Rising cylinder rheometer and tabulated previously (6). These same mucilages were now studied in the EMY and found to have little or no yield values. The mucilages had been aging for 6 months. It can be concluded therefore that the structural properties of Carbopol-934 mucilages is relatively permanent, while those of tragacanth mucilages deteriorate rapidly with time.

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Inhibitory Effect of Gums and Adsorbants Upon the Migration of FD&C Blue No. 1 in Lactose

By JONAH JAFFE* and IRWIN LIPPMANN

The degree of migration of FD&C Blue No. 1 on a lactose granulation column was determined. Additives to act as dye migration inhibitors were uniformly incorporated into lactose granulation columns (prior to wetting with aqueous dye solutions) and evaluated. Tragacanth (1 per cent), acacia (3 per cent), attapulgite (5 per cent), and talc (7 per cent) were effective dye inhibitors at their respective levels. Solka-Floc was shown to have dye migration tendencies, but was still not completely effective at the 9 per cent level.

VISUAL OBSERVATIONS of commercially available uncoated colored tablets reveal the need, in many instances, for an examination into the cause of lack of uniformity of the tablet color and into a method for its improvement. In a recent article (1), the operation of fluidized bed drying was reported to be an effective procedure in improving color uniformity of colored granulations. Aside from the manufacturing techniques involved in mixing, blending, and drying colored granulations, the problem appears to center on those factors influencing the uniformity of application of dyes to a granulation, the most pronounced phenomenon being migration of the dye.

When a powder or granulation previously wetted with an aqueous dye solution is being air dried, the solvent rises through the powder by means of capillary action and diffusion and evaporates at the surface. Under these conditions, a hydrophilic dye with little affinity for the powder or granulation would, if present, rise with the aqueous solvent and be deposited at the solid-gas interface. This process then could be likened to adsorption chromatography wherein a water soluble dye applied to a weak adsorbant passes through the column with the aqueous eluent.

Although texts on tableting (2, 3) recognize the problem of dye migration, no evaluation or treatment of the subject appears to have been reported. It was therefore of interest to determine quantitatively the degree of migration of a dye in a granulation being dried and to examine the inhibitory influence of several additives on dye migration during the drying process.

EXPERIMENTAL

An aqueous solution of FD&C Blue No. 1 at 0.1% concentration was prepared. To 500 Gm. of lactose U.S.P. (in a Hobart mixer, model N-50) 60 ml. of the dye solution was slowly added and mixed to a reasonably uniform color dispersion. (This required about 5 minutes.) The colored and dampened material was then hand screened through a 16-mesh stainless steel screen. Clear glass vials of uniform internal diameter (29 mm.) and of uniform height (69 mm.) were then rapidly filled in duplicate to within 5 mm. of the top of the vial with approximately 29 Gm. of colored granulation. The material was settled by gently tapping the underside of the vial. The uncovered vials were then placed to dry in a dehumidified room held to a relative humidity of 20% and a temperature of 25°.

FD&C Blue No. 1, having a relatively large degree of water solubility, was chosen as representative of the FD&C dyes commonly employed. The adsorptive abilities of the powder and its constituents must be taken into consideration, as we were

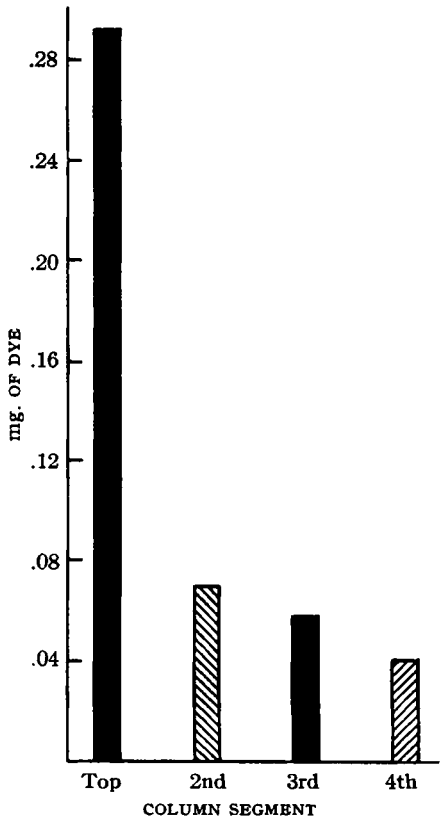


Fig. 1.—Concentration in mg./Gm. of FD&C Blue No. 1 in various segments of a lactose column.

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interested in examining the migration aspects of the dye. It has been indicated by several authors (4, 5) that an inverse relationship between solubility and adsorbability appears to exist. The triphenylmethane group of dyes generally shows better water and polar solvent solubility than the azo, indigoid, and xanthene dyes. FD&C Blue No. 1, a triphenylmethane dye with three sulfonic acid groups, should serve as a dye more difficult to adsorb onto a column than other triphenylmethane dyes with fewer sulfonic acid groups. The inverse relationship of adsorption from aqueous solutions and the number of sulfonic acid groups present on a dye molecule has been reported (6).

As adsorption takes place better at lower temperatures (7), a temperature of 25° was chosen in preference to higher oven drying temperatures. A slow uniform rate of drying also precludes the possibility

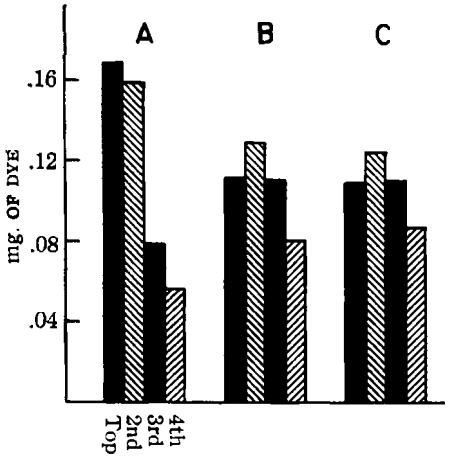


Fig. 2.—Concentration in mg./Gm. of FD&C Blue No. 1 in various segments of lactose columns containing different levels of acacia. Key: A = 2%, B = 3%, C = 4%.

of "case hardening" and subsequent retention of moisture within the column.

After a period of 5 days, to permit the moisture level to drop to insignificant levels (less than 0.4% measured on a Cenco moisture balance) and to assure contact of the dye with any adsorbent that may be present, the columns of material were removed by inverting the vials and tapping the sides and glass bottoms smartly. The intact formed cylinder of granulation was then measured off into equal sections and cut with a sharp blade. Each section was then weighed and dissolved in 50 ml. of distilled water. Absorbance readings, using a Beckman model DU spectrophotometer at a wavelength of 630 mμ, were recorded and the dye concentration in milligrams per gram of lactose were calculated (see Fig. 1).

Since the dye solution is absorbed by the lactose in a sponge-like manner and since the color can be readily washed out of the granulation (as exhibited by the rapid migration of the dye), the process of coloring the lactose is said to be one of "imbibition" and not one of true dyeing (8). This suggested that "colloids which imbibe to their fullest" (9) might act to localize the color and prevent migration. "Thickeners," exemplified by acacia and tragacanth, have

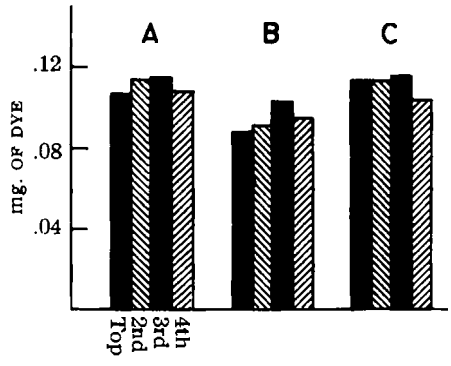


Fig. 3.—Concentration in mg./Gm. of FD&C Blue No. 1 in various segments of lactose columns containing different levels of tragacanth. Key: A = 1%, B = 2%, C = 3%.

been employed in textile dyeing for just this purpose (10). As these gums readily form colloidal dispersions, they were incorporated into the columns of lactose in an attempt to inhibit the migration of the dye.

Solka-Floc, a purified wood cellulose, has been listed as an organic adsorbant (11); this material also has the property of absorbing moisture. These properties led to the inclusion of Solka-Floc as an additive to be evaluated.

Adsorptions of dyes on mineral substances have been amply described in the literature (12). Based on their dispersed position throughout a lactose granulation, it was thought that inert adsorbants, such as talc and activated attapulgite, might adsorb and hold the dyes in place, thereby preventing migration of a hydrophilic dye as the water surfaced and evaporated.

The experimental procedures employed to eval-

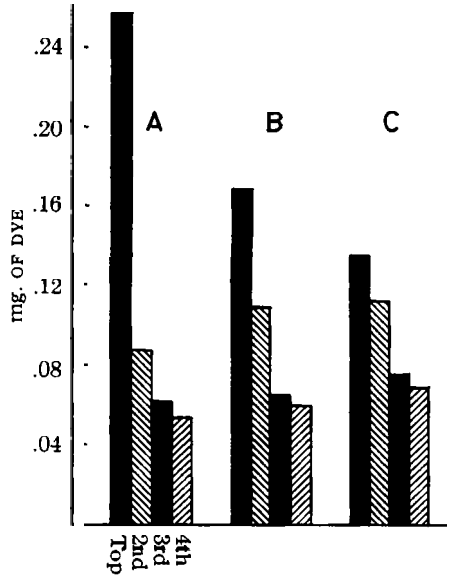


Fig. 4.—Concentration in mg./Gm. of FD&C Blue No. 1 in various segments of lactose columns containing different levels of Solka-Floc. Key: A = 3%, B = 6%, C = 9%.

uate the effect of gums and adsorbants on the migration of dyes were the same as described above, except that each column now contained a uniformly incorporated additive. The calculated concentrations of the prepared dye solutions added to the different granulations was not exactly 0.1% for each solution prepared. It is the relative dye concentration found within each individual column, however, that points to the degree of migration. A level of 3% was first chosen to establish a basis for comparison. Additional concentration levels of the additives were then incorporated and evaluated in an attempt to determine that level which would be effective in inhibiting the migration of the dye.

RESULTS AND DISCUSSION

Figures 2–6 offer a visual representation of the results. Uniformity of height of the bars indicates

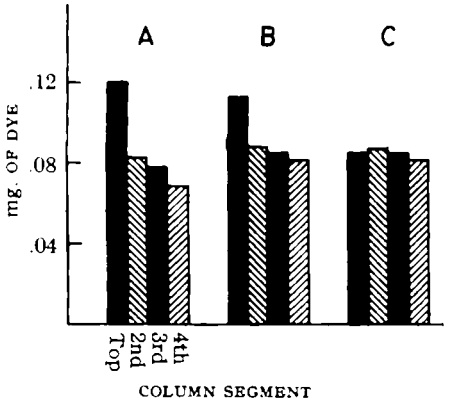


Fig. 5.—Concentration in mg./Gm. of FD&C Blue No. 1 in various segments of lactose columns containing different levels of talc. Key: A = 3%, B = 5%, C = 7%.

the ideal situation of uniformity of color throughout the columns.

Figure 4 reveals the concentration of dye found in the upper portions of the column expressed as per cent of total dye recovered from the column.

Acacia, at the 3% level, inhibited the migration of FD&C Blue No. 1 in our lactose column. Tragacanth appears to be equally effective at the 1% level. The adsorbants, talc and attapulgite, were also effective, but required a greater concentration of additive than that required for the gums. Approximately 5% of activated attapulgite was comparable in effect to 7% talc. Solka-Floc exhibited migration inhibiting tendencies as shown by the inverse relationship between concentration of additive and con-

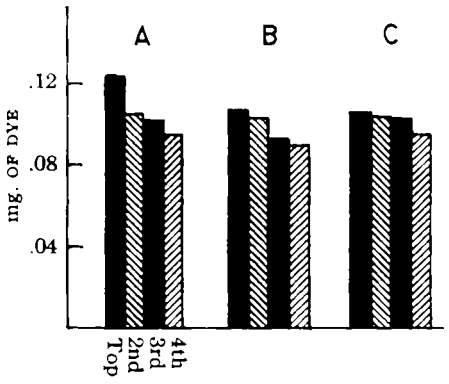


Fig. 6.—Concentration in mg./Gm. of FD&C Blue No. 1 in various segments of lactose columns containing different levels of attapulgite. Key: A = 3%, B = 4%, C = 5%.

centration of dye into the upper portions of the column. However, complete inhibition was not found at the 9% level of additive. Surface area of adsorbant may be a factor influencing the relative abilities of the various additives to inhibit dye migration, but this has not yet been evaluated.

It would seem from the above that gums and adsorbants can be successfully employed to inhibit the migration of FD&C Blue No. 1 on a lactose column. In the order or effectiveness, it has been found that 1% tragacanth is roughly equivalent to 3% acacia, which is approximately equivalent to 5% attapulgite or 7% talc. Extrapolation of the data in Fig. 4 indicates that Solka-Floc at the 12–15% level might also be an effective dye migration inhibitor. Thus, it appears that incorporation of selected gums and mineral adsorbants into formulations of wet granulations inhibits the migration of dyes.

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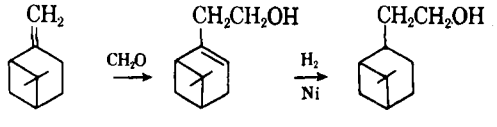
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Hydronopol and Its Derivatives as Possible Sources for New Medicinal Agents

By HEINO A. LUTS, W. ZUCCARELLO, J. F. GRATTAN, and W. LEWIS NOBLES*

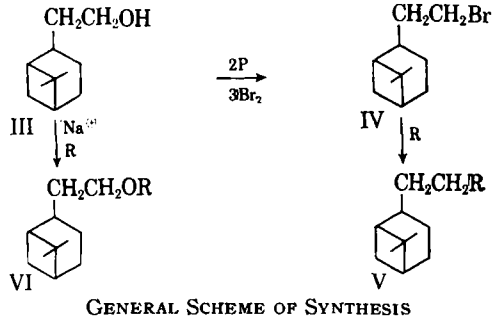
A group of compounds prepared from nopol has been synthesized for pharmacological evaluation. Preliminary studies indicate that they have only a very limited potential in this area, but further studies are in process.

HYDRONOPOL was first prepared (1) by condensing (−) β-pinene (I), a constituent of turpentine, with paraformaldehyde, thus obtaining an alcohol (II), which was named nopol. Hydrogenating nopol over Raney nickel yielded a saturated alcohol (III), the hydronopol.



I II III

Reviewing the literature for new avenues, we found that some nopol and hydronopol derivatives have been made (2, 3), but these were not screened for physiological activity; an exploratory study was thus begun.



GENERAL SCHEME OF SYNTHESIS

In this study compounds 1, 2, and 3 (Table I) had been previously reported (1, 2); the compounds of Type V were prepared by reacting hydronopol bromide with hydrazine in xylene. The compounds of Type VI were prepared by reacting compound III with a corresponding chloro derivative in the presence of sodium.

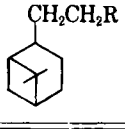
1. Spontaneous Motor Activity in Mice and Rats.—Animals receiving intraperitoneal injections of these derivatives exhibited a biphasic motor response which was characterized by a brief stimulatory phase preceding a period of depression. During the latter stage, the animals exhibited varying degrees of ataxia and sedation, but loss of the righting reflex did not occur. Derivatives III and VII appeared to produce the most profound degree of depression, although none of the hydronopol compounds exerted a significant hypothermic effect.

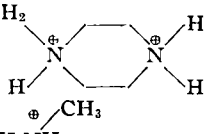
2. Effect of Barbiturate or Ethanol Hypnosis.—Among the various derivatives examined, only V was found to potentiate hexobarbital hypnosis, to effect reinduction of hexobarbital-induced sleep, and to augment ethanol narcosis. The remaining derivatives were inconsistent with regard to the ability to alter the depressant effects of either evipal or ethanol. Derivatives IV, VI, and VII produced varying degrees of mydriasis at high dose levels, but this response was unrelated to their actions on barbiturate or ethanol-induced sleep.

3. Anticonvulsant Action in Mice.—The ability of these compounds to antagonize strychnine-induced convulsions in mice was studied. At the high dose levels employed, only compound II exerted a definite protective effect against strychnine.

4. Analgesic Activity.—The analgesic activity of the hydronopol derivatives was assessed through the use of the following tests: (*a*) phenylquinone-induced writhing, and (*b*) rat tail-flick. Possible analgesic activity was detected in the phenylquinone writhing test for derivatives IV, V, and VI, although none of the compounds was active at the levels used

TABLE I.—HYDRONOPOL AND ITS DERIVATIVES SYNTHESIZED AND TESTED



Compd. No.	R	
1	OH	
2	Br	
3	OCH_2CH_2OH	
4	$OCH_2CH_2\overset{\oplus}{N}(CH_3)_2CH_3$	$I^{\ominus}$
5	$\overset{\oplus}{N}H_2\,\overset{\oplus}{N}H_3$	$2\,Br^{\ominus}$
6		$2\,Cl^{\ominus}$
7	$OCH_2CH_2\overset{\oplus}{N}H(CH_3)CH_3$	$Cl^{\ominus}$

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in the rat tail-flick test. Further, none of the derivatives appeared to possess either antipyretic or anti-inflammatory activity.

5. Anticholinergic Action, In Vitro.—Compounds IV, VI, and VII effectively antagonized the ileal contractures induced by acetylcholine. Compounds III and V were less active, while II was without anticholinergic action even at high concentrations.

6. Cardiovascular Effects.—Hydronopol derivatives IV and VII elicited an immediate, transient, dose-dependent hypotensive response in the chloralosed cat. Both compounds potentiated the pressor response consequent on the intravenous injection of epinephrine, but neither compound altered the depressor responses following the administration of either acetylcholine or histamine. Other studies indicated that these actions reflected the ganglionic-blocking property of these derivatives.

Compounds V and VI also elicited a weak, transient hypotensive response in the anesthetized cat. Like IV, compound V caused relaxation of the nictitating membrane and mydriasis. Neither V nor VI altered the cardiovascular changes after acetylcholine or histamine.

The hypotensive actions of these compounds were not altered when studied in the bilaterally vagotomized, atropinized cat.

7. Local Anesthetic Action.—Derivative IV exerted a local anesthetic effect when applied to the cornea of the rabbit. Associated with this effect, however, was a marked irritation of the ocular mucosal membrane. No attempt was made to titrate these responses.

EXPERIMENTAL[1]

2-Hydronopoxy-1-ethyldimethylamino Methiodide.—Seven grams (0.03 mole) of 2-hydroxy-1-ethylmethylamino hydrochloride was converted to the corresponding base by dissolving it in 15 ml. of water and made basic with sodium carbonate. After salting with sodium chloride, the mixture was extracted twice with 50 ml. of ether. Ether extracts were combined and dried over sodium carbonate. The ether was then evaporated; 15 ml. of dry acetone was added with 5 ml. of methyliodide and refluxed for 5 minutes. The acetone was evaporated and 15 ml. of dry ether added. The formed crystalline product was separated by filtration and washed twice with 10 ml. of ether, m.p. 169–170°, yield 85%.

Anal.—Calcd. for $C_{16}H_{32}INO$: C, 50.04; H, 8.48; I, 33.03. Found: C, 49.89; H, 8.41; I, 33.24.

[1] Analyses performed by G. Robertson, Florham Park, N. J. All melting points are uncorrected.

Hydronopolhydrazide Dihydrobromide.—A 22.99-Gm. (0.1 mole) quantity of hydronopol bromide and 3.7 Gm. (0.1 mole) of hydrazine (85%) in 100 ml. of ethanol were refluxed for 20 hours; then the reaction mixture was evaporated to 1/5 volume and 100 ml. of ether added. Then the mixture was cooled, and the formed crystals were separated by filtration and washed twice with 15 ml. of ether, m.p. 200°, yield 12%.

Anal.—Calcd. for $C_{11}H_{24}Br_2N_2$: C, 38.39; H, 7.02; N, 8.10. Found: C, 38.21; H, 7.17; N, 7.88.

2-Hydronopoxy-1-ethylpiperazine Dihydrochloride.—A 6.5-Gm. (0.05 mole) quantity of hydroxyethylpiperazine was refluxed with 1.14 Gm. (0.05 mole) of sodium in 35 ml. of xylene for 30 minutes. To the formed sodium salt was then added 11.45 Gm. (0.05 mole) of hydronopol bromide; the mixture was refluxed for 20 hours. The reaction mixture was then filtered and the filtrate evaporated to dryness in high vacuum. Seventy milliliters of dry ether was added and acidified with dry HCl. The formed crude product was collected by filtration and recrystallized from the ethanol-ether mixture, m.p. 169°, yield 78%.

Anal.—Calcd. for $C_{17}H_{34}ClN_2O$: C, 64.22; H, 10.77; N, 7.99. Found: C, 64.01; H, 10.69; N, 8.25.

2-Hydronopoxy-1-ethyldimethylamino Hydrochloride.—A 8.9 Gm. (0.1 mole) portion of dimethylaminoethanol was refluxed with 2.29 Gm. (0.1 mole) of sodium in 100 ml. of xylene. After the salt formation was complete, 22.99 Gm. (0.1 mole) of hydronopol bromide was added; the mixture was refluxed for 18 hours. The formed sodium bromide was filtered off and the filtrate evaporated to dryness. To the residue was then added 100 ml. of dry ether, and it was acidified with dry HCl. The formed product was separated by filtration and washed twice with 15 ml. of ether, m.p. 113°, yield 82%.

Anal.—Calcd. for $C_{15}H_{23}NO$: C, 67.17; H, 8.64; N, 5.14. Found: C, 67.02; H, 8.59; N, 5.13.

SUMMARY

A brief, preliminary evaluation of the pharmacologic activity of several hydronopol derivatives indicates that the compounds studied in the reported series have only limited potential in this area.

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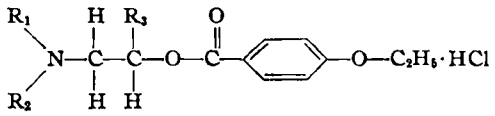
Synthesis of Alkyl Amino Alkanol Esters of *p*-Ethoxy Benzoic Acid

By F. F. MILLIKAN and A. E. WADE

A series of eight alkyl amino alkanol esters of *p*-ethoxy benzoic acid were prepared having hydrogen, methyl, ethyl, isopropyl, butyl, *t*-butyl, benzyl, and benzylmethyl groups so attached to give a secondary nitrogen in five of the compounds. Some physical properties were determined for comparative purposes in connection with the pharmacological studies.

EARLY WORK in the field of local anesthetics established that these compounds were composed of three parts: (*a*) an acid portion, generally a derivative of benzoic acid, (*b*) an alkyl chain, and (*c*) a basic nitrogen group. This combination is achieved by the esterification of the appropriate acid with an amino alcohol, usually a dialkyl alcohol. Of the various acids employed for the acid portion (*a*) of the local anesthetic molecule, derivatives of *p*-ethoxy benzoic acid have exhibited satisfactory pharmacological activity. Some of these derivatives have been patented and are currently being used clinically (1). With this data in mind, the synthesis of some derivatives of *p*-ethoxy benzoic acid have been prepared for pharmacological studies. The compounds and their physical properties (given in Table I) were prepared from secondary and tertiary amino alcohols by esterification with *p*-ethoxy benzoyl chloride.

TABLE I.—DATA FOR THE ESTERS PREPARED



Ester	Substituent Groups R_1	R_2	R_3	M.p., °C.[a]	pH[b] 1% Solution	0.03*M*, pH	Surface[c] Tension, 1% Dynes/cm.	Yield
I	CH_3	H	H	165.0	5.65	5.6	61.7	50.0
II	CH_3	CH_3	CH_3	170.0	4.50	5.4	59.2	91.0
III	C_2H_5	H	H	157.0	5.80	5.5	59.6	19.9
IV	iso C_3H_7	H	H	212.0	6.65	5.6	60.18	31.0
V	*n*-C_4H_9[d]	H	H	140 to 141.0	4.80	5.3	48.4	23.1
VI	*t*-C_4H_9	H	H	222.0	4.80[e]	[e]	[e]	29.0
VII	Benzyl	H	H	176 to 177.0	5.05	5.05	60.9	23.0
VIII	Benzyl	CH_3	H	168.5 to 170.0	4.40	4.40	53.1	70.0

[a] Fisher-Johns uncorrected. [b] Beckman pH meter model G. [c] Solubility did not permit 1% solution. [d] Prepared originally, U. S. pat. 2,372,116(1945). [e] Fisher model 20 DuNouy tensiometer.

EXPERIMENTAL

Acid Chloride.—*p*-Ethoxy benzoic acid and excess thionyl chloride were refluxed for 3 hours. The residue remaining after removal of last traces of sulfur dioxide, hydrogen chloride gas, and excess thionyl chloride, was distilled. The portion boiling at 128–130°/6 mm. was collected.

Alkyl Amino Alkanols.—The following alcohols[1] were used: methylethanolamine (I); 1-dimethylamino-2-propanol (II); ethylethanolamine (III); isopropylethanolamine (IV); *n*-butylethanolamine (V); *t*-butylethanolamine (VI); benzylethanolamine (VII); and benzylmethylethanolamine (VIII).

Esters.—A slight modification of the method described in Mannich's patent (2) was used to prepare the esters. In this work the alcohols (0.05 *M*) were dissolved in a volume of chloroform that equaled the weight of the particular alcohol; this cooled solution was added slowly dropwise to a solution of the acid chloride (0.05 *M* + 10% excess) in a volume of chloroform equal to its weight. The mixture was maintained at 0° throughout the reaction.

After all alcohol was added, the reaction mixture was allowed to stand for about 15 hours. This mixture was then heated for 2 hours at 45–50°, after which the solvent was removed under reduced pressure without heat. The residue in the flask was treated with dry ether and those which were oily to semisolid solidified with stirring. This solid material was collected, washed with dry ether, dissolved in isopropyl alcohol, and heated (not boiled) with Darco. The crystals forming after removal of carbon were then further purified by recrystallization from isopropyl alcohol.

DISCUSSION

The first pH values using a 1% w/v solution showed little relationship to each other when plotted against molecular weight. However, a 0.03-*M* solution of those compounds having a secondary nitrogen, except for Compound IV, gave a straight line when the pH was plotted against milligrams of chemical per 100 ml. (Fig. 1). The graph showed that the pH decreased in value as the molecular weight increased.

The ultraviolet absorption maximum of the esters as hydrochlorides (5×10^{-5} *M*) in water using a Beckman DU spectrophotometer occurred at 260 mμ (Fig. 2). The absorption peak of *p*-

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[1] The authors thank the following companies for the alcohols: I, II, III, IV, and V, Pennsalt Chemical Corp., VI, Rohm and Haas, VII and VIII, Sumner Chemical Co.

TABLE II.—ANALYTICAL DATA FOR ESTERS

Compd.	C Found	C Calcd.	H Found	H Calcd.	N Found	N Calcd.	HCl Found	HCl Calcd.	
I	55.75	55.46	6.91	6.98	5.44	5.39	13.77	14.07	$C_{12}H_{17}NO_3 \cdot HCl$
	55.68		6.71		5.38		13.79		
II	58.45	58.40	7.53	7.71	4.97	4.86	12.68	12.67	$C_{14}H_{21}NO_3 \cdot HCl$
	58.55		7.47		4.81				
III	57.12	57.01	7.29	7.36	5.19	5.11	13.21	13.33	$C_{13}H_{19}NO_3 \cdot HCl$
	57.17		7.27		5.02				
IV	58.31	58.40	7.95	7.71	4.81	4.86	12.66	12.67	$C_{14}H_{21}NO_3 \cdot HCl$
	58.26		7.62		5.09				
V	59.59	59.66	7.76	8.01	4.73	4.64	11.82	12.08	$C_{15}H_{23}NO_3 \cdot HCl$
	59.73		8.01		4.58				
VI[a]	59.98	59.66	7.85	8.01	4.68	4.64	11.82	12.08	$C_{15}H_{22}NO_3 \cdot HCl$
VII	64.22	64.35	6.67	6.60	4.38	4.17	10.46	10.85	$C_{18}H_{21}NO_3 \cdot HCl$
	64.18		6.39		4.12				
VIII	65.03	65.21	6.75	6.91	3.89	4.00	10.42	10.42	$C_{19}H_{23}NO_3 \cdot HCl$
	64.88		6.64		4.04				

[a] Insufficient sample for check analysis.

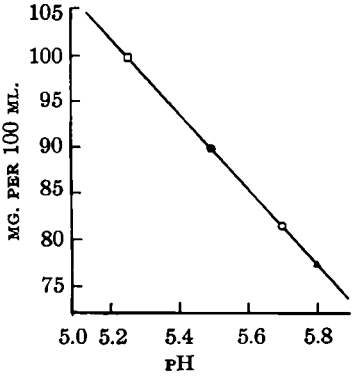


Fig. 1.—Plot of pH values *vs.* 0.03 *M* solution milligrams per 100 ml. Key: △, β-methylaminoethyl-*p*-ethoxybenzoate HCl (I); ○, β-ethylaminoethyl-*p*-ethoxybenzoate HCl (III); ●, β-*n*-butylaminoethyl-*p*-ethoxybenzoate HCl (V); and □, β-benzylaminoethyl-*p*-ethoxybenzoate HCl (VII).

ethoxy benzoic acid (5×10^{-5} *M*) in isopropyl alcohol came at 255 mμ. The alcohol of Compound VII (5×10^{-5} *M*) in isopropyl alcohol gave an absorbance reading of 0.40 at 205 mμ; the absorbance dropped rapidly before reaching 220 mμ, and at 230 mμ approached zero and remained there. The alcohol in Compound VII contained a benzyl group; since it did not change the general curve, it may be assumed that the alcohols just shift the maximum peak toward the longer wavelength. The shoulder or irregularity in the curve between

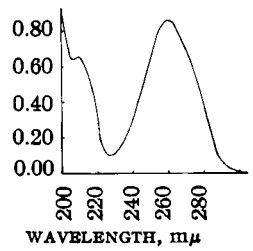


Fig. 2.—General curve which all compounds followed.

200 and 215 mμ may be due in part to the alcohol portion of ester. Six of the compounds had a molecular extension coefficient of 17,200, and Compounds V and IV had values of 17,100 and 17,800, respectively.

The surface tension results seem to follow the observations of Weiser (3) that the surface tension of water is lowered most strongly by organic compounds with a long chain of carbon atoms or with one or more benzene rings. The values obtained indicate that the substituent groups on the nitrogen influence the surface tension more than the benzoic acid group does.

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Modified U.S.P. Tablet Disintegration Apparatus

By LEONARD L. KAPLAN

IN AN EARLIER REPORT (1) a modification of the U.S.P. tablet disintegration apparatus was described which creates more uniform attrition on all sides of the test tablet surfaces and serves to obviate the need for the disks in tablet disintegration testing.

Received June 3, 1963, from Walker Laboratories, Mount Vernon, N. Y.
Accepted for publication July 17, 1963.

The modification consists of a thin flexible insert placed inside the glass tubes of the apparatus at the lower end in contact with the bottom stainless steel wire cloth. The dimensions of this insert are external diameter 21.5 mm., internal diameter 15 mm., wall thickness 3.25 mm., and insert thickness 3.5 mm. The U.S.P. XVI specifies a basket rack assembly consisting of six open glass tubes,

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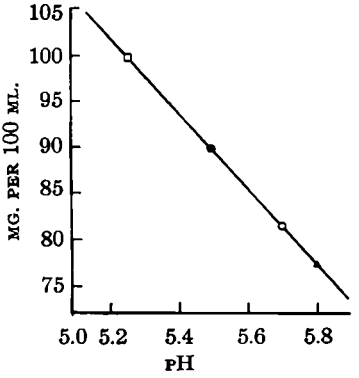


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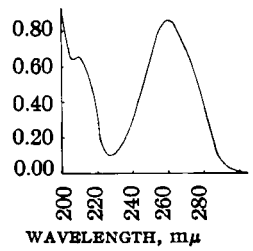


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TABLE I.—PRODUCTS USED IN THIS STUDY

Product	Therapeutic Class	Tablet Wt., mg.	Tablet Diam., in.	Product	Therapeutic Class	Tablet Wt., mg	Tablet Diam., in.
1	Enzyme	600	7/16 flat face	13	Antihistamine	150	5/16 reg. concave
2	Lipotropic	570	13/32 reg. concave	14	Vasodilator	730	1/2 reg. concave
3	Vitamin B_1	240	5/16 reg. concave	15	Digestant	415	3/8 reg. concave
4	Vitamin B_1	198	5/16 reg. concave	16	Multivitamin	480	7/16 square
5	Vitamin C	270	5/16 reg. concave	17	Muscle relaxant	480	7/16 reg. concave
6	Tranquillizer	205	5/16 reg. concave	18	Energizer	103	6/32 reg. concave
7	Analgesic	425	13/32 reg. concave	19	Digestant	890	1/2 reg. concave
8	Multivitamin	395	13/32 reg. concave	20	Multivitamin	450	1/2 oval
9	Enzyme	620	1/2 reg. concave	21	Vasodilator	600	13/32 reg. concave
10	Vasodilator	550	7/16 reg. concave	22	Multivitamin	850	15/32 oval
11	Diuretic	560	7/16 flat face	23	Digestant	1000	5/8 flat face
12	Antacid	777	15/32 flat face	24	Antihistamine	135	9/32 flat face
				25	Calcium pantothenate	200	5/16 reg. concave

each 7.75 cm. long, and each having an inside diameter of approximately 21.5 mm. (2). Thus, the net effect of the insert is to decrease the inside diameter of the lower end of the glass tube in contact with the wire cloth to 15.0 mm.

For any fluid of constant density passing through a stream tube, the product of the cross-sectional area and velocity is constant at all points along the stream tube (3). This is shown by

$$Q = A_1V_1 = A_2V_2 = K \qquad \text{(Eq. 1)}$$

where Q is the flow rate constant K, A is the cross-sectional area of the stream tube, and V is the velocity of the fluid in motion. This equation of continuity states that in steady flow, the mass of fluid passing all cross sections of a stream tube per unit of time remains constant. Therefore, as the cross-sectional area, A_1, of the tube is decreased by the insert to A_2, the velocity of the fluid passing through the tube at that point increases proportionately from V_1 to V_2 to maintain the constant flow rate Q. This increase in fluid velocity at the constricted point creates a turbulence as the basket rack assembly rises and falls. This turbulence causes the tablets to "bob" up and down with greater action, resulting in more uniform attrition to all of the tablet surfaces and prevents adhesion of the tablets to the bottom wire cloth.

EXPERIMENTAL

Twenty-five commercially available uncoated compressed tablet products were subjected to tablet disintegration testing by three methods: (*a*) U.S.P. XVI apparatus, (*b*) U.S.P. XVI apparatus with disks, and (*c*) U.S.P. apparatus with insert

TABLE II.—RANGE OF DISINTEGRATION RESULTS[a]

Product	Minutes — Method A[b]		Method B[c]		Method C[d]	
1	15–18	(3)	12–30	(18)	10–11	(1)
2	27–36	(9)	10–20	(10)	12–16	(4)
3	2–4	(2)	2–3	(1)	1–2	(1)
4	1–3	(2)	1/2–4	(3–1/2)	3/4–1–1/2	(3/4)
5	4–11	(7)	3–7	(4)	2–3	(1)
6	7–11	(4)	5–5–1/2	(1/2)	5–5–1/2	(1/2)
7	8–70+		3–70+		3–70+	
8	1–8	(7)	2–5	(3)	1–5	(4)
9	29–38	(9)	17–22	(5)	20–23	(3)
10	3–6	(3)	3–5	(2)	3–5	(2)
11	1/2–1	(1/2)	1/2	(0)	1/2	(0)
12	1–1–1/2	(1/2)	1–2	(1)	1/2–1	(1/2)
13	2–4	(2)	1–4	(3)	1/2–3	(2–1/2)
14	42–70+		20–47	(27)	23–29	(6)
15	70+		14–17	(3)	12–15	(3)
16	31–60	(29)	4–11	(7)	17–26	(9)
17	6–10	(4)	7–10	(3)	4–6	(2)
18	2–1/2	(0)	2–2–1/2	(1/2)	2	(0)
19	35–40	(5)	12–13	(1)	25–32	(7)
20	50–70	(20)	15–23	(8)	28–40	(12)
21	70+		10–49	(39)	48–57	(9)
22	43–63	(20)	15–22	(7)	25–32	(7)
23	70+		8–11	(3)	17–30	(13)
24	1/2–2	(1–1/2)	1/2–1	(1/2)	1/2–2	(1–1/2)
25	3–4	(1)	2	(0)	2–3	(1)

[a] Figures in parentheses represent the range spread in minutes for each product tested by each of the three methods. [b] U.S.P. apparatus. [c] U.S.P. apparatus with disks. [d] U.S.P. apparatus with insert modification.

TABLE III.—STATISTICAL ANALYSIS

Product	Test Method	Arithmetic Mean, min.	S.D.	Coefficient of Variation, %
1	A[a]	16.7	1.2	7.3
	B[b]	19.3	6.6	34.2
	C[c]	10.8	0.4	3.9
2	A	30.7	3.5	11.5
	B	12.6	3.6	28.6
	C	13.6	2.4	17.2
5	A	5.9	2.3	38.1
	B	4.3	1.1	25.1
	C	2.6	0.6	21.2
6	A	8.4	0.9	10.2
	B	5.1	0.4	7.8
	C	5.3	0.3	4.9
9	A	32.8	3.0	9.3
	B	20.3	1.5	7.3
	C	21.9	0.4	1.8
16	A	39.0	14.8	37.8
	B	6.4	3.5	54.4
	C	20.2	2.9	14.1
17	A	7.4	1.0	13.2
	B	7.9	1.2	15.4
	C	4.9	0.6	11.6
19	A	37.3	0.6	1.6
	B	12.2	0.8	6.5
	C	28.3	1.3	4.5
20	A	63.4	4.5	7.2
	B	18.1	2.6	14.2
	C	34.0	3.5	10.4
22	A	55.5	7.8	14.1
	B	18.6	2.2	12.0
	C	28.8	1.2	4.2

[a] U.S.P. XVI apparatus. [b] U.S.P. XVI apparatus with disks. [c] U.S.P. XVI apparatus with insert modification.

modification. Water maintained at 37° was used as the disintegrating medium as specified in the U.S.P. XVI monograph for uncoated tablets. Eighteen tablets of each product were tested using each method. The products used in this study and their therapeutic class, tablet weight, and tablet diameter are listed in Table I.

The results of these disintegration studies in terms of range for the 18 tablets tested for each product are reported in Table II. Observations were terminated at the 70-minute period for the purpose of this study. The figure in the parenthesis represents the range spread in minutes for each product tested by each of the three methods.

ANALYSIS OF RESULTS

Those products in which the mean average disintegration times were less than 5 minutes for all three methods and those products in which the disintegration time end points exceeded 70 minutes are omitted from the statistical analysis of this report. From a practical standpoint, the disintegration times in fractions of minutes recorded for the quickly disintegrating products could not be precisely noted, and therefore would not be valid in the statistical analysis. For purposes of laboratory expediency, the longer disintegrating tablet studies were terminated at 70 minutes and therefore also are not included in this analysis. This left ten products, identified in Table I as products 1, 2, 5, 6, 9, 16, 17, 19, 20, and 22 for the statistical analysis.

The arithmetic mean, standard deviation, and coefficient of variation for the ten products tested by each of the three disintegration methods are reported in Table III. The consistently lower standard deviation and coefficient of variation obtained with the insert modified apparatus (*Method C*) compared to the U.S.P. apparatus (*Method A*) and the U.S.P. apparatus with disks (*Method B*) indicates the degree of variance in the disintegration results caused by the apparatus. The coefficient of variation as a determinant of the relationship between the arithmetic mean and the standard deviation permits comparison of the relative variability introduced by each of the three disintegration methods. The chi-square test determining the goodness of fit of the actual data to the hypothesized distribution was applied to the coefficients of variation and standard deviations of the ten products ranked for each of the three test methods.[1] With four degrees of freedom, the tabled 1% value of chi-square is 13.277. For the coefficient of variation data, chi-square is 13.2, with a probability value of $p \backsimeq .01$. For the standard deviation data, chi-square is 17.4, for which p is less than 0.01. Hence, the hypothesis of a uniform distribution is rejected, the acceptable alternative is that the insert modification provides a disintegration method with significantly less apparatus induced variance than the present U.S.P. apparatus, with and without disks.

CONCLUSIONS

Disintegration studies conducted on 25 commercially available uncoated, compressed tablet products indicate a wide range in results for each product.

Comparative determinations of the apparatus induced variance of the three disintegration methods indicate that the U.S.P. XVI apparatus, with or without disks, introduces a significantly greater degree of variance than the insert modified apparatus.

Based upon the results of this study, the insert modified apparatus warrants consideration as an improved laboratory control method for the *in vitro* testing of tablet disintegrations.

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[1] A formula for chi-square algebraically simplified for this study is

$$\chi^2 = \frac{n}{N}(\Sigma f^2 - N^2)$$

where n is the number of methods ranked, N is the number of products tested, and f is the frequency of results per ranking column.

Oral Absorption with Various Preparations of Spironolactone in Dogs

By C. M. KAGAWA, D. J. BOUSKA, and M. L. ANDERSON

Gastrointestinal absorption of various spironolactone preparations was investigated in acute tests with dogs treated with an aldosterone-like steroid. Absorption was determined by the appearance in plasma of aldadiene, a major spironolactone metabolite with fluorogenic properties, and by renal manifestations of mineralocorticoid antagonism. Variable absorption was noted with tablet forms of spironolactone with particle size controlled to suggest a profound influence of pharmaceutical formulation. Available data would indicate that the assay using dogs is valuable for clinical prediction of absorption differences between spironolactone preparations.

SPIRONOLACTONE[1] is a steroidal spirolactone (1) with the ability to inhibit competitively the renal electrolyte effects of aldosterone (2, 3). Therefore, it has been of interest as a diuretic agent in clinical edema characterized by hyperaldosteronism (4–6). Recently, clinical evidence was presented to suggest differences in gastrointestinal absorption of various pharmaceutical formulations of spironolactone, as judged by the concentration of its fluorogenic Δ^6-metabolite, aldadiene, in plasma (7–9) or by renal properties of the preparations (7, 8, 10–14). Noteworthy in these observations is that relatively poor absorption occurred with commercial tablets of spironolactone, which contrasted with the view developed independently from data on mineralocorticoid-blocking properties of the pure drug in the laboratory (2). All these observations have suggested the need for further studies on absorption characteristics with various pharmaceutical formulations of spironolactone. This report indicates that satisfactory absorption data may be derived from plasma aldadiene and urinary electrolyte measurements in acute tests with dogs.

EXPERIMENTAL

Healthy female mongrel dogs (14 to 19 Kg. body weight), maintained under constant laboratory conditions of temperature (22–25°) and humidity (25–50%), were used for the experiments. A standard diet consisting of 300 Gm. of homogenized dog meal reconstituted to contain 75 meq. Na was fed each day to the animals; water was provided *ad libitum*. All animals had been previously used for other tests and were acquainted with procedures of bleeding from the jugular vein and bladder catheterization. Spironolactone given either as the milled powder or tablet form was administered orally to dogs treated with a mineralocorticoid, deoxycorticosterone acetate (DCA). Each animal was injected intramuscularly in the thigh with 0.25 mg. of DCA in oil at the time of oral treatment with spironolactone, then placed in a metabolism cage for 6 hours. At intervals of 2 hours, a urinary specimen was obtained by catheterization, followed by a 6-7-ml. blood sample from the jugular vein using a heparinized syringe. Preliminary time-response tests with spironolactone (powder or tablets) indicated that there was no advantage in extending the 6-hour period of assay, considering the time factor for maximal plasma aldadiene response and schedule of a working day. DCA was used in conjunction with spironolactone treatment to induce a relative hyperaldosterone-like state, a condition required for demonstrating renal properties of a mineralocorticoid antagonist. DCA alone was expected to reduce the ratio of urinary Na/K, a frequently used criterion of its aldosterone-like activity (15), by inducing Na retention and K loss. Antimineralocorticoid effects were measured by reversal of the Na/K response to DCA. Spironolactone was given in gelatin capsules as the milled powder, as whole 100-mg. tablets[2] wherein spironolactone is compacted in the tablet core, or as whole 25-mg. uncoated tablets[3] which differ in pharmaceutical compounding from the press-coated tablets by dispersion of the drug in tablets with a water-soluble matrix. Both tablet forms were prepared from the powder of spironolactone, with mean particle diameter of 20 μ measured by a Coulter counter. Standard U.S.P. disintegration tests have revealed that the uncoated and coated tablets of spironolactone disintegrate in 1.5 and 20–30 minutes, respectively. Aldadiene concentration in plasma was determined by a fluorometric technique using the Aminco-Bowman spectrophotometer (16), and urinary Na and K determined on the Beckman flame photometer. Relative potencies of various spironolactone preparations were calculated by the method of Irwin as discussed by Pugsley (17).

RESULTS AND DISCUSSION

Table I describes the mean plasma aldadiene and urinary Na/K response following oral administration of spironolactone (powder form) in animals treated with DCA.[4] In general, increasing doses of spironolactone induced progressively higher levels of plasma aldadiene at each 2-hour interval of measurement; accordingly, the aldadiene level over the total period of test, 0–6 hours, determined by averaging the values obtained at 2, 4, and 6 hours for each of the animals, showed a stepwise increment with dosage of spironolactone. Noteworthy in these 0–6-hour data is that each doubling of the dose produced an average change of 0.28 units (antilog of which is 1.9) or approximately a twofold increment, to indicate a direct proportionality between dosage and plasma level of aldadiene. Furthermore, the time-response data demonstrate that maximal levels of plasma aldadiene were achieved more frequently in the fourth hour of study than with measurements

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[1] 3-(3-Oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)-propanoic acid lactone.

[2] Marketed as Aldactone by G. D. Searle and Co., Chicago, Ill.

[3] Marketed as Aldactone-A by G. D. Searle and Co., Chicago, Ill.

[4] Transformation of the original values to logarithms provided homogeneous variances with the various treatments as determined by Bartletts' test (18) and, thereby, a statistically useful index for evaluation of the data.

TABLE I.—PLASMA ALDADIENE CONCENTRATION AND URINARY Na/K RESPONSE FOLLOWING ORAL ADMINISTRATION OF GRADED DOSES OF SPIRONOLACTONE IN DOGS TREATED WITH A MINERALOCORTICOID

Treatment Total Dose, mg. DCA	Spironolactone	Animals, No.	Plasma Aldadiene[a] 2 Hr.	4 Hr.	6 Hr.	0-6 Hr.	Urinary Na/K[b]
...	...	9	...	...	...	...	1.36
0.25	...	6	...	...	...	...	0.77[c]
0.25	25	6	0.79	0.86	0.69	0.78	0.93
0.25	50	6	0.64	1.27	1.12	1.01	1.02
0.25	100	9	1.46	1.57	1.42	1.48	1.23[d]
0.25	200	6	1.66	1.60	1.53	1.60	1.26[d]
0.25	400	6	1.61	2.02	2.01	1.88	1.56[d]

[a] Mean log micrograms of aldadiene per 100 ml. plasma; pooled s = 0.232 at 2, 4, or 6 hours (degrees of freedom or DF = 60) and 0.210 at 0–6 hours (DF = 20). [b] Mean log (Na × 10)/K in the 0–6-hour measurement. [c] $p < 0.05$ relative to untreated controls (pooled s = 0.268, DF = 23). [d] $p < 0.05$ relative to DCA alone.

TABLE II.—RELATIVE POTENCIES OF VARIOUS SPIRONOLACTONE PREPARATIONS GIVEN ORALLY IN DOGS TREATED WITH DCA

Assay	Spironolactone	Relative Potency[a] Plasma Aldadiene	Urinary Na/K
A	Powder form	1	1
	Uncoated tablets	0.99 (0.45 to 2.18)	0.81 (0.31 to 2.12)
B	Coated tablets	1	1
	Uncoated tablets	6.68 (2.81 to 15.89)	3.73 (1.50 to 9.20)

[a] Potency (95% confidence interval), as judged by the 0–6-hour measurements; X^2-tests for parallelism of the dose-response curves indicated validity of the assays ($X^2 \leq 0.878$, which is far below value of 3.841 required for significance at p of 0.05).

at 2 or 6 hours. To circumvent the problem of quantitating time-response data statistically, the 0–6-hour response—an index determined from multiple measurements—was selected as the criterion for the assay of absorption differences. The effects of spironolactone on the ratio of urinary Na/K in the total period of test are summarized in the table, together with reference data of untreated and DCA-treated controls. DCA alone gave a relatively low ratio of Na/K; but, co-administration of spironolactone in graded doses progressively reversed the response to DCA, with significant inhibition occurring at the 100, 200, and 400-mg. doses. These data demonstrate the association of antimineralocorticoid effects and plasma aldadiene levels, not unlike that found in man (7, 8) and suggest the value of using dogs for absorption studies with spironolactone.

Quantitative data on absorption differences with the spironolactone powder form and uncoated tablets using procedures of the 0–6-hour assay are summarized in Table II (*Assay A*). In this evaluation both preparations were tested simultaneously at two dosage levels predetermined by preliminary tests and the experiments replicated to obtain crossover data using a group of nine animals. With a weight dosage consideration of spironolactone, the uncoated tablets showed a relative potency that was statistically indistinguishable from that of the powder form of spironolactone, judged by plasma aldadiene levels and urinary Na/K. Interpretation of these data suggests that absorption was either complete for the tablet and powder form or, with particle size and disintegration time presumed equal, rate-limited by a common dissolution property. In contrast, similar data described an important difference in absorption characteristics between the two tablet forms of spironolactone witnessed by data in *Assay B, e.g.*, the uncoated tablets demonstrated a potency of approximately 7 and 4 relative to that of the coated tablets, using plasma aldadiene and urinary Na/K, respectively, as the criterion of absorption. The difference of potencies was highly significant in both comparisons ($p < 0.01$). Thus, it would appear from the available data that pharmaceutical formulation, expressed possibly by ultimate differences in disintegration and/or dissolution rates of the tablets (8, 19), may be an important variable for absorption characteristics of coated tablets and uncoated tablets of spironolactone. The laboratory data with these tablet forms parallel closely those of clinical studies based on plasma aldadiene levels (8, 9), mineralocorticoid-antagonism (10, 11), and diuretic efficacy in edematous patients (12–14).

The assay procedures in dogs provide, therefore, a convenient means for study of absorption differences with various pharmaceutical preparations of spironolactone. Validity of the procedures is in good agreement with the laboratory data and clinical observations.

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Browning of Spray-Processed Lactose

By CHARLES A. BROWNLEY, JR., and LEON LACHMAN

The relationship that exists between the discoloration of spray-processed lactose and the presence of 5-(hydroxymethyl)-2-furaldehyde (HMF) was investigated. The HMF was determined by reaction with 2-thiobarbituric acid using procedures developed by Keeney and Bassette. The results obtained indicate that spray-processed lactose contains significant quantities of HMF, while conventionally processed lactose contains essentially no HMF.

FOLLOWING THE appearance of spray-processed lactose on the market in 1956, a serious problem soon developed for the users of this common pharmaceutical excipient. This sugar, unlike the lactose prepared by the conventional recrystallization methods, was occasionally received from the supplier brown instead of white, or else turned brown in storage or in systems where such color change had not been previously experienced.

The literature on browning reactions has, in general, emphasized the complexity of the subject and the lack of specific knowledge of the chemical reactions and intermediates involved (1, 2). It is recognized today that browning of food products can be broadly classified into three types.

The most common type—carbonyl-amine reactions—includes the reactions of aldehydes, ketones, and reducing sugars with amines, amino acids, peptides, and proteins. The term "Maillard reaction" (3) has been attached to this type of browning in honor of the man who made the first definitive study of the phenomenon. This type of browning requires a relatively low order of energy for its initiation and exhibits autocatalytic qualities once it has begun.

Another type, called caramelization, occurs when polyhydroxycarbonyl compounds (sugars, polyhydroxycarboxylic acids) are heated to relatively high temperatures in the absence of amino compounds. Dutra, Tarassuk, and Kleiber (4) found that this type of discoloration characteristically requires more energy to get started than carbonyl-amino reactions, other conditions being equal. Both acids and bases are known to catalyze caramelization reactions, but little more than this is known. Patton (5) made the interesting commentary that pure caramelization is rarely encountered in the food field.

A third type of browning frequently encountered by the food processor is the group of oxidative reactions which, for example, convert ascorbic acid and polyphenols into dicarbonyl and polycarbonyl compounds. Oxidative browning is exemplified by the so-called enzymatic browning in fruits and vegetables, but is not necessarily limited to enzyme-initiated systems.

The literature of the dairy chemists and food technologists is abundant with experiments concerning discoloration and degradation of manufactured dairy products, especially dried milk products (4–15). A considerable amount of evidence has implicated Maillard-type browning as the main contributing factor in these products.

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It becomes apparent, after considering the literature on the types of browning, that in studying the browning experienced with lactose U.S.P. prepared by spray drying, investigation of the Maillard-type reaction provides a good starting point.

It is the purpose of this paper to show that a relationship exists between the concentration of 5-(hydroxymethyl)-2-furaldehyde (HMF), a reaction product of the Maillard-type browning, and the degree of browning of spray-processed lactose. In addition, an attempt is made to relate the presence of HMF to the relative stabilities of spray-dried and conventionally processed lactose.

EXPERIMENTAL

Materials

Lactose U.S.P., prepared by spray drying and conventional drying after recrystallization; 5-(hydroxymethyl)-2-furaldehyde, (Aldrich Chemical Co. Inc.), purified by recrystallization from ether and petroleum ether, m.p. 31.5°; 0.3*N* oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) (Fisher Scientific Co.); 40% trichloroacetic acid (Fisher Scientific Co.); and 0.05*M* 2-thiobarbituric acid (Bios Laboratory, Inc.) were utilized.

2-Thiobarbituric (TBA) solution is prepared by warming to 60° and cooling to room temperature before use. The reagent must be prepared fresh. It remains relatively unchanged for a 6-hour period. After this period of time, the absorbance values decrease.

Equipment

Water baths at 40 and 100°, Beckman model DK-1 recording spectrophotometer, Beckman zeromatic pH meter, and a Beckman model DU spectrophotometer with fitted reflectance attachment were employed.

Test Methods

TBA Reaction.—The analytical procedure used for determining the concentration of HMF in lactose includes the selective digestion procedure developed by Keeney and Bassette (15) to convert the early intermediates of the Maillard-type browning reaction into HMF. The HMF is then determined by spectrophotometric measurement of its 2-thio barbituric acid (TBA) reaction product.

The TBA combines with furfural compounds by simple condensation reaction to yield yellow pigments. The reaction provides a sensitive method of measuring the extremely small quantities of the decomposed products of milk—namely, the carbonyl compounds.

The analysis is performed as follows. Dissolve 5 Gm. of lactose in sufficient purified water to make 100 ml. of solution. Pipet 10 ml. of the solution into a 50-ml. test tube, and add 5 ml. of 0.3 *N* oxalic acid.

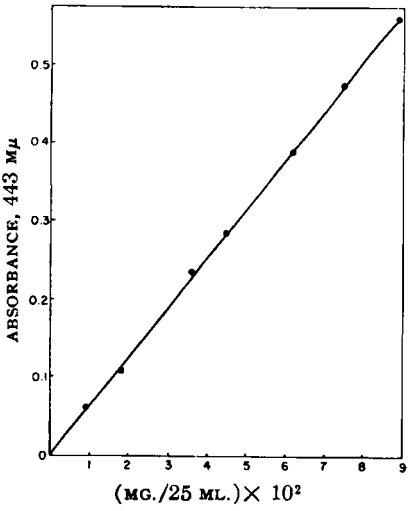


Fig. 1.—Colorimetric assay for 5 -(hydroxymethyl)-2-furaldehyde showing Beer's law relationship.

TABLE I.—CONCENTRATION OF HMF IN LACTOSE U.S.P. PREPARED BY SPRAY DRYING

Sample Age, Mo.	Mg. Free HMF/Kg. of Lactose	Mg. Total HMF/Kg. of Lactose	Relative Degree of Browing[a]	Reflectance, %
2	11.71	13.73	0	93.0
18	14.98	19.98	+	...
20	19.31	20.91	+	75.6
22	17.95	20.60	+	...
24	19.98	25.60	+++	69.8
26	21.23	26.84	+++	...
27	18.73	23.43	+	75.0
27	26.54	36.84	+++	...
29	25.77	33.74	++	70.4
29	32.78	41.52	+++	...
30	23.43	31.24	+++	72.7
30	29.66	39.34	+++	...

[a] From 0 to +++ shows increase in browning.

Cover the tube with an inverted 20-ml. beaker, and place it into a boiling water bath. Immerse it so that the top of the tube and the cover can be kept cool by a gentle flow of air. Remove the tube after 1 hour; cool with cold water to room temperature. (This heating step is omitted in the determination of the free HMF.)

Add 5 ml. of 40% trichloroacetic acid to the digested solution. Mix the solution and filter through Whatman No. 42 paper. Collect the filtrate into a 25-ml. volumetric flask; add 2.5 ml. of 0.05 *M* TBA to the flask. Wash the filter paper with sufficient purified water to bring the volume to 25 ml. Heat the flask and its contents to 40° in a water bath, and keep at this temperature for 30 to 40 minutes. Remove the flask and cool to room temperature. Measure the absorbance of the solution at 443 mμ against a blank prepared in the same manner as the sample, substituting purified water for the lactose solution. Figure 1 shows that the absorbance of TBA/HMF reactant conforms with Beer's law.

The HMF in the sample is converted to milligrams per kilogram of lactose by mg. HMF/Kg. lactose = (absorbance/6.36) × 2000.

Test solutions for spectrophotometric measurement are stable for approximately 1 hour.

The concentrations of HMF found in the sample of lactose are expressed as free HMF or as total HMF. Free HMF value is the quantity found by direct analysis. The total HMF value is a measure of the free HMF plus HMF developed as a result of the selective digestion procedure used.

Reflectance.—Color change in the powder samples was measured by reflectance at 480 mμ. The reference standard was a block of reagent grade magnesium carbonate, and the results are given as per cent reflectance.

pH.—The pH of the test solutions were checked periodically. In the test procedures, the oxalic acid is employed to provide an optimum pH of 3.2 to produce the maximum amount of HMF from its precursors. The trichloroacetic acid provides a pH of 0.8 to 1.0, which is the acidity necessary to carry out the TBA/HMF reaction.

RESULTS AND DISCUSSION

The relationship that exists between the presence of HMF and the browning of spray-processed lactose was investigated. The data presented in Tables I and II show the HMF concentration and extent of browning for various samples of spray-processed and conventionally dried lactose, respectively. These samples are representative of materials obtained from quality control reference stock and have been stored under ambient conditions for varying time intervals.

It is evident from the data in Table I that as the concentration of free HMF increases, the spray-processed lactose darkens in color. The data in Table II for conventionally processed lactose show the presence of no free HMF or color change for storage up to 36 months.

The analysis for early browning intermediates

TABLE II.—CONCENTRATION OF HMF IN LACTOSE U.S.P. PREPARED BY CONVENTIONAL RECRYSTALLIZATION TECHNIQUES[a]

Sample Age, Mo.	Mg. Total HMF/Kg. of Lactose	Reflectance, %
2	6.87	94.7
11	13.42	92.4
12	16.39	93.0
13	15.36	94.3
17	16.39	90.5
20	11.23	93.1
28	8.12	91.9
30	11.71	92.3
34	12.49	91.9
36	13.49	92.9

[a] No free HMF was detected and the lactose remained white with all samples.

TABLE III.—CONCENTRATION OF HMF AND PER CENT REFLECTANCE FOR SPRAY-PROCESSED LACTOSE STORED AT 80° C.

Time, Hr.	Mg. Free HMF/Kg. of Lactose	Mg. Total HMF/Kg. of Lactose	Reflectance, %
0	3.12	18.73	94
2	9.37	24.98	91
4	12.49	28.10	78
6	18.73	32.78	73
8	24.98	39.03	71
16	46.83	...	62
20	56.20	...	59
24	62.44	73.34	59

capable of being converted to HMF produced a positive TBA reaction for spray-processed and conventionally processed lactose. This is evident by the total HMF content of up to 16.39 mg./Kg. lactose for conventionally prepared and up to 41.52 mg./Kg. of lactose for spray-processed lactose. A definitive pattern of increase in total HMF with storage time for spray-processed lactose appears to exist, but no such relationship exists for conventionally processed lactose.

The data in these tables attempt to relate total HMF concentration to color development measured by reflectance readings for both conventionally and spray-processed lactose stored at ambient conditions. It is evident from these data that total HMF does not appear to be the criterion for color development since samples of conventionally processed lactose have considerably higher total HMF values than spray-processed lactose without affecting the color of the lactose. It would appear from these results that it is the concentration of free HMF in the samples that is related to color change, since none of the samples of conventionally processed lactose contained any detectable free HMF, while the spray-processed lactose samples contained varying amounts of free HMF.

It appears from these data that the presence of free HMF is an indication that the complex Maillard-type reaction has begun, and browning will take place once the concentration of HMF reaches a critical value. The data obtained by other investigators appear to substantiate this reasoning. Keeney and Bassette (14) made the general observation that once HMF in dry milk increased to a certain level, flavors associated with color change develop. Craig (12) reported that a quantitative relationship existed between storage stability and initial HMF concentration for vacuum foam-dried whole milk. Newth (16) also reported such a relationship for glucose solutions.

To evaluate the effect of the presence of HMF precursors in conventionally and spray-dried processed lactose on color development, samples of both sugars were heated at 80° for 24 hours. At the end of 24 hours, the total HMF present in conventionally processed lactose was approximately equal to that determined initially. However, in spray-dried lactose an increase in free and total HMF took place as illustrated in Table III. In addition, these samples exhibited a significant degree of darkening, evidenced by the decrease in reflectance values. The data presented in this table illustrate that, as HMF is formed, a concurrent production of coloring matter results.

In an attempt to determine whether the process of spray drying influences the HMF content of lactose, a sample of conventionally processed lactose, containing no free HMF and 13.42 mg./Kg. of total HMF, was spray dried. Testing of this spray-dried material for 1 year at ambient conditions gave no darkening. Spray drying a slurry containing 50% lactose, 5% dextrose U.S.P., and 5% galactose also showed no increase in total HMF after drying and no darkening after storage for 1 year.

These preliminary experiments indicate that the physical operations of spray drying and the addition of the common hydrolysis products of lactose give no additional quantities of HMF. It would appear that the simple hydrolysis products of lactose are not the major precursors of HMF in spray-processed lactose.

SUMMARY

The results obtained in this investigation implicate the presence of free HMF in lactose with resultant browning. Samples of conventionally processed lactose containing no free HMF and heated for 24 hours at 80° and stored at ambient conditions for 36 months showed no darkening. On the other hand, samples of spray-processed lactose, treated similarly, containing free HMF, darkened significantly.

The TBA reaction provides a suitable quantitative method for determining free HMF and should serve as a suitable technique for the quality control of lactose relative to browning possibilities.

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Centrifugally Accelerated Thin-Layer Chromatography

By B. P. KORZUN and S. BRODY

Centrifugal force has been used to accelerate the development of thin-layer chromatograms and the results compared with data on the standard ascending development technique.

CENTRIFUGAL force has been employed to accelerate paper chromatographic separation of compounds in mixtures (1–4). This method was applied to thin-layer chromatography on circular glass plates or aluminum plates. Comparisons were made between standard thin-layer and accelerated thin-layer chromatography. The accelerated method was completed in about 10 minutes, compared to the 30 to 40 minutes required for the standard ascending method.

Circular glass or aluminum plates 26 cm. in diam-

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To evaluate the effect of the presence of HMF precursors in conventionally and spray-dried processed lactose on color development, samples of both sugars were heated at 80° for 24 hours. At the end of 24 hours, the total HMF present in conventionally processed lactose was approximately equal to that determined initially. However, in spray-dried lactose an increase in free and total HMF took place as illustrated in Table III. In addition, these samples exhibited a significant degree of darkening, evidenced by the decrease in reflectance values. The data presented in this table illustrate that, as HMF is formed, a concurrent production of coloring matter results.

In an attempt to determine whether the process of spray drying influences the HMF content of lactose, a sample of conventionally processed lactose, containing no free HMF and 13.42 mg./Kg. of total HMF, was spray dried. Testing of this spray-dried material for 1 year at ambient conditions gave no darkening. Spray drying a slurry containing 50% lactose, 5% dextrose U.S.P., and 5% galactose also showed no increase in total HMF after drying and no darkening after storage for 1 year.

These preliminary experiments indicate that the physical operations of spray drying and the addition of the common hydrolysis products of lactose give no additional quantities of HMF. It would appear that the simple hydrolysis products of lactose are not the major precursors of HMF in spray-processed lactose.

SUMMARY

The results obtained in this investigation implicate the presence of free HMF in lactose with resultant browning. Samples of conventionally processed lactose containing no free HMF and heated for 24 hours at 80° and stored at ambient conditions for 36 months showed no darkening. On the other hand, samples of spray-processed lactose, treated similarly, containing free HMF, darkened significantly.

The TBA reaction provides a suitable quantitative method for determining free HMF and should serve as a suitable technique for the quality control of lactose relative to browning possibilities.

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Centrifugally Accelerated Thin-Layer Chromatography

By B. P. KORZUN and S. BRODY

Centrifugal force has been used to accelerate the development of thin-layer chromatograms and the results compared with data on the standard ascending development technique.

CENTRIFUGAL force has been employed to accelerate paper chromatographic separation of compounds in mixtures (1–4). This method was applied to thin-layer chromatography on circular glass plates or aluminum plates. Comparisons were made between standard thin-layer and accelerated thin-layer chromatography. The accelerated method was completed in about 10 minutes, compared to the 30 to 40 minutes required for the standard ascending method.

Circular glass or aluminum plates 26 cm. in diam-

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TABLE I.—R_f VALUES AND TIME OBTAINED FOR SEPARATION OF DYES USING THE STANDARD AND ACCELERATED METHODS OF THIN-LAYER CHROMATOGRAPHY

Dyes	R_f Value—Accelerated	R_f Value—Standard
Light green SF yellowish	0.00	0.00
Sudan Black B	0.10	0.15
Indophenol blue	0.25	0.35
Sudan R	0.40	0.45
Oil Red O	0.55	0.65
Dimethylaminoazo benzene	0.65	0.70
Adsorbent—aluminum oxide G		
Solvent benzene-hexane (9:1)		
Development time accelerated—10 min.		
Development time standard—35 min.		

eter, 2.5 mm. thick, and with a 1.2-cm. centered hole to fit the Precision hi-speed chromatography[1] apparatus were used. The plates were coated with aluminum oxide G or silica gel G (with binder) by the spray technique of Bekersky (5) or the technique of Davidek and Prochazka (6). The plates were activated in the usual manner.

[1] Precision Scientific Co., Chicago, Ill.

The samples are spotted at a point 2.5 cm. from the center hole. Ten spots may be applied per plate. The inside of the apparatus was lined with heavy filter paper saturated with the solvent used for developing. The plate is secured to the spindle of the apparatus, and the rotation of the plate set at 500–700 r.p.m. The solvent flow was adjusted to permit a constant flow without overloading and possible washing away of the adsorbent.

Table I shows the R_f values and time obtained for separation of dyes using the standard and accelerated methods of thin-layer chromatography.

Accelerated thin-layer chromatography provides a method of decreasing the time necessary to achieve separation.

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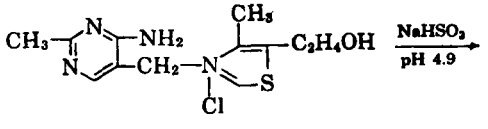
Estimation of Thiamine by Inverse Isotope Dilution

By ANTOINE A. NOUJAIM†, WAYNE V. KESSLER, JOHN E. CHRISTIAN, and ADELBERT M. KNEVEL

Thiamine hydrochloride was determined quantitatively by inverse isotope dilution in the presence of ascorbic acid, riboflavin, and nicotinic acid. The results obtained, using 100-mg. quantities, indicated an average error of 0.1 per cent and a reproducibility of 0.27 per cent.

THE PURPOSE OF THIS investigation was to study a thiamine assay by inverse isotope dilution analysis. Several advantages are inherent in this technique, including speed and simplicity. However, the most outstanding advantage is that quantitative isolation is not required at any point in the analysis. Thus, thiamine may be determined in the presence of other substances without quantitative extraction.

It was necessary to choose a suitable labeling reagent to react with thiamine to produce a chemically stable derivative. Williams, *et al.* (1), proved the structure of thiamine by a quantitative sulfite cleavage into two moieties. This reaction is

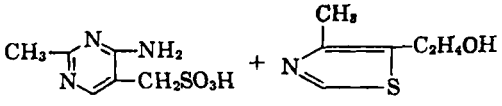


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The specific activity of the derivative was determined indirectly by carrying out inverse isotope dilution analysis with a known amount of thiamine. The labeled derivative was then mixed with a known amount of carrier. The specific activity of the carrier-diluted derivative was determined by measuring the weight and radioactivity of a portion of the purified material. The weight of the derivative before adding the carrier was calculated by employing the standard inverse isotope dilution formula. The weight of thiamine analyzed was calculated from the weight of the derivative by a gravimetric factor.

EXPERIMENTAL

Optimum Conditions for Cleavage Reaction.—



Thiamine hydrochloride was cleaved under different conditions of temperature, pH, and reaction time. Cleavage at room temperature for 15 hours at a pH of 4.9 was chosen. These cleavage conditions are confirmed by the literature (1).

Physical Properties of Derivative.—The derivative crystallized as fine needle-like crystals insoluble

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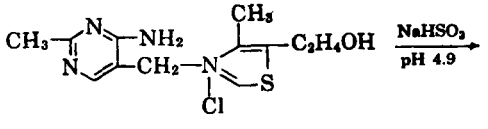
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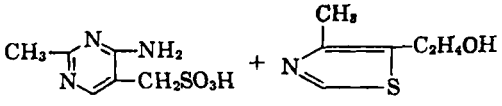


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in cold water and organic solvents such as pyridine, ethanol, methyl ethyl ketone, and chloroform. The derivative was readily soluble in small amounts of ammonia T.S. and did not precipitate upon dilution with water. The derivative was soluble to the extent of about 1.6% in boiling water after prolonged boiling; it precipitated upon cooling slowly. The compound had no sharp melting point, but began to char at about 410°. These properties match those of the compound separated by Williams, *et al.* (2).

Preparation of Carrier.—Fifty grams of thiamine hydrochloride (Merck, manufacturing grade) was dissolved in 600 ml. of water containing 125 Gm. of anhydrous sodium sulfite (Mallinkrodt, A.R.). Hydrochloric acid, 2.6 *N*, was added dropwise to adjust the pH to 4.95. The mixture was set aside for 2 days; the product was then collected with suction filtration. The product was dissolved in the least amount of ammonia T.S., and glacial acetic acid was then added dropwise until the mixture was distinctly acid to litmus paper. The mixture was set aside for another 2 days to allow for complete crystallization. The supernatant was decanted, and the product was dissolved in 1500 ml. of water by boiling on a hot plate for about 5 minutes. The hot plate was turned off; the solution was allowed to crystallize by cooling slowly to room temperature. The mixture was set aside for 2 days. The supernatant was decanted, and the product was dissolved again in 1500 ml. of boiling water. The product formed after cooling was removed with suction, oven-dried at 120°, and stored in a tightly closed container.

Because the carrier did not have a definite melting point, it was assayed potentiometrically to determine its purity. A weighed portion of the carrier was dissolved in 0.1 *N* sodium hydroxide, and this solution was back titrated with 0.1 *N* hydrochloric acid. The results showed an average of 101.2% carrier for three determinations.

Measurement of Radioactivity.—The radioactivity of all samples was measured with a Tri-Carb liquid scintillation spectrometer, model 314-X (Packard Instrument Co., La Grange, Ill.). The instrument was operated with a 10-90 v. window and the freezer temperature was 1°. The XDC scintillator (3), modified by substituting toluene in place of xylene, was used. The counting vials were 20-ml. screw cap, low potassium glass vials (Packard Instrument Co.).

Sample activities were corrected for variation in counter efficiency by use of a C-14 standard. The energy of the C-14 β particle was considered sufficiently similar to that of the S-35 β particle for this purpose. Sample activities were corrected mathematically for decay. All background measurements were made with a blank containing 15 ml. of scintillator, 1.0 ml. of ammonia T.S., and 1.0 mg. of carrier.

A study of the quenching effect of the carrier was performed; the results indicated that quenching did not occur up to 4.0 mg. of carrier in 1.0 ml. ammonia T.S. per sample.

Determination of the Reproducibility of the Cleavage Reaction.—To determine whether the cleavage reaction was reproducible with the cleavage conditions selected, it was carried out with sodium sulfite-S-35 (New England Nuclear Corp., Boston, Mass.). The cleavage reaction was run with 100 mg. of thiamine hydrochloride, and 500 mg. of carrier was added. One milligram of the carrier-diluted derivative was counted, the specific activity of which was approximately 20,000 d.p.m./mg. The specific activity of the sodium sulfite used was approximately 300,000 d.p.m./mg. A 25-ml. 1 *M* solution with the above specific activity was prepared with sodium sulfite-S-35 and carrier (anhydrous sodium sulfite, Mallinkrodt, A.R.).

The cleavage reaction was run three times with a 2% aqueous solution of thiamine hydrochloride (Roche, U.S.P.). The reaction procedure used was similar to that for the analysis of thiamine hydrochloride in the presence of other vitamins, except that the vitamin mixture was not added, and the carrier-diluted derivative was not recrystallized from ammonia-acetic acid.

For each run, 50.0 mg. of the carrier-diluted derivative was dissolved in 50.0 ml. of ammonia T.S. Six samples of each of these solutions, each containing 1.0 ml., were prepared and counted; the results are shown in Table I. The coefficient of variation for the three runs was 0.20%.

Recrystallization Procedure Necessary to Obtain a Constant Final Specific Activity.—Two methods of recrystallization were employed to purify further the carrier-diluted derivative. The remaining parts of the carrier-diluted derivative from the three runs of the previous experiment were dissolved separately in 5 ml. of ammonia T.S. The pH was adjusted to 4.9 to 5.0 with glacial acetic acid; the mixtures were set aside overnight to complete crystallization. The crystals were collected on a sintered-glass funnel, washed with water, and oven-dried at 120° for 2 hours. A solution containing 50.0 mg. of the purified material in 50.0 ml. of ammonia T.S. was prepared from each run. Six samples from each of these solutions, each sample containing 1.0 ml., were prepared for counting.

The remaining parts of the solids from the ammonia-acetic acid recrystallization were boiled separately on a hot plate with sufficient water to dissolve them. The solutions were allowed to cool slowly overnight. The crystals that formed were

TABLE I.—REPRODUCIBILITY OF THE CLEAVAGE OF THIAMINE HYDROCHLORIDE

Run No.	Av. Sample[a,b] Activity, c.p.m.	Coeff. of Variation, %
1	1746	1.20
2	1752	1.26
3	1752	0.80

[a] Average of six samples, each counted for 4 minutes. [b] Samples were counted 5 months after sodium sulfite-S-35 reagent was prepared.

TABLE II.—DETERMINATION OF THE RECRYSTALLIZATION PROCEDURE NECESSARY TO OBTAIN A CONSTANT SPECIFIC ACTIVITY

Run No.	Noncrystallized,[a,b,c] c.p.m.	Recrystallized[a,c] from Ammonia-Acetic Acid, c.p.m.	Recrystallized[a,c] from Water, c.p.m.
1	1746 ± 21	1708 ± 28	1704 ± 23
2	1752 ± 22	1710 ± 21	1707 ± 23
3	1752 ± 14	1697 ± 25	1723 ± 30

[a] Average of six samples, each counted for 4 minutes. [b] From Table I. [c] The limits of each mean are expressed as the standard deviation.

TABLE III.—INVERSE ISOTOPE DILUTION ANALYSIS OF THIAMINE HYDROCHLORIDE IN PRESENCE OF OTHER VITAMINS

Run No.	Prepn.	Av. Activity,[a] c.p.m.	Coeff. of Variation, %	Recovery,[b] %
1	Standard 1	8072	0.63	...
	Standard 2	8038	0.20	...
	Mixture 1	8072	0.21	100.2
	Mixture 2	8074	0.31	100.2
2	Standard 1	7982	0.66	...
	Standard 2	8067	0.88	...
	Mixture 1	8058	0.45	100.3
	Mixture 2	8006	0.77	99.7

[a] Average of three samples, each counted for 5 minutes. [b] Based on 99.6 mg. thiamine hydrochloride actually present.

collected on a sintered-glass funnel, washed with water, and oven-dried at 120° for 2 hours. Samples were prepared for counting in the manner described above.

The results are shown in Table II. The average activities of the three runs recrystallized from ammonia-acetic acid and water were 1705 and 1711 c.p.m., respectively. The conclusion was that recrystallization from ammonia-acetic acid was sufficient for determination of the final specific activity.

Analysis of Thiamine Hydrochloride in the Presence of Other Vitamins.—The following stock solutions were prepared.

1. A 2% aqueous solution of thiamine hydrochloride (Roche, for ampul use) which was oven-dried at 105° for 2 hours before use.

2. A 5% solution of the carrier in ammonia T.S. oven-dried at 120° for 2 hours before use.

3. An aqueous vitamin mixture containing 25.0 mg. ascorbic acid, 0.50 mg. riboflavin, and 10.0 mg. nicotinic acid in each 2 ml. was prepared by warming a suspension of riboflavin and nicotinic acid in water until a clear solution was obtained, then cooling, and adding the ascorbic acid.

4. A 25-ml. 1 *M* sodium sulfite-S-35 solution was prepared so that the final specific activity of the carrier-diluted derivative would be approximately 15,000 d.p.m./mg., calculated on the basis of using five parts of carrier for each part of thiamine hydrochloride cleaved. These solutions were stored in a refrigerator.

Two runs were carried out on consecutive days under the same conditions. For each run, two standards consisting of 5.0 ml. of the 2% thiamine hydrochloride solution and two mixtures consisting of the above solution plus 2.0 ml. of the vitamin mixture stock solution were used. The assay procedure for the mixtures is described. Into a 30-ml. beaker were pipeted 5.0 ml. of the 2% thiamine hydrochloride solution, 2.0 ml. of the vitamin mixture solution, and 2.0 ml. of the 1 *M* sodium sulfite-S-35 solution. The pH was adjusted to 4.9 to 5.0 by adding 2.6 *N* hydrochloric acid dropwise from a buret. The pH was measured with a pH meter. The beaker was then covered with aluminum foil and set aside overnight. Then, 10.0 ml. of the 5% carrier solution was added. The precipitate already present dissolved in the ammoniacal solution. The pH was readjusted to 4.9 to 5.0 with 2.6 *N* hydrochloric acid. After 3 hours, crystallization was almost complete. The mixture was set aside for 10 hours after which time the supernatant was decanted. The crystals were dissolved in 5.0 ml. of ammonia T.S., and the pH was adjusted to 4.9 to 5.0 with glacial acetic acid. The mixture was set aside to crystallize for 10 hours, then the crystals were collected with a suction flask and a sintered-glass funnel and washed with five 10-ml. portions of distilled water. The funnel and crystals were oven-dried at 120° for 2 hours; the crystals were transferred to a weighing bottle and cooled in a dessicator.

For each mixture and standard, 50.0 mg. of the carrier-diluted derivative was dissolved in 50.0 ml. of ammonia T.S. Three samples of each of these solutions, each containing 1.0 ml., were prepared and counted.

The average activities of both standards for each run were averaged. These averages were used to calculate the initial specific activity of the labeled derivative for each run. The amount of thiamine hydrochloride present in each mixture was calculated. The results are shown in Table III. The average of the four analyses was 99.7 mg. (100.1% recovery), and the standard deviation was 0.27 mg.

SUMMARY

An inverse isotope dilution method for the analysis of thiamine hydrochloride in the presence of other vitamins was studied. The procedure involved cleavage with sodium sulfite-S-35 to form an S-35 labeled derivative. The average error and precision were 0.1 and 0.27%, respectively, using 100-mg. quantities. The method should be applicable to the analysis of considerably smaller quantities of the vitamin contained in complex mixtures.

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Prolonged Administration of Glycyrrhetinic Acid in Rats

By SHIRLEY D. KRAUS

Prolonged administration of glycyrrhetinic acid was studied in immature female rats, castrated male donor parabionts, and mature cyclic rats. Glycyrrhetinic acid treatment for 7 days interfered with the uterotrophic response of immature rats to estradiol benzoate but had no effect on the pituitary-ACTH content. It did not alter gonadotropin release or estradiol-induced gonadotropin inhibition in male castrated donors. One month of treatment did not alter the estrus cycles. It is apparent that the antiestrogenic effect of glycyrrhetinic acid is limited to exogenous estrogen and its effect on the female accessory reproductive tract.

THIS LABORATORY previously reported that β-glycyrrhetinic acid restricted the response of the uterus (1, 2) and the vagina (2) to exogenous steroidal estrogens. It was not capable of interfering with the uterotrophic response to endogenous sex steroids in the pregnant mare serum stimulated animal (2) or female recipient parabionts (1). Short term administration (3 days) which depressed the uterine growth response of immature intact (1, 2), adrenalectomized (1), and ovariectomized (2) rats had no effect in hypophysectomized rats (2). The pituitary-adrenal axis was not affected by short term administration (3). Prolonged administration (7 days) interfered with the pituitary-adrenal axis (3) and also increased the uterine response of female recipient parabionts (2).

Further investigations on the effect of long term administration were undertaken in an attempt to clarify mode of action. These studies consisted of the effect of (*a*) 7 days of treatment on the uterotrophic response as well as pituitary-ACTH of immature female rats, (*b*) 8 days of treatment on gonadotropin release in male castrated donor parabionts with and without estrogen, and (*c*) 1 month of treatment on the estrus cycles of mature cyclic rats.

MATERIALS AND METHODS

Rats were maintained under constant lighting (14 hours artificial light) at 25° and fed Purina laboratory chow and water *ad libitum*. Hypophysectomized rats were given 5% glucose in the drinking water. All experimental animals were of the Wistar strain. Hypophysectomized Sprague-Dawley male rats (120–140 Gm.) used in the ACTH assay were received 24 hours after surgery and used 48 hours postoperatively. Both β-glycyrrhetinic acid[1] and estradiol benzoate[2] were prepared in an ethanol–sesame oil vehicle (1) and administered subcutaneously.

Immature Female Rats.—Immature female rats (35–50 Gm.) were divided into four groups—controls, glycyrrhetinic acid only, estradiol benzoate only, and glycyrrhetinic acid plus estradiol benzoate. The 7 days of glycyrrhetinic acid treatment was the previously determined pituitary-adrenal blocking dose (3). Glycyrrhetinic acid (2 mg.) or vehicle (0.2 ml.) was administered daily for 6 days with a double dose on the seventh day. Estradiol benzoate was administered to two groups as 0.1 mcg. daily during the last 3 days of treatment. Animals were sacrificed on the eighth day (72 hours after the first estradiol dose), and the uteri were weighed. The pituitaries of two groups (controls and glycyrrhetinic acid only) were removed for ACTH assay. Each pituitary was placed in 0.5 ml. of 0.1 *N* HCl and finely macerated with a motor driven Potter tissue grinder. The final concentration of pituitary suspension was made by adding 4.5 ml. of physiological saline to each 0.5 ml. of pituitary suspension, thus producing a suspension of 0.1 pituitary per 0.5 ml. of 0.01 *N* HCl in saline. The dose of 0.1 pituitary (0.5 ml.) per 100 Gm. of body weight was administered subcutaneously. Animals were sacrificed after 3 hours and the adrenal ascorbic acid determined (4). Each sample was administered to two test animals. The adrenal ascorbic acid was compared with that of untreated hypophysectomized rats to determine the degree of depletion.

Gonadotropin Release in Male Castrated Donor Parabionts With and Without Estrogen.—Parabiotic rats (23–25 days old) consisted of a castrated male donor and an immature female recipient. Rats were anesthetized with pentothal (50 mg./Kg. i.p.). After castrating the male partner, a skin incision was made laterally from the ear to the tail region (right side of the male and left side of the female). The pair was joined anteriorly by a mattress suture through the left scapula of the female and the right scapula of the male and posteriorly by a mattress suture through the gluteus maximus muscle. The two ventral skin flaps were closed with skin clips; a small lateral incision was made in the peritoneum of each animal. The four cut surfaces were sutured together with fine silk thread and the dorsal skin flaps were then closed with skin clips. Each parabiont was given 20,000 units of penicillin intramuscularly immediately postoperatively and again on the third and fifth days. The parabiotic rats were divided into six groups. Male parabionts were treated for 8 days with either vehicle (0.2 ml.) glycyrrhetinic acid (2 mg.), estradiol benzoate (0.25 mcg. or 0.5 mcg.), or glycyrrhetinic acid (2 mg.), plus either dose of estradiol benzoate. Animals were sacrificed on the ninth postoperative day and the ovaries were weighed.

Mature Cyclic Rats.—Estrus cycles were determined by wet vaginal smears taken daily at 9:00 a.m. in mature cyclic rats. Animals were only used after three consecutive normal cycles had been observed. Glycyrrhetinic acid (4 mg.) or vehicle (0.4 ml.) was administered three times a week for 1 month. There were 16 animals in each group.

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[1] β-Glycyrrhetinic acid, supplied by S. B. Penick and Co., New York, N. Y.

[2] Estradiol benzoate, supplied by Organon, Inc., Nutley, N. J.

TABLE I.—EFFECT OF PROLONGED ADMINISTRATION OF GLYCYRRHETINIC ACID ON THE UTEROTROPHIC RESPONSE OF IMMATURE RATS

Group	G.A.,[a] mg.	E.B.,[b] mcg.	N	Uterine Wt., mg. ± S.E.	p
1	0	0	7	40.69 ± 3.85	...
2	16	0	8	42.34 ± 3.27	...
3	0	0.3	16	110.45 ± 4.54	...
4	16	0.3	15	72.20 ± 6.59	<0.001

[a] Glycyrrhetinic acid (2 mg.) or vehicle (0.2 ml.) s.c. daily for 6 days with a double dose on the seventh day. [b] Estradiol benzoate 0.1 mcg. s.c. daily during the last 3 days of treatment. Sacrificed on eighth day.

TABLE II.—ASSAY OF ACTH FROM PITUITARIES OF UNTREATED AND GLYCYRRHETINIC ACID TREATED IMMATURE FEMALE RATS

Pituitary,[a] 0.1/100 Gm. B.W.	N[d]	Adrenal Ascorbic Acid, mcg./100 mg.
None	8	412.11 ± 17.94
Controls[b]	7	325.11 ± 21.27
G.A. treated[c]	8	333.90 ± 27.93

[a] Pituitary suspension of 0.1 pituitary/0.5 ml. of 0.01 *N* HCl in saline made by grinding one pituitary in 0.5 ml. of 0.1 *N* HCl and diluting to 5 ml. with 4.5 ml. of physiological saline. Administered s.c. to hypophysectomized Sprague-Dawley rats 48 hours postoperatively. Adrenal ascorbic acid determined 3 hours after injection. [b] Pituitaries from animals receiving vehicle (0.2 ml. daily for 6 days with double dose on seventh day). [c] Pituitaries from animals receiving glycyrrhetinic acid (2 mg. daily for 6 days with double dose on seventh day). [d] N represents the number of pituitaries tested. Each pituitary was administered to two test animals.

TABLE III.—OVARIAN RESPONSES OF FEMALE RECIPIENT PARABIONTS

Group	G.A.,[a] mg.	E.B.,[a] mcg.	N	Ovarian Wt., mg. ± SE.
1	0	0	6	42.67 ± 2.86
2	2	0	6	37.03 ± 8.47
3	0	0.5	9	12.64 ± 0.80
4	2	0.5	7	14.11 ± 0.63
5	0	0.25	6	14.00 ± 1.40
6	2	0.25	5	11.72 ± 2.88

[a] Daily dose administered s.c. to male castrated donor starting on the day of surgery. Controls received an equivalent volume of vehicle. Sacrificed on ninth postoperative day.

RESULTS AND DISCUSSION

Immature Female Rats.—The uterine growth response to estradiol benzoate was significantly reduced by prolonged administration of glycyrrhetinic acid (Table I). This previously determined pituitary-adrenal blocking dose (3) did not reverse the antiestrogenic effect and had no effect on the unstimulated uterus (Table I).

Seven days of treatment with glycyrrhetinic acid had no effect on the pituitary-ACTH content (Table II). Pituitaries from the treated animals produced the same degree of adrenal ascorbic acid depletion as pituitaries from control animals (Table II). An increase in the resting adrenal ascorbic acid reported at this dosage schedule (3) might have been due to a decreased secretory rate or decreased adrenal responsiveness to the ACTH secretory rate. It certainly cannot be attributed to decreased ACTH reserve or synthesis.

Gonadotropin Release in Male Castrated Donor Parabionts With and Without Estrogen.—Ovarian weights of female recipients receiving gonadotropin from untreated male donors were equal to those receiving gonadotropins from glycyrrhetinic acid treated donors (Table III). Estradiol benzoate administered to male donors depressed the ovarian response of female recipients even when glycyrrhetinic acid had been simultaneously administered (Table III). Glycyrrhetinic acid therefore did not interfere with gonadotropin release nor the estrogenic inhibition of gonadotropin release in male donors. It can only exert an antiestrogenic effect on the response of the female accessory reproductive tract and not the pituitary target organ. This confirms the previous results (1) of lack of interference with estrogenic adrenal stimulation and estrogenic testicular atrophy.

Mature Cyclic Rats.—One month of glycyrrhetinic acid treatment did not alter the estrus cycles of mature rats. Normally established cycles continued throughout the entire period of treatment. Alteration of the estrus cycle could not have been anticipated on the basis of gonadotropin inhibition. These results indicate that glycyrrhetinic acid does not interfere with the response of the vaginal mucosa to endogenous sex steroids. The lack of antagonism of endogeneous sex steroids previously reported (2) and reproduced here infers that the mechanism of action as an estrogen antagonist lies in interference with absorption or metabolism of exogenous estrogen. This apparentiy can only influence the target organs of the accessory reproductive tract. For some reason still unexplained, there is no alteration in the response of the pituitary target organ to exogenous estrogen.

SUMMARY AND CONCLUSIONS

Prolonged administration of glycyrrhetinic acid to immature female rats did not reverse the antiestrogenic effect, did not alter the pituitary-ACTH content, had no effect on the unstimulated uterus, and antagonized the uterine response to exogenous estrogen.

Glycyrrhetinic acid administered to male castrated donor parabionts for 8 days did not interfere with gonadotropin release nor estradiol inhibition of gonadotropin release.

One month of glycyrrhetinic acid treatment did not alter the estrus cycles of mature cyclic rats.

Glycyrrhetinic acid therefore restricts the response of the uterus to exogenous estrogen regardless of duration of treatment. Its antagonism is limited to the response of the female accessory reproductive tract to exogenous estrogen and does not extend to other target organs such as the pituitary. It cannot alter estrus cycles either through interference with gonadotropin release or alteration in the response of the vaginal mucosa to exogenous sex steroids.

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Analysis of the Fixed Oil of *Clitocybe illudens* Schw.

By AGUSTIN MATSON, A. H. SMITH, and A. G. PAUL

The fixed oil of the poisonous Jack O'Lantern mushroom has been analyzed, and the usual physical and chemical constants for fats have been determined. The fatty acids, as methyl esters, were resolved by gas chromatography using a liquid phase of 25 per cent diethyleneglycolsuccinate polyester and 2 per cent phosphoric acid on Chromosorb W and a thermal conductivity detector. The fatty acids present in the oil include myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, and linoleic acids. The unsaponifiable fraction was analyzed by gas chromatography using a liquid phase of 0.4 per cent silicone gum rubber on Chromosorb W and a hydrogen flame ionization detector. Ergosterol accounted for 76.2 per cent of this fraction.

CLITOCYBE ILLUDENS, known as the "Jack O' Lantern" because of its phosphorescent glow, has been classified as a poisonous mushroom which causes nausea, vomiting, diarrhea, and prostration (1). Ford (2, 3) found no hemolysins in this species, but reports that acute intoxication in guinea pigs is fatal in 1 to 7 days. Clark and Smith (4) claim that muscarine or a muscarine-like compound is present in extracts of the plant based on atropine-reversal of the effect on frog heart. Though still classed as a poisonous mushroom (5, 6), no chemical investigation of the plant has been reported.

These investigations report results of analysis of the petroleum ether extract of *C. illudens*. Studies of other fractions are currently under investigation.

EXPERIMENTAL

The plants were collected from a single location in Ann Arbor, dried at 45°, and reduced to a 20-mesh powder in a Wiley mill. The milled plant (1.865 Kg.) was extracted with petroleum ether (b.p. 30–60°) for 5 days in a Soxhlet apparatus. The extract was transferred to a weighed flask, and the solvent was removed by distillation. The oil was freed from traces of solvent and moisture by heating the flask at 110° under reduced pressure. A total yield of 51.6 Gm. of oil, or 2.8%, was obtained; it was stored under nitrogen at refrigerator temperature.

Physical Constants.—The specific gravity was determined by the pycnometer method and the refractive index, using an Abbe refractometer.

Sp. gr. $\frac{20°}{20°}$ = 0.9611; $n^{20.8}_{D}$ = 1.4634 (average of 5). The oil showed no optical rotation.

Chemical Constants.—Except for the iodine value, chemical constants were determined by standard procedures (7). Because of the limited amount of oil available, the micro method of Toms (8) was used to determine the iodine value. Table I lists the chemical constants determined for the oil.

Isolation of the Fatty Acids.—Two grams of the oil was saponified in 25 ml. of 0.5 *N* alcoholic KOH by refluxing on a steam bath for 2 hours. The soap solution was separated from the unsaponifiable material, concentrated to approximately half its volume, diluted with 10 ml. of water, and the solution washed with three 20-ml. portions of ether. The free acids were regenerated with HCl solution and extracted with ether until the aqueous layer was neutral to Congo red paper. The ether extracts were combined, dried over anhydrous sodium sulfate, filtered, and the solvent removed under reduced pressure at 45°. The fatty acids were freed of traces of solvent by bubbling nitrogen through the liquid. The mixture was stored under nitrogen at refrigerator temperature.

TABLE I.—CHEMICAL CONSTANTS OF THE FIXED OIL

Sample	I	II
Saponification value	149.7	149.8
Unsaponifiable matter, %	11.4	11.3
Acid value	50.3	51.1
Iodine value	138.4	138.2
Reichert value	1.0	1.0
Polenske value	5.1	5.2
Kirschner value	0.5	0.5

TABLE II.—RETENTION TIMES OF KNOWN METHYL ESTERS OF FATTY ACIDS

Methyl Ester	Retention Time, min.
Caproate	1.62
Caprylate	2.22
Caprate	3.24
Laurate	4.68
Myristate	7.08
Myristoleate	8.76
Palmitate	11.04
Palmitoleate	13.32
Stearate	17.40
Oleate	20.40
Linoleate	26.05
Arachidate	27.79

Gas Chromatography of the Fatty Acids.—The fatty acids were converted to their methyl esters by the acid catalyzed esterification method (9). Twelve known fatty acids[1] were esterified in like manner.

The apparatus used was the F&M Scientific Corp. model 300 gas chromatograph equipped with a thermal conductivity cell. The column, 10-ft. × 1/4-in. (O.D.) stainless steel, was packed with 25% diethyleneglycolsuccinate polyester and 2% phosphoric acid on Chromosorb W, 60–80 mesh.

Helium was used as the carrier gas at a flow rate of 60 ml./minute with an inlet pressure of 50 p.s.i. The column temperature was maintained at 205°, the injection port at 250°, and the detector block at 275°. The bridge current was maintained at 150 ma., the attenuation at a setting of 2; the recorder chart was driven at a speed of 20 in./hour.

A Hamilton 10-μl. capacity syringe was used for injection of samples. One-microliter quantities of the known methyl esters and both 2-μl. and 3-μl. quantities of the unknown mixture of methyl esters were used without dilution.

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[1] Purchased from K & K Laboratories, Inc., Jamaica, N. Y.

TABLE III.—GAS CHROMATOGRAPHIC RESULTS OF THE ANALYSIS OF THE ESTERIFIED FATTY ACID FRACTION OF *C. illudens* OIL

Peak No.	Retention[a] Time, min.	Confirmed by Enrichment as	Methyl Ester, %[a]	% Expressed as Free Acid
1	7.15 ± 0.02	Myristate	0.4 ± 0.1	0.4
2	8.84 ± 0.12	Myristoleate	0.6 ± 0.2	0.6
3	11.08 ± 0.19	Palmitate	17.2 ± 1.1	17.2
4	13.40 ± 0.21	Palmitoleate	1.1 ± 0.2	1.1
5	17.64 ± 0.27	Stearate	3.9 ± 0.2	3.9
6	20.81 ± 0.32	Oleate	36.1 ± 1.7	36.2
7	26.24 ± 1.27	Linoleate	40.7 ± 0.8	40.7

[a] Average of eight determinations.

Comparison of the retention times of the unknown compound with those of the known compounds was used for identification. Each unknown peak was confirmed by the enrichment method. Table II lists the retention times of the known methyl esters.

Quantitative estimates of the proportion of each compound present in the unknown mixture were based on calculation of the area under each peak. Table III lists the averages of eight gas chromatographic determinations of the unknown mixture of methyl esters. Standard deviations were calculated in the usual manner. The percentages of free acids were calculated from the molecular weights of the acids compared with their methyl esters and the proportion of each acid then calculated as per cent.

Gas Chromatography of the Unsaponifiable Matter.—The fraction used for the determination of unsaponifiable matter was analyzed for steroid content. The method of Horning, *et al.* (10, 11), was utilized in these investigations.

The F & M Scientific Corp. model 609 gas chromatograph, equipped with a flame ionization detector, was used. A 6-ft. × 1/4-in. (O.D.) stainless steel column was packed with 0.4% silicone rubber (SE-30) on Chromosorb W, 60–80 mesh. The operating conditions were—flow rates as indicated on the rotometer—helium, 8.5; hydrogen, 9.0; air, 8.5; column temperature, 222°; injection port temperature, 302°; detector block temperature, 350°; chart speed, 20 in./hour, attenuation, 8; range, 1000.

A saturated solution of ergosterol (<2%) and 2% solutions of β-sitosterol and stigmasterol in benzene were used as controls.[2] Samples were injected in 5 or 7-μl. quantities using a 10-μl. capacity Hamilton syringe; a 2% solution of the unsaponifiable matter in benzene was analyzed in the same manner. Comparison of the retention times and confirmation by the enrichment method were used for identification.

Two peaks appeared in chromatograms of the unsaponifiable fraction. Peak 1, with an average retention time of 9.0 minutes, was broad and did not compare with tested or reported steroids. Peak 2, with an average retention time of 17.62 minutes, compared with ergosterol (retention time, 17.46 minutes). This was confirmed by the enrichment method.

On prolonged storage of solutions of the unsaponifiable matter, peak 1 appeared larger on chromatograms at the expense of peak 2. Cochromatography of the unsaponifiable matter with calciferol and viosterol[3] indicated that peak 1 could be attributed to calciferol. The broadness of the peaks, the impurity of these latter two compounds, and apparent decomposition of them during chromatography prevented confirmation.

The quantitative estimates were determined by calculation of the area under the peaks. The average of four determinations indicated that a quantity of 76.2 ± 1.5% ergosterol occurred in the unsaponifiable fraction.

SUMMARY

The petroleum ether extract of *C. illudens* has been analyzed. The plant contains 2.8% fixed oil on a dry weight basis.

Specific gravity, refractive index, per cent unsaponifiable matter, acid, iodine, Reichert, Polenske, and Kirschner values are reported.

The methylated fatty acid fraction, analyzed by gas chromatography, contained myristic acid, 0.4%; myristoleic acid, 0.6%; palmitic acid, 17.2%; palmitoleic acid, 1.1%, stearic acid, 3.9%; oleic acid, 36.2%; and linoleic acid, 40.7%.

The unsaponifiable fraction, analyzed by gas chromatography, contains 76.2% ergosterol; the remainder appears to be calciferol, but this has not been confirmed.

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[3] Purchased from General Biochemicals, Chagrin Falls Ohio.

Occurrence of 5-Hydroxytryptamine and 5-Hydroxytryptophan in *Panaeolus sphinctrinus*

Sir:

The status of *Panaeolus sphinctrinus* (Fr.) Quél. as a hallucinogenic mushroom is currently a matter of dispute. Specimens of this species collected in Mexico by Schultes approximately 25 years ago were said to be employed there as psychotomimetic agents (1). The possibility of such application was seemingly verified when Heim and Hofmann reported the isolation of psilocybin from cultivated sporocarps of the fungus (2). However, their recent investigations of other samples of *P. sphinctrinus* failed to detect psilocybin (3). In neither case did the authors present experimental details or sources of the fungi examined, and herbarium reference specimens were apparently not retained.

An investigation of nine species and forms of *Panaeolus* by Tyler and Smith (4) revealed the presence of 5-substituted tryptamine derivatives but no 4-substituted derivatives. Because of its relative scarcity, *P. sphinctrinus* was not included in that study, but the possibility that it might contain psilocybin or psilocin appeared remote on chemotaxonomic grounds. Investigation of an authentic sample of the fungus was clearly required to settle the ethnological, phytochemical, and chemotaxonomic problems raised by this apparent discrepancy in the type of tryptamine derivatives reported to be contained in a single species of the genus *Panaeolus*.

Recently, we obtained through the courtesy of Dr. R. Singer[1] a small sample of *P. sphinctrinus* (Fr.) Quél. collected in Argentina (collection No. M 3514) and determined by Singer to be the same species as the Mexican material originally collected by Schultes (1). The powdered specimen (95 mg.) was extracted with 5 ml. of methanol and 100–150-μl. quantities of the extract subjected to circular chromatography on S. and S. 2043-bm. filter paper in three solvent systems essentially as described by Benedict, *et al.* (5). Spraying the chromatograms with *p*-dimethylaminobenzaldehyde solution (PDAB) or with Pauly's reagent revealed two principal zones (violet-PDAB, reddish orange-Pauly's) with R_f values in all three solvent systems corresponding to reference samples of 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT). The average R_f values of the zones are listed in Table I. Urea was also identified by its R_f value and distinctive yellow with PDAB.

TABLE I.—R_f VALUES OF INDOLE DERIVATIVES IDENTIFIED IN *P. sphinctrinus* EXTRACT AND OF REFERENCE COMPOUNDS

Compd. Spotted	R_f Values			
	5-HTP	5-HT	Psilocybin	Psilocin
	n-Butanol:Acetic Acid:Water (4:1:1)			
P. sphinctrinus extract	0.31	0.51	...	...
Reference	0.33	0.53	0.45	0.71
	n-Butanol (Water-saturated)			
P. sphinctrinus extract	0.18	0.34	...	...
Reference	0.19	0.33	0.09	0.54
	n-Propanol:1*N* Ammonium Hydroxide (5:1)			
P. sphinctrinus extract	0.21	0.59	...	...
Reference	0.22	0.59	0.16	0.90

The methanol extract was then concentrated to 2 ml., 200–400 μl. quantities were applied as 2-cm. streaks on thin-layer plates prepared with Merck silica gel G (according to Stahl), and the chromatograms developed twice in a solvent mixture composed of chloroform: 5% methanol, saturated with concentrated ammonium hydroxide (6). After spraying with PDAB, the chromatograms showed two principal violet spots with mobilities equivalent to 5-HTP and 5-HT and which failed to separate from these compounds when spotted in combination with them. Several other spots giving bluish colors with PDAB were noted on the chromatograms but did not prove to be identical with available reference compounds. None of the spots corresponded to psilocybin or psilocin.

From these observations in four different chromatographic systems, it was concluded that the sample of *P. sphinctrinus* examined contained neither psilocybin or psilocin. In agreement with the numerous species of this genus previously analyzed (4), it contained relatively large amounts of 5-HTP, 5-HT, and urea. The reported isolation of psilocybin from *P. sphinctrinus* sporocarps by Heim and Hofmann may be

[1] The authors gratefully acknowledge the valuable contribution made to this study by Dr. R. Singer, Department of Botany, University of Buenos Aires, Buenos Aires, Argentina.

attributed to the existence of different chemical races of the species; however, this possibility now appears remote. A more likely conclusion is that that report, like numerous others attributing hallucinogenic properties to *Panaeolus* species, was based on a misidentification of the specimens examined.

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Dielectric Constants and Solubility

Sir:

A recent publication of Sorby, Bitter, and Webb (1) reporting the dielectric constants of the water-ethanol-glycerin and water-ethanol-propylene glycol systems has prompted this communication. Since we are also presently engaged in studies of this nature, we wish to indicate briefly some additional observations concerning the possible relation between dielectric constant and the solubility of nonelectrolytes. These observations will be the subject of detailed reports at a later date.

Our studies have led us to believe that the dielectric constant principle of solvent blending outlined by Moore (2) is fortuitous. Implicit in Moore's suggestion is the presumption that solvent systems of approximately the same dielectric constant will show the same solvent properties for given drugs. Goyan (3) has recently pointed out the limitations of this assumption. Sorby, *et al.* (1), have suggested that Moore's method may fail in practice because of the approximations made in computing rather than directly measuring dielectric constants of complex mixtures. In a previous communication (4) we had pointed out that Moore had not considered cosolvency phenomena in his treatment.

A detailed examination of one of Moore's examples, the solubility of phenobarbital as a function of dielectric constant, proves illustrative. Figure 1 shows the solubility data of phenobarbital in several binary systems which were originally reported by Krause and Cross (5) and Peterson and Hopponen (6). The peak solubilities in different blends are seen to fall within a narrow range of dielectric constant (27–30). This observation led to our suggestion (4) that a dielectric requirement (DR) for solubility exists, *i.e.*, the dielectric constant at which peak solubility is observed in a solvent blend. At constant temperature, this requirement should be independent of the actual nature of the solvents in the blend and dependent only upon the nature of the drug. The existence of such a requirement has been confirmed in the cases of salicylic acid (DR: ~15) and theobromine (DR: ~20 and ~42) in binary systems of widely differing composition (7).

If Moore's technique is valid, then its application should not critically depend upon the selection of the solvent which determines the value of the approximate dielectric constant (A.D.C.)

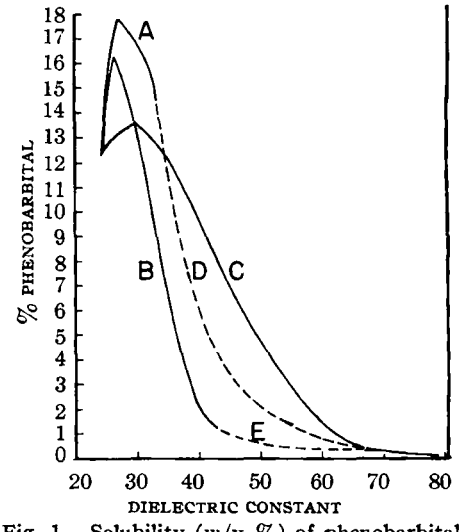


Fig. 1.—Solubility (w/v %) of phenobarbital in various binary systems at 25° plotted as a function of dielectric constant. Key: A, propylene glycol-ethanol; B, glycerin-ethanol; C, water-ethanol; D, propylene glycol-water; E, glycerin-water.

attributed to the existence of different chemical races of the species; however, this possibility now appears remote. A more likely conclusion is that that report, like numerous others attributing hallucinogenic properties to *Panaeolus* species, was based on a misidentification of the specimens examined.

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Dielectric Constants and Solubility

Sir:

A recent publication of Sorby, Bitter, and Webb (1) reporting the dielectric constants of the water-ethanol-glycerin and water-ethanol-propylene glycol systems has prompted this communication. Since we are also presently engaged in studies of this nature, we wish to indicate briefly some additional observations concerning the possible relation between dielectric constant and the solubility of nonelectrolytes. These observations will be the subject of detailed reports at a later date.

Our studies have led us to believe that the dielectric constant principle of solvent blending outlined by Moore (2) is fortuitous. Implicit in Moore's suggestion is the presumption that solvent systems of approximately the same dielectric constant will show the same solvent properties for given drugs. Goyan (3) has recently pointed out the limitations of this assumption. Sorby, *et al.* (1), have suggested that Moore's method may fail in practice because of the approximations made in computing rather than directly measuring dielectric constants of complex mixtures. In a previous communication (4) we had pointed out that Moore had not considered cosolvency phenomena in his treatment.

A detailed examination of one of Moore's examples, the solubility of phenobarbital as a function of dielectric constant, proves illustrative. Figure 1 shows the solubility data of phenobarbital in several binary systems which were originally reported by Krause and Cross (5) and Peterson and Hopponen (6). The peak solubilities in different blends are seen to fall within a narrow range of dielectric constant (27–30). This observation led to our suggestion (4) that a dielectric requirement (DR) for solubility exists, *i.e.*, the dielectric constant at which peak solubility is observed in a solvent blend. At constant temperature, this requirement should be independent of the actual nature of the solvents in the blend and dependent only upon the nature of the drug. The existence of such a requirement has been confirmed in the cases of salicylic acid (DR: ~15) and theobromine (DR: ~20 and ~42) in binary systems of widely differing composition (7).

If Moore's technique is valid, then its application should not critically depend upon the selection of the solvent which determines the value of the approximate dielectric constant (A.D.C.)

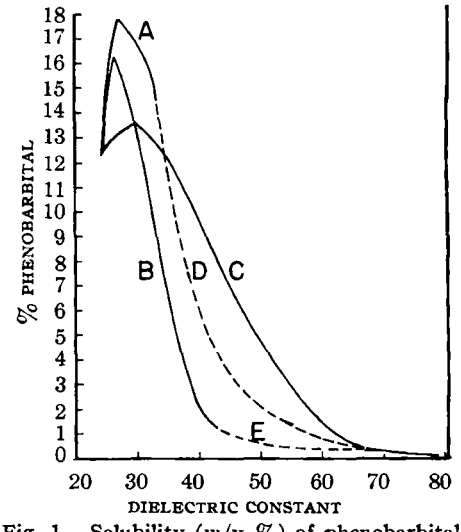


Fig. 1.—Solubility (w/v %) of phenobarbital in various binary systems at 25° plotted as a function of dielectric constant. Key: A, propylene glycol-ethanol; B, glycerin-ethanol; C, water-ethanol; D, propylene glycol-water; E, glycerin-water.

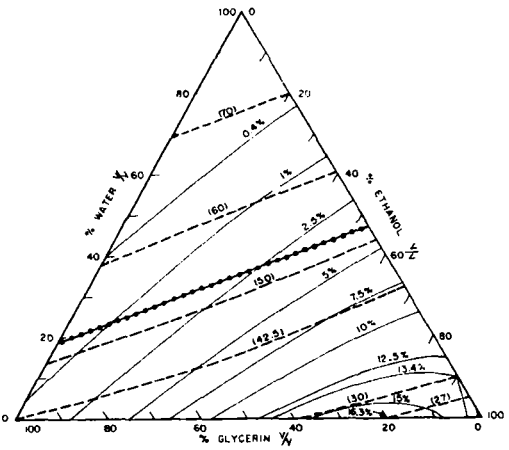


Fig. 2.—Phenobarbital solubility and dielectric constant-volume percentage relationships for the water-ethanol-glycerin system. Key: ——, tie lines representing constant solubilities (w/v %) of phenobarbital at 25°; ----, tie lines representing isodielectric mixtures where figures in ( ) are dielectric constants; -●-●-●-, tie line representing dielectric constant of 50 reported by Sorby, *et al.* (1).

at which a certain solubility is to be maintained. It is evident from Fig. 1 that no apparent correlation can be made between dielectric constant and actual solubility. Solubilities differ significantly in blends of the same dielectric constant (isodielectric blends). If we desired to prepare a 2% solution of phenobarbital, our choice of A.D.C. would depend on whether we happened to use a glycerin-alcohol, propylene glycol-water, alcohol-water, or some other solvent for its measurement.

Correlation of solubility and dielectric constant in tertiary solvent blends is more complex. Figure 2 shows the solubility of phenobarbital in the water-ethanol-glycerin system. This is represented on the ternary diagram as tie lines of constant solubility which were interpolated from the data of Krause and Cross (5). The isodielectric lines shown on the chart were computed from our own measurements of dielectric constant. They were made using a technique similar to that described by Sorby, *et al.* (1), except that dioxane-water (8) instead of acetone-water mixtures were used to standardize the oscillometer. However, our values for systems with dielectric constants less than 60 (*e.g.*, compare the isodielectric 50 lines in Fig. 2) are significantly greater than those reported by Sorby (1). The dielectric constant values of the water-acetone system which they used (9) are lower in this region than those reported by Albright (10).

In any case, it is again evident that considerably different solubilities will be observed in isodielectric mixtures. Optimum solvent power, however, will be observed in blends with an apparent DR of 27–30. If the solubility of phenobarbital in the system water-ethanol-propylene glycol (6) was analyzed in the same fashion, similar conclusions could be drawn.

We suggest that the maximum utility of the dielectric constant as an aid to formulation of solvent blends for nonelectrolytes is gained from knowledge of the dielectric requirement. One may readily measure this for any drug by determining its solubility in dioxane-water mixtures of known dielectric constant and noting the dielectric constant(s) at which peak solubility is observed. Furthermore, studies directed toward the interpretation of solubility behavior of drugs in terms of the dielectric character of solvent systems will prove most fruitful if solubilities are compared at the dielectric requirement where solute-solvent interaction is a maximum. Our interest in this area of research lies in establishing the validity of these statements.

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Review Article

Photometric Titrations

By STUART P. ERIKSEN and KENNETH A. CONNORS

INTRODUCTION

Scope of This Review

An increased interest in the photometric titration technique has resulted in the publication of several reviews in recent years. Among these are articles by Underwood (1), Goddu and Hume (2), and Headridge (3), and a book by Headridge (4). These authors have given adequate attention to the history of photometric titration methods—the summary by Goddu and Hume (2) is particularly recommended—and this material will not be repeated here. Our treatment will emphasize the principles upon which photometric titrations are based, with special attention given to techniques developed in the past few years. Examples illustrating each type of titration have been drawn from the literature or from our unpublished results.

Experimental Operations[1]

One advantage of end-point detection by photometric means is simplicity of the experimental operation, which merely requires the evaluation of the suitable optical property—which is accomplished with a spectrophotometer, a fluorimeter, or a polarimeter—as a function of titrant volume. Any commercially available instrument may be employed. Two types of titration cells have been used.

The first and most widely used arrangement places the titration cell directly in the light path of the instrument; it thus takes the place of the usual sample cell. Titration is carried out directly in the cell. Some means of stirring the solution must be provided; this may be a mechanical or a magnetic stirrer. The presence of a magnet in the vicinity of a photoelectric cell may affect the response of the cell, and this possible source of error must be kept in mind. The titration cell may be an ordinary glass beaker for titrations using the visible region of the spectrum. Ultraviolet radiation requires special cell walls of silica. Provision must be made for the buret tip and, where necessary, a source of nitrogen for flushing the solution and cell compartment.

Many modifications of this basic assembly have been reported. The examples given by Goddu and Hume (2), Headridge (4), and Bobtelsky (5) illustrate the types of arrangement possible.

The second kind of titration system consists of a titration cell external to the instrument and connected to a flow-through photometer cell with some kind of pump to circulate the solution. Both mechanical and magnetic circulation have been used. An advantage of this method is that the magnet can be removed from the vicinity of the photocell. Moreover, it is very easy to carry out a simultaneous photometric and potentiometric titration by inserting electrodes in the titration flask. Further advantages are the ease with which the solution may be thermostated and protected from the atmosphere. The photometer does not require any modification other than a small hole in the cell compart-

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[1] Throughout this review the spectrometric nomenclature preferred by *Analytical Chemistry* will be used (6).

ment cover to permit passage of the tubing connecting the flow-through cell and the titration flask. A convenient assembly has been described by Rehm, *et al.* (7).

Several commercial photometric titrators are available. Headridge (4) has discussed several instruments designed primarily for use in absorbance titrations, while Howerton and Wasilewski (8) have described one for use in studying either absorbance or fluorescent end points. Some of these provide a means for recording, manually or automatically, the photocell response as a function of titrant volume. Other more sophisticated instruments can terminate the titration at the end point. Although these titrators can save much time and effort in routine analyses, for developmental and investigative work we recommend use of an ordinary spectrophotometer or fluorimeter and one of the titration cell assemblies discussed above.

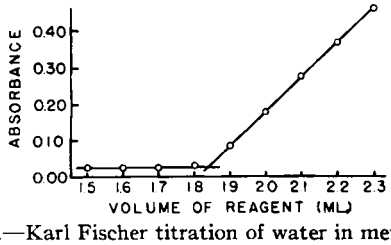


Fig. 1.—Karl Fischer titration of water in methanol solution; absorbance measured at 525 mμ (9).

ABSORBANCE MEASUREMENTS IN HOMOGENEOUS SYSTEMS

In this section we consider titrations in homogeneous solutions which are accompanied by changes in the light absorbing properties of the solution. All such photometric titrations are based upon Beer's law, $A = abc$, which states that the absorbance, A, of the solution is directly proportional to the concentration, c, of the absorbing substance; b is the path length, and a is the absorptivity. Absorptivity is a function of wavelength, solvent, temperature, and molecular structure. If the solution contains more than one absorbing component, the observed absorbance is the sum of the absorbances of the individual components. These general statements are of course subject to exceptions, but unless otherwise stated we shall assume adherence to Beer's law in these systems.

Self-Indicator (Direct) Titrations

Single Samples.—The simplest systems are those in which the sample, the product of the titration reaction, the titrant, or a combination of these, absorbs light of the selected wavelength,

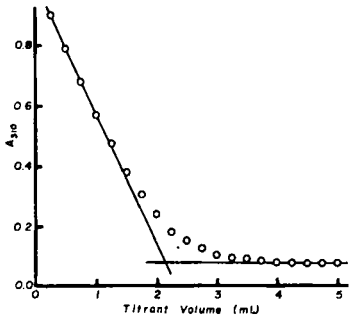


Fig. 2.—Titration of aniline with acetic anhydride (10).

such that a change in absorbance occurs during the titration. Since absorbance is directly proportional to concentration, while electromotive force is logarithmically related to concentration, photometric titration may be expected to be more sensitive than potentiometric titration (2) (assuming suitable absorption characteristics of the components of the titration solution). If the concentrations and absorptivities of all species are known, the titration plot may be quantitatively predicted. It will consist of straight-line segments. The end point is marked by the intersection of two such lines. For the titration of a single substance the titration may take one of several forms, depending upon the absorption characteristics of the solution components.

If the titrant, but neither the sample nor the product, absorbs at the selected wavelength, the plot will look like Fig. 1, which shows the Karl Fischer titration of water followed at 525 mμ (9). By extrapolation of the two line segments, the end point is located. A classic example of this type of titration is the titration of ferrous ion with potassium permanganate.

If the sample absorbs, but the product and titrant do not, as the titration proceeds the absorbance will fall to zero. Figure 2 shows an acylation reaction—the titration of aniline with acetic anhydride (10). At the wavelength selected, only the aniline absorbs (actually the

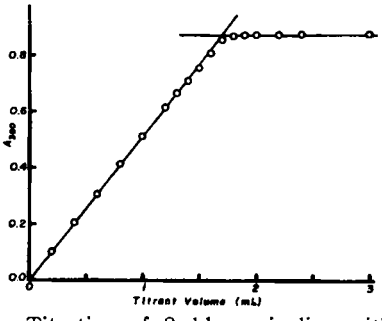


Fig. 3.—Titration of 8-chloroquinoline with perchloric acid in acetic acid (11).

product does absorb slightly) so the absorbance falls. (Sources of curvature will be discussed later.)

If the sample and the titrant do not absorb, but the product of the titration reaction does, a plot will be obtained like that shown in Fig. 3. The acid form of 8-chloroquinoline absorbs strongly at 380 mμ, so the absorbance rises linearly with titrant volume until the titration is complete, when it levels off (11). Another example would be the titration of *p*-nitrophenol with a strong base, the absorbance being followed near 400 mμ.

If the sample does not absorb, but both titrant and product do (but to different extents), then a break will again be observed. If the titrant is a much stronger absorber than the product, a graph like that shown in Fig. 4 will be obtained. In this titration the product absorbed strongly

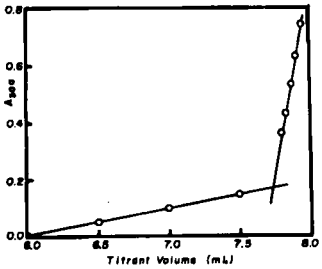


Fig. 4.—Titration of *o*-cresol with bromate-bromide solution (12).

enough that for convenience the absorbance was arbitrarily set at zero about 2 ml. prior to the end point (12). In general, these direct titration plots require only relative absorbance values because the end point is detected through the change in absorbance.

If the product is a stronger absorber than the titrant, the slope of the line following the end point will be less than that before it. Of course, other possible combinations of absorbers exist but examples of these are quite rare.

Mixtures.—One of the advantages of self-indicator photometric titrations appears to be their ability to distinguish between closely related components in a mixture. Hume and his co-workers have explored the applicability of the method (11, 13, 14). Restricting attention to acid-base titrations (though similar conditions should be applicable to other systems), two kinds of binary mixtures described below may be encountered.

First, the weaker compound (acid or base) exhibits absorption at longer wavelengths. Then the wavelength can be selected such that the weaker compound absorbs while the stronger one does not. The resulting titration curve will look

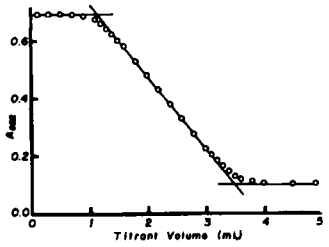


Fig. 5.—Titration of 2-methyl-5-nitroaniline and 4-methyl-2-nitroaniline with perchloric acid in acetic acid (11).

like Fig. 5 (11), which shows the nonaqueous titration of 2-methyl-5-nitroaniline [pKb (H_2O) 12.0] and 4-methyl-2-nitroaniline [pKb (H_2O) 13.5]. The *ortho*-nitroaniline, which is the weaker base, is the only compound which absorbs at around 520 mμ. Therefore, no absorbance change is seen in the initial stage of the titration, for the stronger component is being titrated. At the conclusion of this stage, the weaker base begins to consume titrant, and the titration is accompanied by a decrease in absorption. Finally, when both components have been titrated, the absorbance drops to a low constant value.

The second situation is when the stronger compound absorbs at the longer wavelengths; this is less favorable. The titration of the stronger component is accompanied by a fall in absorbance if its spectrum is shifted appropriately on titration. However, in order to follow the titration of the weaker component it is now necessary to shift to a lower wavelength. Since the stronger compound probably absorbs intensely here, and traces of it remain untitrated, curvature of the titration plot may result.

In favorable instances multicomponent mixtures may be susceptible to analysis, particularly if the wavelength shift technique is applied. Thus, Hummelstedt and Hume (14) titrated the four component mixture of (in descending order

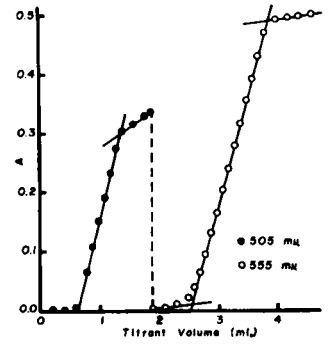


Fig. 6.—Titration of diphenyl phosphate, 2,4-dinitrophenol, *p*-nitrophenol, and *m*-nitrophenol with tetrabutylammonium hydroxide in isopropyl alcohol (14).

of acid strength) diphenyl phosphate, 2,4-dinitrophenol, *p*-nitrophenol, and *m*-nitrophenol (Fig. 6). At 505 mμ, diphenyl phosphate does not absorb. This strong acid is titrated first. Then the 2,4-dinitrophenol is titrated, the anion absorbing strongly. This accounts for the second segment of the curve, and allows extrapolation back to yield the end point for the diphenyl phosphate titration. (In a sense, therefore, 2,4-dinitrophenol may be considered an indicator for the photometric titration of the colorless diphenyl phosphate.) The next component to be titrated is the *p*-nitrophenol, the anion of which also absorbs at 505 mμ, but with sufficiently different intensity from the dinitrophenolate to permit location of the second end point.

Since *m*-nitrophenolate absorbs very strongly at 505 mμ, and its titration would give rise to extremely high absorbances, the wavelength is now shifted to 555 mμ, and the titration is continued to yield the third and fourth end points.

Sources of Error.—In the most satisfactory instances the direct photometric titration plot consists of straight-line segments intersecting at the end point. Many titrations, however, yield lines which exhibit more or less curvature, and which do not permit thoroughly reliable extrapolation to the end point. One source of this curvature is the dilution effect. If the volume of the titration solution is significantly

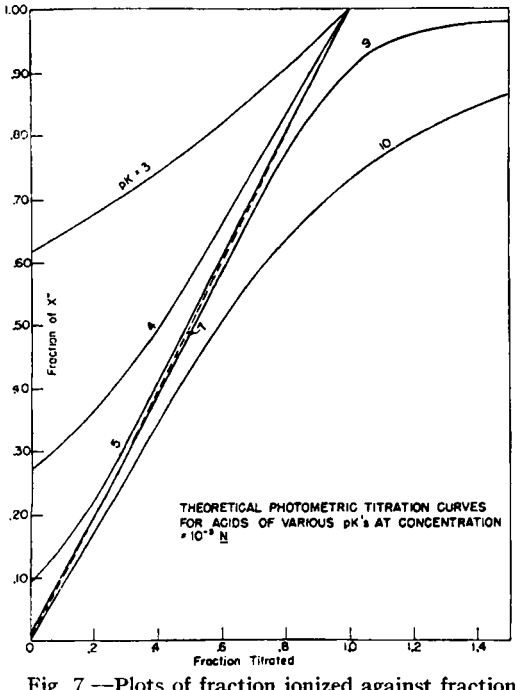


Fig. 7.—Plots of fraction ionized against fraction titrated for acids of various pKa's at 10^{-3} *M* concentration (13). [Courtesy of *Analytical Chemistry*.]

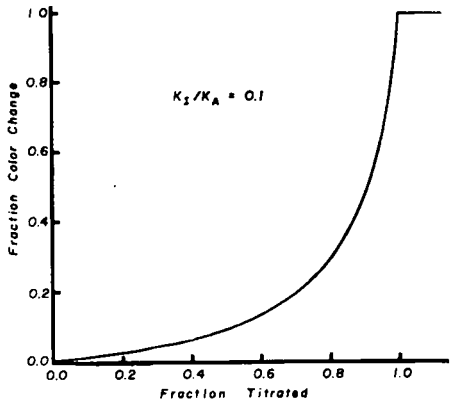


Fig. 8.—Theoretical color change curve for titration of weak acid with strong base in presence of indicator (16).

increased by the addition of titrant, evidently the concentration of each absorbing species will decrease due solely to this volume change. The result is an apparent failure of Beer's law, with negative curvature over the entire length of the line segment. Large errors may result. This dilution effect may be entirely compensated for by multiplying each observed absorbance value by the correction factor $(V + v)/V$, where V is the initial volume of the sample solution and v is the volume of titrant solution added. To render the dilution effect negligible, it is common practice to employ highly concentrated titrants.

A second effect which leads to apparent deviations from Beer's law is more specific in nature. If one or more of the absorbing species is involved in a molecular interaction with another component of the solution so that a change in its absorption spectrum results, a curvature may be observed in the titration plot. Thus, Hummelstedt and Hume (14) observed curvature in the titration of some phenols and ascribed it to phenol-phenolate complex formation. Such effects are sensitive to the nature of the solvent, which may be changed to eliminate them.

The most common cause of curvature is incompleteness of the titration reaction. This is manifested by curvature in the vicinity of the end point, and it obviously makes an accurate extrapolation more difficult. For example, considering acid-base reactions, it is possible to construct a graph of the fraction of the acid in the ionized state as a function of the fraction of the acid titrated, as done by Goddu and Hume (13), Fig. 7. This plot is made for acids of various pKa values, all at $10^{-3}M$ concentration in aqueous solution. In the case of acids whose absorption is due solely to the anion form, these graphs are equivalent to the absorbance-volume plots.

Of particular interest here are the curves for the weaker acids. An acid of pKa 7 gives the expected straight line. As the acid becomes weaker, say pKa 9, a pronounced curvature is observed in the region of the end point. Evidently, extrapolation in such a case is less certain. This source of curvature—namely, incomplete reaction (that is, "hydrolysis of the salt" in the present case)—is responsible for most of the curvature observed in this type of photometric titration plot. Goddu and Hume (13) estimate that satisfactory titrations can be made in aqueous solution if the product $C_{HA}Ka$ is equal to or greater than 10^{-12}. Grunwald (15) has presented a mathematical correction procedure which employs the data taken in the curved portion of the plot to yield the correct end point.

Indicator Titrations

A major limitation to the application of the direct or self-indicator photometric titrations just discussed is of course the requirement on the absorption spectra of the solution components. By incorporating an indicator in the solution, the titration becomes nearly general in its applicability and in this section we consider the types of photometric indicator titration plots which have been developed.

Absorbance-Volume Plots.—Visual indicator titrations are the most convenient of all, but sometimes the color change is too gradual to permit accurate location of the end point. It seems reasonable to expect that photometric observation of the color change might lead to more accurate and precise titrations. It is possible to predict the curve to be found. Figure 8 shows the curve relating the fraction of indicator color change to the fraction of weak acid titrated by a strong base for the case in which $K_I/Ka = 0.1$; in other words, the indicator is a weaker acid than is the sample. It is assumed that the titration reaction is complete. If the base form of the indicator were colored, Fig. 8 would be equivalent to the absorbance-volume plot (16). The end point is marked by the sharp change in direction. Note that the approach to the end point is characterized by a line which approximates to a straight line, but is really a curve. In any actual case we may expect that this will be a possible source of error. It is not possible to extrapolate from a great distance prior to the end point because the line is curved.

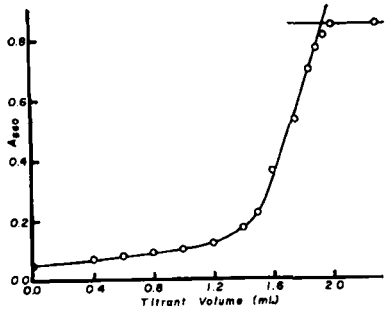


Fig. 9.—Titration of magnesium with EDTA, using Eriochrome black T indicator (18).

Though Fig. 8 has been calculated for an acid-base reaction, the same considerations will hold for complexometric titrations, though in this case conditional stability constants would apply. Fortuin, *et al.* (17), have presented a graph for a complexometric indicator titration which is similar to Fig. 8, and these authors have examined some of the factors which influence the accuracy of the end-point determination. Complexometric titration has in fact been the area of greatest use of absorbance-volume plots in indicator titrations. Figure 9 shows a typical example—the EDTA titration of magnesium with Eriochrome black T indicator—obtained by Zak, *et al.* (18). The experimental curve approximates to the theoretical shape, but some curvature is noted near the end point. Ringbom, *et al.* (19), have carried out similar calculations and experiments for the complexometric titration of very dilute solutions.

Such plots have not been widely used in acid-base titrations. An ingenious application of indicators to these reactions has been made by Goddu and Hume (13), who carried out matched-strength indicator titrations. The colorless acid sample was titrated in the presence of an indicator of the same acid strength and of known concentration. The titration plot resembles that for the colored acid alone, being a direct type of plot, but the apparent intensity of absorption is diminished because the colorless acid is titrated simultaneously. The end point is for the sum of the two acids, and the colorless acid is found by difference. Another kind of absorbance-volume indicator titration plot has already been encountered in the discussion of Fig. 6, where it was pointed out that the weaker acid 2,4-dinitrophenol served as an indicator for the titration of diphenyl phosphate. Goddu and Hume (13) and Reilley and Schweizer (20) have employed such systems in acid-base titrations. The two types of titration discussed in this paragraph differ from the kind represented by Figs. 8 and 9 in that the indicator concentration in the solutions considered here is comparable to the concentration of the sample substance, while for titrations of the kind in Figs. 8 and 9 the indicator

concentration is generally negligible in terms of titrant consumption.

A variation of the absorbance-volume plot has been introduced by Bruckenstein and Gracias (21), who evaluate (for the case of aqueous acid-base titrations) the point at which the second derivative $\Delta^2 pH/\Delta V^2$ is zero; this is taken as the end point. The method employs differential spectrophotometry of solutions containing an indicator, the data being taken in the region of the end point. The original paper should be consulted for details.

Indicator Ratio Methods.—The absorbance-volume plot is limited primarily by its dependence upon data obtained in the vicinity of the end point, and these are often the poorest data for extrapolation purposes. Moreover, it is necessary to collect data on both sides of the end point for such extrapolation. Recently, some related methods have been developed by Higuchi and his co-workers which utilize data further from the end point for linear extrapolation; in these methods data are necessary on only one side of (that is, before or after) the end point. The treatment given here will consider the titration of a base with an acid. The titration system is pictured as a competition between the sample base B and indicator base I for the titrant acid HA (Eqs. 1 and 2).

$$BHA + I \rightleftharpoons IHA + B \qquad \text{(Eq. 1)}$$

$$K_{ex} = \frac{[IHA][B]}{[I][BHA]} \qquad \text{(Eq. 2)}$$

The reaction between B and HA (the titration reaction) is considered to proceed to completion, and the amount of titrant consumed by the indicator is assumed negligible. Now let X' be the total concentration of acid added to the system at any time prior to the end point, and let S' be the total concentration of base initially present.

$$X' = [BHA] \qquad \text{(Eq. 3)}$$

$$S' = [B] + [BHA] \qquad \text{(Eq. 4)}$$

The concentrations X' and S' can be converted to the corresponding volumes X and S with the relationships $X' = XN/V$ and $S' = SN/V$, where N is the titrant normality, V is the total volume of the titration solution, X is the volume of titrant added at any point, and S is the volume of titrant required to react stoichiometrically with the total base present. Combination of these relations with Eqs. 2, 3, and 4 gives the fundamental titration equation

$$K_{ex} = \frac{I_a}{I_b} \cdot \frac{S - X}{X} \qquad \text{(Eq. 5)}$$

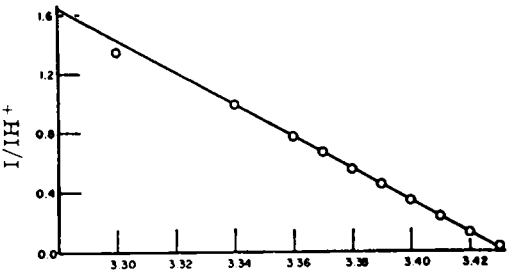


Fig. 10.—Type I plot for titration of triphenylguanidine with perchloric acid in acetic acid, using quinaldine red indicator (16). [Courtesy of *Analytical Chemistry*.]

where I_a/I_b, the indicator ratio, is written for the quantity [IHA]/[I]. K_{ex} is called the exchange constant, and evidently it is a measure of the relative basicity of I and B.

The indicator ratio is calculated from the spectrophotometric data by

$$I_a/I_b = \frac{A_b - A}{A - A_a} = \frac{A - A_b}{A_a - A} \qquad \text{(Eq. 6)}$$

where A_b is the absorbance of the solution when the indicator is totally in the base form, A_a is the absorbance when the indicator has been completely converted to the acid form, and A is the absorbance when it is in any mixture of the two forms; all solutions must contain the same concentration of indicator. Usually the wavelength is chosen so that either A_a or A_b is zero. To render negligible the effect of dilution by the titrant, it is common practice to incorporate the indicator in the titrant in the same concentration it has in the sample solution; the dilution effect is thus eliminated. Several uses of Eq. 5 will be considered below.

Type I Systems.—The titration Eq. 5 can be rearranged to $XK_{ex}(I_b/I_a) = S - X$. Now consider a system in which K_{ex} is very small, of the order 10^{-2}. This means that B is a much stronger base than is I. The indicator color change will all occur in the final few per cent of the titration. In such a case, therefore, it is permissible to make the substitution $S = X$

$$SK_{ex}(I_b/I_a) = S - X \qquad \text{(Eq. 7)}$$

This is a linear equation in I_b/I_a and X; the intercept on the X axis is S, the end-point volume. An example of this method of titration, which is termed the Type I plot, is shown in Fig. 10 (16). The Type I method is capable of great precision, and it should find use in the analysis of high-purity chemicals and the development of primary standards. Because of the nature of the assumption made in the derivation of Eq. 7 data can only be taken close to the end point. It should

be noted that with this plot all data are collected before the end point is reached.

TYPE II SYSTEMS.—In the more general case the basic titration Eq. 5 must be employed (16). This equation can be placed in three forms which are linear in the titration variables (22). (For convenience K is written for K_{ex}.)

$$1/X = (K/S)(I_b/I_a) + 1/S \qquad \text{(Eq. 8)}$$

In this form the indicator ratio I_b/I_a is linear with $1/X$, the reciprocal of the titration volume (16). An example is shown in Fig. 11 (22). From the slope and either intercept the end-point volume, S, and the exchange constant, K,

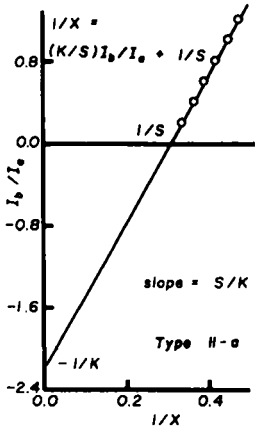


Fig. 11.—Type II-a plot for titration of urea with perchloric acid in acetic acid, using Nile blue A indicator (22).

can be determined.[2] This photometric titration plot, called the Type II-a plot, has been applied to acid-base titrations in aqueous and nonaqueous systems (16, 23–26). It has enabled successful titration to be made of very weakly acidic and basic substances not otherwise accessible to acid-base titration.

Equation 9 gives another form of Eq. 5. The

$$X(I_b/I_a) = S/K - X/K \qquad \text{(Eq. 9)}$$

corresponding plot, known as the Type II-b plot, is shown in Fig. 12 for the same data graphed in Fig. 11 (22). Again both S and K are readily determined from any two of the three features of the line (two intercepts and one slope). The third form (Eq. 10) is graphed in Fig. 13; this is the Type II-c plot.

$$I_a/I_b = (I_a/I_b)S/X - K \qquad \text{(Eq. 10)}$$

All Type II plots employ data taken before the end point, and in fact can utilize information taken much earlier in the titration than is possible with absorbance-volume plots. Besides permitting the use of much more data, some special advantage may result. For example, precipitation of the titration product, which would vitiate the absorbance-volume plot, may not prevent accurate location of the end point by the Type II plot.

In addition to providing a value of S, the end-point volume, these methods give a value for the exchange constant. In the case of acid-base reactions the exchange constant is a measure of acidity or basicity, and many such constants have been evaluated by this means (16, 27–29). The same equations will apply to 1:1 complexation reactions, and it seems that one of the most promising uses of the Type II plots may be in the evaluation of complex stability constants.

TYPE III SYSTEMS.—If the indicator base is so weak that it does not begin to change color until essentially all of the sample base has been titrated, then the color change is governed not by the competition (1) but by

$$I + HA \rightleftharpoons IHA \qquad \text{(Eq. 11)}$$

The free acid concentration [HA] is given by

$$[HA] = (I_a/I_b)(1/K') \qquad \text{(Eq. 12)}$$

where the significance of K' depends upon the nature of the reaction and the titration conditions; thus in an aqueous acid-base reaction K' is the reciprocal of the dissociation constant of the conjugate acid form of the indicator. Equation 12 can be transformed into

$$X - S = (I_a/I_b)(V/K'N) \qquad \text{(Eq. 13)}$$

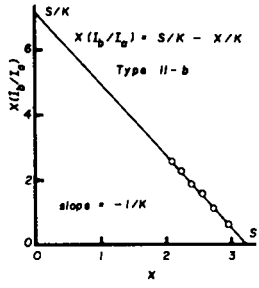


Fig. 12.—Type II-b plot; see Fig. 11 for details (22).

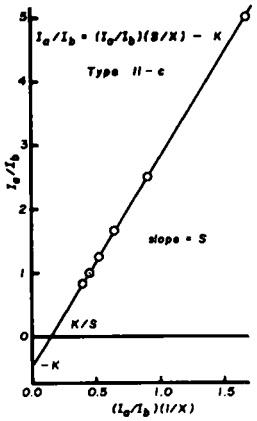


Fig. 13.—Type II-c plot; see Fig. 11 for details (22).

[2] The plot as shown places the independent variable on the horizontal axis, as is customary. This is why the slope is S/K rather than K/S, indicated by Eq. 8.

where the quantity $(X - S)N/V$ has been substituted for [HA]; in this case X is greater than S. This equation is linear in X and I_a/I_b, and the X axis intercept is equal to the end-point volume, S. From the slope, the equilibrium constant can be evaluated. Figure 14 shows an example of this kind of titration, which Rehm and Higuchi called the Type III plot (30). Note that with the Type III plot all data are taken after the end point.

Sources of Error.—In many systems application of the indicator ratio methods just discussed reveals curvature in the titration plots. This deviation from the behavior predicted on the basis of Eq. 5 can be accounted for by a more complete treatment of the system. Suppose that the equality expressed by Eq. 3 fails to hold; in other words, some of the acid added to the system is present in a form other than BHA. (The reasons why this may be so will be discussed shortly.) Let the concentration equivalent to this acid be denoted by R'. Then Eqs. 3 and 4 may be more accurately given as

$$X' = [BHA] + R' \qquad \text{(Eq. 14)}$$

$$S' = [B] + [BHA] \qquad \text{(Eq. 15)}$$

These equations can be combined with Eq. 2 as before to give the general titration equation

$$K_{ex} = \frac{I_a}{I_b} \cdot \frac{S - (X - R)}{(X - R)} \qquad \text{(Eq. 16)}$$

where R is the volume of titrant acid equivalent to that added but not consumed in the actual titration reaction. In the limiting case in which R is negligible Eq. 16 becomes equivalent to Eq. 5. When R becomes significant compared

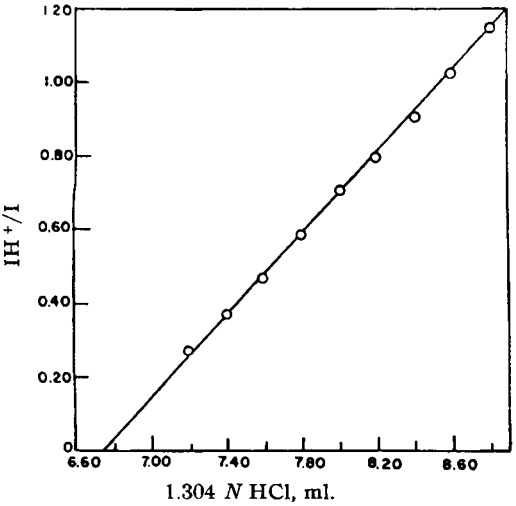


Fig. 14.—Type III plot for titration of aniline in water with hydrochloric acid, using Metanil yellow indicator (29). [Courtesy of *Analytical Chemistry*.]

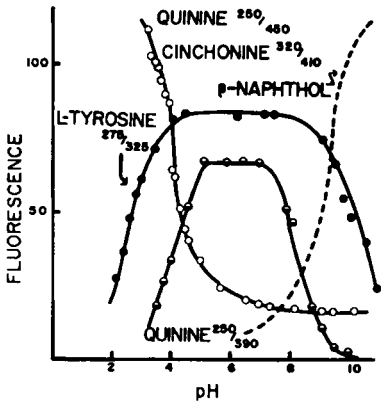


Fig. 15.—Representative pH-fluorescence curves for the three most common types of fluorescent substances. Key: ---, acid type (β-naphthol); —○—, basic type (quinine, activation/fluorescent wavelength 250/450 mμ); —◉—, bifunctional type (quinine 250/390 mμ).

with X, then curvature is observed in the conventional Type II plots as described. There may be several sources of this deviation:

(*i*) The indicator is present in sufficient concentration that it accounts for the consumption of a significant volume of titrant acid; then $R' = [IHA]$.

(*ii*) The solvent contains a basic impurity C which consumes titrant; then $R' = [CHA]$.

(*iii*) Most important, the titration reaction does not go to completion (that is, the product of the reaction undergoes solvolysis).[3] Then $R' = [HA]$.

A practical experimental technique has been developed which eliminates all three sources of error (27). The procedure involves the titration of two samples identical except for the amount of sample base. Let sample 1 be the smaller of the two. Then Eq. 16 represents the titration of this sample, the volumes being denoted X_1 and S_1. A similar equation describes the titration of sample 2. The quantity R is identical (or practically so) in the two titrations at a given value of the indicator ratio. The two equations are now subtracted, at constant I_a/I_b, thus eliminating R. The resulting equation can be placed in the usual Type II forms; for example, the Type II-b equation becomes $(X_2 - X_1)(I_b/I_a) = (S_2 - S_1)/K - (X_2 - X_1)/K$ which may be compared with Eq. 9. The end point represents the titration of the difference between samples 1 and 2. This technique is called the modified Type II titration (27). It is effective in eliminating the sources of error listed above. Some special cases of mixture analysis have also

[3] The limitations thus imposed on the use of Eq. 5 have been analyzed in detail by Bruckenstein and Nelson (24).

been treated (27). Details for its application will be found in the original papers (22, 27).

The general application of indicator ratio titration plots to the analysis of mixtures has not yet been attempted. It must be kept in mind that a second basic substance will cause an interference which cannot be circumvented by the modified Type II technique unless this impurity is introduced in the solvent. If the impurity is present in the sample its amount cannot be varied independently of the sample base and its influence cannot therefore be subtracted out.

Although the discussion throughout has treated the titration of a base with an acid, the reverse case is analogous, the ratio I_a/I_b appearing as its reciprocal, I_b/I_a.

FLUORESCENCE MEASUREMENTS IN HOMOGENEOUS SYSTEMS

Fluorescence is a function of the molecular species present in solution and may often therefore be used as a means of determining the change in ionic form occurring during a titration. The fluorescence of a dissolved substance is very nearly a linear function of the concentration of the fluorescing ion so that it may be used in a manner quite analogous to absorbance. Fluorescence varies with concentration according to

$$F = PC \qquad \text{(Eq. 17)}$$

where F is fluorescence (in relative photometer units), C is the concentration of the fluorescing species, and P is a constant relating the two which is a function of the structure of the molecule, the incident light power, and the Beer's law constants for the system.[4]

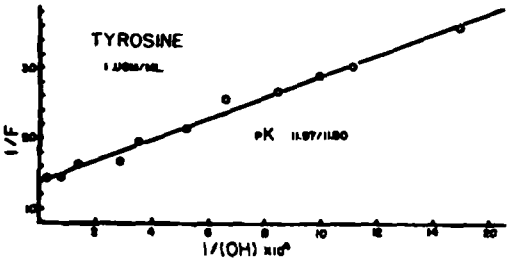


Fig. 16.—Titration of 1 mcg. tyrosine (1 mcg./ml.), pK (found/lit.) 11.97/11.80.

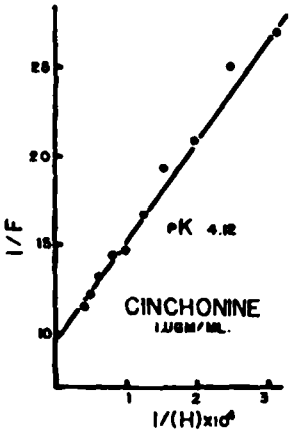


Fig. 17.—Titration of 1 mcg. cinchonine (1 mcg./ml.), pKfd 4.12.

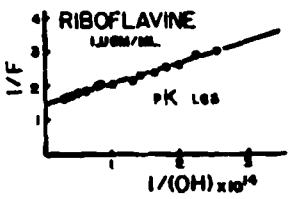


Fig. 18.—Titration of 1 mcg. riboflavin (1 mcg./ml.), pK fd 1.63.

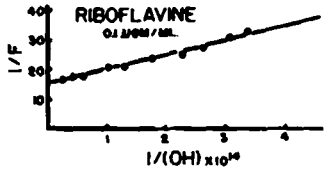


Fig. 19.—Titration of 0.1 mcg. riboflavin (0.1 mcg./ml.).

Just as with absorbance, titrations involving fluorescence may be performed using either the activity of the compound itself as a measure of ion concentration (self-indicator or direct titration) or the change of the fluorescence of an indicator to determine the effect of added titrant (indicator titration).

Self-Indicator Direct Titrations

Fluorescence-pH curves may assume three general shapes (see Fig. 15). Those molecules with two functional groups generally show a humplike curve as shown for tyrosine and one wavelength combination for quinine. The acid type is represented by naphthol and the basic type by quinine and cinchonine. As may be expected, any of these fluorescence-pH slopes may be used for titration.

Plots to determine the end points in direct titrations using fluorescence suffer from the same problems that are observed in absorbance "self-indicating" titrations, in that there is a lower concentration limit at which the effect of

[4] The exact expression is $F = ZI_0(1 - e^{-abc})$, where the term F is the observed fluorescence, Z is a constant relating the efficiency of the conversion of absorbed light to that fluoresced, both terms being measured in relative photometer units, and I_0 incident light power.

The complete expansion of the above is

$$F = ZI_0\left(abc - \frac{(abc)^2}{2!} + \frac{(abc)^3}{3!} - \ldots\right)$$

so that the assumption of linearity, i.e., $F = (ZI_0ab)\,C = PC$, involves an error of about 1 per cent or less for molecular weights of about 300, 1-cm. cells, absorptivities of about 1000, and concentrations of about 10^{-6} M.

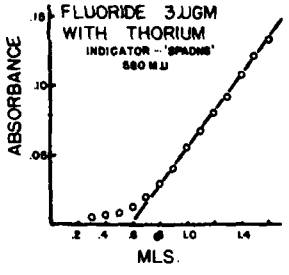


Fig. 20.—A fluorescent indicator titration of fluoride with thorium (35).

the solvent swamps that of the sample (11). In the extremely dilute solutions measurable by fluorescence, data may easily be obtained well beyond the limiting product $C_{HA}Ka = 10^{-12}$, but the end point is not readily observed. Data from titrations of these dilute solutions can, however, be used to obtain pKa values for fluorescent compounds.

Rearrangement of the general equations for the dissociation constants for acids and bases, using the approximation in Eq. 17 to estimate the concentration of the fluorescing species, yields equations suggesting linear plots for easily obtaining Ka values for fluorescing substances

$$1/F = Ka/PC(1/H^+) + 1/PC \quad \text{(Eq. 18)}$$

$$1/F = K_w/KaPC(1/H^+) + 1/PC \quad \text{(Eq. 19)}$$

for either the case where the conjugate acid (Eq. 18) or the conjugate base (Eq. 19) is fluorescent. By the simultaneous measurement of pH and fluorescence, the quotient of slope and intercept of the appropriate graph will yield values for the Ka (31) even in very dilute solution; see Figs. 16–19. Quantities of material as small as 0.1 mcg. and probably lower can be determined reproducibly as only 1 ml. of solution is required for titration.

Indicator Titrations

Fluorescence titrations with indicators are much more commonly used than are direct titrations; many examples are available, particularly those in which metal complexation or chelation is involved, *e.g.*, see (4). Their prime use has been for metal titrations, but they need not be so limited. Fluorescent compounds can be quite effective as pH indicators, for instance, although they would seem to offer little advantage over standard indicators except for their intense color. Their ability to fluoresce brightly in very thin layers, however, has suggested their use in titrations involving very dark or opaque solutions and suspensions (32). Many fluorescent pH indicators are available (33, 34), and the entire pH range is accessible.

Despite the considerable application of fluorescent indicator titration, it appears that investigators using this technique have not made the most of the recent advances in the field of data handling. A scan of the likely literature indicates that, although they are amenable to such processing, fluorescent indicator titrations are not being evaluated through the use of the "indicator ratio" equations, despite the strong evidence (from absorbance titration results) of their superiority over direct fluorescence *versus* volume plots. Figure 20 (35) shows the results of a thorium titration of fluoride, using SPADNS (a phenylazonaphtholene sulfonate) as an indicator, plotted as a direct titration. After total precipitation, the color of the thorium-SPADNS complex develops. While this is a satisfactory end point, the data prior to the end point could have been used to get the same end point value using a Type I plot (Fig. 21). If the addition of thorium had been carried on far enough to permit the top of the complex-color curve to be observed, this system probably would be better graphed as a Type III plot.

The sharpness of break at the end point in some of the direct titration plots in the literature hides the fact that the end points are not always so clearcut. Considerable rounding often occurs near the end point, making extrapolation dif-

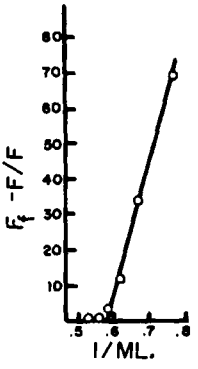


Fig. 22.—A replot of the data of Howerton (8) for the 80 μl. Labtrol 4 × 10^{-4} EDTA titration of zinc (calcein), according to Type II mathematics.

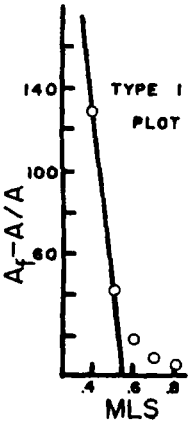


Fig. 21.—A replot of the data of Fig. 20 according to Type I mathematics.

ficult. The back-titration of EDTA with zinc in a calcium determination with calcein indicator, by Howerton and Wasilewski (8), was such a situation. The data obtained by these authors, replotted with Type II mathematics, yielded Fig. 22, showing clearly the applicability of such end-point extrapolations.

The sensitivity of fluorometric titrations is great. Titrations of very fluorescent substances (using Eqs. 18 or 19) in the 0.1 to 1 mcg. range can easily be duplicated to within 2 to 5 per cent without special precautions. The indicator method without plotting improvements yields 1 to 5 per cent accuracy on samples of about 1 mcg./ml. The more sophisticated data handling methods should afford better results.

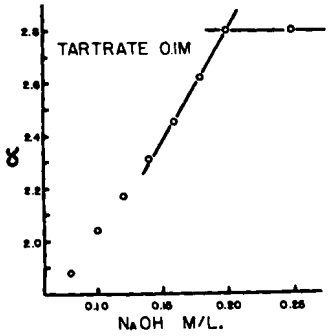


Fig. 23.—The titration of optically active tartrate with NaOH using optical rotation to locate the end point (37).

METHODS BASED ON OPTICAL ROTATION

End points depending upon changes in optical rotation can be used only for compounds having rather specific structural features. They must be optically active, and their activity must change, preferably by a great deal, during the course of the titration.

Self-Indicator (Direct) Titrations

While a considerable amount of work has been published on the effects of pH on optical rotation, especially by Britton and his co-workers (36, 37), these were not actually titrations. These workers were mainly interested in using optical rotation to detect the ionic species present during complexing. Their data were obtained as in a titration, however, and can be used to demonstrate the methods. Examples of some of their original data, replotted as for a titration, indicate that (although these workers apparently did not see the value of it at the time) polarimetric titration end points can be obtained (Figs. 23 and 24). Britton and co-workers do not appear to have been the first to use polarimetric-pH data, although they seem to have been the most prolific (38). These figures show the titration of tartrate and lactate, their own rotation being used as an indication of the degree of titration (self-indicator). The change in the case of tartrate is quite spectacular, while the lactate case leaves something to be desired; but both are usable.

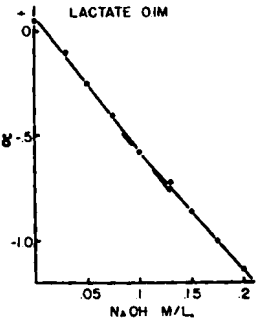


Fig. 24.—The titration of optically active lactate with NaOH using optical rotation to locate the end point (36).

The recent availability of spectropolarimeters has made it convenient to select the wavelengths most suitable for titration studies, creating possibilities for selective titrations of mixtures, and for maximizing end-point breaks by selecting appropriate wavelengths.

Indicator Titrations

Recently, Kirschner and Bhatnagar (39) have described work done with a Beckman DU modified by the Rouy method (40) and enlarged to take a 10-cm. cell. They report sample curves for an acid-base titration (HCl with NaOH) using about 0.04 *M* D-tartaric acid as the indicator. The sharpness of the end point is notable, but is largely due to the very high indicator concentration (see Fig. 25). The reactant concentrations were 0.1 *N* HCl and 5 *N* NaOH, so that one might expect sharp end points. The authors assert that the second break is due to the second ionization constant of tartaric acid; *i. e.* this is a "self-indicator" titration that they suggest can be used to find the concentration of the indicator present. While the end point shown on the figure and in their paper is clear enough, a con-

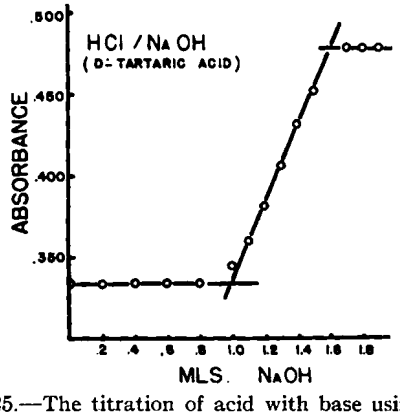


Fig. 25.—The titration of acid with base using an optically active indicator (39).

siderable improvement in the data treatment for this type of titration, which should be helpful when less definite end points are obtained, can be achieved by using the Type III "linear plot"—the one designed for indicators that change after the end point. The results of this type of plotting are shown in Fig. 26. The authors have also shown this procedure to be useful in the study of the interaction of metals and optically active complexers, *l*-histidine in the case shown in their paper, although this use has been developed to a much greater degree by Britton and his co-workers (37) and others, *e.g.*, see (41) and (42).

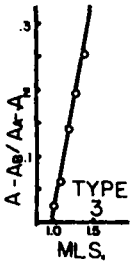


Fig. 26.—A replot of the data of Fig. 25, according to Type III mathematics.

It would not seem that this would be a particularly sensitive method, although it is certainly specific, and very likely would yield good pK values for specific groups even when associated with other groups with similar pK's. The large volume and high indicator concentration that seem to be required would appear to limit its usefulness. All that is required for improvement is the discovery of some really active pH-sensitive compounds.

One important point that should be recognized is the rather large temperature effect on optical rotation of some optically active molecules—up to 10 per cent /° C. for tartaric acid, for instance (43)—and the relative ease with which the racemization of isomers can occur, particularly in strong acid or base. Thus, if one proposes to develop a very accurate and sensitive method using this technique, temperature control is necessary.

ABSORBANCE MEASUREMENTS IN HETEROGENEOUS SYSTEMS

Self-Indicator (Direct) Titration

Titrations using the separation of another phase to signal the end point have been grouped into three categories: turbidimetry, nephelometry, and heterometry. These are all characterized by measurements of the effects of a dispersed phase on the passage of light through the system, and the first two (turbidimetry and nephelometry) while requiring different equipment are, from the standpoint of visually detected end points, the same. The end points for heterogeneous titrations are always made at the point of complete precipitation and visual detection of this point is some small problem, as one must determine the titrant volume that causes the last bit of precipitation in a cloudy solution.

Several very clever methods have been devised to overcome this limitation, GayLussac's "equal turbidity" method being one (44). All of the other schemes devised to sharpen the observation of the faint opalescence produced in the supernatant by "one more drop of titrant" have encountered the problem of the common ion effect. As a point of interest, it was to determine this "amount still in solution" in the AgCl titration that led Richards and Wells (45) to develop the nephelometer during their early studies on the atomic weight of silver.

Instrumentally, turbidimetry and nephelometry differ; transmitted light is measured in turbidimetry, scattered light in nephelometry. While both are instrumentally similar save for geometry, nephelometry (in which one measures a small change against a dark background) should be fundamentally more sensitive than turbidimetry, which, like colorimetry, involves measuring a small change against a bright background.

Heterometry is a field in itself. Largely pioneered by Bobtelsky and co-workers (46), it is not actually a titration tool at all, but rather an investigational tool for studying multiphase relationships and phase changes. It would seem from Bobtelsky's earlier work that quantitative titration was a valuable side product rather than an aim of the development of heterometry.

Heterometry differs from turbidimetry more in point of view than in any profound manner. Its development stemmed, it seems, from the desire to study multiphase systems without additives (such as polymers to retard crystal growth and influence particle size) and it produced a simplified turbidimetric titration procedure by ignoring "principles" considered to be absolute in turbidimetry and nephelometry. Thus, in many cases, Bobtelsky has found that differing rates of nucleation and growth, admittedly problems, affect the absolute values of the absorbance or light transmission but not the critical or "break" points which are of interest in titration.

For the moment we shall ignore the theoretical difference between scattered light measurements and transmitted light measurements (we will merely assume that one need only change ordinates to plot one or the other). Figure 27 shows the eight types of precipitation titrations. Graph 1 depicts the classical one obtained from

the ideal case where the precipitate is virtually insoluble. Reagent A plus reagent B creates a nearly insoluble precipitate AB. Additional A has no effect on the state or amount of precipitate.

Graph 2 describes the case in which the precipitate forms but, at the equilibrium point B, a second 1:1 solubilizing reaction begins which proceeds to completion at about A_2B. Graphs 4 and 7 show similar situations where the dissolution is either nonstoichiometric or involves other ratios of titrant to titrand than that in Graph 2. Point B in Graphs 1 and 2 represent stoichiometric formation of a product and thus are usable end points.

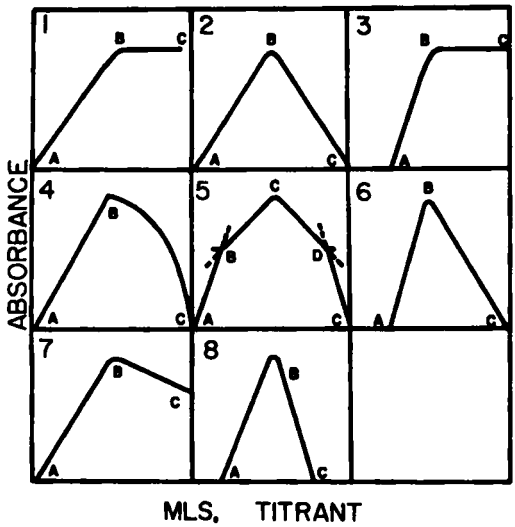


Fig. 27.—The eight general types of multiphase titrations, after Bobtelsky.

Graph 3 shows the formation of either a partially soluble product or a soluble interaction product (complete at point A) which secondarily interacts with another molecule of titrant to produce complete precipitation at point B. This is much like the Ag(AgCN) reaction. Graphs 6 and 8 show the same situation, but include quantitative precipitation followed by further solubilization of the precipitate; different ratios of titrant-titrand are required to achieve the same result in the two cases shown. The initial point A then represents either the solubility of the product or the stoichiometric formation of the soluble interaction product.

Graph 5 shows the formation of insoluble intermediates which rearrange to more stable forms upon addition of more titrant.

There is a great number of possible combinations of these basic systems, and in addition no limit to the possible number of intermediates. The detection of a quantitative end point depends upon a knowledge of the reaction in order to know which portion or portions of the curve can be used, although in theory any "critical point" should be usable. Complicating the analytical problem are practical limitations such as partial solubility of the precipitate, rate of precipitation at the end point, and coagulation caused by excess titrant which produces nonlinear portions that defy extrapolation.

The problems of speed of nucleation and whether crystal growth is occurring rather than new nucleation have been theoretically discussed by Fischer and co-workers (47). Slow crystallization is a prime concern because it is just at the end point, where the degree of supersaturation is lowest, that the rate of precipitation is also the slowest, making equilibration at this point of some concern. In this connection Bobtelsky asserts that equilibration is usually reached "after a few seconds to one minute" as evidenced by stable photometer readings on his "heterometer." Because light scattering and turbidity should both be functions of particulate surface area, and the mass of precipitate a function of titrant volume, calculated data for surface area as a function of added titrant should closely approximate absorbance-volume data from titrations. Fischer (47) was able to perform this calculation for the two extremes to be expected for absorbance *versus* titrant volume plots (Fig. 28). The upper (straight) line in Fig. 28 represents the situation where only nucleation occurs without crystal growth. The surface area increase is roughly linear with the mass of preicpitate (light absorbance thus should be a linear function of the added titrant volume). When the worst possible situation occurs, an initial seeding followed only by crystal growth and no further nucleation, the parabolic lower curve of Fig. 28 results. All possible variations

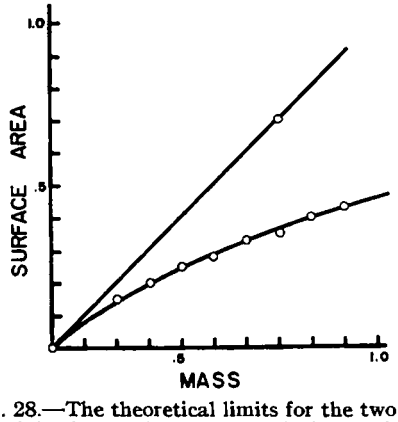


Fig. 28.—The theoretical limits for the two types of precipitation to be expected during a titration (47). The upper line, nucleation without crystal growth, lower line, growth only without nucleation. All combinations should lie between these two extremes.

and degrees of these two should lie between these two extremes. The more curved (crystal growth) cases will produce titration end points which are hard to detect, being marked by the intersection of a nearly horizontal curved line with the horizontal one occurring after the end point.

The solution to this problem often simply requires the addition of a protective colloid. While this is usually effective, as one might expect, crystal-face poisoning is a highly selective phenomenon and no universal nonionic protective colloid has been found. Protective colloids have one other beneficial effect: they inhibit the adsorption and premature precipitation of the ions being titrated onto the surface of the precipitate, a troublesome problem that can produce early end points.

Despite the problems outlined, turbidometric end points can in many cases be quite straightforward. Consider the titration of 0.585 mg. of sulfate with $1.6 \times 10^{-3}M$ barium ion [Fig. 29 (48)] or the titrations of calcium with phthalate by Bobtelsky [Fig. 30 (49)].

An interesting nonmetallic, organic titration was published by Lambert (50). An anionic surfactant was titrated with a cationic agent to produce an almost perfect reproduction of one of Bobtelsky's theoretical curves (Fig. 25) involving a slightly soluble interaction product suddenly achieving saturation, then redissolving (or in this case reacting further to produce a soluble compound); see Fig. 31. These curves are endlessly repeated in the polymer literature, but this is an interesting pharmaceutical example.

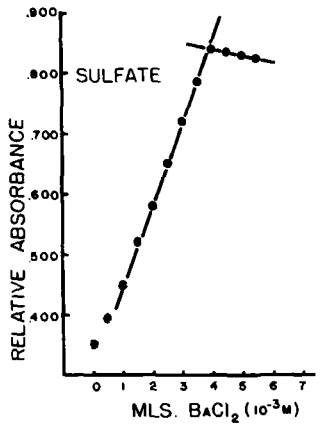


Fig. 29.—The nephelometric titration of sulfate (0.585 mg.) with barium ion (48).

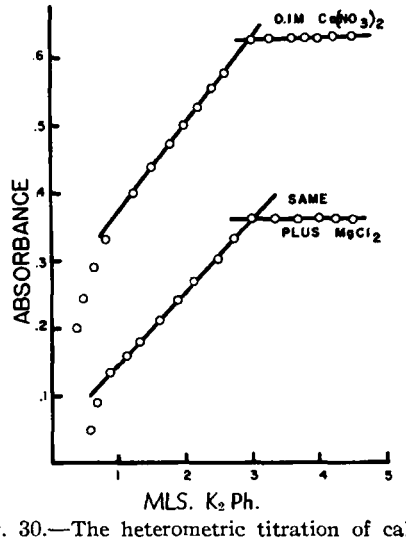


Fig. 30.—The heterometric titration of calcium ion with phthalate in the presence and absence of magnesium (49).

Indicator Titrations

There was a flurry of research done several decades ago on added indicators whose function was to precipitate at the end point of a titration (51). This "new principle," as it was termed by its inventor in 1925, seems to have died a very undeserved death. Several series of potentially useful indicators were tested, of which the most promising seemed to be the rhodamines and the *p*-aminoazobenzenes. Many of the derivatives of these parent compounds were quite effective and were able, according to the author, to precipitate in an extremely narrow pH range. These indicators were effectively used for the titration of several common acids (along with barbital, boric, and arsenous acids) with excellent results. The sharp change in the pH-solubility profile of these indicator compounds makes them somewhat more difficult to use than the usual indicator that telegraphs its end point by the color changes produced as the titrant is added. Fairly dilute solutions must be used as concentrated titrant produces an immediate precipitate which is only very slowly redissolved; thus high local concentrations should be avoided. The true end points for these titrations should be determinable photometrically using a Type III plot as discussed before.

Photoelectric titration using nephelometry has several pitfalls—the effects of stirring in particular—that are not present in other types of titration. In Fig. 32 the results of a fluorometric precipitation titration are plotted both with and without stirring during photoelectric readings (52). The stirred titration solution always results in higher photometer readings (fluorescence or absorbance) than the unstirred. Because the line after the end point is the same for both and the line before the end point is much steeper and thus less sensitive to suspended particles, stirring results in a poorer end point.

Nephelometric titration methods can be quite sensitive. Bobtelsky feels that his heterometric

method is capable of using solutions of $10^{-6}M$ concentration. In absolute amounts, 1–3 mcg. seems to be the current extreme, with the precision ranging from 0.1 to 5 per cent. Naegli and co-workers obtained a precision of about 0.2 per cent using isonitroso-acetyl-paraminazobenzene as a precipitation indicator (51). This was without instrumental aid; with the improved plotting procedures now available for indicator titrations, an instrumental titration using his indicators should be capable of much greater sensitivity and at least equal precision.

CONCLUSION

The literature of the past few years has recorded a great increase in both developmental work and applications of photometric titrations (though the same may be said of almost every analytical technique). Some of the advantages peculiar to these methods may be expected to lead to their continued wide use. The simplest types of photometric titrations are those utilizing direct plots of photometer readings against titrant volume to locate the end point, and when such plots yield sufficiently accurate results they should certainly be employed. The limitations of such procedures have been outlined, and the alternate procedures, involving indicators and the evaluation of an indicator ratio function, have been presented in sufficient detail to permit their use. The application of these more general methods to complexometric and precipitation indicator titrations should be especially fruitful.

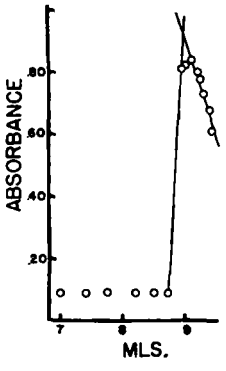


Fig. 31.—The nephelometric titration of an anionic surfactant (Igepon, 10^{-3}) with a cationic agent (Ceepryn, 10^{-3}) (50).

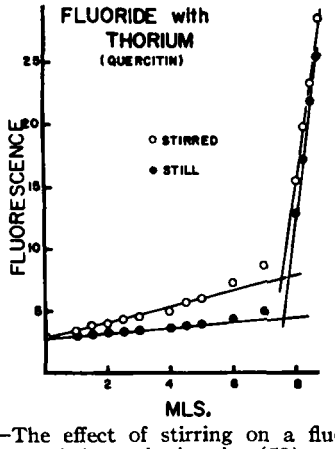


Fig. 32.—The effect of stirring on a fluorescent-nephelometric titration (52).

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Synthesis of Some Substituted Piperidones

By PETER A. RATTO* and ARNOLD A. LIEBMAN‡

5,5-Dimethyl-6-hydroxy-6-phenyl-2-piperidone was obtained from a side reaction of a synthetic sequence aimed at the production of analgesics. A series of these hydroxypiperidones has been prepared and their stability toward dehydration studied.

DURING THE preparation of compounds designed to contain the structural requirements for analgesia (1), a side reaction led to a piperidone structure having possible hypnotic or stimulatory properties. This piperidone was prepared by cyanoethylation of isobutyrophenone (I) according to the procedure of Campbell, *et al.* (2), to give 4-benzoyl-4-methylvaleronitrile (II). Hydrolysis of this ketonitrile to the corresponding acid (III) was effected in the described manner (2), and the acid was then treated with thionyl chloride, followed by cold concentrated ammonia solution. The amide that had formed cyclized into a 6-hydroxypiperidone structure (IV), evidenced by infrared and ultraviolet spectra. In hydroalcoholic solution, the material evidently existed as an equilibrium mixture of the δ-ketoamide and the 6-hydroxypiperidone since a semicarbazone derivative was obtainable.

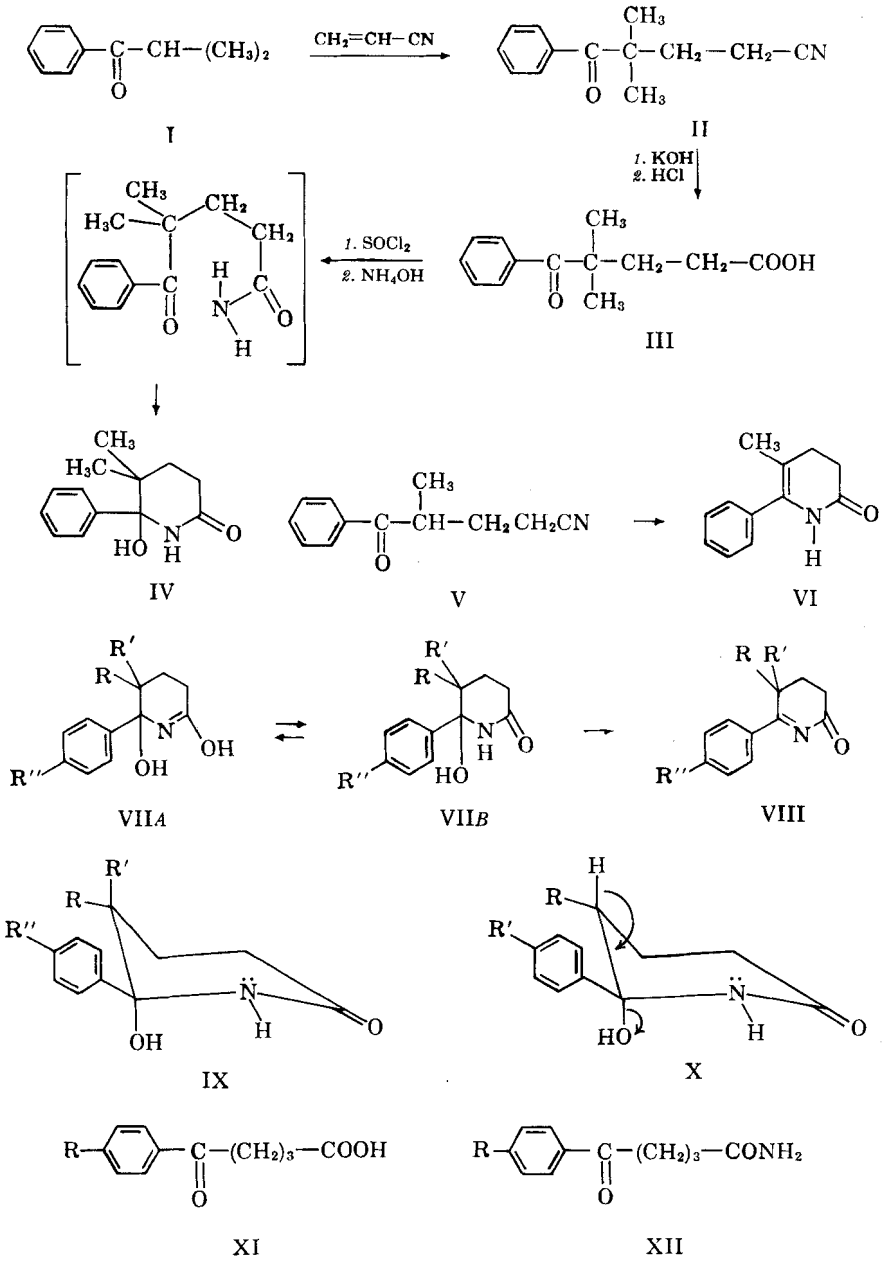
Comparing the structure of the 6-hydroxypiperidone (IV) to phenobarbital or other hypnotics and anticonvulsants, it is noted that the position of the lactam relative to the alkyl substituents suggests possible hypnotic or anticonvulsant activity. On the other hand, the 6-hydroxy-6-phenyl structure, coupled with the hypnotic-producing moiety, could confer convulsive or hypnotic inhibiting activity. In addition to the pharmacological possibilities, the stability of the compound toward dehydration was also of interest.

Several additional ketones were used as starting materials including *p* substituted propiophenones, *p*-substituted isobutyrophenones, α-methylbutyrophenone, and hexahydrobenzophenone. These substances readily underwent cyanoethylation (2, 3) (Table I).

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To obtain the desired piperidones from the δ-ketonitriles, several approaches were available. Where applicable, treatment of the nitrile with alkaline peroxide represents a quick method (4, 5). Using this procedure, the expected hydroxylactam (IV) was obtained only with 4-benzoyl-4-methylvaleronitrile (No. 4, Table I); the others in this series exhibiting an inertness toward this reagent. However, sulfuric acid hydrolysis of these δ-ketonitriles yielded the expected hydroxylactams as did complete hydrolysis to the acid, followed by acid chloride formation and treatment with aqueous ammonia. Finally, lactams of this type have been realized directly from the ketone by replacing acrylonitrile with acrylamide (6); however, this procedure was not applied to these compounds.

When propiophenone was used as the starting ketone, cyanoethylation yielded the expected product (V). Either by alkaline hydrolysis of the nitrile followed by amidation or direct sulfuric acid hydrolysis, a cyclic dehydro product resulted (VI). The nitriles prepared from *p*-substituted propiophenones (Table I, nos. 2, 3) gave analogous results when converted to their corresponding amides (Table III). In the case of each δ-ketonitrile containing two alkyl groups in the position α to the aroyl substituent, no dehydration occurred with the cyclization. It is conceivable, but improbable, that inhibition of dehydration of these 5,5-dialkylpiperidones could be attributed solely to tautomerization of the lactam. Since the tautomeric structures must include some *VIIB*, dehydration could occur and result in the formation of the highly conjugated system (VIII). In the case of those compounds that underwent dehydration with the cyclization, infrared spectra showed the presence of NH stretching bands, thereby confirming the formation of a carbon-carbon rather than a carbon-nitrogen double bond (VI). Dehydration of those compounds containing two alkyl groups in the 5 position (IX) could not result in the formation of



a similar carbon-carbon double bond without migration of an alkyl group. Therefore, the only hydrogen atom available for direct participation in dehydration of these latter compounds is the lactam hydrogen. Conformationally, the aromatic substituent, by virtue of its greater size, should be predominantly in an equitorial position, placing the hydroxyl group in the axial position. The lactam hydrogen atom may be considered as existing in an equatorial position relative to the unshared pair of electrons about the nitrogen. The combination of this hydrogen's *cis* relationship to the hydroxyl group and the resonance of the lactam carbonyl precludes dehydration under these reaction conditions. On the other hand, with those compounds containing one alkyl substituent in the 5 position of the piperidone ring (X), an axial hydrogen *trans* to the 6-axial hydroxyl group is available, and cyclization also results in dehydration.

Several δ-ketoacids were prepared which contained no alkyl substituents (XI). Subsequent treatment of these compounds with thionyl chloride or ethyl chloroformate, followed by concentrated ammonia solution, yielded the open chain δ-ketoamides (XII) (Table IV). Failure

TABLE I.—δ-KETONITRILES

No.	R	R′	R″	Formula	B.p., °C.[a]	Yield, %	Semicarbazone Formula	M.p., °C.[a]	Carbon, % Calcd.	Carbon, % Found[b]	Hydrogen, % Calcd.	Hydrogen, % Found
1	CH_3	H	H	$C_{12}H_{13}NO$	163–164/10 mm.	60	...	...	77.00	77.27	7.00	6.96
2	CH_3	H	Cl	$C_{12}H_{12}ClNO$	145–147/0.5 mm.	25	$C_{13}H_{15}ClN_4O$	167–168	56.02	56.22	5.43	5.58
3	CH_3	H	CH_3	$C_{13}H_{15}NO$	132–135/0.4 mm.	37	$C_{14}H_{18}N_4O$	134–135	65.10	64.96	7.06	7.18
4	CH_3	CH_3	H	$C_{13}H_{15}NO$	194–200/23 mm.[c]	...	...	...	...	...	7.4[d]	7.0
5	CH_3	CH_3	Cl	$C_{13}H_{14}ClNO$	174–179/2.6 mm.	66	$C_{14}H_{17}ClN_4O$	179–180	57.43	57.57	5.85	6.01
6	CH_3	CH_3	CH_3	$C_{14}H_{17}NO$	147–150/0.8 mm.	54	$C_{15}H_{20}N_4O$	160–161	66.15	66.12	7.40	7.19
7	CH_3	CH_3CH_2	H	$C_{14}H_{17}NO$	148–160/1.2 mm.	62	$C_{20}H_{21}N_5O_4$[e]	130–131	60.75	60.61	5.35	5.41
8	CH_3	CH_3	CH_3O	$C_{14}H_{17}NO_2$	179–186/3.8 mm.	53	$C_{15}H_{20}N_4O_2$	183–185	62.48	62.45	6.99	6.73
9	—$(CH_2)_5$—		H	$C_{16}H_{19}NO$	190–195/2 mm.	39	...	...	79.63	79.64	7.93	7.98

[a] Boiling points and melting points are uncorrected. Melting points were determined with a Thomas Hoover capillary tube melting point apparatus. [b] Microanalyses were performed by the Microanalytical Laboratories, Department of Chemistry, University of California, Berkeley, and by Weiler and Strauss Microanalytical Laboratory, Oxford, England. [c] This is the reported boiling point (2). [d] Reported (2) values for nitrogen. [e] Characterized as the 2,4-dinitrophenylhydrazone derivative.

of these compounds to cyclize may be explained by their lack of alkyl substituents which in turn facilitate ring formation (7).

EXPERIMENTAL[1]

Cyanoethylation.—The appropriate ketone (0.1 mole), potassium hydroxide, 300 mg. (50% aqueous solution), and 75 ml. of *tert*-butanol were treated with 7 Gm. (0.13 mole) of freshly distilled acrylonitrile according to the procedure of Cason and Chang (3). Distillation yielded up to 66% of the δ-ketonitriles (Table I, Nos. 4–9). With propiophenone and *p*-substituted propiophenones, the same procedure was followed; however, a 2:1 *M* ratio of the ketone to acrylonitrile was employed. The yields of cyanoethylated product from these ketones were slightly less (Table I, Nos. 1–3).

Semicarbazone derivatives were prepared in the usual manner (Table I).

Alkaline Hydrolysis.—The appropriate δ-ketonitrile, 0.05 mole, was heated under reflux with 200 ml. of 20% aqueous potassium hydroxide solution until the evolution of ammonia ceased (16–20 hours). The solution was cooled and extracted with 25 ml. of chloroform. The aqueous layer was acidified with concentrated hydrochloric acid and extracted with three 50-ml. portions of chloroform which were then combined and dried with anhydrous calcium chloride. The chloroform was evaporated under reduced pressure leaving 85–90% of the crude acid which was not further purified.

The acid was treated with 11.9 Gm. (0.1 mole) of thionyl chloride dissolved in 50 ml. of anhydrous benzene. The solution was heated under reflux for 1 to 2 hours and the benzene and excess thionyl chloride then removed by distillation under reduced pressure. The residue was cooled and poured slowly with stirring into 25 ml. of cold ammonia solution (28%). The product (60–70%) was filtered, washed with water, and recrystallized from methanol or methanol-water. (Tables II, III.) Infrared spectra of these compounds showed the following peaks. In the case of 5,5-dimethyl-6-hydroxy-6-(*p*-methoxyphenyl)-2-piperidone (No. 5, Table II), 2.8μ (hydroxyl), 3μ (lactam NH), and 6.09μ (lactam carbonyl). Similar spectra were obtained from those compounds in this series containing two alkyl substituents in the 5 position of the piperidone ring. For those compounds containing one alkyl substituent in the 5 position of the piperidone ring, *i.e.*, 5-methyl-6-(*p*-tolyl)-Δ5-tetrahydro-2-pyridone (No. 3, Table III), 2.99μ (lactam NH), and 6.05μ (lactam carbonyl).

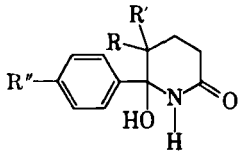
5,5-Dimethyl-6-hydroxy-6-phenyl-2-piperidone Semicarbazone.—The first member of this series (No. 1, Table II) readily formed a semicarbazone derivative in the usual manner. After recrystallization from ethanol, the derivative melted at 219–220°.

Anal.—Calcd. for $C_{14}H_{20}N_4O_2$: N, 20.29. Found: N, 20.33.

Sulfuric Acid Hydrolysis.—One gram of the appropriate δ-ketonitrile was treated with 5 Gm. of concentrated sulfuric acid. The solution was allowed to stand at room temperature for 15 minutes, then poured with stirring into 150 ml. of ice water.

[1] The procedures listed are typical of those used for the preparation of compounds listed in Tables I–IV.

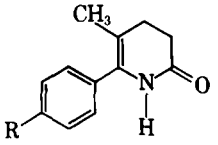
TABLE II.—6-ARYL-5,5-DIALKYL-6-HYDROXY-2-PIPERIDONES



No.	R	R′	R″	Formula	Yield,[a] %	Recrystallizing Solvent	M.p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
1	CH_3	CH_3	H	$C_{13}H_{17}NO_2$	60.5	Methanol	175–176	71.20	71.45	7.81	7.90
2	CH_3	CH_3	Cl	$C_{13}H_{16}ClNO_2$	64	Methanol	175–176	61.53	61.60	6.36	6.43
3	CH_3	CH_3CH_2	H	$C_{14}H_{19}NO_2$	72	60% Methanol	160–161	72.07	72.44	8.21	8.42
4	CH_3	CH_3	CH_3	$C_{14}H_{19}NO_2$	72	Methanol	172–174	72.07	71.89	8.21	8.13
5	CH_3	CH_3	CH_3O	$C_{14}H_{19}NO_3$	63	60% Methanol	188–190	67.44	67.25	7.69	7.71
6	—$(CH_2)_4$—		H	$C_{16}H_{21}NO_2$	67	Methanol	185–186	74.11	74.39	8.16	8.31

[a] Yields calculated from the nitriles.

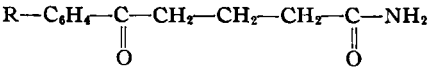
TABLE III.—6-ARYL-5-METHYL-Δ5-TETRAHYDRO-2-PYRIDONES



No.	R	Formula	Yield, %	Recrystallizing Solvent	M.p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
1	H	$C_{12}H_{13}NO$	72[a]	Methanol	136–136.5	77.00	77.00	7.00	6.96
2	Cl	$C_{12}H_{12}ClNO$	60[b]	Methanol	159–160	65.03	65.09	5.46	5.22
3	CH_3	$C_{13}H_{15}NO$	60[b]	Methanol	146–147	77.60	77.54	7.52	7.60

[a] Yield calculated from the acid. [b] Yields calculated from the nitriles.

TABLE IV.—4-AROYL-BUTYRAMIDES



No.	R	Formula	Yield,[a] %	Recrystallizing Solvent	M.p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
1	H	$C_{11}H_{13}NO_2$	65	Water	156–157	69.10	69.40	6.85	6.81
2	CH_3	$C_{12}H_{15}NO_2$	90	Water	170–171	70.21	69.96	7.37	7.55
3	CH_3O	$C_{12}H_{15}NO_3$	90	Water	145–146	65.14	65.24	6.83	6.96

[a] Yields calculated from the 4-aroyl-butyric acids.

The crude product (50–60%) was filtered and washed with 5% sodium hydroxide solution. Recrystallization was effected from methanol or methanol-water. Since the yield of product from this reaction was less than that obtained from the two-step procedure previously described, only compounds 2 and 3 of Table III were prepared by this latter procedure.

4-Aroyl-butyric Acids.—The appropriate aromatic hydrocarbon was condensed with glutaric anhydride in the presence of anhydrous aluminum chloride as described by Berliner (8).

4-Aroyl-butyramides.—The appropriate acid (0.005 mole), prepared in the above experiment, was dissolved in 10 ml. of chloroform and the solution neutralized with 0.005 mole of triethylamine. The resulting mixture was chilled to 0°. A solution of 0.005 mole of ethyl chloroformate dissolved in 5 ml. of chloroform was added slowly so that the temperature of the mixture did not rise above 10° (9). The reaction mixture was kept in an ice bath for 30 minutes, after which time 3 ml. of cold concentrated ammonium hydroxide solution was added. A gas evolved followed by the formation of a precipitate. The precipitate was collected and recrystallized from water (Table IV).

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Determination of Solubility of a Metastable Polymorph

By GEORGE MILOSOVICH

A method based on dissolution rate measurement was developed to obtain solubility data for rapidly reverting solid states. Experimental tests of the method showed it to be valid. Solubility and thermodynamic data for sulfathiazole II and I in 95 per cent alcohol were obtained. Other applications of the method are discussed.

RECENT LITERATURE reports (1–3) have demonstrated the feasibility of using metastable polymorphic crystal forms to improve the pharmacologic performance of drug compounds possessing low water solubility. Several drugs are presently marketed in their metastable polymorphic forms. Stability problems associated with such products have also been noted (1).

Both increase in drug absorption rate and physical instability are functions of the increased solubility of the metastable state. In order to study such systems it is advantageous to have solubility data on each solid state, preferably at several temperatures. Usual methods for solubility determination are not suitable for many polymorphic forms because reversion can occur during the time period necessary for the measurement. Such is the case for the phenacetin, diphenylsulfone, and sulfathiazole systems. Data reported by Higuchi and Shefter (4) on dissolution from solvate forms also show reversion occurring during the experimental time period, the concentration in solution passing through a maximum with time. It was the purpose of this investigation, therefore, to develop a method suitable for the determination of the solubility of metastable solid states that rapidly revert.

Because the relatively long experimental time required for an equilibrium solubility measurement increases the probability for nucleation and growth of more stable forms, it was felt that the desired data would have to be obtained in the shortest possible time period. Also, it is known that nucleation probability is a function of the size of the physical system, supersaturation increasing with decrease in volume of solution. These considerations suggested a procedure based on a measurement of dissolution rate since such data can be obtained in time periods less than 1 minute (5) with minimum volume of supersaturated solution due to the limited boundary region in contact with the dissolving solid.

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The Nernst equation (6) relates the rate of concentration increase to solubility for a dissolving solid

$$\frac{dC}{dt} = \frac{SD}{V\delta}(C_s - C_t) \qquad \text{(Eq. 1)}$$

where S is the area of the dissolving interface, D is the diffusion coefficient of the solute in the solvent, V is the volume of solvent, δ is the thickness of the diffusion layer, and C_s and C_t are concentrations at saturation and at time, t, respectively. Equation 1 reduces to

$$\frac{dW}{dt} = \frac{SD}{\delta} C_s \qquad \text{(Eq. 2)}$$

for the experimental condition that $C_s \gg C_t$ and V constant. Since D is a property of the solute molecule and the solvent, it is independent of solid-state form. S and δ can be maintained constant by suitable control of the experiment. If these conditions obtain, the rate of dissolution is directly proportional to solubility, and the solubility of higher energy states can be calculated from dissolution rate data and solubility data of the most stable form. Thus $dW/dt = KC_s$.

EXPERIMENTAL

Sulfathiazole U.S.P. was chosen as the model compound for use in this investigation. It can be obtained in at least two crystal forms, I melting at 174–175° and II melting at 200–201°. The II→I system is enantiotropic, I being the lower energy state at room temperature. Form I was obtained by slow recrystallization from warm alcohol. Form II was obtained by heating form I to 180° where form I melted and form II crystallized. The particle sizes of these states were reduced to approximately the same range by mortar and pestle. Normal solubility determinations showed both forms to have the same solubility, indicating that the higher energy state reverted to form I during the experiment. This was confirmed by X-ray diffraction measurements.

To conduct the dissolution rate experiments under conditions of constant surface area and constant δ the apparatus shown in Fig. 1 was built from aluminum and stainless steel. The sample holder was a $^3/_8$-in. hardened steel die obtained from a rotary tablet press. The surface of the die was ground to remove the tapered shoulder. The stirrer

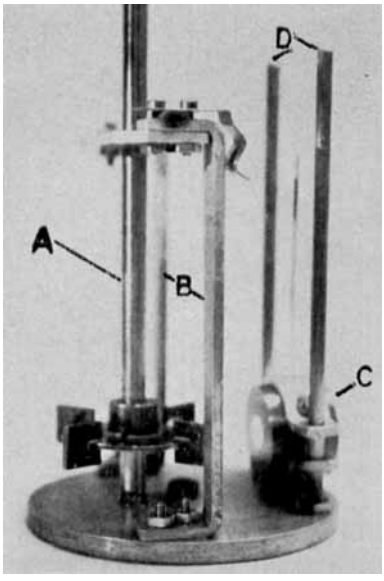


Fig. 1.—Apparatus used to determine dissolution rate under conditions of constant S and δ. Key: A, stirrer shaft; B, baffles; C, rotary tablet die and holder; and D, aluminum support rods for positioning the die.

was driven with a Bodine model B2246E-06 300 r.p.m. constant speed motor. Previous experiments using a variable speed motor showed 300 r.p.m. to be well within the range for diffusion control (a prerequisite for Eq. 2). The sample was prepared by compressing approximately 300 mg. of powder in the die between a $^3/_8$-in. punch and a hardened steel block using a Carver press. One surface of the formed tablet was flush with the die face, and the opening in the opposite face was corked. This procedure has the advantage over similar ones reported in the literature (7, 8) that a good tablet is not necessary so long as the surface is uniform. The apparatus was mounted in a 1-L. stainless steel beaker which was partially emersed in a constant temperature water bath. Dissolution was measured using the method of Niebergall and Goyan (5). Because of the limited surface available for dissolution, a "good" solvent was necessary so that sufficient sulfathiazole would dissolve in a short time period for assay. Ninety-five per cent v/v alcohol was chosen as the solvent for this study.

Since a rapid flow rate was desired, the solution was returned to the beaker to maintain the volume and agitation constant. At the beginning of an experiment, 500 ml. of solvent was added to the beaker, and the circulating pump was started. A 65-ml. quantity of solvent was contained in the pump and the flow lines to make the total volume 565 ml. The stirrer was started, and the temperature within the beaker was monitored until it became constant. At that time the recorder was balanced to 0 and 100% transmission; the sample was introduced by sliding the die holder down the two alignment rods. Figure 2 shows a typical recording obtained from these experiments. The per cent transmission data were converted to absorbance and then to concentration using a standard Beer-Lambert plot of known concentrations. Corrections were made for solvent expansion at temperatures above 25°.

RESULTS

Effect of Compression Pressure.—Dissolution data obtained from a tablet have meaning only if it is assumed that the tablet dissolves as though it were a single crystal. It was felt that if the dissolution rate from a tablet were independent of compression pressure this assumption would be valid. Figure 3 shows data obtained from a pressure series using lubricated (die walls swabbed with an ether solution of stearic acid) and unlubricated dies. While the lubricated die series showed the expected pressure independence, it is interesting to note the greater variation and apparent increase in dissolution rate with pressure for the unlubricated series. This latter effect will be reported in more detail in a future publication. In further support of the validity of using tablet surfaces for dissolution experiments, rate data were determined using tablets made from different particle sizes of sulfathiazole I. No size effect was observed. The conclusion was that as long as lubricated dies were used the tablet surface could be assumed to behave as a single crystal. Subsequent experiments were conducted with surfaces compressed under 21,000 p.s.i. pressure.

Effect of Solid State.—Dissolution rates were determined for each of the two crystal forms of sulfathiazole at six temperatures. Table I shows the data obtained from these experiments.

Figure 4 shows an absorbance *versus* time plot for form II at 29.8°. The change in slope occurring between 40–60 seconds demonstrates reversion of

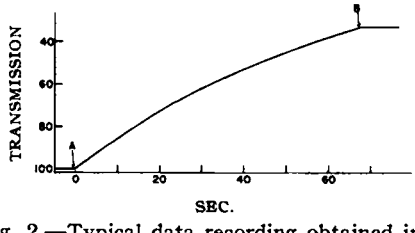


Fig. 2.—Typical data recording obtained in this study; form II, 48.8°C. Key: A, tablet added; B, tablet removed.

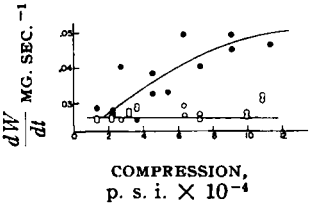


Fig. 3.—Effect of compression on dissolution rate of tablet surfaces. Key: ●, unlubricated die wall; ○, lubricated die wall.

TABLE I.—DISSOLUTION RATE AND SOLUBILITY DATA FOR SULFATHIAZOLE I AND II IN 95% ALCOHOL

Temp., °C.	Dissolution Rate, mg. cm.$^{-2}$ sec.$^{-1}$		Solubility, Gm./1000 Gm. Solvent	
	Form I	Form II	Form I	Form II
59.1	.185	.239	31.50	40.7
48.8	.102	.145	19.80	28.1
39.4	.0598	.0913	14.00	21.4
29.6	.0355	.0597	9.93	16.7
24.1	.0237	.0413	8.15	14.2
20.4	.0201	.0371	7.10	13.1
14.5	...	...	5.70	...

form II to form I, the ratio of the slopes being a measure of the ratio of solubilities for these two forms. This effect was not time reproducible and in most instances was not observed. It should be pointed out that this would be a function of nucleation and would not be expected to be reproducible. Also, experiments were terminated after 90 seconds for most runs, so that it would not be observed if it occurred beyond this time period. From this rapid and complete change in slope, it appeared highly probable that solid-state reversion occurred. Otherwise, an intermediate slope corresponding to an intermediate steady state concentration would have been expected over a longer time period.

Calculation of Solubilities.—The solubility of form I was determined at seven temperatures as follows. A large excess of form I powder was added to about 500 ml. of 95% alcohol in the beaker and was held at the desired temperature with stirring until equilibrium was obtained. Stirring was stopped, and samples were pipeted through glass wool to remove suspended particles. The samples were weighed and quantitatively diluted with 95% alcohol for spectrophotometric assay at 288 $m\mu$. Solubility data are given in Table I as grams dissolved per 1000 Gm. of solvent. Also included in Table I are the solubilities of the form II state calculated from the dissolution rate data. Figure 5 shows the van't Hoff plots for these data. Both plots deviate from linearity, showing an increasing negative slope with increase in temperature.

Test of Solubility Data.—Since the II→I system is enantiotropic, proper plotting of the solubility data should predict the transition temperature However, the van't Hoff plots were nonlinear and could not be extrapolated with accuracy. As was pointed out by Higuchi, *et al.* (3), if it is assumed that deviation from linearity is due mainly to solvent effects and Henry's law applies, then ratios of solubilities can be plotted in the same manner [log (solubility ratio) *versus* $1/T$] and the intersection at ratio = 1 would give the transition temperature. Figure 6 is such a plot for these data and predicts that the transition temperature is 94.5 ± 2.7°. That this predicted transition temperature is in the correct range was verified by equilibrating both forms in glycerin solution at 100° and at 90° on a Köfler hot stage. Figure 7 presents time photomicrographs showing the dissolution of I and growth of II at 100° and dissolution of II and growth of I at 90°.

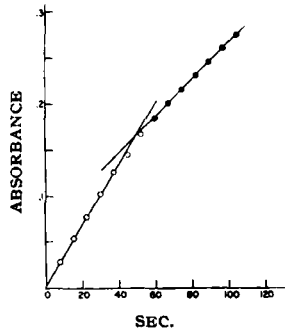


Fig. 4.—Dissolution of form II showing reversion to form I. Key: O, form II; ●, form I. 29.8°C.

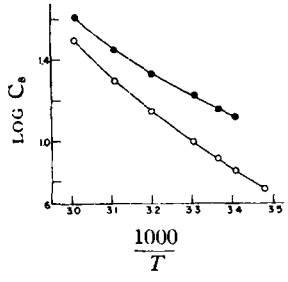


Fig. 5.—van't Hoff plots for sulfathiazole solid states. Key: O, form I; ●, form II.

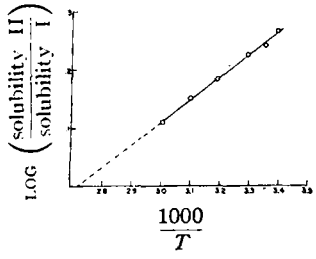


Fig. 6.—Plot of log solubility ratio against $1/T$. Transition temperature, 94.5±2.7°C.

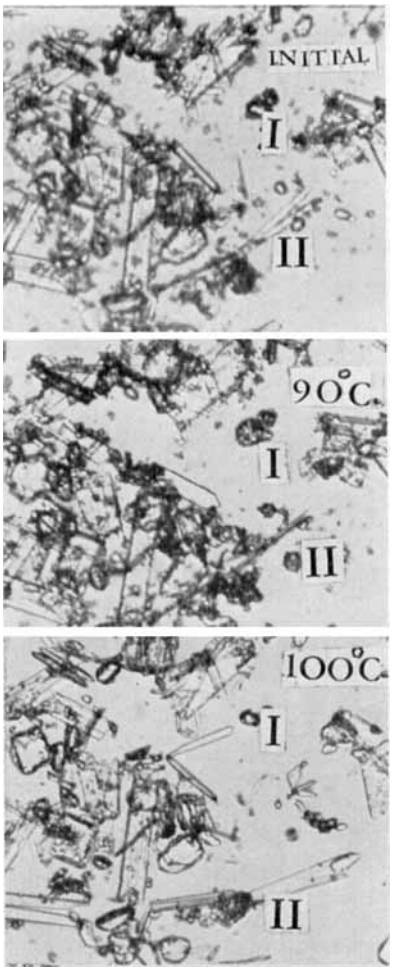


Fig. 7.—Photomicrographs showing reversion of II to I at 90°C. and I to II at 100°C. in glycerin suspension.

Thermodynamic Differences Between I and II.—The enthalpy and entropy differences between forms I and II can be calculated from the solubility data (3). The slope of the log (solubility ratio) *versus* $1/T$ plot was calculated to be 381.07. From this the enthalpy difference was calculated to be 1744 cal./

mole. This value along with the predicted transition temperature gave a value for the entropy difference equal to 4.75 e.u.

DISCUSSION

The method developed in this study to determine solubility of rapidly reverting polymorphic states was shown to work very well for sulfathiazole II. Even though the II→I reversion is rapid enough to preclude equilibrium measurement, it is sufficiently slow to allow use of this method. However, it is conceivable that reversions in other systems can be so rapid that this method will not be applicable. Thus, in systems where one form may be 10 times more soluble, the supersaturation obtained in the boundary layer may be such that instantaneous crystallization of less soluble forms will result.

The method has other applications. It can be used to measure rapidly the effect of nucleation and crystallization inhibitors in reverting systems. It can also be used for studying dissolution mechanism for systems that dissolve through acid-base reaction. It may also have value for studying the other factors influencing the dissolution process. More data will be forthcoming to test the general applicability of this method.

CONCLUSIONS

A method suitable for the rapid determination of solubility has been developed and used to obtain solubility and thermodynamic data for sulfathiazole II. The transition temperature predicted from these data was confirmed by an independent experiment.

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Sustained-Release Aspirin Tablet Using an Insoluble Matrix

By MAHENDRA S. VORA, ARTHUR J. ZIMMER, and PAUL V. MANEY

The mode of administering drugs in a sustained-release form has recently assumed considerable importance in the pharmaceutical industry. Various resins, plastics, and polymers were investigated in this study. Polyvinyl chloride was selected as the base for the insoluble matrix. Evaluation of the sustained-release aspirin tablet was by *in vitro* and *in vivo* methods. Evidence is presented that the *in vivo* release rates provide a uniform blood level over a predictable period of time.

SUSTAINED-RELEASE tablets may provide the desired release rates by employing one of several techniques (1): (*a*) compressing coated pellets into a soft matrix, (*b*) mixing several granulations (each containing different retarding agents), (*c*) combining different layers of sustained-release granules, (*d*) forming an insoluble complex by ion-exchange methods, and (*e*) distributing medication in fatty bases. Oral sustained-action preparations are described in a Swedish patent (2) which outlines the use of synthetic resins and polymers for the purpose of imparting sustained-release properties to various drugs.

A comprehensive publication "Formulation and Experimental Evaluation of Oral Sustained Release Medication Based on the Principle of Delayed Diffusion," by Simoons (3), has recently been released. This paper advocates the use of several components to obtain the desired release rate.

A recent study by Levy (4) indicated that aspirin is rapidly absorbed from all parts of the gastrointestinal tract. Thus, aspirin can serve as an excellent tracer to assess the effect of certain formulation and dosage form characteristics upon absorption rate. It is understood that other drugs may give different results with these formulations.

Various *in vitro* methods for the evaluation of sustained-release products indicated that the use of radioisotopes could be of value (5). In this work an attempt was made to incorporate aspirin with the resin polyvinyl chloride, then to prepare a two-layer product—one layer to give immediate and the other time-delayed release.

The use of *in vitro* methods of testing by de-

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The authors acknowledge the technical assistance of Z. Khatoon, M.D., City Hospital, St. Louis, Mo., and Mr. M. A. Ghafoor, St. Louis College of Pharmacy. The cooperation of volunteers, who served as test subjects in this study, is also acknowledged. The authors are grateful to Dr. B. A. Barnes of the St. Louis College of Pharmacy for the pharmacological and pathological examinations and interpretation.

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Abstracted from a thesis submitted by Mahendra S. Vora to the St. Louis College of Pharmacy, St. Louis, Mo., in partial fulfillment of Master of Science degree requirements.
The authors acknowledge the technical assistance of Z. Khatoon, M.D., City Hospital, St. Louis, Mo., and Mr. M. A. Ghafoor, St. Louis College of Pharmacy. The cooperation of volunteers, who served as test subjects in this study, is also acknowledged. The authors are grateful to Dr. B. A. Barnes of the St. Louis College of Pharmacy for the pharmacological and pathological examinations and interpretation.

TABLE I.—RELEASE OF ASPIRIN BY ROTATING BOTTLE METHOD[a]

Simulated Fluid	pH	Time	Mean Av., mg.	Mean, % Release	Mean Cumulative
Gastric	1.5	1 hr.	312	31.6	31.6
Intestinal	4.5	1 hr.	155	15.7	47.3
Intestinal	6.9	2 hr.	230	23.4	70.7
Intestinal	6.9	1 hr.	110	11.15	81.85
Intestinal	7.2	2 hr.	122	12.40	94.25
Intestinal	7.5	1 hr.	33	3.35	97.60
Intestinal	7.5	45 min.	8	.810	98.41

[a] Figure 1 and Table I show averages of 10 determinations.

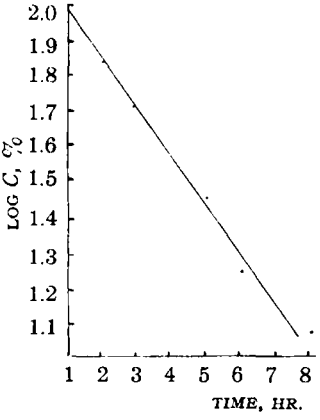


Fig. 1.—Linear relationship between log C (residue) and time.

termining the amount of drug released in simulated gastric juices is of questionable value in correlating actual performance in man. However, the results obtained justify the use of *in vitro* methods as a preliminary study. The value of the *in vitro* tests are considerably enhanced by adequate *in vivo* studies.

In this work we have compared three *in vitro* analytical procedures (one chemical and the other two using radioactive tracers) and determined pH effects before using *in vivo* testing.

In producing a sustained-release product, the objective should be to provide the desirable release rate which is independent of the many aforementioned factors and to develop suitable *in vivo* and *in vitro* procedures.

PHYSIOLOGICAL CONSIDERATIONS

Attempts to explain the physiological factors which determine the attainment of a therapeutic drug level in the blood and tissues have been published (5–8). The rate at which a substance enters the blood stream must be constant and continuous, so that the amount absorbed per unit time would effectively control the concentration level. In Wagner's review and several other papers the various factors affecting absorption in the gastrointestinal tract have been covered (9–12).

In vivo studies conducted in connection with the study of enteric coated tablets have shown that some tablets have remained in the stomach for 8 hours or more before passing into the intestinal tract. On the other hand, it was shown that some tablets passed into the intestinal tract within 15 minutes (13).

The consideration of the many physiological variables that a sustained-release product must encounter while releasing the drug on a predetermined basis presents a problem that may never be completely resolved. Our objective in producing a sustained-release product was to provide a desirable release rate, independent of the environmental conditions of the gastrointestinal tract, and provide suitable testing procedures.

EXPERIMENTAL

Materials.—Polyethylene, ethylcellulose, zein, polyvinyl chloride,[1] aspirin (No. 80 mesh), and isopropyl alcohol.

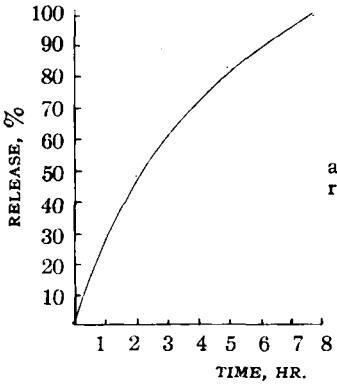


Fig. 2.—Percentage of aspirin tablet released *vs.* time.

TABLE II.—RADIOISOTOPE[a] EVALUATION OF RELEASE RATE[b]

Simulated Fluid	pH	Time, min.	Av. Counts	Total % Released
Gastric	1.5	30	1890	9.95
Gastric	1.5	60	3600	18.95
Intestinal	4.5	30	1575	27.25
Intestinal	4.5	60	3690	38.35
Intestinal	6.9	30	1680	47.20
Intestinal	6.9	60	3810	58.35
Intestinal	6.9	90	5400	66.75
Intestinal	6.9	120	6600	73.05
Intestinal	6.9	30	1230	79.50
Intestinal	6.9	60	2100	84.10
Intestinal	7.2	30	505	86.75
Intestinal	7.2	60	975	89.23
Intestinal	7.2	90	1260	90.75
Intestinal	7.2	120	1950	94.35
Intestinal	7.5	30	225	95.53
Intestinal	7.5	50	450	96.70

[a] Radioisotope used was S^{35}-sodium sulfate. [b] Figure 2 and Table II show averages of 10 determinations.

Equipment.—Stokes modified BB2 rotary tablet machine, Stokes model F tablet machine, Twin-Shell dry blender, Tracer/Matic scale and gas flow detector model SC73, Beckman model B spectrophotometer, Stokes model 43A oscillating granulator, Day model 71989 mixer, and Strong-Cobb hardness tester.

Preparation of Tablets.—The procedure was to prepare a two-layer tablet which consisted of the A layer and the B layer. The A layer consisted of 640.0 mg. of aspirin and 90.0 mg. of polyvinyl chlo-

[1] Supplied as Opalon by Monsanto Chemical Co., St. Louis, Mo.

TABLE III.—COMPARISON OF CHEMICAL AND S^{35} RADIOISOTOPE METHODS OF RELEASE RATE[a]

Simulated Fluid	pH	Time	Chemical Process Av. in mg.	Chemical Process Cumulative %	Radioisotope Total Av. Counts	Radioisotope Cumulative %
Gastric	1.5	1 hr.	120	18.7	3600	18.9
Intestinal	4.5	1 hr.	125	38.3	3690	38.25
Intestinal	6.9	2 hr.	195	68.7	6600	73.0
Intestinal	6.9	1 hr.	90	82.7	2100	84.2
Intestinal	7.2	2 hr.	72	94.2	1950	94.5
Intestinal	7.5	50 min.	17	96.6	450	96.7

[a] Figure 3 and Table III show averages of 10 determinations.

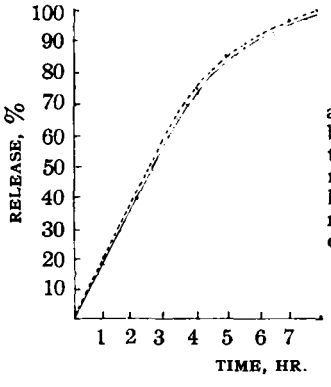


Fig. 3.—Comparative results of Tables II and III with the chemical method (broken line) and the S^{35} radioisotope method (solid line).

ride for the sustained-release portion. For the *B* layer, 320.0 mg. of aspirin (10% starch granulation) was compressed on the *A* layer. The *A* layer granulation was prepared by mixing the polyvinyl chloride and the aspirin in the Twin-Shell dry mixer for 20 minutes, and then granulating in the Day mixer, using 200.0 ml. of isopropyl alcohol for each kilogram of the described mixture. The dry granulation was passed through a No. 12 screen, 0.3% magnesium stearate was added as the lubricant, and the dry granulation was then compressed on a Stokes model BB2 modified tablet machine, using 7/16-in.-diameter punch at a pressure of 6 to 8 Kg.

EVALUATION

In Vitro Evaluation of Release Rate

Equipment.—Rotating bottle apparatus as described by Souder and Ellenbogen (14), 75.0-ml. cylindrical bottles with plastic screw cap, U.S.P. simulated gastric and intestinal fluid, ferric nitrate reagent (1% ferric nitrate in 1% nitric acid), 10% potassium hydroxide solution, 6 *N* hydrochloric acid solution, Beckman model B spectrophotometer, and pH indicator papers were employed.

Procedure.—One tablet with 60.0 ml. of simulated gastric fluid at pH 1.5 was placed in a 75.0-ml. bottle (described above) and rotated in the apparatus at 40 r.p.m. at 37 ± 2° for 1 hour. The liquid was removed (leaving the undissolved core in the bottle), placed in 100.0-ml. volumetric flask; the bottle was rinsed with 15.0 ml. of distilled water which was added to the contents of the flask.

Simulated intestinal fluid (60.0 ml.) at pH 4.5 was placed in the same bottle (containing the core) and rotated for 1 hour. The fluid was collected as described above.

Simulated intestinal fluid (60.0 ml.) at pH 6.9 was placed in the bottle and rotated for 2 hours. The fluid was collected as before.

Simulated intestinal fluid (60.0 ml.) at pH 6.9 was placed in the bottle and rotated for 1 hour. The fluid was collected as before.

TABLE IV.—COMPARISON OF CHEMICAL AND C^{14} ISOTOPE METHODS OF RELEASE RATE[a]

Simulated Fluid	pH	Time	Chemical Process Av. mg.	Chemical Process Cumulative %	Radioisotope Total Av. Counts	Radioisotope Cumulative %
Gastric	1.5	1 hr.	103	16.1	8000	16.6
Intestinal	4.5	1 hr.	100	31.7	7000	31.2
Intestinal	6.9	2 hr.	167	57.8	12000	56.4
Intestinal	6.9	1 hr.	75	69.5	5200	67.0
Intestinal	7.2	2 hr.	112	87.0	7900	83.5
Intestinal	7.5	1 hr.	32	92.0	2600	89.0
Intestinal	7.5	45 min.	34	97.2	2800	95.0

[a] Table IV and Fig. 4 show averages of 10 determinations.

Experimentally, 1.0% ethylcellulose in acetone solution for granulating the materials in the *A* layer, instead of the isopropyl alcohol, provided a release rate of longer duration in the tablet described above. The use of 15% zein solution in isopropyl alcohol as the granulating agent in the preparation of the *A* layer provided a release rate of shorter duration than the ethylcellulose granulation. Substituting polyethylene (No. 80 mesh) for the polyvinyl chloride in the same proportion in the matrix provided a sustained-release tablet with the desired release rate when tested *in vitro*. The scope of this study permitted only detailed experiments on the formulation containing the polyvinyl chloride as the insoluble matrix.

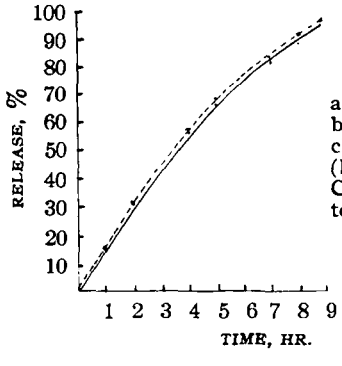


Fig. 4.—Comparative results of Table IV with the chemical process (broken line) and C^{14} aspirin radioisotope (solid line).

Simulated intestinal fluid (60.0 ml.) at pH 7.2 was placed in the bottle and rotated for 2 hours. The fluid was collected as before.

Simulated intestinal fluid (60.0 ml.) at pH 7.5 was placed in the bottle and rotated for 1 hour. The fluid was collected as before.

Simulated intestinal fluid (60.0 ml.) at pH 7.5 was placed in the bottle and rotated for 45 minutes. The fluid was collected as before.

The total salicylate of each volumetric flask was determined by the method of Pankratz and Bandelin (15).

Table I shows the *in vitro* release of the aspirin in the simulated gastric and intestinal fluid. Figure 1 explains the linear relationship between the percentage log *C versus* time. Figure 2 represents percentage release *versus* time.

In Vitro Evaluation Using Soluble Isotope S^{35}

Equipment.—Tracer Laboratories, Inc., Versamatic II scale with a Geiger-Müller tube end window detector, 1-in. stainless-steel planchets, infrared lamp, and Stokes model F single punch tablet machine.

Procedure.—Sixty-four grams of No. 80 mesh aspirin powder was mixed thoroughly with S^{35} radioisotope solution containing 1,900,000 total counts. The mixture was dried under an infrared lamp and mixed with 8.0 Gm. of polyvinyl chloride. It was then granulated with isopropyl alcohol, passed through a No. 12 mesh screen again, lubricated with 0.3 Gm. magnesium stearate, and compressed on a Stokes model F single punch tablet machine using 7/16-in. diameter punch at a pressure of 6 to 8 Kg. The tablets were compressed to 720 mg., giving approximately 19,000 counts per tablet.

Evaluation.—As stated in the chemical process, this core was rotated with 60.0 ml. of simulated gastric and intestinal fluid. The fluid was changed at the intervals shown in the chemical process (13). Every 30 minutes, 0.4 ml. fluid was withdrawn and the same amount of the fluid was replaced. Samples were mounted on stainless-steel planchets and a drop of 2% sodium sulfosuccinate solution was used to spread them evenly. Drying of the samples was carried out under the infrared lamp. The counts per 10 minutes were determined using the Tracer/-Matic scale with the Geiger-Müller tube detector.

Table II shows the average count based on 30-minute intervals and the total percentage released over a period of 8 hours. Table III represents the comparison of the described chemical determination and the S^{35} radioisotope experiment. Each experiment shows the cumulative percentage release in the simulated gastric and intestinal fluid over the period of approximately 8 hours. Figure 3 represents the comparative results of Tables II and III.

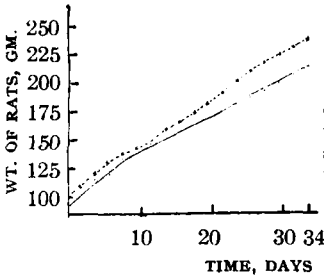


Fig. 5.—Toxicity study showing comparative growth rate of test animals (solid line) and control animals. Average polyvinyl chlorine consumed 9.9 Gm./2 days.

TABLE V.—SALICYLATE CONTENT (MCG./ML.) OF PLASMA WITH SUSTAINED-RELEASE TABLET

Subject	Age	Wt.	Blood Samples at Intervals of Hr. 1	4	7	10	24
1	31	160	36	52.2	69.6	69.6	6
2	30	150	89.6	85.2	67.2	67	9.6
3	31	170	65	81.6	70	67	...
4	45	188	55.2	42	30	24	18
5	28	160	50.2	52.8	66	60	46
6	29	140	65	69.6	72	72	36
7	32	150	40	60	66	...	23
8	30	150	60	72	70	50	37
9	29	165	41	41	39	45	12
10	30	160	55	62.4	64.8	64.8	...

TABLE VI.—SALICYLATE CONTENT (MCG./ML.) OF PLASMA WITH REGULAR ASPIRIN TABLET

Subject	Age	Wt.	Blood Samples at Intervals of Hr. 1	4	7
1	27	160	42	32.4	24
2	26	140	55.2	48.0	24
3	30	180	32.4	48.0	16.8
4	31	160	48.0	37.2	24.0
5	30	163	48.0	50.4	42.0
6	23	115	67.2	48.0	22.0

In Vitro Evaluation Using Insoluble Isotope Aspirin C^{14}

Procedure.—Aspirin radioisotope (activated at carboxyl group C^{14}) was dissolved in 90.0 ml. of isopropyl alcohol which contained 5.0 Gm. aspirin. The alcohol was evaporated with the infrared lamp. The triturate represents 1400 counts per minute/mg.

A 2570-mg. quantity of the triturate was mixed with 45.730 Gm. of aspirin. Enough of the alcohol was added to make a thin slurry. It was mixed thoroughly, 6.75 Gm. of polyvinyl chloride added, and the triturate was mixed again; the alcohol evaporated.

This material was then granulated and prepared into tablets as described in the first step of this experiment.

Evaluation.—The procedure for evaluation was identical to the one described using the soluble isotope, except the gas flow counter was used and the counting time was measured as shown in Table IV and Fig. 4. Table IV presents a comparison of the percentage release of the described chemical process and that released from C^{14} isotope. Plotting these results (Fig. 4) shows nearly identical curves.

Toxicity Study on Rats

Although there are reasons to believe that high molecular weight compounds, such as polyvinyl chloride, are physiologically inert, we considered it advisable to investigate the toxicity of the polyvinyl chloride used in this study.

A group of 16 Sprague-Dawley rats was maintained for 34 days on a diet of laboratory chow.[2] Nine of the rats were on a diet of polyvinyl chloride and seven were fed the regular laboratory chow. They were weighed every other day, and a record of the food consumed was recorded. There were no significant effects, either on the growth rate or the appetite, of the rats being fed the polyvinyl chloride.

Upon completion of the feeding tests, five of the

[2] Ralston-Purina Co., St. Louis, Mo.

TABLE VII.—SALICYLATE CONTENT (MCG./ML.) OF PLASMA WITH SIX 0.32-GM. REGULAR ASPIRIN TABLETS

Subject	Age	Wt.	Blood Samples at Intervals of Hr. 1	4	6	7	10.5	13.5	24
1	30	160	126	100	100	174	175	153	90
2	29	140	156	105	100	...	185	186	95
3	29	165	102	84	80	125	156	140	82
4	30	150	110	108	90	145	168	140	...

rats receiving polyvinyl chloride were sacrificed, and a gross pathological examination was made on the liver, kidneys, G. I. tract, heart, spleen, and lungs. No abnormalities were observed

These data correspond with a much more detailed study on the toxicity of a mixture of 95% polyvinyl chloride and 5% polyvinyl acetate. The scope included feeding tests on 150 rats over a period of 400 days (16).

Figure 5 illustrates the growth rate and the average consumption of polyvinyl chloride in the test animals.

Evaluation In Vivo

Sixteen healthy adult males served as test subjects. Their weight and ages are listed in Tables V, VI, and VII. The subjects were divided into three groups *A*, *B*, and *C*. In group *C*, the subjects participated in both the *A* and *C* groups.

The protocol for group *A* was: (*a*) 10 subjects selected had no salicylates for 48 hours, (*b*) overnight urine was emptied from the bladder and determined for the presence of salicylates, (*c*) two 960-mg. sustained-release aspirin tablets were swallowed with 100 ml. of water, and (*d*) blood and urine samples were collected at 1, 4, 7, 10, and 24 hours (Table V).

The protocol for group *B* was: (*a*) six different subjects were selected and followed the procedure as above, (*b*) same as above, (*c*) each subject swallowed two 0.32-Gm. aspirin tablets whole with 100 ml. of water, and (*d*) blood samples were collected (Table VI) at 1, 4, and 7 hours.

The protocol for group *C* was: (*a*) four subjects were selected and followed the procedure as above, (*b*) same as above, (*c*) each subject swallowed six 0.32-Gm. whole aspirin tablets with 200 ml. of water, (*d*) blood samples were collected at 1, 4, 6, 7, 10.5, 13.5, and 24 hours. (*e*) At the end of 6 hours, a second dose of six 0.32-Gm. aspirin tablets was taken with 200 ml. of water (Table VII).

Each of the above tests was conducted 4 days apart. All subjects were denied food but permitted water for 2 hours after initiation of tests.

Analytical Methods

Salicylates in the urine and blood were determined by methods outlined by Trinder (17). Figure 6

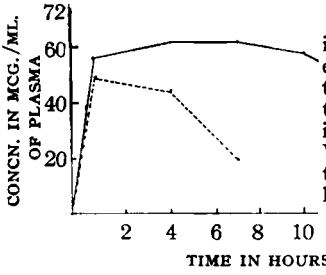


Fig. 6—Comparison of plasma levels (Table V) obtained with sustained-release (solid line) and (Table VI) regular aspirin tablets (broken line).

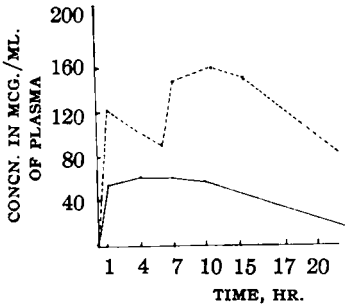


Fig. 7—Comparison of plasma levels (Table V) obtained with sustained - release (solid line) and (Table VII) regular aspirin tablets (broken line).

illustrates graphically a comparison of the micrograms per milliliter plasma level interpreted from results of groups *A* and *B*. Table VII outlines the results from group *C*. Figure 7 shows the comparative results interpreted from groups *A* and *C*.

RESULTS AND DISCUSSION

The use of polyvinyl chloride as described in the preparation of sustained-release tablets appears to have desirable characteristics. A method for the tableting is presented that will give reproducible results when tested *in vitro*. In the development of this sustained-release aspirin, our experiments included the use of polyethylene, ethylcellulose, zein, and polyvinyl chloride[1] with varying degrees of success. The scope of this project permitted only a detailed study of the agent polyvinyl chloride. The information obtained from this work confirms reports (18) of earlier investigations to the extent that physical and chemical properties of each drug require a particular type of matrix in order to provide the desired sustained-release characteristics. The amount of pressure exerted in the compression of the tablets was important.

The salicylate plasma level, according to the data presented with the sustained-release tablet, was uniform for over 10 hours. There was a significant amount of salicylate in the plasma after 24 hours. On the other hand, the urine excretion studies indicated a significant amount of salicylates present after 36 hours.

In vivo studies indicated that with the regular commercial aspirin tablets a peak level was reached within 1 hour and then dropped rapidly. When a massive dose (1.92 Gm.) was administered, the blood plasma level reached a high concentration within 1 hour, then dropped rapidly. A second identical dose was given at the end of 6 hours, and the plasma level showed the cumulative effect for a period of 6 hours, then gradually dropped. Figure 7 shows the comparative effect on plasma level by the 1.92 Gm. of aspirin in sustained-release form and the same dose with commercial aspirin tablets.

SUMMARY

A method for the preparation of a sustained-re-

lease aspirin tablet was developed. The tablets were evaluated by three methods *in vitro*. Absorption studies were made comparing the salicylate plasma level from the sustained-release tablets with that obtained from the regular commercial aspirin tablets.

The base polyvinyl chloride appears to be nontoxic according to extensive data available. Further *in vivo* evaluation by a double blind technique would be valuable in predicting the clinical efficacy of the developed sustained-release tablet.

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Rheology and Suspension Activity of Pseudoplastic Polymers I

Quantification of Pseudoplastic Viscosity as a Second Order Function of the Rheogram and the Relationship of This Parameter to Concentration

By SHIVACHANDRA P. KABRE, H. GEORGE DEKAY, and GILBERT S. BANKER

The apparent viscosity (pseudoplastic viscosity) was determined for a number of natural and synthetic gums in aqueous solution, based on flow curves obtained with a multiple point instrument. The power expression $F^N = n'G$ was used to calculate the apparent viscosity from the rheograms. The relationship between the resulting apparent viscosities and the concentrations of the suspending agents studied which were required to produce these viscosities was determined and reduced to the mathematical expression, $n' = e^{KC+b}$, where n' is apparent viscosity, C is concentration, and K and b are material constants. The usefulness of the concentration–apparent viscosity relationship to formulation and product development is discussed.

TWO APPROACHES to the attainment of suspension stability have been discussed recently. Martin and Haines (1) have suggested that controlled flocculation is an approach to the problem of suspension stability, and Samyn (2) suggests that suspensions may be stabilized by preventing phase separation through the careful selection of the rheological properties desired in the suspension media. To achieve this latter goal, Samyn advocated the use of a combination of pseudoplastic and plastic suspending agents in the formulation of suspensions.

A knowledge of the flow properties and rheological parameters of pharmaceutical suspending media is of interest from at least three stand points: (*a*) effect on suspension stability, *i.e.*, ability to retain insoluble particles in a suspended or substantially suspended easily redispersed state; (*b*) effect on the flow, pourability, and measurability of the final product required for pharmaceutical use; and (*c*) effect on process design and methods of manufacture in large scale production.

In practically all industrially made pharmaceutical suspensions, including flocculated systems, suspending agents of some type are used to stabilize the suspension product. The mucilages of natural and synthetic gums, which include a large majority of suspending agents, are pseudoplastic. However, for reasons of convenience, cost, or due to a lack of knowledge of a better approach, these mucilages are very often evaluated in pharmaceutical practice using a single

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lease aspirin tablet was developed. The tablets were evaluated by three methods *in vitro*. Absorption studies were made comparing the salicylate plasma level from the sustained-release tablets with that obtained from the regular commercial aspirin tablets.

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Rheology and Suspension Activity of Pseudoplastic Polymers I

Quantification of Pseudoplastic Viscosity as a Second Order Function of the Rheogram and the Relationship of This Parameter to Concentration

By SHIVACHANDRA P. KABRE, H. GEORGE DEKAY, and GILBERT S. BANKER

The apparent viscosity (pseudoplastic viscosity) was determined for a number of natural and synthetic gums in aqueous solution, based on flow curves obtained with a multiple point instrument. The power expression $F^N = n'G$ was used to calculate the apparent viscosity from the rheograms. The relationship between the resulting apparent viscosities and the concentrations of the suspending agents studied which were required to produce these viscosities was determined and reduced to the mathematical expression, $n' = e^{KC+b}$, where n' is apparent viscosity, C is concentration, and K and b are material constants. The usefulness of the concentration–apparent viscosity relationship to formulation and product development is discussed.

TWO APPROACHES to the attainment of suspension stability have been discussed recently. Martin and Haines (1) have suggested that controlled flocculation is an approach to the problem of suspension stability, and Samyn (2) suggests that suspensions may be stabilized by preventing phase separation through the careful selection of the rheological properties desired in the suspension media. To achieve this latter goal, Samyn advocated the use of a combination of pseudoplastic and plastic suspending agents in the formulation of suspensions.

A knowledge of the flow properties and rheological parameters of pharmaceutical suspending media is of interest from at least three stand points: (*a*) effect on suspension stability, *i.e.*, ability to retain insoluble particles in a suspended or substantially suspended easily redispersed state; (*b*) effect on the flow, pourability, and measurability of the final product required for pharmaceutical use; and (*c*) effect on process design and methods of manufacture in large scale production.

In practically all industrially made pharmaceutical suspensions, including flocculated systems, suspending agents of some type are used to stabilize the suspension product. The mucilages of natural and synthetic gums, which include a large majority of suspending agents, are pseudoplastic. However, for reasons of convenience, cost, or due to a lack of knowledge of a better approach, these mucilages are very often evaluated in pharmaceutical practice using a single

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point viscosity measurement, which essentially treats them as if they were Newtonian.

No part of the rheogram curve for a pseudoplastic is linear, and the viscosity cannot thus be expressed as a single value corresponding to a single point on the curve. The single point determinations for two pseudoplastic materials could nearly coincide, even though rheograms for the materials had markedly different curvatures or even crossed each other at higher shearing rates if plotted as superimpositions. In such cases, depending on where the one point determination was taken, entirely different conclusions might be reached in comparing product viscosities. In practice, at the lower shearing stresses, it is not usually possible to obtain reproducible shearing rates with pseudoplastics and a one point measurement.

The hysteresis effects as reflected in the rheogram, particularly in the downcurve where molecular redisorientation is occurring, can be obtained only with the multipoint instrument. Data of this latter type can be expected to be much more valid in predicting the stability of suspension systems than any one point determination.

The following four rheological properties alone and in combination are of primary importance in determining the stability of suspension systems: (*a*) yield value, (*b*) thixotropy, (*c*) elastic components, and (*d*) the decrease of the proportionality factor of shearing stress/shearing rate, with an increase in the applied shearing stress. The third and fourth listed properties, and sometimes thixotropy, are present in all pseudoplastic materials.

The mathematical representation of pseudoplastic flow has been a serious problem to rheologists. No theoretical equation has been derived from physical concepts, but an empirical power function has been proposed by Porter and Rao (3) to fit the flow curves produced by pseudoplastic materials. Their expression, in the form of more than one constant, is

$$F^N = \eta' \frac{dv}{dy} \qquad \text{(Eq. 1)}$$

where F = shearing stress, dv/dy = velocity gradient per unit area, N = is the constant, and η' = is "the apparent viscosity." The apparent viscosity, being different from the coefficient of viscosity, does not have the dimensions of viscosity and is not expressed in poises. N is a characteristic constant which depends on the substance and its concentration and also indicates the structural change.

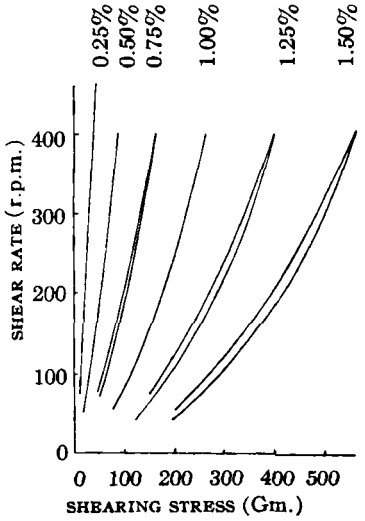


Fig. 1.—Rheograms of Kelcosol mucilages of various concentrations.

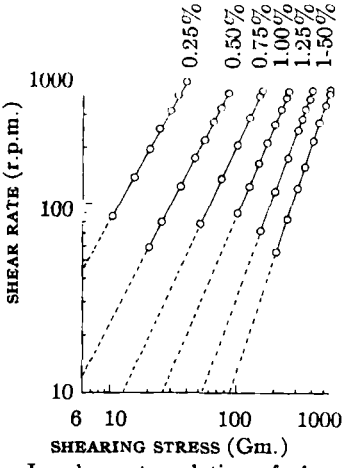


Fig. 2.—Log–log extrapolation of rheograms of Kelcosol mucilages (for calculation of "apparent" viscosity).

Farrow, *et al.* (4), studied the rheological behavior of starch paste and found that Newton's law could not be maintained. Their modified equation is

$$F^N = \eta' G \qquad \text{(Eq. 2)}$$

where G = shearing rate. This equation is also written

$$\log G = N \log F + \log 1/\eta' \qquad \text{(Eq. 3)}$$

The above equation gives a straight line where N is the slope, and $\log 1/\eta'$ is the Y intercept. The value N is considered to be an index which describes the behavior of the liquid. When $N = 1$ the flow is Newtonian, when greater than 1 it is pseudoplastic, and when less than 1 the flow is dilatant.

Work which would define a method of expressing apparent viscosity (pseudoplastic viscosity) and which would permit the establishment of the relationship between the apparent viscosity of pseudoplastic materials and concentration would provide (*a*) a systematic method for calculating the apparent viscosity of various suspending agents used in pharmaceutical preparations, and (*b*) a well planned experimental procedure for the evaluation and comparison of the suspension activity of the various pseudoplastic suspending agents.

EXPERIMENTAL

Suspending Agents.—The following pseudoplastic suspending agents were used for the rheological study: Kelcosol,[1] high viscosity sodium alginate; Kelmar,[1] potassium derivative of alginic acid; Kelzan,[1] microbiological polysaccharide derived from glucose; Kelgin,[1] medium viscosity sodium alginate; Keltone,[1] sodium alginate, calcium tartrate, and sodium phosphate; tragacanth (U.S.P. powdered); CMC-7 MP (sodium carboxymethylcellulose); and Methocel 1500 cps. (methylcellulose). The first five of the materials mentioned

[1] Kelco Co., Los Angeles, Calif.

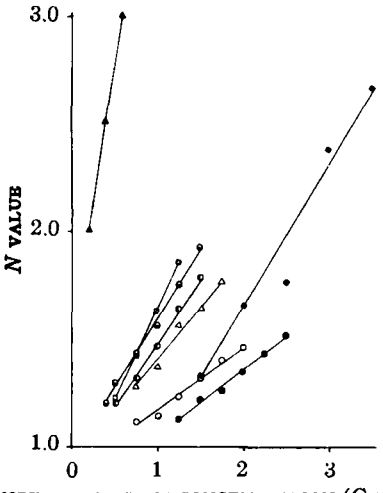


Fig. 3.—Concentration–N value relationship of the various suspending agents. Key: O, Methocel 1500 cps.; ◐, Keltone; ◒, Kelcosol; ●, CMC-7 MP; □, Kelmar; ■, tragacanth; △, Kelgin, and ▲, Kelzan.

above are highly purified derivatives of alginic acid. Tragacanth was selected because it is a U.S.P. natural gum, while CMC-7MP and Methocel 1500 cps. are synthetic gums.

Preparation of Samples.—An accurately weighed amount of suspending agent was added to a 4-ounce bottle containing 100 ml. of distilled water, and the bottles were shaken occasionally to produce the mucilages. Six different concentration ranges of the various gums were used to prepare products having approximately the same range in apparent viscosity (0.1 to 480) for each product. The concentration ranges required to produce this viscosity range varied, of course, according to the material used. Preservatives were not added to the mucilages of the suspending agents, and freshly prepared (24-hour-old) samples were used to obtain the initial rheograms.

Rheological Study.—The rheology of the mucilages was studied utilizing a modified Stormer viscometer (5) in which the bob or inner cylinder rotated, and the cup containing the sample remained stationary. A constant temperature water bath maintained the sample temperature at 25 ± 0.1°. The cup and solid aluminum bob were calibrated with N.B.S. standard viscosity oils (6). The sample of the mucilage or the suspension was placed in the cup and deaerated by gentle suction. The cup and bob were mounted in the viscometer making certain that no air was entrapped within the sample. The sample was allowed to remain undisturbed for 90 minutes before beginning a run. The rheograms were obtained by adding successively increasing weights to the hanger. A point on the curve was obtained by noting the time required for a given weight to drive the bob through 100 revolutions. This value was converted to revolutions per minute. When a maximum of 380 to 410 r.p.m. was reached, the weights on the hanger were reduced in the same order in which they were increased. An interval of 60 seconds was allowed to elapse between each observation. The data for the downcurve obtained from the above runs were plotted with the shearing stress *versus* shearing rate on log-log paper in instrumental units (grams *versus* revolutions per minute).

Assuming a suspension product can be converted to a substantially uniform dispersion by shaking or agitation, the subsequent stability of the product reflected by any change in uniformity of dispersion would depend on the rate and degree of redisorientation of the molecules of the suspension media. Therefore, we would be more interested in redisorientation rheology than orientation rheology from a suspension stability standpoint and any subsequent attempts to relate apparent viscosity and suspension activity. The downcurve of a rheogram represents the redisorientation and the upcurve the orientation of the molecules in the suspension system, and thus in this experiment downcurves were used for the calculation of apparent viscosity. To determine product "pourability," the upcurve would, of course, be of more significance.

RESULTS AND DISCUSSION

The rheological data obtained with the modified Stormer viscometer were plotted as rheograms of shearing stress (Gm.) *versus* shearing rate (r.p.m.). Characteristic flow curves of one of the alginate suspending agents (Kelcosol) are shown in Fig. 1. In this study a modified Newton's equation (Eq. 3) was used for the calculation of the apparent viscosity of pseudoplastic flow. The rheological data, which were obtained, were plotted on log-log paper for various percentages of suspending agents (Fig. 2). The slopes of the straight lines (Fig. 2) are N and were calculated using the least squares method for each concentration of the suspending agent. The exponential constant (index) N increased as the concentration of the suspending agent increased and was always greater than 1. The relationship between N and concentration (Fig. 3) was a zero-order function for all of the materials studied except tragacanth.

The apparent viscosity, η', of each material was

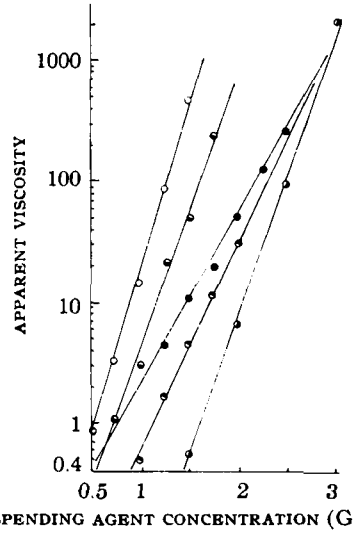


Fig. 4.—Apparent viscosity–concentration relationship. Key: O, Kelcosol; ◒, Kelgin; ●, CMC-7 MP; ◓, Methocel 1500 cps.; and ◑, tragacanth.

TABLE I.—CALCULATED MATERIAL CONSTANTS OF THE PSEUDOPLASTIC AGENTS STUDIED

Suspending Agent	Material Constants K	b
Kelcosol	2.7042	−1.4533
Kelmar	2.7271	−1.5443
Kelzan	7.5840	−2.4834
Kelgin	2.2744	−1.6153
Keltone	2.7046	−1.4424
Tragacanth	2.2858	−3.6653
CMC-7 MP	1.4222	−2.1351
Methocel 1500 cps.	1.8363	−2.0426

determined from the intercept, log $1/\eta'$, of the log-log plots (Fig. 2), using the least squares method.

A first-order relationship existed between the apparent viscosity of the pseudoplastics and their concentration (Fig. 4) and may be expressed mathematically

$$\eta' = e^{KC+b} \qquad \text{(Eq. 4)}$$

where η' = apparent viscosity, and C = concentration of the suspending agents.

In Eq. 4, b and K are constants depending upon the suspending agent (Table I). The value b can be calculated from the graph as the interception point, while the constant K is the slope. Once K and b are calculated for a suspending agent, then these values are the fixed constants for that particular suspending agent according to the method of viscosity determination. The effect of lot to lot trace chemical variation or processing variation on these constants was not determined. The concentration required to produce the desired apparent viscosity for a material can thus be computed by using the above constants in Eq. 4 or by the standard curve.

SUMMARY AND CONCLUSIONS

Eight pseudoplastic suspending agents, including five alginates, a vegetable gum, and two synthetic cellulose derivatives, were rheologically evaluated. The apparent viscosity was calculated using the equation $F^N = \eta' G$. This equation was expressed in logarithmic form in order to obtain a straight line relationship of shearing stress and shearing rate.

The exponential constant N was calculated using the least squares method, and these values varied from one material to another and were related (zero-order function) to the concentration of each suspending agent studied.

The N values for all of the suspending agents used for this study were greater than 1 and increased with the concentrations of the suspending agent. This further demonstrates the pseudoplastic natures of the suspending agents.

A first-order relationship between apparent viscosity, η', and concentration, C, was exhibited by all of the mucilages prepared for this study, and is expressed as $\eta' = e^{KC+b}$ where K and b are material constants.

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(1) Haines, B. A., and Martin, A. N., THIS JOURNAL, 50, 228, 753(1961).
(2) Samyn, J. C., *ibid.*, 50, 517(1961).
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(5) Fischer, E. K., "Colloidal Dispersions," John Wiley and Sons, Inc., New York, N. Y., 1950, pp. 159–168.
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Rheology and Suspension Activity of Pseudoplastic Polymers II

Comparison of Suspension Activity of Pseudoplastic Polymers at Fixed Apparent Viscosities

By SHIVACHANDRA P. KABRE, H. GEORGE DEKAY, and GILBERT S. BANKER

According to a previously described method of expressing the apparent viscosity of pseudoplastic materials and of relating this parameter to concentration, eight pseudoplastic suspending agents were prepared at four levels of apparent viscosity and were studied for their suspension activity for two insoluble drugs (zinc oxide U.S.P. and sulfamethazine U.S.P.). The drug which was compatible with the suspending agents studied was stabilized in the various suspension media at an apparent viscosity of 10 or above. Both insoluble drugs demonstrated an exponential sedimentation rate, while zinc oxide showed a zero order and sulfamethazine an exponential separation rate in the various pseudoplastic suspension media.

A PRECEDING REPORT (1) described a method of characterizing pseudoplastic materials according to calculated values derived from their rheograms. The relationship between the resulting apparent viscosity (pseudoplastic viscosity) and concentration of the suspending agents required to produce that viscosity has also been determined and reduced to a mathematical expression. In this study the suspension activity of

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eight pseudoplastic suspending agents which have been rheologically characterized was studied for two insoluble drugs at four apparent viscosity levels.

EXPERIMENTAL

The formula for the suspension vehicles (mucilages) was:

Concentration of the suspending agent yielding the desired apparent viscosity	(see Table I)
Methylparaben as preservative (in all algins)	1.5 Gm.
(in CMC-7 MP and Methocel 1500 cps.)	0.5 Gm.
Amaranth solution U.S.P.	10 ml.
Distilled water q.s. to	1000 ml.

The weighed amount of preservative was dissolved in 90% of the final volume of distilled water in a 1-L. beaker, and the concentration of suspending agents required to produce the desired apparent viscosity (Table I) was added. A lightnin mixer was used to mix the preservative solution during the gradual addition of the suspending agent. After formulation of the mucilage, the amaranth solution U.S.P. was added. The dye was used to facilitate the observation of sedimentation and/or separation of the insoluble drugs in these latter studies. The tragacanth and methylcellulose solutions were prepared according to the U.S.P. method.

Two suspensions were prepared, one containing 16% zinc oxide and the other 10% sulfamethazine U.S.P., in the mucilages of each suspending agent at each of the four apparent viscosity levels. The zinc oxide and sulfamethazine suspensions were passed through a Tri-Homo-Disperser[1] at a setting of 0.002 in. to obtain a more uniform particle size of the dispersed solid.

Storage of the Samples.—Separate 100-ml. samples of each suspension product were stored in two glass tubes, 2 cm. in diameter and 45 cm. in length, for the determination of sedimentation and separation. In addition, 100 ml. of each suspension sample was stored in a 4-ounce bottle for the resuspendibility evaluation.

Reproducibility.—The concentrations required to produce the four selected apparent viscosities for each material were determined from the standard curves (1). The measured viscosities of the samples thus prepared were within a range of ±10% of the expected values. The apparent viscosity is dependent on the value N, which is the slope of the curve of the log of shearing stress *versus* the log of shearing rate. The apparent viscosity is calculated from the interception point, log $1/\eta'$. The shearing stress-shearing rate curves should be carefully prepared using an adequate number of points, since a slight change in the slope produces a substantially different interception point from which apparent viscosity is calculated.

The apparent viscosity of the mucilages prepared by the hand shaking bottle method in the previous study (1) was initially slightly greater (10 to 15%) than the viscosity of the mucilages prepared using a laboratory propeller mixer in this study. After storage for 2 to 3 days, however, the rheological replication was good (no appreciable difference in apparent viscosity according to the method of preparation). This also indicated that the preservative used had no significant effect on the viscosity, since no preservative was used in the first study.

[1] Tri-Homo Corp., Salem, Mass.

TABLE I.—PER CENT CONCENTRATION OF SUSPENDING AGENTS USED FOR THE STUDY OF SUSPENSION ACTIVITY AT FOUR SELECTED APPARENT VISCOSITIES

Suspending Agents	Apparent Viscosity 1	5	10	20
Kelcosol	0.53	0.80	0.90	1.00
Kelmar	0.57	0.82	0.94	1.05
Kelzan	0.33	0.42	0.46	0.50
Kelgin	0.70	1.00	1.15	1.28
Keltone	0.54	0.78	0.89	1.00
Tragacanth	1.60	1.90	2.05	2.17
CMC-7 MP	1.50	2.00	2.20	...
Methocel 1500 cps.	1.10	1.50	1.65	...

RESULTS AND DISCUSSION

Evaluation of Pseudoplastic Suspension Vehicles

In an attempt to evaluate the advantages of more precisely describing the rheology of the pseudoplastic suspension vehicles, the following parameters of the suspension activity of the various systems were determined: (*a*) sedimentation rate, (*b*) separation rate, and (*c*) resuspendibility.

In all preparations containing alginates and CMC, the sulfamethazine separated very rapidly. The sulfamethazine suspensions in alginates and Methocel foamed considerably with processing. Channeling was observed in all of the sulfamethazine suspensions. Coagulation of the zinc oxide was observed in the suspensions in the Kelzan vehicle. The particles of zinc oxide appeared as agglomerates upon visual observation in Kelzan; this phenomenon was also noticed in the zinc oxide–tragacanth preparations with apparent viscosities of 1 and 5. The comprehensive summary of the evaluation of the suspending agents is shown in Table II.

Sedimentation Rate (2).—Sedimentation was observed in all zinc oxide suspension series and in the sulfamethazine suspensions prepared in the Methocel vehicles.

The sedimentation rate can be defined as the accumulation of the solid below the bulk suspension in the form of a dense cake, per unit time.

The sedimentation ratio is defined as the ratio between the sedimented column length and the length of the original total suspension column (diameter of the columns are the same). The observations for sedimentation were made daily for the first week and then weekly for 12 weeks. The sedimentation ratios were calculated using

$$\text{Sedimentation ratio} = \frac{Hs_1}{Ho}$$

where Hs_1 = height of sedimented volume in mm., and Ho = original height of sample column in mm.

The calculated sedimentation ratios were plotted on linear coordinate graph paper *versus* time of

TABLE II.—COMPREHENSIVE SUMMARY OF THE EVALUATION OF SUSPENDING AGENTS

Suspending Agents	Apparent Viscosity	After 3-Mo. Storage Period: Sedimentation Ratio	Separation Ratio	Shakes for Resuspension, No.	Appearance[a]	Vehicle Suspendibility[b]
		ZINC OXIDE SUSPENSIONS				
Kelcosol	1	0.0935	0.2569	15	Fair	U
	5	0.0800	0.1444	35	Good	Q
	10	0.0700	0.0475	35	Good	A
	20	0.0549	0.0262	35	Good	A
Kelmar	1	0.0985	0.2433	25	Fair	U
	5	0.0601	0.1445	50	Good	A
	10	0.0268	0.0354	35	Good	A
	20	0.0412	0.0072	35	Good	A
Kelzan	1	0.2394	0.7581	5	Poor	U
	5	0.2581	0.7441	5	Poor	U
	10	0.3431	0.6525	5	Poor	U
	20	0.3651	0.6258	20	Poor	U
Kelgin	1	0.0952	0.2150	30	Poor	U
	5	0.0554	0.0459	80	Good	A
	10	0.0303	0.0303	70	Good	A
	20	0.0093	0.0116	55	Good	A
Keltone	1	0.0926	0.2537	20	Fair	U
	5	0.0750	0.1573	45	Good	A
	10	0.0666	0.0860	55	Good	A
	20	0.0547	0.0642	55	Good	A
Tragacanth	1	0.0000	0.3743	15	Poor	U
	5	0.0000	0.0779	45	Fair	Q
	10	0.0000	0.0318	10	Fair	Q
	20	0.0271	0.0169	10	Good	A
CMC-7 MP	1	0.0227	0.0579	20	Good	Q
	5	0.0160	0.0114	15	Good	A
	10	0.0162	0.0093	15	Good	A
Methocel 1500 cps.	1	0.2185	0.1115	20	Fair	U
	5	0.1649	0.0318	20	Fair	Q
	10	0.0000	0.0314	20	Fair	Q
		SULFAMETHAZINE SUSPENSIONS				
Kelcosol	1		0.6481	5	Poor	U
	5		0.6482	5	Poor	U
	10		0.6221	5	Poor	U
	20		0.6299	5	Poor	U
Kelmar	1		0.7171	5	Poor	U
	5		0.6254	5	Poor	U
	10		0.6842	5	Poor	U
	20		0.6223	5	Poor	U
Kelzan	1		0.6564	5	Poor	U
	5		0.5772	5	Poor	U
	10		0.5204	5	Poor	U
	20		0.4541	5	Poor	U
Kelgin	1		0.6595	5	Poor	U
	5		0.6053	5	Poor	U
	10		0.5880	5	Poor	U
	20		0.5689	5	Poor	U
Keltone	1		0.6393	5	Poor	U
	5		0.6369	5	Poor	U
	10		0.6332	5	Poor	U
	20		0.6021	5	Poor	U
Tragacanth	1		0.1813	5	Poor	U
	5		0.1278	5	Poor	U
	10		0.0594	5	Poor	Q
	20		0.0272	5	Poor	A
CMC-7 MP	1		0.5551	5	Poor	U
	5		0.2078	5	Poor	U
	10		0.0164	5	Poor	Q
Methocel 1500 cps.	1	0.1484	0.0653	25	Fair	U
	5	0.1826	0.0201	20	Fair	U
	10	0.2075	0.0165	20	Fair	Q

[a] Good: uniform smooth appearing suspension with some separation possible, but no distinct sedimentation. Fair: suspension may be quite stable with slight or no separation and no distinct sediment, but particles appear to be flocculated; products appeared less smooth than those classified as good. Poor: Samples evidenced considerable separation with sediment formation and possible channeling (vertical clear areas) or layering (horizontal clear areas). [b] Vehicle suspendibility indicates the adequacy of each suspension system at a set apparent viscosity to suspend zinc oxide or sulfamethazine satisfactorily. A, Acceptable: product does not sediment or materially separate and is probably of commercial quality. Q, Questionable: product separates and/or sediments to a moderate degree, and the acceptance of the vehicle for drug suspension is doubtful. U, Unacceptable: product separates considerably and/or sediments.

storage. Table II summarizes the sedimentation ratios calculated for all the preparations studied.

Kelzan was a poor suspending agent for zinc oxide and sulfamethazine and demonstrated both channelling and layering, making the measurement of the sediment portion difficult. In tragacanth and Methocel, no sedimentation of zinc oxide was observed during the storage period.

Figure 1 shows the sedimentation rate as a ratio for the zinc oxide suspensions in Keltone observed for a period of 12 weeks. Similar curves of the sedimentation rate were obtained with zinc oxide suspensions in Kelcosol, Kelgin, Kelmar, CMC, and for sulfamethazine in Methocel (Table II). The sedimentation rate decreased exponentially with the time of storage and equilibrated at a sedimentation ratio of about 0.05 at an apparent viscosity of 20 and at a ratio of about 0.09 at an apparent viscosity of 5 (Fig. 1). The sedimentation ratios of zinc oxide in Kelcosol, Kelgin, Kelmar, and CMC at an apparent viscosity of 5 were all approximately twice the ratio values at an apparent viscosity of 20.

Separation Rate.—The measurements of phase separation were made at 1-day intervals for the first week and weekly thereafter for 10 weeks for the sulfamethazine suspensions. With zinc oxide suspensions, the measurements of phase separation were made weekly for 12 weeks. The separation ratios were calculated in the same way as the sedimentation ratio, the only difference being in the definition of the numerator, which is the height of clear separated phase in mm. The calculated separation ratios are shown in Table II.

Separation of Suspensions During the First Week.—The suspensions of zinc oxide did not show noticeable phase separations during the first week, while the majority of the sulfamethazine suspensions showed appreciable separation. The pattern of separation obtained for Kelcosol, Keltone, Kelgin, and Kelmar, corresponding to the fixed apparent viscosities 1, 5, 10, and 20, are exemplified by the Kelcosol data in Fig. 2. Sulfamethazine suspensions in tragacanth and Methocel did not show separation during the first week. The curves indicate an exponential decrease in the separation rate over the period of 7 days.

Separation of Suspensions Over a 10 to 12-Week Period.—The rate of separation of the sulfamethazine suspensions (Fig. 3) was of a different order than that of the zinc oxide suspensions (Fig. 4). The sulfamethazine suspensions separated at an exponential rate, while the zinc oxide suspensions separated at a zero-order rate. All the zinc oxide suspensions separated according to the relationship exemplified by the Kelmar data (Fig. 4), except for the Kelzan system which was incompatible. In the zinc oxide systems, exemplified by Fig. 4, there was a great change in the separation rate as the apparent viscosity changed from 1 to 10, with much less change between 10 and 20. The suspensions having an apparent viscosity of 20 were very stable to separation. They usually sedimented the least; except for the most uniform systems on aging (tragacanth, Methocel, and CMC), they were not the easiest to redisperse (Table

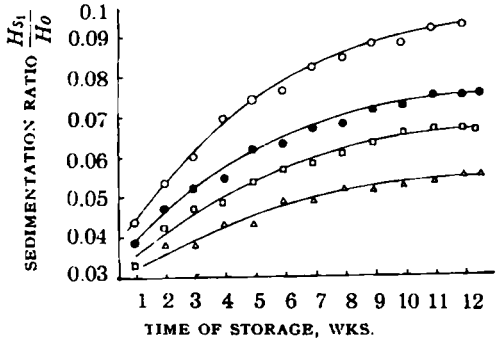


Fig. 1.—Weekly sedimentation rate of zinc oxide suspensions (Keltone as the suspending agent). Key: ○, vehicle apparent viscosity, 1; ●, vehicle apparent viscosity, 5; □, vehicle apparent viscosity, 10; △, vehicle apparent viscosity, 20.

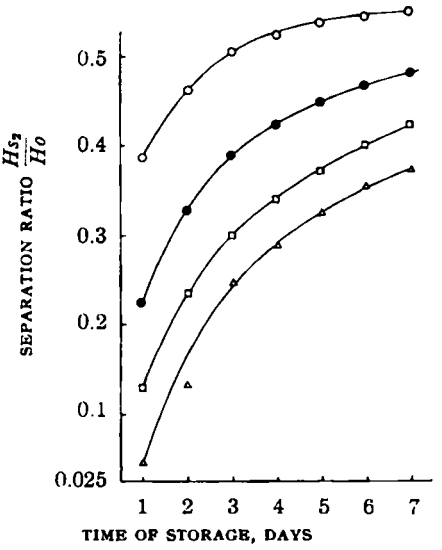


Fig. 2.—Daily separation rate of sulfamethazine suspensions (Kelcosol as the suspending agent). Key: ○, vehicle apparent viscosity, 1; ●, vehicle apparent viscosity, 5; □, vehicle apparent viscosity, 10; △, vehicle apparent viscosity, 20.

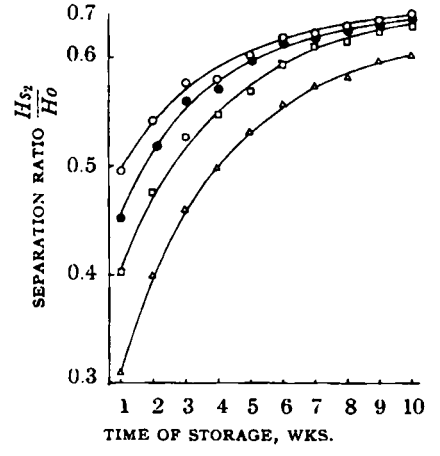


Fig. 3.—Weekly separation rate of sulfamethazine suspensions (Keltone as the suspending agent). Key: ○, vehicle apparent viscosity, 1; ●, vehicle apparent viscosity, 5; □, vehicle apparent viscosity, 10; △, vehicle apparent viscosity, 20.

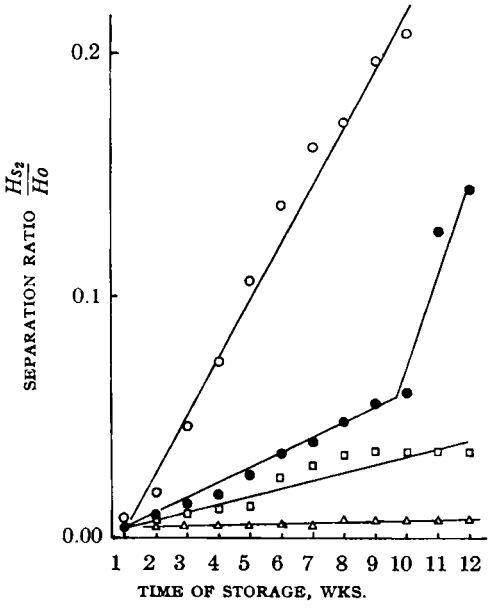


Fig. 4.—Weekly separation rate of zinc oxide suspensions (Kelmar as the suspending agent). Key: O, vehicle apparent viscosity, 1; ●, vehicle apparent viscosity, 5; □, vehicle apparent viscosity, 10; △, vehicle apparent viscosity, 20.

II). The resuspendibility of the suspensions was expressed in terms of the number of shakes produced by a mechanical shaker (3) required to resuspend the sediment resulting from a 3-month storage period at room conditions.

The sulfamethazine suspensions separated more rapidly initially and (in general) to a much greater extent than the zinc oxide suspensions, particularly at the higher viscosity levels. However, the zinc oxide suspensions caked, while the sulfamethazine suspensions did not. There are many factors, alone or in combination, which are responsible for dispersed particle behavior in suspension systems. These factors include (*a*) wettability of the insoluble particles, (*b*) electrokinetic properties of the system, (*c*) free surface energy of the system, (*d*) concentration of insoluble material, (*e*) particle size distribution, (*f*) particle interaction, (*g*) flocculation, and (*h*) density of each phase.

The microscopically observed flocculated condition of the sulfamethazine suspensions is probably a major factor in accounting for the difference in the separation and sedimentation behavior between these and the zinc oxide suspensions (which showed no flocculation).

While it is virtually always impossible to make absolute correlations between apparent viscosity and suspension stability due to the complexity of the systems and the numerous physical factors involved, there are advantages to determining apparent viscosity (pseudoplastic viscosity) and the relationship of this parameter to concentration in product development. These advantages include comparing the suspension activity of pseudoplastic polymers at fixed apparent viscosities for any insoluble drug of interest, calculation from the viscosity-concentration relationship of the concentration corresponding to acceptable suspension stability for any extrapolated viscosity value, and better comparisons of vehicle costs based on calculated polymer concentration requirements required to achieve any viscosity level. In addition, certain physical studies of suspension stability would be facilitated by being able to calculate rather precise concentrations of various pseudoplastic polymers to achieve constant apparent viscosity levels.

SUMMARY AND CONCLUSIONS

Pseudoplastic suspension vehicles of natural and synthetic polymeric suspending agents having apparent viscosities of 1, 5, 10, and 20 were used for a study of their suspension activity for zinc oxide and sulfamethazine. The suspension stability was evaluated according to sedimentation and separation rates and resuspendibility. The following conclusions may be made: (*a*) The alginates when used alone are poor suspending agents for sulfamethazine, based on a rapid separation rate. (*b*) The sedimentation rate and separation rate of the sulfamethazine preparations and sedimentation rate of the zinc oxide preparations in this study were exponential. The separation rate of the zinc oxide preparations was zero order. (*c*) The pseudoplastic suspending agents which were compatible with the insoluble materials studied provided adequate particle suspension at an apparent viscosity of 10 and above, according to separation and sedimentation ratio data.

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Factorial Study of Thiourea Adjuvants on Quinine and Quinidine Absorption

By PATRICK W. RAGOZZINO* and MARVIN H. MALONE

A factorially designed experiment using the single perfusion technique in rats indicated that thiourea consistently enhanced absorption of quinine from the intestine while being ineffective for quinidine. *N,N'*-Diethylthiourea increased absorption of both alkaloids. 1-Phenyl-2-thiourea was an inhibitor of alkaloidal absorption, except when tested in fasted animals. With this exception, fasting routinely decreased the ability of the animals to absorb these isomers. Strong and weak buffer systems did not interfere with alkaloidal absorption.

PERFUSION STUDIES conducted by Ragozzino and Malone (1) indicated that thiourea, *N,N'*-diethylthiourea, 1-phenyl-2-thiourea, and 2-benzyl-2-thiourea were all effective as adjuvants in increasing the absorption of quinine from the small intestine of anesthetized rats. *N,N'*-Diethylthiourea was the only significant adjuvant reported for quinidine. These adjuvants appeared to be specific and apparently operating on some enzyme-dependent transport process. Most absorption studies that have appeared in the literature (2) utilized experimental animals that had been fasted for 20 to 24 hours prior to experimentation. In view of reports indicating that fasting for as little as 16 hours nullified certain enzyme-dependent detoxication systems (3), it was considered important to study the effect of fasting on the enzyme-dependent adjuvant transport system. A factorial experiment was designed to examine the relative effects of thiourea, *N,N'*-diethylthiourea, 1-phenyl-2-thiourea, fasting, ionic strength, and some of their interactions on the absorption of quinine and quinidine as measured by the disappearance of these drugs from the rat small intestine.

EXPERIMENTAL

Methods and Materials.—Wistar strain albino rats of each sex were obtained from E. G. Steinhilber, Oshkosh, Wis., and housed separately in the animal quarters of this laboratory for at least 1 week prior to use, with Purina Lab Chow and water being allowed *ad libitum*. Weights of the animals varied from 120 to 160 Gm. at the time of testing. When fasting was specified, food was withheld from the rats for 24 hours prior to experimentation, but water was permitted *ad libitum*. Animals were housed in cages having wide-mesh screen floors to prevent coprophagy. The rats were anesthetized lightly with pentobarbital sodium, 50 mg./Kg. i.p., to implant the perfusion cannulae surgically into the duodenal and ileal ends of the small intestine. The stomach and cecum were closed by ligatures.

The perfusion experiments with quinine and quinidine were carried out according to the "single perfusion technique" described by Schanker, *et al.* (2), with the following minor changes. Inulin recoveries were based on the colorimetric method of Smith (4). Six experiments were run concurrently so that each of six rats would receive exactly the same treatment. Three of these animals received perfusate warmed to 37° from waterbath *A* and the remaining three from waterbath *B*. A Phipps and Bird perfusion pump was utilized, delivering fluid at the constant rate of 1.5 ml./minute. The perfusion solution termed "Buffer A" in these experiments consisted of the drug in a concentration of 1 mmole/L. in a weakly buffered saline solution containing the following salts in millimoles per liter: NaCl, 145; KCl, 4.56; $CaCl_2$, 1.25; Na_2HPO_4, 1.33; and NaH_2PO_4, 0.33. "Buffer B" differed only with respect to the concentration of ingredients: Na_2HPO_4 was changed to 72 mmole/L. and NaH_2PO_4 to 28 mmole/L., so that the total phosphate concentration would be 0.10 *M*. Sodium ion concentration was maintained at 145 mmole/L., and the chloride concentration reduced in proportion to the added buffer anions. The initial pH of all perfusion solutions was adjusted to 7.2 ±0.01 before experimentation. After perfusion, the pH of solutions containing Buffer A was 6.6 and for perfusion solutions containing Buffer B:7.1.

Quinine and quinidine were estimated spectrophotometrically using a modification of the method of Brodie (5). Following the extraction of the alkaloid with the polar solvent ethylene dichloride, the alkaloid was returned to the aqueous phase by the addition of 0.1 *N* H_2SO_4 and re-extracted with a less polar solvent, benzene. This procedure was repeated twice, since it was found that complete extraction of the alkaloids without their metabolic or degradation products was achieved. Using this double extraction procedure, the biological blank was negligible in every case.

In analyzing the results of this study, the general statistical methods used were those described by Snedecor (6), except that the analysis of variance was performed as described by Cochran and Cox (7). These experiments were planned to have a $2^4 \times 4$ factorial design involving five factors, four of them at two levels each, and the fifth one at four levels. In

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most cases the low level of a factor occurring at two levels indicated that none of that factor was present. In the case of the four-level factor, each level actually represented a different mode of administration of the treatment. Only one-half of the 64 possible combinations were used. However, the 32 chosen combinations were selected in such a way that information on the effects of interest would not be sacrificed. The following scheme (Table I) lists the factors studied together with the "levels" of each. In the *Code* column, the customary notation for a two-level factorial is used, the presence or absence of the code letter for a particular factor indicating the level of that factor. In the case of the four-level factor, two code letters are required to specify the level.

TABLE I.—FACTORIAL DESIGN OF PERFUSION STUDY

Factor	Level	Code
Drug	Quinine	a
	Quinidine	No a
Thiourea	Present	b
	Absent	No b
N,N'-Diethylthiourea	Present	c
	Absent	No c
1-Phenyl-2-thiourea	Present	d
	Absent	No d
Mode of administration	Buffer A (weak)	No e, no f
	Buffer B (strong)	e alone
	Fasted 24 hours	f alone
	Not fasted	e and f

TABLE II.—EFFECT OF VARIOUS ADJUVANTS ON RAT INTESTINAL ABSORPTION OF QUININE AND QUINIDINE: A FACTORIAL EXPERIMENT

Test Group Code[a]	% Absorbed[b]
ab	49.9 (48.9 to 51.1)[c]
ad	20.7 (17.9 to 25.5)
ac	30.2 (28.9 to 31.5)
abcd	47.9 (45.7 to 50.4)
abde	47.6 (46.1 to 49.2)
acde	32.9 (31.2 to 37.0)
ae	15.2 (13.0 to 18.7)
abce	53.1 (53.0 to 53.2)
abdf	52.6 (49.6 to 55.0)
acdf	30.0 (27.2 to 32.5)
af	13.1 (12.3 to 13.9)
abcf	53.4 (51.6 to 55.2)
abef	29.0 (15.4 to 39.5)
acef	31.0 (28.9 to 33.4)
adef	19.9 (9.9 to 27.3)
abcdef	50.5 (46.4 to 54.2)
[d]	17.2 (16.6 to 18.2)
bc	99.5 (98.7 to 100.0)
bd	17.6 (16.3 to 19.2)
cd	99.1 (98.7 to 100.0)
de	20.6 (17.3 to 23.4)
bcde	88.7 (87.8 to 89.6)
be	16.8 (12.9 to 19.1)
ce	99.0 (97.4 to 100.0)
df	14.8 (11.1 to 18.1)
bcdf	80.4 (77.8 to 83.4)
cf	99.6 (98.7 to 100.0)
bf	17.0 (15.4 to 19.1)
ef	17.1 (15.2 to 18.4)
bcef	99.5 (98.4 to 100.0)
bdef	16.7 (12.5 to 31.7)
cdef	98.4 (96.4 to 100.0)

[a] For identification see Table I. [b] The per cent absorbed is expressed as the mean for a total of six animals run concurrently, three males and three females. Four determinations were made per animal, one each for the four 10-minute samples collected after the 30-minute drug perfusion period which followed the initial 30-minute drug-free cleansing perfusion. [c] Observed range of the mean values per group. [d] No coded factor present.

TABLE III.—ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Mean Square	F Ratio
Experiments	1	24,708.34	10.3[a]
Main effects			
A: Quinine *vs.* quinidine	1	19,436.63	8.1[a]
B: 1-Phenyl-2-thiourea	1	32,701.03	13.7[a]
C: Thiourea	1	37,282.14	15.6[a]
D: N,N'-Diethylthiourea	1	35,350.56	14.8[a]
M: Methods of treatments	3	55,205.26	23.1[a]
Interaction between main effects			
A × *B*	1	1,119.25	0.4
A × *C*	1	21,053.34	8.8[a]
A × *D*	1	15,678.31	6.5[b]
B × *C*	1	2,032.12	0.8
B × *D*	1	6,526.80	2.7
C × *D*	1	13,484.89	5.6[b]
A × *M*	3	1,196.87	0.5
B × *M*	3	12,650.52	5.3[b]
C × *M*	3	5,765.63	2.4
D × *M*	3	1,541.73	0.6
Interactions with experiments			
A × Experiments	1	6,186.34	2.5
B × Experiments	1	3,154.03	1.3
C × Experiments	1	629.74	0.2
D × Experiments	1	4,584.63	1.9
M × Experiments	3	1,076.92	0.4
Remainder or pooled error	30	2,385.81	...
Further comparisons			
AC: quinine with thiourea	1	74,569.57	31.2[a]
Quinidine, quinidine with thiourea, quinine without thiourea	2	719.71	0.3
AC × *M* × Experiments	3	84,244.23	35.3[a]
AC × *M*	3	2,926.47	1.2
AC × Experiments	1	7,309.13	3.0

[a] Statistically significant at the 1% probability level. [b] Statistically significant at the 5% probability level.

TABLE IV.—INTERACTION BETWEEN QUININE AND QUINIDINE AND PRESENCE *versus* ABSENCE OF THIOUREA EXPRESSED IN ABSOLUTE UNITS

Thiourea	Quinidine	Quinine	Av.	Difference
Absent	144.82	143.50	144.16	44.24
Present	154.97	221.82	188.40	
Av.	149.90	182.66		
Difference	32.76			

RESULTS AND DISCUSSION

Interpretation of the Factorial.—The results of these experiments are shown in Table II. The mean per cent absorbed for each group represents a total of 24 separate determinations. Amount absorbed was calculated from the difference in the concentration entering and leaving the intestine. The analysis of variance in Table III shows that all the main effects were statistically significant as well as four of the first-order interactions. These will be considered separately.

For the purpose of analysis, the data could be arranged so that an internal comparison could be made concerning the duplicatability of the results. The results of the analysis indicated that the three

rats on one side of the perfusion apparatus supplied by water bath *A* responded to a different degree than the three rats on the other side of the apparatus supplied by water bath *B*, possibly due to slight but inherent differences between the two constant temperature water baths employed. To determine whether this effect was significant, the analysis of variance was determined as if two concurrent experiments had been carried out—three rats per run or a total of 12 analyses per group. For the simplicity of calculation and to demonstrate more dramatically the variation or similarity in response of the various factors and levels, all the data were coded so that the values represented absolute units ranging from 0 to 400.

The main effect *A* (quinine *versus* quinidine) and the main effect *C* (presence *versus* the absence of thiourea) were studied together because their interaction, $A \times C$, is statistically significant. The 2 × 2 table or Table IV represents the four averages involved, each value being an average of 16 items, eight from one experiment and eight from the other experiment. The explanation of the significant main effects (*A*, *C*) and the interaction $A \times C$ is now apparent. Averaged over the quinidine and the quinine, the addition of thiourea caused a mean increase of 44.24 units. Quinine was higher than quinidine by 32.76 units, when averaged over the presence and absence of thiourea. However, the difference between thiourea and lack of thiourea was much greater with quinine than with quinidine, and the difference between the quinine and quinidine existed only when thiourea was present—thus causing the significant $A \times C$ interaction. Further inspection of Table IV confirms this conclusion since there appears to be no difference between the two isomers when thiourea is absent, and there appears to be no effect of thiourea on quinidine. This suggests that instead of looking at the *A*, *C*, and $A \times C$ effects, one should examine the effect of quinine with thiourea contrasted with the other three combinations (quinidine, quinidine with thiourea, and quinine without thiourea) averaged together. This comparison is listed at the bottom of the analysis of variance in Table III. This comparison is, in fact, statistically significant to a high degree (F ratio = 31.2), while the variation among the remaining three means is well within experimental error (F ratio = 0.3).

Considering the effect resulting from the combination of quinine and thiourea as the one of greatest interest, it seemed worthwhile to determine if this effect would be exhibited by both experiments and with all four modes of treatment. For this purpose, the remaining comparisons listed among the *Further comparisons* in Table III were made. There is a moderately large but not significant interaction between this effect and *Experiments* and also a significant (F ratio = 35.3) second-order interaction among this effect, *Experiments* and the methods of treatments. The reason for this is shown in Table V. The combinations of quinine and thiourea when averaged over the four methods of treatments was 50.81 units better than the others in *Experiment* 1, and 97.19 units better in *Experiment* 2. Thus, the two experiments agree on the superiority of this combination, but they disagree on its magnitude. This is the cause of the rather large interaction of $AC \times$ Experiments. However, this interaction was not

TABLE V.—INTERACTION BETWEEN QUININE–THIOUREA AND METHODS OF TREATMENTS EXPRESSED IN ABSOLUTE UNITS

Factor	Food	Fasting	Buffer A	Buffer B	Av.
Experiment 1:					
Quinine with thiourea	177.01	118.45	242.19	206.04	185.93
Others	171.64	44.63	192.10	132.04	135.12
Differences	5.37	73.82	50.09	74.00	50.81
Experiment 2:					
Quinine with thiourea	356.15	180.72	230.10	262.75	257.63
Others	159.82	87.05	216.53	178.61	160.44
Differences	196.33	93.67	13.57	84.14	97.19

TABLE VI.—INTERACTION BETWEEN METHODS OF TREATMENTS AND PRESENCE *vs.* ABSENCE OF 1-PHENYL-2-THIOUREA IN ABSOLUTE UNITS

1-Phenyl-2-thiourea	Food	Fasting	Buffer A	Buffer B	Av.
Absent	243.87	75.68	229.58	200.92	187.51
Present	137.94	97.90	195.19	149.15	145.05
Differences	105.93	−22.22	34.39	51.77	42.46

statistically significant and the interaction $AC \times$ methods of treatments was not significant. Observation of the individual differences listed in Table V indicates that in *Experiment* 1 the superiority of the combination of quinine and thiourea was uniformly substantial, except for the nonfasted animals. On the other hand, in *Experiment* 2 there was a large difference for the nonfasted animals, but the difference was rather small with Buffer A. This discrepancy between the results of the two experiments was the cause of the significant $AC \times M \times$ Experiments interaction. It should be noted that the mean values listed in Table V for quinine with thiourea are averages of only two items, and they are subject to large experimental errors.

The reason for the statistically significant main effect *B* (presence *versus* the absence of 1-phenyl-2-thiourea) is that the average of the 32 items with 1-phenyl-2-thiourea absent was 187.51 units (Table VI) compared with 145.05 units for the average of 32 results with 1-phenyl-2-thiourea present. Apparently this adjuvant was detrimental to the absorption of the alkaloids. However, there is a significant interaction between this effect and the methods of treatments. Table VI shows averages of eight items per entry. The reason for the significant interaction with methods of treatments is evident—the 1-phenyl-2-thiourea was not detrimental when administered to fasted animals but was detrimental when given with Buffer A, Buffer B, and to rats that had not been fasted.

The presence *versus* the absence of *N,N'*-diethylthiourea, or the main effect *D*, was statistically significant according to the analysis of variance (Table III). In Table VII the average of 32 items with this adjuvant present was 188.31 units; the average of 32 items without *N,N'*-diethylthiourea was 144.21 units. This is a difference of 44.10 units in favor of the addition of this adjuvant. However, there is a significant $C \times D$ interaction, indicating that the effect of this adjuvant may depend upon whether thiourea is present. This interaction is shown in Table VII, which indicates that the effect

TABLE VII.—INTERACTION BETWEEN PRESENCE *vs.* ABSENCE OF *N,N'*-DIETHYLTHIOUREA AND PRESENCE *vs.* ABSENCE OF THIOUREA

N,N'-Diethylthiourea	Thiourea	No Thiourea	Differences
Present	196.86	179.83	17.03
Absent	179.93	108.40	71.53
Differences	16.93	71.43	

TABLE VIII.—EFFECTS OF METHODS OF TREATMENTS ON ALKALOID ABSORPTION EXPRESSED IN ABSOLUTE UNITS

Methods	Av.
Food	190.95
Fasting	86.79
Buffer A	212.39
Buffer B	174.99

of *N,N'*-diethylthiourea is greater when thiourea is absent (71.43 units contrasted to 16.93). There is a large statistically significant effect for the interaction $A \times D$. Gross inspection of the lower half of Table II indicates that there is a large effect between *N,N'*-diethylthiourea and quinidine as previously reported (1), and this is the reason for the significant $A \times D$ interaction.

The *Methods of treatments* comparison was statistically significant as indicated in the analysis of variance of Table III. Table VIII indicates that this was almost entirely due to the 24-hour fasting of the rats prior to the test. Fasting definitely hinders absorption of the alkaloids in these experiments. Each figure in Table VIII is the mean of 16 items. The inhibitory effect of fasting can be seen as a uniform phenomenon in Tables V and VI. Inspection of Table VI indicates that 1-phenyl-2-thiourea in a fasted animal encourages alkaloidal absorption as reported earlier (1); yet in a nonfasted animal its presence actually discourages absorption.

SUMMARY

A factorial experiment was carried out in rats to study the effects of thiourea, *N,N'*-diethylthiourea, 1-phenyl-2-thiourea, ionic strength, *ad libitum* food, and 24-hour fasting on the absorption of quinine and quinidine. Thiourea administered in equal weight with quinine consistently gave enhancement of absorption, while absence of effect from thiourea was observed when given with quinidine. 1-Phenyl-2-thiourea administration resulted in lowering the rate of absorption in nonfasted animals while increasing absorption in fasted animals. *N,N'*-Diethylthiourea routinely increased the absorption of both quinine and quinidine. There was no noticeable interference with alkaloidal absorption from either the strongly buffered or the weakly buffered vehicles. Fasting exerted a strong inhibitory effect on quinine and quinidine absorption.

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Influence of Adrenergic Receptors on Blood Sugar and Lactic Acid Levels in the Rat

By BENJAMIN W. LEI* and ROB S. McCUTCHEON

Six treatment combinations were compared for their production of blood sugar and lactic acid in rats. The challenging drugs—saline, epinephrine, and levarterenol-isoproterenol combined—were tested against saline (without adrenergic blockers) and against DCI combined with hydergine. Epinephrine and levarterenol-isoproterenol, the challenging amines, were both effective in increasing the production of blood sugar and lactic acid. The adrenergic blockade produced by DCI combined with hydergine was effective in inhibiting both hyperglycemia and hyperlacticacidemia. Since a specific blockade of both α and β adrenergic receptors prevented the glycogenolytic effects of epinephrine and levarterenol-isoproterenol, it is concluded that this effect is mediated through these receptors.

IT IS WELL known that the sympathetic amines will cause an increased production of both blood sugar and lactic acid (1); there is, however, some uncertainty as to which adrenotropic receptors, if any, are responsible for the mediation of this response. Glycogenolysis was first attributed to the α receptors by Ahlquist (2), then to both receptors by Van der Pol (3) and Claasen and Noach (4). Mayer, *et al.* (5), and McCutcheon (6) took exception to this and suggested that the β receptors—or possibly some unknown receptors

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TABLE VII.—INTERACTION BETWEEN PRESENCE *vs.* ABSENCE OF *N,N'*-DIETHYLTHIOUREA AND PRESENCE *vs.* ABSENCE OF THIOUREA

N,N'-Diethylthiourea	Thiourea	No Thiourea	Differences
Present	196.86	179.83	17.03
Absent	179.93	108.40	71.53
Differences	16.93	71.43	

TABLE VIII.—EFFECTS OF METHODS OF TREATMENTS ON ALKALOID ABSORPTION EXPRESSED IN ABSOLUTE UNITS

Methods	Av.
Food	190.95
Fasting	86.79
Buffer A	212.39
Buffer B	174.99

of *N,N'*-diethylthiourea is greater when thiourea is absent (71.43 units contrasted to 16.93). There is a large statistically significant effect for the interaction $A \times D$. Gross inspection of the lower half of Table II indicates that there is a large effect between *N,N'*-diethylthiourea and quinidine as previously reported (1), and this is the reason for the significant $A \times D$ interaction.

The *Methods of treatments* comparison was statistically significant as indicated in the analysis of variance of Table III. Table VIII indicates that this was almost entirely due to the 24-hour fasting of the rats prior to the test. Fasting definitely hinders absorption of the alkaloids in these experiments. Each figure in Table VIII is the mean of 16 items. The inhibitory effect of fasting can be seen as a uniform phenomenon in Tables V and VI. Inspection of Table VI indicates that 1-phenyl-2-thiourea in a fasted animal encourages alkaloidal absorption as reported earlier (1); yet in a nonfasted animal its presence actually discourages absorption.

SUMMARY

A factorial experiment was carried out in rats to study the effects of thiourea, *N,N'*-diethylthiourea, 1-phenyl-2-thiourea, ionic strength, *ad libitum* food, and 24-hour fasting on the absorption of quinine and quinidine. Thiourea administered in equal weight with quinine consistently gave enhancement of absorption, while absence of effect from thiourea was observed when given with quinidine. 1-Phenyl-2-thiourea administration resulted in lowering the rate of absorption in nonfasted animals while increasing absorption in fasted animals. *N,N'*-Diethylthiourea routinely increased the absorption of both quinine and quinidine. There was no noticeable interference with alkaloidal absorption from either the strongly buffered or the weakly buffered vehicles. Fasting exerted a strong inhibitory effect on quinine and quinidine absorption.

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—were responsible for this glycogenolytic effect. However, Furchgott (7) contends that this response cannot be assigned to either the α or β receptors, and he has suggested a separate class—γ receptors—for glycogenolysis.

Despite this controversy, some observations regarding glycogenolysis have been consistently reported. First, epinephrine, a potent α and β stimulant, produced the greatest increase in blood sugar and lactic acid, whereas isoproterenol, a specific β stimulant, produced the smallest effect. The potency ratio in this regard for the three classical amines are: *l*-epinephrine > levarterenol > *dl*-isoproterenol (8, 9) (Claasen and Noach(4)). Second, no complete blockade of catecholamine-induced glycogenolysis has been demonstrated when using either α or β blocking agents independently. The reported inhibition of this response has varied from relatively ineffective to relatively effective. Third, of all the adrenergic blocking agents used to inhibit this response, the derivatives of the ergot alkaloids have been shown to be the most effective. This class of drugs is considered primarily blockers of the α receptors with some blocking action on the β receptors (10). Fourth, the rise in blood sugar produced by the conjoint administration of levareterenol and isoproterenol, independent α and β stimulants, was greater than that of either alone at the same dosage level (3).

These observations are best explained if it is assumed that glycogenolysis was mediated through the synergistic effects of both α and β receptors. Thus, further evidence for such a hypothesis would be advanced if data could be presented to demonstrate that the combined blockade of the α and β adrenotropic receptors completely inhibited this response.

METHODS

l-Epinephrine bitartrate, levarterenol bitartrate, and *dl*-isoproterenol HCl (Sterling-Winthrop Research Institute) were made up in 0.001 *M* solutions in 0.1% sodium bisulfite and 0.1% chlorobutanol as preservatives. Dichloroisoproterenol, *dl*-1-(3′,4′-dichlorophenyl)-2-isopropylaminoethanol HCl (DCI, Eli Lilly and Co.) was preserved in the same manner. Hydergine (Sandoz, Inc.) was used from ampuls containing 0.1 mg. each of the dihydrogenated ergot alkaloids in 1 ml.

Rats, fasted for 16–18 hours, were anesthetized intraperitoneally with pentobarbital (Abbott Laboratories), 35 mg./Kg. Three rats received 0.1 ml. of saline, and three other rats received the conjoint administration of DCI (10 mg./Kg.) and hydergine (0.2 mg./Kg.). Thirty minutes after pretreatment of the three rats with saline, they were given, (I) saline 0.1 ml., (II) epinephrine (0.2 mg./Kg.) and isoproterenol (0.2 mg./Kg.). The three rats pretreated with the combined α and β adrenergic blockers were treated in exactly the same manner: (IV) saline 0.1 ml., (V) epinephrine (0.2 mg./Kg.), and (VI) levarterenol (0.2 mg./Kg.) conjointly with isoproterenol (0.2 mg./Kg.). Since all doses were given intraperitoneally, the doses used by Van der Pol (3) and Claasen and Noach (4) were reduced to give approximately the same activity according to Ellis (8). The chosen dose of hydergine (0.2 mg./Kg.) was successful in antagonizing the hyperglycemic effect without gross signs of side effects (11).

Chemical Methods.—One hour after treatment, blood collected by cardiac puncture with a 1-ml. tuberculin (precision) syringe coated with heparin (5, 12) was ejected directly into the deproteinizing solution (13). After deproteinization by the method of Van Slyke and Hawkins (14), blood sugar was determined according to the method of Nelson (15), in which an alkaline copper solution is reduced by glucose, an arsenomolybdate color reagent added, and the resulting degree of change determined in a colorimeter. The lactic acid was determined by the method of Barker and Summerson (16), in which glucose and other interfering substances are removed from the protein free filtrate with copper sulfate and calcium hydroxide. The lactic acid is converted to acetaldehyde with sulfuric acid and determined colorimetrically after reaction with *p*-hydroxydiphenyl in the presence of copper ions.

Statistical Method.—This experiment utilized a 2 × 3 factorial design in randomized complete blocks. The combined α and β adrenergic blocking agents (DCI-hydergine) and saline constituted one factor. The other factor was composed of saline, *l*-epinephrine, and levarterenol–*dl*-isoproterenol combined. Thirteen replications were made; each replication or block represented an individual experiment with six treatment combinations with one rat per treatment. The random numbers table (17) was used to group the rats into blocks, to determine the sequence of treatments, and to determine the assignment of treatment to each rat. *F* values were calculated at 1 and 55 degrees of freedom and all results indicated as significant are at the 5% level or less (17).

TABLE I.—SUMMARY OF SIX-TREATMENT COMBINATIONS FOR BLOOD SUGAR[a]

Pretreatment	Treatment	Mean Body Wt., Gm.	Mean Blood Sugar Values, mg. %	Mean Deviation from Control	S.E. of the mean[b]
Saline	Saline	230	95	0	± 4.64
Saline	*l*-Epinephrine	226	210	115	±11.75
Saline	Levarterenol–*dl*-isoproterenol	237	138	43	± 7.17
DCI-Hydergine	Saline	232	109	14	± 4.95
DCI-Hydergine	*l*-Epinephrine	251	126	31	± 7.08
DCI-Hydergine	Levarterenol–*dl*-isoproterenol	247	105	10	± 2.75

[a] Thirteen replicates were run on each treatment combination. [b] Significant at the 5% level (17).

TABLE II.—SUMMARY OF SIX-TREATMENT COMBINATIONS FOR BLOOD LACTATE[a]

Pretreatment	Treatment	Mean Body Wt., Gm.	Mean Blood Lactate Values, mg. %	Mean Deviation from Control	S.E. of the Mean[b]
Saline	Saline	230	9.2	0.0	±1.19
Saline	*l*-Epinephrine	226	26.6	17.4	±3.72
Saline	Levarterenol–*dl*-isoproterenol	237	17.5	8.3	±2.57
DCI-Hydergine	Saline	232	14.2	5.0	±2.30
DCI-Hydergine	*l*-Epinephrine	251	15.1	5.9	±2.60
DCI-Hydergine	Levarterenol–*dl*-isoproterenol	247	16.1	6.9	±4.75

[a] Twelve replicates were run on each treatment combination. [b] Significant at the 5% level (17).

RESULTS

Blockade of Catecholamine-Induced Hyperglycemia by Conjoint Administration of Dichloroisoproterenol-Hydergine.—To measure the degree of inhibition, it was of interest to sample only the period during which the maximum increase occurred. According to the reports of Claasen and Noach (4), Ellis (8), and Mayer (5), the maximum output was determined to be approximately 1 hour after intraperitoneal injection. Thus, a sample taken during this period would be most significant in demonstrating the degree of inhibition.

A summary for the six treatment combinations is shown in Table I. These values show that the combined DCI-hydergine is a potent antagonist to the effects of both epinephrine and levarterenol-isoproterenol induced hyperglycemia. The small increase in blood sugar due to the blocking agents alone is approximately 14 mg. %. The subtraction of this value from the values found in treatments (V) and (VI) reflects the true blocking ability of this combination. The adjusted value for treatment (V) is 112 mg. %, which is 17 mg. % above the simultaneous control of treatment (I). This increase is not statistically significant, and it represents an inhibition of 85%. The increase in blood sugar after this treatment combination would be more accurate at 7 mg.%, for the additional 10 mg.% is the result of two observations with large deviations. The degree of inhibition would then be 94%. These values were included as such to maintain the completeness of each block. The rise in blood sugar found in treatment (VI) shows the increase in blood sugar due to the adrenergic blocking agents alone. The values obtained by these two treatment combinations justifies the conclusion that a complete blockade has been demonstrated. The *F* test in the analysis of variance also supports the contention that a complete blockade has been demonstrated.

Blockade of Catecholamine-Induced Hyperlacticacidemia by Conjoint Administration of DCI and Hydergine.—Table II contains a summary of the six treatment combinations. A comparison of treatment (I) and (IV) shows that the blocking agents produced an increase in blood lactate which is statistically significant. Consequently, the observations for treatments (IV), (V), and (VI) had to be adjusted by subtracting this increase, 5 mg. %, before computing the analysis of variance. The similarity of the values found in treatments (IV), (V), and (VI) indicate that the adrenergic blockers have prevented sympathomimetic amine–induced hyperlacticacidemia. By using treatment (IV) as an internal control with 14.2 mg.% as 100% inhibition, the degree of inhibition in treatments (V) and (VI) were 94% and 87%, respectively. The similarity of these three treatment effects and the computed *F* values demonstrated the completeness of this blockade.

DISCUSSION

Because both levarterenol and isoproterenol produced a small but significant increase in blood sugar and because the conjoint administration of these two amines produced an effect greater than either alone, Van der Pol (3) suggested that the glycogenolytic effect was due to the synergistic interactions of both α and β receptors. To confirm this hypothesis, Claasen and Noach (4) studied the glycemic effect of epinephrine, levarterenol, and isoproterenol against the pretreatment of saline and DCI. Their investigation demonstrated that the hyperglycemic effect of levarterenol was unaffected by DCI, while the effect of epinephrine was partially inhibited, and the effect of isoproterenol was completely inhibited. This clearly shows that while the β receptors were blocked, the α receptors continued to mediate glycogenolysis.

Mayer, *et al.* (5), however, took exception to this premise and suggested that the glycogenolytic effect was mediated by the β receptors because (*a*) DCI blocked epinephrine-induced augmentation of cardiac contraction, heart phosphorylase, and hyperglycemia. (*b*) Ergotamine and phenoxybenzamine, both potent α blockers, were unable to prevent the increase in blood sugar and lactic acid. (*c*) DCI partly blocked epinephrine-induced hyperlacticacidemia, but ergotamine had no such effect.

Further evidence for this proposal was advanced by McCutcheon (6) whose investigation of adrenergic amines after adrenergic blockade confirmed those observations made by Mayer, *et al.* (5).

To reconcile the findings of these two groups of investigators, the most obvious means would be to attribute these differences to (*a*) species variation (between dogs and rats), (*b*) differences in route of drug administration (between infusions and subcutaneous injections), or (*c*) the actions of some unknown receptors.

Despite the differences in animals and technique, the results of these two groups can be best explained if the hypothesis is accepted that glycogenolysis is mediated through the interactions of both receptors. Thus, the blockade produced by DCI may be partial because the α receptors were still free to mediate its effects. The partial inhibition of epinephrine-induced hyperlacticacidemia reported by Mayer (5) could also be explained on this basis. Since muscle is the main source of lactic acid and since the receptors here are of the β type (18), this inhibition would be expected.

Inhibition of glycogenolysis with DCI alone has failed to abolish this response completely. This

indifferent antagonism is equally true with the ergot alkaloids. This partial inhibition would be expected if glycogenolysis was mediated through both α and β adrenotropic receptors. If these α and β adrenergic blockers were given together, then the increase in blood sugar and lactic acid can be abolished.

If the contention is true that epinephrine has a greater glycogenolytic effect than either levarterenol or isoproterenol because it stimulates both α and β receptors, then it is reasonable to assume that the combined blockade of both α and β receptors will completely inhibit glycogenolysis. This has been shown to be the case. The results of this study give only an incomplete insight into the mechanisms involved in glycogenolysis, but do suggest that the receptors involved include both the α and the β adrenotropic receptors.

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Polarography of Diastereoisomeric *N*-Nitrosoephedrines

By LOUIS MALSPEIS and NELL G. M. HUNG

The polarographic reductions of *N*-nitrosoephedrine (*erythro* isomer) and *N*-nitrosopseudoephedrine (*threo* isomer) were examined in the pH range 0.6 to 10. Below pH 3.2, the limiting currents of both compounds are diffusion controlled and identical. In the pH range 3.2 to 8.2, the limiting currents of the diastereoisomers are controlled by diffusion and rate of reaction. At a given pH value in this region, under identical conditions, the limiting current for *N*-nitrosoephedrine is greater than that for *N*-nitrosopseudoephedrine. The kinetic currents are catalyzed both by hydrogen ion and general acids. With Delahay's equation, the heterogeneous rate constants of protonation of the diastereoisomers were calculated. The rate of protonation of *N*-nitrosoephedrine is greater than that of *N*-nitrosopseudoephedrine. These relative rates are interpreted in terms of structural considerations. The rate of protonation is greater with that isomer in which there is a lesser degree of intramolecular hydrogen-bonding. Kinetic currents can be used to distinguish between diastereoisomers.

LIMITING POLAROGRAPHIC currents which are controlled by the rate of a chemical reaction and by diffusion are generally referred to as kinetic currents (1, 2). One type of kinetic current is controlled by the rate of reaction in the electrode reaction layer of an electro inactive species which diffuses to the electrode surface from the bulk of the solution. Since the relative reaction rates of diastereoisomers depend upon the population of the possible conformations (3), kinetic currents could be used to distinguish between diastereoisomers. At a given concentration that diastereoisomer which undergoes reaction at the higher rate should afford the higher kinetic current. The primary object of this investigation is to determine the effect of configurational differences on the kinetic currents of diastereoisomers.

The compounds chosen for study are the *N*-nitroso derivatives of (−)-ephedrine and (+)-pseudoephedrine, diastereoisomers having well established configurations (4–7) (see Scheme I). These compounds satisfy the requirement that a center of asymmetry must not be involved in the reaction at the electrode surface, a requirement which increases the likelihood that the reduction mechanisms of the two compounds under comparable conditions will be the same.

EXPERIMENTAL

Materials.—The diastereoisomeric *N*-nitrosoephed-

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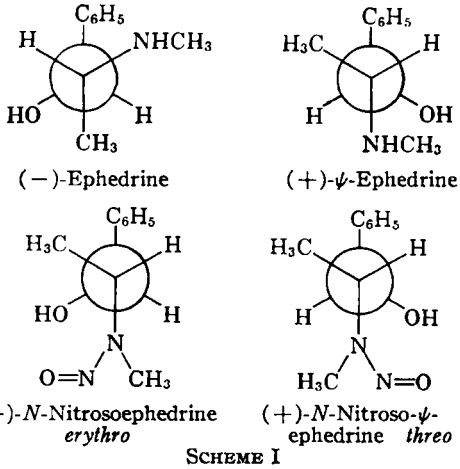
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SCHEME I

rines were prepared from a commercial sample of (−)-ephedrine hydrochloride and a research sample of (+)-pseudoephedrine hydrochloride kindly furnished by Burroughs Wellcome and Co., using a modification of the method of Mitchell (8). The rate of nitrosation using 10% hydrochloric acid was extremely slow; the rate was rapid when concentrated hydrochloric acid was used. To the alkaloidal salt (5.0 Gm.) dissolved in 13 ml. water was added a solution of 3.0 Gm. of sodium nitrite in 5 ml. of water. To this mixture was added 2.0 ml. of concentrated hydrochloric acid with constant stirring for 10 minutes. The reaction mixture was stirred for 1 hour longer and the resulting yellow oil was separated and purified according to Mitchell's procedure. There was obtained 2.58 Gm. of *N*-nitrosoephedrine, m.p. 93.8 to 94.2°, $[\alpha]_D^{25}$ + 80.7° (reported (8) m.p. 93°, $[\alpha]_D^{20}$ + 80.5°) and 3.03 Gm. of *N*-nitrosopseudoephedrine, m.p. 88.0 to 88.5°, $[\alpha]_D^{22.5}$ + 126.7° (reported (8) m.p. 86.0°, $[\alpha]_D^{20}$ + 124.5°).

The buffer systems were prepared from reagent grade chemicals which were used without further purification. The composition of the buffers for the pH dependence study are listed in Table I.

Equipment.—Polarograms were recorded with a Leeds and Northrup Electro-Chemograph, type E; the electrolysis vessel was an H-type cell containing a saturated calomel reference electrode separated from the solution compartment by a potassium chloride–agar plug and a sintered-glass disk. The polarographic cell was thermostated at 25 ± 0.1°.

TABLE I.—BUFFER COMPOSITION, IONIC STRENGTH = 0.29

Buffer	pH[a]	Composition, moles/L.		
1		HCl		KCl
	0.60	0.300		...
	1.60	0.030		0.260
	2.61	0.003		0.287
2		H_3Citrate	NaH_2Citrate	KCl
	3.11	0.005	0.00175	0.2882
	3.23	0.005	0.00250	0.2875
	3.39	0.005	0.00345	0.2866
	3.53	0.005	0.00400	0.2860
	3.70	0.005	0.00477	0.2853
3		$HC_2H_3O_2$	$NaC_2H_3O_2$	KCl
	3.94	0.005	0.001	0.289
	4.38	0.005	0.002	0.288
	4.75	0.005	0.005	0.285
	5.11	0.005	0.012	0.278
	5.20	0.005	0.015	0.275
	5.47	0.005	0.020	0.270
	5.70	0.005	0.045	0.235
4		KH_2PO_4	K_2HPO_4	KCl
	5.57	0.005	0.0003	0.2841
	5.82	0.005	0.0006	0.2832
	6.15	0.005	0.0011	0.2817
	6.45	0.005	0.0021	0.2787
	6.65	0.005	0.0033	0.2751
	6.74	0.005	0.0041	0.2727
	6.82	0.005	0.0050	0.2700
	6.89	0.005	0.0061	0.2667
	7.00	0.005	0.0075	0.2625
	7.11	0.005	0.0093	0.2571
	7.21	0.005	0.0116	0.2502
	7.33	0.005	0.0150	0.2400
	7.48	0.005	0.0200	0.2250
	7.62	0.005	0.0283	0.2001
	7.84	0.005	0.0450	0.1500
	7.95	0.005	0.0600	0.1050
	8.22	0.005	0.0950	...
5		NH_4OH	NH_4Cl	KCl
	8.95	0.005	0.0117	0.2783
	9.33	0.005	0.0050	0.2850
	9.61	0.005	0.0021	0.2879
	10.03	0.005	0.0006	0.2894

[a] pH values of the buffer solutions after the addition of 10% by volume of 95% ethanol.

The dropping mercury electrode had values of $m^{2/3}t^{1/6}$ of 2.165 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.092 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.35 v. *versus* S.C.E. in orthophosphate buffer, 2.165 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.084 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.35 v. *versus* S.C.E. in acetate buffer, 2.140 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.045 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.70 v. *versus* S.C.E. in ammonia buffer, and 2.170 mg.$^{2/3}$sec.$^{-1/2}$ at pH 3.70 and 2.144 mg.$^{2/3}$ sec.$^{-1/2}$ at pH 3.11 open circuit in citrate buffer. The values of $m^{2/3}t^{1/6}$ are at a corrected mercury head of 80.9 cm., except in the experiments concerned with the variation of average limiting current with the head of mercury.

Applied voltages were measured with a Rubicon model 2730 potentiometer and the *i*R drop in the circuit was measured with an Industrial Instruments model RC conductivity bridge. A Beckman model G pH meter was used.

Polarographic Procedure.—A stock solution of the nitrosamine was prepared daily by dissolving a weighed amount of the compound in 95% ethanol. The sample solution was made by the dilution of 5.00 ml. of the stock solution to 50.00 ml. with the appropriate buffer solution. The polarographic cell was rinsed with the sample solution and then filled. The solution was deaerated in the cell by bubbling nitrogen gas through the solution in accordance with standard practice, and the polarographic wave was recorded at a constant head of mercury (80.9 cm. corr.). The initial potential of the polarogram was measured and the voltage span of the polarograms was calibrated with the Rubicon potentiometer. The reported half-wave potentials are corrected for ohmic drop; the reported limiting currents are corrected for the residual current. Damping "2" was used throughout the study. The reported limiting currents are average currents.

The ritual used for determining half-wave potentials involved bisecting the distance between the residual current and the tangent to the wave and constructing a parallel to the residual current through this point; the voltage corresponding to the point of intersection of the rising wave with the parallel was taken as the half-wave potential.

RESULTS

Wave Characteristics.—Both nitrosamines gave a single wave in acidic solutions up to pH 3.7. A second wave was observed in the pH range 3.9 to 6.9. In alkaline solution, pH 8.95 to 10.03, only

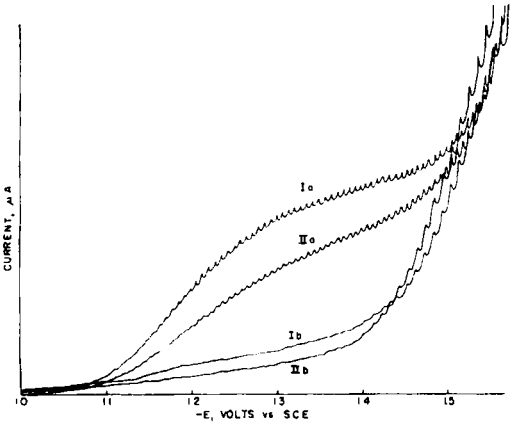


Fig. 1.—Polarograms of *N*-nitrosoephedrine (I) and *N*-nitrosopseudoephedrine (II) in 0.41 *M* acetate buffer (*a*) at pH 5.69 and in 0.0045 *M* phosphate buffer (*b*) at pH 7.48. Depolarizer concentration 4×10^{-4} *M*.

TABLE II.—WAVE HEIGHTS AND HALF-WAVE POTENTIALS IN 4×10^{-4} *M* SOLUTIONS OF THE DIASTEREOISOMERIC *N*-NITROSOEPHEDRINES AT VARYING pH AT 25°

pH	*N*-Nitrosoephedrine $-E^{1/2}$, v. *vs.* S.C.E.	*N*-Nitrosoephedrine Wave Ht.,[a] μA.	*N*-Nitrosopseudoephedrine $-E^{1/2}$, v. *vs.* S.C.E.	*N*-Nitrosopseudoephedrine Wave Ht.,[a] μA.
0.60	0.730	5.51	0.728	5.51
1.60	0.843	5.51	0.837	5.51
2.61	0.958	5.51	0.952	5.51
3.11	1.005	5.51	0.996	5.51
3.23	1.018	5.51	1.010	5.51
3.39	1.033	5.51	1.027	5.47
3.53	1.052	5.47	1.046	5.39
3.70	1.060	5.43	1.042	5.35
3.94	1.084	5.24	1.081	4.96
4.38	1.108	4.80	1.115	4.57
4.75	1.125	4.61	1.136	4.13
5.11	1.146	4.09	1.156	3.62
5.20	1.151	3.94	1.163	3.39
5.47	1.166	3.31	1.178	2.60
5.70[b]	1.175	2.80	1.184	2.21
5.57	1.165	1.52	1.163	1.22
5.82	1.174	1.35	1.174	1.03
6.15	1.178	1.06	1.177	0.79
6.45	1.181	0.77	1.189	0.57
6.65	1.184	0.60	[c]	0.47
6.74	1.186	0.53	[c]	0.41
6.82	1.190	0.47	[c]	0.35
6.89	[c]	0.43	[c]	0.33
7.00	[c]	0.41	[c]	0.27
7.11	[c]	0.41	[c]	0.26
7.21	[c]	0.33	[c]	0.25
7.33	[c]	0.40	[c]	0.22
7.48	[c]	0.27	[c]	0.19
7.62	[c]	0.22	[c]	0.17
7.84	[c]	0.21	[c]	0.18
7.95	[c]	0.20	[c]	0.13
8.22	[c]	0.18	[c]	0.12
8.95	1.522	2.87	1.524	2.48
9.33	1.524	2.70	1.532	2.30
9.61	1.532	2.52	1.531	2.22
10.03	1.540	2.30	1.542	2.09

[a] Data were secured at −1.35 v. *vs.* S.C.E. in the pH range 0.60 to 8.22 and at −1.70 v. *vs.* S.C.E. in the pH range 8.95 to 10.03. Currents in the pH range 0.60 to 3.70 are extrapolated values. [b] Buffer 3. [c] Poorly defined wave.

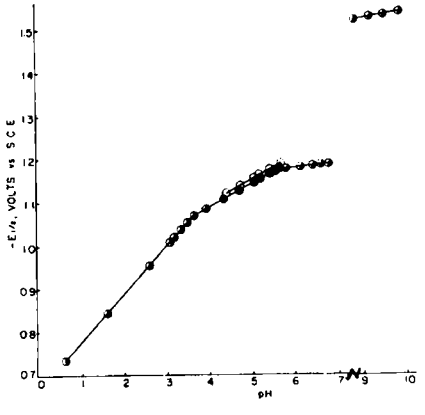


Fig. 2.—Variation of half-wave potential, $E_{1/2}$, with pH for ● *N*-nitrosoephedine and ○ *N*-nitrosopseudoephedrine. Identical half-wave potentials for both diastereoisomers are denoted by ◑.

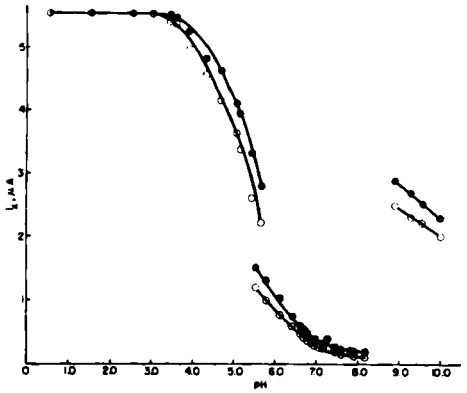


Fig. 3.—Variation of limiting current with pH at 25°C. and ionic strength 0.261. Key: ●, 4×10^{-4} *M* *N*-nitrosoephedrine; ○, 4×10^{-4} *M* *N*-nitrosopseudoephedrine. pH range 0.60 to 8.22, −1.35 v. *vs.* S.C.E.; pH range 8.95 to 10.03, −1.70 v. *vs.* S.C.E. Currents in the pH range 0.60 to 3.70 are extrapolated values. Identical currents for both diastereoisomers are denoted by ◑. pH 0.60 to 2.60, buffer 1; pH 3.10 to 3.65, buffer 2; pH 3.94 to 5.70, buffer 3; pH 5.57 to 8.22, buffer 4; pH 8.95 to 10.03, buffer 5.

one wave was seen. The reported values of potential and current refer to the first wave in the pH range where two waves occur.

The second wave possessed a prominent maximum. The total height of the first and second waves remained constant in both the acetate (16.4 μA.) and orthophosphate buffer systems (13.5 μA.). Accurate values of the half-wave potentials could not be determined because of the maximum. Within the experimental error, the half-wave potentials of the second wave and *total* wave heights for both diastereoisomers appeared to be the same in both buffer regions.

The first wave in strongly acidic solution is well defined, and up to pH 3.23 the waves appear to be identical for both isomers. With increasing pH, the characteristic wave shape of the compounds becomes increasingly drawn out. Figure 1 shows that the current-potential curve of the first wave for *N*-nitrosopseudoephedrine is more irreversible than the curve for *N*-nitrosoephedrine at pH 5.69 in acetate buffer. At pH 6.65 for the *threo* isomer and pH 6.89 for the *erythro* isomer, the waves become poorly defined. Typical poorly defined waves at pH 7.48 are given in Fig. 1.

The irreversible character of the reduction was demonstrated by the results of the logarithmic analysis of the first wave. At pH 0.6 and 1.6, the plot of log $(i/i_d - i)$ *versus* E exhibited two straight line segments. At pH 2.6, the log plot yielded a single straight line with a slope of 11.8 $v.^{-1}$ for both diastereoisomers. At pH 3.1, straight line log plots with slopes of 12.0 $v.^{-1}$ for *N*-nitrosoephedrine and 11.0 $v.^{-1}$ for *N*-nitrosopseudoephedrine were obtained. At higher pH values, the log plots showed two and three straight-line segments and occasionally single straight lines. Similar results have been reported for the reduction of aromatic *N*-nitrosohydroxylamines (9).

pH Dependence.—The half-wave potentials and limiting currents of the diastereoisomeric nitrosamines at different pH values are listed in Table II.

The half-wave potentials of both compounds shift to more negative values with increase in pH. It is evident from Fig. 2 that the linear variation of half-wave potential with pH in the acid region (pH 0.60 to 6.82) is divided into three pH ranges. In the pH range 0.60 to 3.53, the half-wave potentials of the isomers are approximately the same. The half-wave potential varies with pH according to the expression $E_{1/2}(E) = -0.668 - 0.110$ pH for *N*-nitrosoephedrine and $E_{1/2}(\psi) = -0.663 - 0.110$ pH for *N*-nitrosopseudoephedrine, where the intercepts are obtained by extrapolation. The half-wave potential of the *erythro* isomer is clearly more positive than that of the *threo* isomer in the pH range 3.70 to 5.70 (acetate buffers); however, the slopes of the $E_{1/2}$ *versus* pH plots are identical, so that $E_{1/2}(E) = -0.863 - 0.055$ pH and $E_{1/2}(\psi) = -0.873 - 0.055$ pH. The half-wave potentials of both isomers are virtually identical in the pH range 5.57 to 6.82 (phosphate buffers), and the linear variation of $E_{1/2}$ with pH is slight; the expression $E_{1/2} = -1.073 - 0.016$ pH applies to both diastereoisomers.

In the alkaline region, only the pH range 8.95 to 10.03 (ammonia buffers) was examined in the present study. The half-wave potentials of both isomers are identical in this region, and there is also a

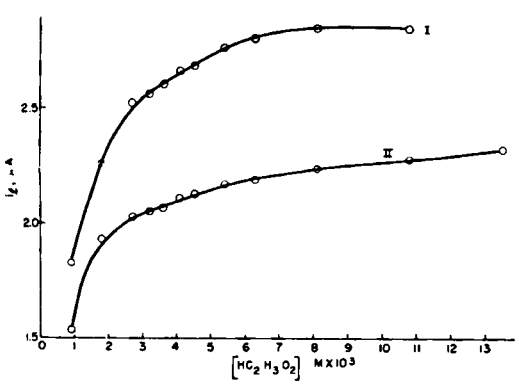


Fig. 4.—Variation of limiting current with concentration of $HC_2H_3O_2$ (−1.35 v. *vs.* S.C.E.). Acetate buffer pH 5.65; ionic strength, 0.261. Curve I: 4×10^{-4} *M* *N*-nitrosoephedrine; curve II: 4×10^{-4} *M* *N*-nitrosopseudoephedrine.

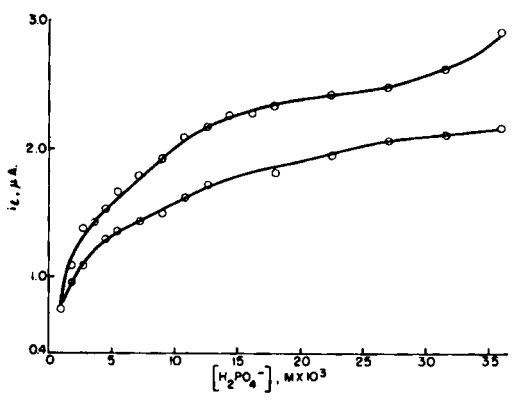


Fig. 5.—Variation of limiting current with concentration of $H_2PO_4^-$ (−1.35 v. *vs.* S.C.E.). Phosphate buffer pH 5.59; ionic strength 0.261. Upper curve, 4×10^{-4} *M* *N*-nitrosoephedrine; lower curve, 4×10^{-4} *M* *N*-nitrosopseudoephedrine.

linear shift of $E_{1/2}$ with pH to more negative values as the pH is increased. The pH-dependence of the half-wave potentials in this region is given by $E_{1/2} = -1.380 - 0.021$ pH.

The effect of pH on the limiting currents is shown in Fig. 3. Since the present study is primarily concerned with kinetic currents in the pH range 3.53 to 8.22, comparisons of limiting currents in the acid region were made at −1.35 v. *versus* S.C.E. Accordingly, it was necessary to extrapolate currents in the pH range 0.60 to 3.70 to −1.35 v. *versus* S.C.E. Comparisons of limiting currents in the alkaline region are at −1.70 v. *versus* S.C.E. The limiting currents for both isomers were identical and constant in the pH range 0.60 to 3.23. With increasing pH, the height of the waves decreased in the manner characteristic of polarographic currents controlled by reaction rate and by diffusion. The striking result is that at a given pH in the range 3.39 to 8.22 under identical conditions, the limiting current for *N*-nitrosoephedrine is invariably greater than the current for *N*-nitrosopseudoephedrine. For each set of buffers in the acid region of the pH-limiting current profile (Fig. 3), the concentration of the buffer acid was maintained constant (Table I).

TABLE III.—DEPENDENCE OF WAVE HEIGHT ON ACETIC ACID CONCENTRATION (−1.35 V. *vs.* S.C.E.) IN ACETATE BUFFERS AT pH 5.65 AND IONIC STRENGTH 0.261

$[HC_2H_3O_2]$ $M \times 10^3$	Wave Ht., μA. *N*-Nitrosoephedrine[a]	Wave Ht., μA. *N*-Nitrosopseudoephedrine[a]
13.5		2.32
10.8	2.84	2.28
8.1	2.84	2.24
6.3	2.80	2.19
5.4	2.76	2.17
4.5	2.68	2.13
4.1	2.66	2.11
3.6	2.60	2.07
3.2	2.56	2.05
2.7	2.52	2.03
1.8	2.26	1.93
0.9	1.83	1.54

[a] Depolarizer concentration 4×10^{-4} *M*.

TABLE IV.—DEPENDENCE OF WAVE HEIGHT ON DIHYDROGEN PHOSPHATE CONCENTRATION (−1.35 V. *vs.* S.C.E.) IN ORTHOPHOSPHATE BUFFERS AT pH 5.59 AND IONIC STRENGTH 0.261

$[H_2PO_4^-]$ $M \times 10^3$	Wave Ht., μA. *N*-Nitrosoephedrine[a]	Wave Ht., μA. *N*-Nitrosopseudoephedrine[a]
36.0	2.93	2.18
31.5	2.64	2.13
27.0	2.50	2.08
22.5	2.44	1.97
18.0	2.35	1.83
16.2	2.30	...
14.4	2.28	...
12.6	2.19	1.73
10.8	2.11	1.63
9.0	1.93	1.51
7.2	1.80	1.44
5.4	1.70	1.36
4.5	1.54	1.30
3.6	1.43	...
2.7	1.38	1.08
1.8	1.09	0.96
0.9	0.77	0.75

[a] Depolarizer concentration 4×10^{-4} *M*.

TABLE V.—DEPENDENCE OF WAVE HEIGHT ON DEPOLARIZER CONCENTRATION (−1.35 V. *vs.* S.C.E.) IN ORTHOPHOSPHATE BUFFER AT pH 7.45 AND IONIC STRENGTH 0.261

Concn., $M \times 10^4$	Wave Ht., μA. *N*-Nitrosoephedrine	Wave Ht., μA. *N*-Nitrosopseudoephedrine
20	0.768	0.673
16	0.626	0.532
12	0.496	0.413
8	0.366	0.307
4	0.248	0.189

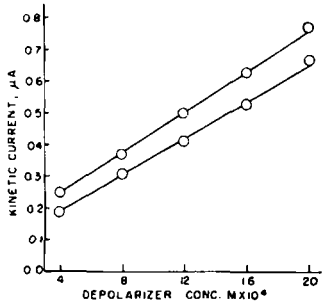


Fig. 6.—The kinetic current in phosphate buffer, pH 7.45, ionic strength 0.261 as a function of *N*-nitrosoephedrine (upper curve) and *N*-nitrosopseudoephedrine (lower curve) concentrations (−1.35 v. *vs.* S.C.E.).

The discontinuity in the pH profile at about pH 5.6 occurs where there is a change from acetate buffers to orthophosphate buffers. It is evident that the limiting current depends on the concentration and type of the buffer acid as well as the hydronium ion concentration.

In the alkaline region, pH 8.95 to 10.03, a linear decrease of limiting current with increasing pH was observed for both isomers. The ammonia concentration was maintained constant in this experiment. It is significant that at a given pH under identical conditions the limiting current of the *erythro* isomer is greater than that of the *threo* isomer.

Variation of Limiting Current with General Acid and Depolarizer Concentration.—The dependence of the limiting current on the concentrations of acetic acid at pH 5.65 and dihydrogen phosphate ion at pH 5.59 are summarized in Figs. 4 and 5 and Tables III and IV. In these experiments, the buffer ratio was maintained constant; constancy of pH was checked throughout the experiment. In each case, at very low concentrations of general acid the limiting currents were very low and increased rapidly with an increase in the general acid concentration. Then followed a range of concentrations of the general acid over which the limiting current proved to be a linear function of the buffer acid concentration. At higher concentrations of the general acid, the rate of change of wave height with concentration of the buffer acid decreased, and the limiting currents appeared to approach a constant value. For a substrate concentration of 4×10^{-4} *M*, it was observed that the limiting current varies linearly with $[HC_2H_3O_2] = 2.7 \times 10^{-3}$ to 5.4×10^{-3} *M* at pH 5.65 and that the limiting current varies linearly with $[H_2PO_4^-] = 4.5 \times 10^{-3}$ to 10.8×10^{-3} *M* at pH 5.59. It is apparent in both cases that the slope of the

TABLE VI.—VARIATION OF AVERAGE LIMITING CURRENT[a] WITH HEAD OF MERCURY FOR *N*-NITROSOEPHEDRINE AT pH 5.59, 7.45, 8.22, AND 9.33 AND IONIC STRENGTH 0.261

	N-Nitrosoephedrine[b]											
h corr.,[c] cm.	pH 5.59[d]		pH 5.59[e]		pH 5.59[f]		pH 7.45[f]		pH 8.22[f]		pH 9.33[g]	
	i_l, μA.	$i_l/h^{1/2}$	i_l, μA.	$i_l/h^{1/2}$	i_l, μA.	$i_l/h^{1/2}$	i_l, μA.	$i_l/h^{1/2}$	i_l, μA.	$i_l/h^{1/2}$	i_l, μA.	$i_l/h^{1/2}$
80.9	2.62	0.291	1.93	0.216	1.55	0.172	0.236	0.0262	0.165	0.0184	2.80	0.311
65.3	2.50	0.309	1.81	0.224	1.44	0.178	0.213	0.0263	0.154	0.0190	2.56	0.317
49.3	2.32	0.330	1.69	0.241	1.38	0.196	0.189	0.0269	0.136	0.0194	2.30	0.328
31.2	2.08	0.371	1.50	0.267	1.26	0.225	0.159	0.0286	0.112	0.0201	1.95	0.349

[a] Data were secured at −1.35 v. *vs.* S.C.E. at pH 5.59, 7.45, and 8.22 and at −1.70 v. *vs.* S.C.E. at pH 9.33. [b] Depolarizer concentration 4×10^{-4} *M*. [c] Mercury height corrected for back pressure; $h_{soln.}$ = 2.5 cm. [d] $[H_2PO_4^-] = 0.036$ *M*. [e] $[H_2PO_4^-] = 0.009$ *M*. [f] $[H_2PO_4^-] = 0.0045$ *M*. [g] $[NH_4OH] = 0.0045$ *M*.

linear plot for the *erythro* isomer is greater than that for the *threo* isomer.

Preliminary experiments were performed to arrive at an estimate of the rate constants of protonation due to water, hydronium ions, and the $H_2PO_4^-$ ion. Several studies of the above type using a constant buffer ratio $[H_2PO_4^-]/[HPO_4^=]$ were made in the narrow pH range 5.59 to 5.91. The slopes of the plot of limiting current against $[H_2PO_4^-]$ were "almost" parallel; slight deviations are undoubtedly due to protonation of the nitrosamines by $HPO_4^=$ ion.

The variation of the limiting currents with the concentration of the substrate was examined at pH 7.45, where the limiting current is largely kinetic current. The limiting current varied linearly with concentration for both diastereoisomers between 4×10^{-4} *M* and 2×10^{-3} *M* (Table V, Fig. 6).

Variation of Average Limiting Current with Head of Mercury.—Wave heights of *N*-nitrosoephedrine were determined at pH values 5.59, 7.45, 8.22, and 9.33 at different mercury column heights from 31.2 to 80.9 cm. (Table VI). The average limiting currents were corrected for variations of residual current with mercury column height, and the mercury head was corrected for back pressure. The experiments in the pH range 5.59 to 8.22 were carried out in phosphate buffers; those at pH 9.33 were done in ammonia buffers. In each experiment a decrease in limiting current and an increase in the ratio $i_l/h^{1/2}$ resulted from a decrease in the height of the mercury column. At pH 5.59, the concentration of dihydrogen phosphate ion was varied from 4.5×10^{-3} to 3.6×10^{-2} *M* to determine the effect of the buffer acid concentration on the variation of the average limiting current with the mercury head. It is seen that with increasing concentration of the buffer acid, the increase in the ratio $i_l/h^{1/2}$ with the decrease in the mercury head becomes more pronounced.

DISCUSSION

Over-all Reactions.—Systematic studies of the polarographic reduction of *N*-nitrosamines of secondary aliphatic and heterocyclic amines were reported by Lund (10) and by Zahradník, Svátek, and Chvapil (11). Based upon controlled potential electrolysis at a macro mercury electrode, Lund proposed that a four-electron reduction yielding a hydrazine derivative occurs in the acid region and that a two-electron reduction yielding the secondary amine occurs in alkaline solution.

$$RR'N{-}N{=}O + 4H^+ + 4e^- \rightarrow RR'N{-}NH_3^+ + 2H_2O$$

$$2RR'N{-}N{=}O + 3H_2O + 4e^- \rightarrow 2RR'NH + N_2O + 4OH^-$$

The same course of the reduction in the acidic region was proposed by Zahradník and co-workers based upon their analysis of the polarographic waves. Analogous to the reaction scheme postulated by Kolthoff and Liberti (12) for the reduction of *N*-nitrosophenylhydroxylamine, Zahradník, *et al.*, suggested that the reduction proceeds in two stages: a two-electron reduction of the nitrosamine to the aminohydroxylamine, followed by a two-electron reduction of the aminohydroxylamine to the hydrazine derivative. In the present study the acid region is considered to extend to pH 8.22. The assumption is made that the hydrazine derivative is the product of the electrochemical reduction.

pH Dependence of Half-Wave Potentials.—The fact that the slopes of the plots of $E_{1/2}$ *versus* pH are the same for both *N*-nitrosoephedrine and *N*-nitrosopseudoephedrine indicates that the number of hydrogen ions which react with the substrate for each electron which is transferred is the same for both diastereoisomers in each of the three pH ranges of the acid region (Fig. 2). It is noted that changes of slope occur with changes of buffer system in the pH region where the current is not entirely diffusion controlled. The first change of slope occurs at about pH 3.5; at this pH the limiting current changes its character from diffusion controlled current to diffusion and kinetic-controlled current. The second change of slope is at about pH 5.7, where the limiting current becomes largely kinetic controlled. At a given pH value in both acetate and orthophosphate buffers, the half-wave potentials were not dependent upon the concentration of the buffer acid, although a slight shift to more negative potentials was observed at high orthophosphate buffer concentrations.

According to Elving (9), no satisfactory interpretation of the magnitudes of the slopes of the $E_{1/2}$ *versus* pH plots is known for the aromatic *N*-nitrosohydroxylamines. The same problem is present in the reduction of the *N*-nitrosamines. The different slopes in the acid region suggest that the reaction mechanisms at the electrode surface change and that the buffer components participate in the reactions.

Current Controlled by Reaction Rate and Diffusion.—It appears from the shape of the pH-limiting current profile (Fig. 3) that the nitrosamine is protonated prior to reduction (11). The unprotonated nitrosamine is either not reduced at the electrode surface, or more likely, it is reduced at greater negative potentials than the protonated compound. The second wave may be due to reduction of the unprotonated compound.

Unlike the kinetic currents of most weak acids which have been studied (13), where the weak acid

and its conjugate base are present in solution, the *N*-nitroso derivatives of aliphatic amines are so weakly basic that the protonated nitrosamine is not present in aqueous solution at pH 0.5 or greater. The ultraviolet absorption spectra of *N*-nitrosoephedrine and *N*-nitrosopseudoephedrine did not change over the pH range 0.60 to 8.22. Hazeldine and Martinson (14) observed that the ultraviolet spectrum of diethylnitrosamine is the same in aqueous neutral, acidic, or alkaline solutions. Zahradník, *et al.* (11), reported that the pK values at 22° of *N*-nitroso derivatives of secondary aliphatic and heterocyclic amines determined in 18.4% sulfuric acid (H_o = −0.80) range from −1.28 to −1.99. Thus, it is concluded that the nitrosamine exists only as the unprotonated species in the bulk of the solution, and that it is only in the field of the electrode that protonation occurs.

The limiting current in the reduction of the protonated nitrosamine is determined by the diffusion of the nitrosamine to the electrode and by the rate of protonation at the electrode surface. In the pH range 0.60 to 3.39 for *N*-nitrosoephedrine and 0.60 to 3.23 for *N*-nitrosopseudoephedrine, the limiting current is invariant with pH change (Fig. 3). The concentration of the hydrogen ion in the electrode double layer is high in this region, and the current is diffusion controlled. It is apparent that the diffusion currents of both diastereoisomers are the same, and it follows that the diffusion coefficients of their conjugate acids are the same. Assuming that four electrons are involved in the reduction, the diffusion coefficient D, was calculated from the classical Ilkovic equation to be 7.36×10^{-6} cm.^{2} sec.$^{-1}$.

With increasing pH, the limiting currents decrease as they are controlled by both diffusion and the rate of protonation. Since the diffusion coefficients of both diastereoisomers are the same, the higher limiting current for *N*-nitrosoephedrine at a given pH value in the range 3.39 to 8.22 under identical conditions indicates that the rate of protonation at the electrode surface of *N*-nitrosoephedrine is greater than that of *N*-nitrosopseudoephedrine.

The fact that the limiting current for the reduction of secondary aliphatic and heterocyclic *N*-nitrosamines in strongly acidic and strongly alkaline solutions is diffusion controlled was established by both Lund (10) and Zahradník, *et al.* (11), who found that the limiting current varied linearly with the square root of the corrected mercury column height. The present study is primarily concerned with the reduction of aliphatic *N*-nitrosamines in aqueous buffers where the current is both diffusion and kinetic controlled. In Table VI, it is seen that the average limiting current decreases and the ratio $i_l/h^{1/2}$ increases as the head of mercury is decreased at pH 5.59, 7.45, 8.22, and 9.33. Clearly, the current is not diffusion controlled in these buffers. The decrease in limiting current with a decrease in the head of mercury is characteristic of currents controlled both by reaction rate and diffusion (15). Unexpectedly, the currents at pH 7.45 and pH 8.22 do not appear to be pure kinetic currents, for the magnitude of the currents are dependent on the mercury head.

At about pH 5.6, there is a discontinuity in the pH-limiting current profile, which indicates that the buffer acids also protonate the nitrosamines at the electrode surface. Since protonation occurs in the potential field of the electrode, the kinetic current due to protonation of the substrate by acetic acid must be larger than the kinetic current, due to protonation by dihydrogen phosphate ion because of the repulsion of the negatively charged ion by the electrode. Consequently, the limiting currents at pH 5.57 in acetate buffers are higher than the limiting currents in orthophosphate buffers at the same concentration of buffer acid.

There are three concentration regions of interest in the plots of limiting currents *versus* general acid concentration (Figs. 4 and 5). At very low concentrations of the general acid, the concentration of the general acid in the electrode double layer is less than that in the bulk of the solution as the electrolysis proceeds. The limiting current in this first region is very low since the catalyst molecule must diffuse to the electrode double layer prior to protonation of the substrate. As the concentration of the buffer acid is increased, the change of the buffer acid concentration in the electrode double layer is relatively small as the electrolysis proceeds, and the concentration of the buffer acid in the electrode double layer can be assumed to be the same as that in the bulk of the solution. The kinetic current in this region varies linearly with the concentration of buffer acid. In the third region, the concentrations of the buffer acid are high, and the limiting current tends to become independent of the buffer acid concentration. The attainment of a constant limiting current is the consequence of reaching a limiting rate of protonation at high catalyst concentrations since the catalyst concentrations are much larger than the substrate concentrations in these systems.

The fact that the slopes of the linear variation of limiting current with the concentrations of acetic acid or dihydrogen phosphate ion are greater for *N*-nitrosoephedrine than for *N*-nitrosopseudoephedrine suggests that the former compound is protonated at a faster rate by these acids.

It is of interest to note that Zahradník and coworkers (11) also observed dependence of the limiting current on phosphate buffer concentration in the polarographic reduction of *N*-nitrosopyrrolidine. However, the reported pH dependency of the wave height showed no discontinuity upon change of the buffer system. In this laboratory, it was observed that the limiting current in the reduction of *N*-nitrosopyrrolidine at pH 5.57 is greater in acetate buffer than in orthophosphate buffer having the same concentration. It is probable that the concentrations of both acetate and phosphate buffers which were used by Zahradník, *et al.* (11), were very high,

TABLE VII—HETEROGENEOUS PSEUDO FIRST-ORDER RATE CONSTANTS OF PROTONATION (−1.35 v. *vs.* S.C.E.) IN ORTHOPHOSPHATE BUFFERS AT pH 5.91 AND IONIC STRENGTH 0.261

$[H_2PO_4^-]$, $M \times 10^3$	*N*-Nitrosoephedrine[c] $(k_r)_h$, cm./sec.$^{-1}$ Exptl.[a]	Calcd.[b]	*N*-Nitrosopseudoephedrine[c] $(k_r)_h$, cm./sec.$^{-1}$ Exptl.[a]	Calcd.[b]
9.0	0.398	0.436	0.306	0.309
7.2	0.388	0.395	0.282	0.280
5.4	0.347	0.353	0.249	0.251
4.5	0.328	0.332	0.238	0.237
3.6	0.309	0.312	0.225	0.223

[a] Experimental values determined by the graphic method from Delahay's equation (15). [b] Calculated values are from the heterogeneous rate constants of protonation of Table VIII. [c] Depolarizer concentration 4×10^{-4} *M*.

TABLE VIII.—ORDERS OF MAGNITUDE OF RATE CONSTANTS OF PROTONATION OF DIASTEREOISOMERIC *N*-NITROSOEPHEDRINES (−1.35 v. *vs.* S.C.E.)

Compd.	$(k_0)_h$, cm. sec.$^{-1}$	$(k_{H^+})_h$, L. cm. moles^{-1} sec.$^{-1}$	$(k_{H_2PO_4^-})_h$, L. cm. moles^{-1} sec.$^{-1}$
N-Nitroso-ephedrine	0.171	4.73×10^4	23.0
N-Nitrosopseudo-ephedrine	0.115	4.07×10^4	15.9

or that the acetate buffer concentration was low and the phosphate buffer concentration was very high.

Orders of Magnitude of Rate Constants of Protonation.—The rate constants of protonation of the nitrosamines can be calculated from the kinetic currents (16), providing the ionization constants of the conjugate acids are known. However, the concept of the ionization constant of the protonated nitrosamines in dilute aqueous solution is meaningless because these compounds are strong acids and are formed only at the electrode surface. Nevertheless, Zahradník, *et al.* (11), using the pK value determined in 18.4% sulfuric acid, calculated the reaction rate constant of protonation of *N*-nitrosopyrrolidine to be 5.0×10^4 L./mole^{-1}/sec.$^{-1}$, and the rate constant of dissociation to be 9.6×10^{15} L./mole^{-1}/sec.$^{-1}$ with Koutecky's equation.

A meaningful estimation of the rate constants of protonation of the diastereoisomeric *N*-nitrosoephedrines can be obtained using the method of Delahay (15), in which heterogeneous rate constants are calculated from the kinetic currents. In this treatment of kinetic polarographic currents, Delahay assumed that the protonation reaction occurs in a monolayer at the electrode surface. The boundary conditions cited apply to the present situation. Delahay's equation for the average current controlled by reaction rate and diffusion is $\bar{\imath} = 1255\beta n m^{2/3} \tau^{2/3} C_0 (k_r)_h$ in which $\bar{\imath}$ is the average current in microamperes, n is the number of electrons involved in the reduction, m is the rate of mercury flow in mg./sec.$^{-1}$, τ is the drop time in second, C_o is the bulk concentration of the substrate in millimoles per liter, $(k_r)_h$ is the heterogeneous rate constant in cm./sec.$^{-1}$, and β is a function $\gamma[1 - \phi\{(k_r)_h\sqrt{\tau/D}\}]$ $\exp[(k_r^2)_h\tau]/D$ in which D is the diffusion coefficient in cm.^{2}/sec.$^{-1}$, where γ is the average current to maximum current ratio, and $\phi\{(k_r)_h\sqrt{\tau/D}\}$ is the error integral $2/\sqrt{\pi} \int_0^{(\tau)kh\sqrt{\tau/D}} e^{-z^2} dz$. With the graphic method (15), rate constants have been calculated for linear segments of the limiting current *versus* $[H_2PO_4^-]$ plots at constant buffer ratios $[H_2PO_4^-]/[HPO_4^=]$ in the pH range 5.59 to 5.91. A typical set of rate constants is presented in Table VII. The rate constant $(k_r)_h$ is the heterogeneous rate constant for a pseudo first-order reaction. For a reaction catalyzed by both hydrogen ions and general acids in orthophosphate buffer $(k_r)_h = (k_o)_h + (k_{H^+})_h[H^+] + (k_{H_2PO_4^-})_h[H_2PO_4^-] + (k_{HPO_4^=})_h[HPO_4^=]$. The slope of the plot of $(k_r)_h$ *versus* $[H_2PO_4^-]$ in the linear region is given by $\{(k_{H_2PO_4^-})_h + (k_{HPO_4^=})_h[HPO_4^=]/[H_2PO_4^-]\}$, and the intercept is given by $\{(k_o)_h + (k_{H^+})_h[H^+]\}$. The pH range 5.59 to 5.91 was selected to yield the best approximation of the rate constant of protonation due to hydrogen ions.

The limiting current in this pH range, while controlled by both diffusion and rate, is largely rate controlled. Delahay (15) determined that when $(k_r)_h\ D^{-1/2}$ is smaller than 0.05 sec.$^{-1/2}$, the current is completely rate controlled, and for $(k_r)_h\ D^{-1/2}$ values between 0.05 and 5 sec.$^{-1/2}$ the current is controlled by both diffusion and rate. The current in these experiments is substantially kinetic current, since the values of $(k_r)_h\ D^{-1/2}$ ranged from 0.083 to 0.187 sec.$^{-1/2}$. It may also be noted that on the pH-limiting current profile (Fig. 3), the current is entirely kinetic current in the pH range 6.65 to 8.22.

The value of $(k_{HPO_4^=})_h$ certainly must be small as a consequence of the repulsion of the doubly charged $HPO_4^=$ ion by the electrode. Nevertheless, the slight deviations from parallel slopes observed in plots of $(k_r)_h$ *versus* $[H_2PO_4^-]$ in the linear region at constant buffer ratios in the pH range 5.59 to 5.91 for both *N*-nitrosoephedrine and *N*-nitrosopseudoephedrine are undoubtedly due to catalysis by $HPO_4^=$ ions. However, the concentrations of $HPO_4^=$ in this pH range are so small that for the purposes of the present approximate calculations, it will be assumed that $(k_{HPO_4^=})_h[HPO_4^=] = 0$ and that $(k_{H_2PO_4^-})_h$ will be taken as an average value of the slopes. From the slopes and intercepts of the $(k_r)_h$ *versus* $[H_2PO_4^-]$ plots, second-order heterogeneous rate constants of protonation were calculated (Table VIII). A further assumption in these calculations is that the reaction at the electrode surface is first order in both hydrogen and $H_2PO_4^-$ ions.

The agreement between experimental and calculated heterogeneous first-order rate constants is satisfactory (Table VII), considering that the average values of the slopes were used to calculate $(k_{H_2PO_4^-})_h$. It can be shown that above pH 5.6, protonation by H_2O molecules represents a major contribution of the kinetic current.

It is of interest to estimate the order of magnitude of the rate constants of protonation of the nitrosamines in terms of "conventional" rather than "heterogeneous" rate constants for comparison purposes. An approximation of the ionization constant K of the protonated nitrosamines ($K = k_d/k_r$, in which k_d and k_r are the rate constants of dissociation and protonation, respectively) is required for the calculation. However, the rate of protonation of the nitrosamine at the electrode surface is quite different from the rate in the bulk of the solution. Accordingly, the requisite ionization constant is the value at the surface of the electrode determined under conditions that the concentrations of hydrogen ions and nitrosamine at the electrode surface are the same as the concentrations in the bulk of the solution. Based upon this interpretation, the value of K to be used in the calculation is the polarographic value; that is, the pK is the pH at which the limiting current is one-half the diffusion current. It should be noted that fluctuation of the hydrogen ion concentration in the vicinity of the electrode surface (15) permits the determination of only an approximate value of the pK.

The polarographic pK could be determined directly from the pH-limiting current profile (Fig. 3) if the reduction were specific acid-catalyzed. How-

TABLE IX.—ORDERS OF MAGNITUDE OF RATE CONSTANTS OF PROTONATION OF DIASTEREOISOMERIC *N*-NITROSOEPHEDRINES (−1.35 V. *vs.* S.C.E.)

Compd.	Polarographic pK	k_o sec.$^{-1}$	k_{H^+} L. mole^{-1} sec.$^{-1}$	$k_{H_2PO_4^-}$ L. mole^{-1} sec.$^{-1}$
N-Nitrosoephedrine	4.67	8.49×10^{-2}	6.50×10^{9}	1.54×10^{3}
N-Nitrosopseudoephedrine	4.61	4.41×10^{-2}	5.52×10^{9}	0.854×10^{3}

ever, the reductions above pH 3.23 are also catalyzed by the buffer acids. In this case, the polarographic pK can be determined from a constructed pH-limiting current profile in which the limiting current at a given pH is the value extrapolated to zero general acid concentration. An alternate procedure is to calculate the pK from the heterogeneous catalytic constants due to hydrogen ion, as described by Delahay (15). According to Delahay, the pH of the solution in which the limiting current is one-half the diffusion current is given by $(pH)_{1/2} = \log (k_{H^+})_h D^{-1/2} - \log [(k_r)_h D^{-1/2}]_{1/2} - 3$, where $(k_{H^+})_h$ is the heterogeneous catalytic constant for H^+, and $[(k_r)_h]_{1/2}$ is the heterogeneous pseudo first-order rate constant for a limiting current equal to one-half the diffusion current. The values of pK, calculated by this latter procedure, were 4.67 and 4.61 for *N*-nitrosoephedrine and *N*-nitrosopseudoephedrine, respectively.

Assuming that the calculated polarographic *K* values are the ionization constants of the protonated nitrosamines at the electrode surface, it is possible to calculate the orders of magnitude of the conventional rate constants of protonation from the heterogeneous rate constants using the relationship (17) $k_r = K/D\ (k_r)_h^2$ in which k_r is in L. mole^{-1} sec.$^{-1}$, when $(k_r)_h$ is in L. cm. mole^{-1} sec.$^{-1}$, and k_r is in sec.$^{-1}$ when $(k_r)_h$ is in cm. sec.$^{-1}$. The conventional rate constants of protonation calculated in this way are listed in Table IX.

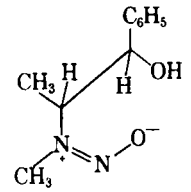
Currents in Alkaline Solution.—Neither Lund (10) nor Zahradník, *et al.* (11), reported currents controlled by reaction rate and diffusion for the reduction of *N*-nitrosamines in alkaline solution. Lund reported that above pH 9, diffusion currents were independent of pH while Zahradník, *et al.*, observed that limiting currents decreased slightly with increasing pH.

A decrease in wave height with increasing pH in the pH range 8.95 to 10.03 was observed in the reduction of both diastereoisomeric *N*-nitrosoephedrines (Fig. 3). Variation of the limiting current with mercury column height (Table VI) indicates that the current in this region is both kinetic and diffusion controlled. The kinetic current for *N*-nitrosoephedrine is greater than that for *N*-nitrosopseudoephedrine. Thus, the diastereoisomers can be distinguished by comparing the limiting currents obtained under identical conditions in alkaline and acidic solutions.

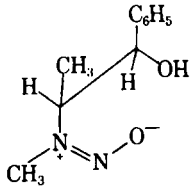
Structural Considerations

While the configurations of (−)-ephedrine and (+)-pseudoephedrine are firmly established, the conformational population of the ground states is still questionable. It is well agreed that the most stable conformation of (+)-pseudoephedrine is the one in which the NH and OH functional groups are gauche. This conclusion is based on crystallographic data (18), infrared (19) and N.M.R. spectra (20), and the results of reaction product studies (21–24). On the other hand, differing views are held regarding the most stable conformation of (−)-ephedrine. The view most widely held, based upon reaction product studies (21–24) and a study of hydrogen bonding in the diastereoisomeric ephedrines by N.M.R. (20), is that the NH and OH groups are in a preferred *trans* conformation. The alternate view, based upon crystallographic data (18) and evidence from infrared spectra that intramolecular hydrogen bonding occurs in ephedrine (19), is that in the most stable conformation of (−)-ephedrine, the NH and OH groups are gauche. The pKa values in 80% cellosolve have been determined to be 9.14 for (−)-ephedrine and 9.22 for (+)-pseudoephedrine; these results have been interpreted in terms of a favored NH, OH *trans* conformation for (−)-ephedrine and a favored NH, OH gauche conformation for (+)-pseudoephedrine (25). However, the very small difference between the pK values suggests that the NH and OH groups are gauche in both diastereoisomers with a greater degree of hydrogen bonding in (+)-pseudoephedrine.

The conformational population of the ground states of the diastereoisomeric *N*-nitrosoephedrines should be the same as that of the diastereoisomeric ephedrines since the conformations are governed by the same nonbonded interactions. Intramolecular hydrogen bonding occurred in both *N*-nitrosoephedrines, suggesting that the N—N = O and OH groups are gauche in both isomers (26), with a higher degree of intramolecular hydrogen bonding in *N*-nitrosopseudoephedrine.



N-Nitrosopseudoephedrine



N-Nitrosoephedrine

It is evident from the structures, that the nonbonded interactions are greater in *N*-nitrosoephedrine. The canonical forms of the *N*-nitrosamines shown above depict the barrier to rotation about the N—N bond. The large contribution of these canonical forms to the structures of the *N*-nitrosamines is indicated by the nonequivalence of the two methyl groups of dimethylnitrosamine, which exhibit two seperate resonances in the room temperature N.M.R. spectrum (27). Intramolecular OH · · · O hydrogen bonding, although involving a seven-membered ring, should stabilize these resonance structures.

Based upon these structural considerations, it is expected that the protonation preceding electron transfer in the electrochemical reduction will take place at the nitroso-oxygen. The compound in which there is the greater degree of hydrogen bonding will be protonated at the slower rate. Accord-

ingly, the kinetic current is expected to be greater for *N*-nitrosoephedrine than that for *N*-nitrosopseudoephedrine. The experimental results confirm this view. In addition, these results suggest that intramolecular hydrogen bonding occurs in both diastereoisomers in dilute aqueous solution, and that the NNO, OH gauche conformation is important in both compounds.

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Coating Pharmaceuticals by Coacervation

By RUSSELL E. PHARES, JR., and G. J. SPERANDIO*

The objectives of this study were to investigate the phenomenon of coacervation as a new means of coating pharmaceuticals and to develop procedures of operation for coating some commonly used drugs. Factors affecting coacervate drop size, some methods of regulating coating thickness, and techniques of controlling the volume of the coacervate produced were determined. Using sodium sulfate solutions and gelatin solutions as the coacervating liquids, five solids and two liquids were coated and processed to produce dry powders.

THE OBJECTIVES of this project were to investigate the phenomenon of coacervation as it is related to the coating of pharmaceuticals and to originate and develop procedures for coating pharmaceuticals by coacervation.

Coacervation is a phenomenon associated with colloidal solutions. A colloidal system is commonly defined as a two-phase system in which one phase is a continuous liquid, while the other phase is a solid which is highly dispersed throughout the liquid as particles ranging in size from 0.001 to 0.5 μ. This definition has been extended to include any (micro) homogeneous system containing colloidal particles or structures derived from them, so that systems containing kinetic units smaller than 0.001 μ may still be considered colloids (1).

In a true solution of macromolecules, that is, a system containing kinetic units smaller than 0.5 μ, certain changes—such as changes of temperature or pH or the addition of substances—can produce a reduction in solubility that will cause a large part of the macromolecules to separate into a new phase. This colloid-rich phase may exist either in a high or low state of dispersion. When a system contains a colloid-rich phase in a low state of dispersion, it is possible to distinguish microscopically and macroscopically between crystallization and coacervation, which is the formation of amorphous liquid drops. These liquid drops constitute the coacervate and under favorable conditions will coalesce in a matter of hours into one clear, homogeneous liquid layer known as a coacervate layer (1).

Coacervates will, in general, accept and surround drops of organic liquids which are immiscible with water, as well as insoluble solid particles, when they are offered in the equilibrium liquid (1). It is this ability of coacervates to coat insoluble materials present in the equilibrium liquid that makes it a method of microencapsulation. "Simple" coacervation as a coating method has been shown to be economically feasible by the National Cash Register Co., Dayton, Ohio,

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ingly, the kinetic current is expected to be greater for *N*-nitrosoephedrine than that for *N*-nitrosopseudoephedrine. The experimental results confirm this view. In addition, these results suggest that intramolecular hydrogen bonding occurs in both diastereoisomers in dilute aqueous solution, and that the NNO, OH gauche conformation is important in both compounds.

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Coating Pharmaceuticals by Coacervation

By RUSSELL E. PHARES, JR., and G. J. SPERANDIO*

The objectives of this study were to investigate the phenomenon of coacervation as a new means of coating pharmaceuticals and to develop procedures of operation for coating some commonly used drugs. Factors affecting coacervate drop size, some methods of regulating coating thickness, and techniques of controlling the volume of the coacervate produced were determined. Using sodium sulfate solutions and gelatin solutions as the coacervating liquids, five solids and two liquids were coated and processed to produce dry powders.

THE OBJECTIVES of this project were to investigate the phenomenon of coacervation as it is related to the coating of pharmaceuticals and to originate and develop procedures for coating pharmaceuticals by coacervation.

Coacervation is a phenomenon associated with colloidal solutions. A colloidal system is commonly defined as a two-phase system in which one phase is a continuous liquid, while the other phase is a solid which is highly dispersed throughout the liquid as particles ranging in size from 0.001 to 0.5 μ. This definition has been extended to include any (micro) homogeneous system containing colloidal particles or structures derived from them, so that systems containing kinetic units smaller than 0.001 μ may still be considered colloids (1).

In a true solution of macromolecules, that is, a system containing kinetic units smaller than 0.5 μ, certain changes—such as changes of temperature or pH or the addition of substances—can produce a reduction in solubility that will cause a large part of the macromolecules to separate into a new phase. This colloid-rich phase may exist either in a high or low state of dispersion. When a system contains a colloid-rich phase in a low state of dispersion, it is possible to distinguish microscopically and macroscopically between crystallization and coacervation, which is the formation of amorphous liquid drops. These liquid drops constitute the coacervate and under favorable conditions will coalesce in a matter of hours into one clear, homogeneous liquid layer known as a coacervate layer (1).

Coacervates will, in general, accept and surround drops of organic liquids which are immiscible with water, as well as insoluble solid particles, when they are offered in the equilibrium liquid (1). It is this ability of coacervates to coat insoluble materials present in the equilibrium liquid that makes it a method of microencapsulation. "Simple" coacervation as a coating method has been shown to be economically feasible by the National Cash Register Co., Dayton, Ohio,

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which produces oil-containing capsules for use in making "carbonless carbon paper" (2–4).

EXPERIMENTAL

Since references to previous pharmaceutical investigations of coacervation as a coating method could not be found, it was often impossible to plan entirely all phases of this work in advance. Many facts which are related to one phase of the work were discovered while working on another aspect of the problem. In this report all experiments and their results have been arranged for the convenience of the reader and therefore are not necessarily presented in the order in which the work was actually done.

In all the experimental investigations, gelatin (Type *A*) solutions were used as the solution of macromolecules. In the first set of experiments the gelatin solutions were coacervated with alcohol. The effects produced by varying the amount of gelatin in a coacervated system while keeping the ratio of water to alcohol constant were first determined. Ten grams, 5 Gm., and 2.5 Gm. of gelatin were each dissolved in 50-ml. portions of distilled water at 50°, and each solution was then coacervated by adding a mixture of 50 ml. of distilled water and 100 ml. of alcohol. After the coacervated systems lwhich were stored at 50°) had separated into two ayers, the only noticeable differences between them were the volumes of their coacervate layers. These volumes varied directly with the amount of gelatin used in the preparation of the original systems.

Experiments in which a constant ratio of gelatin to distilled water was maintained while the amount of alcohol was varied were performed next. The noticeable differences between the systems were again the volumes of the coacervate layers, which in this experiment varied inversely with the volume of ethanol used. A variation in the viscosities of the coacervate layers was also noticed. This variation was directly proportional to the volume of alcohol used in the original systems.

The conditions in the above experiments can be represented on Kruyt's (5) phase diagram for the system gelatin, water, and alcohol (Fig. 1). Line *AE* on the diagram represents all systems having a ratio of ethanol to water of 1:1, and line *DB* represents all systems having a ratio of gelatin to water of 1:19. The farther a point such as *V* is from side *c* of the curve, the smaller will be the proportion of coacervate phase produced in the system that the point represents. Since point *V* lies farther from curve *c* than does point *W*, the system represented by *V* should have less coacervate phase than does the system represented by *W;* and since point *V* represents a system containing a smaller amount of gelatin than does point *W*, the theoretical and experimental results are in agreement. Similarly, it can be seen that the theoretical and actual results for the second experiment are in agreement.

In another series of experiments, factors influencing the size of coacervate drops were studied. The diameters of the coacervate drops were measured using a microscope with a calibrated eyepiece. As soon as a system had been coacervated, it was cooled in an ice bath until it was below 25°. The capsules were thus gelled to prevent the coacervate drops from coalescing on the microscope slide before their size could be measured.

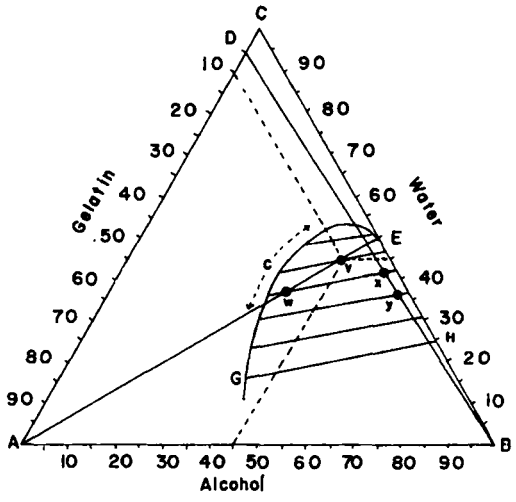


Fig. 1.—Phase diagram of gelatin, water, and alcohol (5).

The proportion of ingredients in a coacervate system had little if any effect on the size of coacervate drops, but the material added to cause gelatin solutions to coacervate could have an effect. When alcohol was added to a gelatin solution, coacervate drops which had a modal diameter of 30–35 μ were formed. Ethanol containing as little as 1% dissolved aspirin produced drops having a modal diameter of 2–3 μ. Solutions of sodium sulfate also produced coacervate drops having a modal diameter of 2–3 μ. Other salt solutions gave the same results as the sodium sulfate solutions. When coacervate drops that had been permitted to coalesce into a single layer were reformed by gentle shaking, they had the same modal diameter as those in a freshly coacervated system. The temperature at which gelatin solutions were coacervated also had an effect on the size of the resulting coacervate drops. As the temperature was raised above 50°, the coacervate droplets became smaller; as the temperature was lowered below 50°, the droplets increased in size.

It is felt that the size of coacervate drops is dependent upon the interaction of many complex physical properties of the entire coacervate system and therefore cannot be attributed to a single controllable factor. The interfacial tension of a coacervated system appeared to have an important effect on the size of coacervate drops; by controlling this property, one may be able to regulate the size of the drops.

Experiments showed that when insoluble drugs were present in a coacervated system, the coacervate drops would surround the suspended particles. When the coacervate drops were gelled by cooling, encapsulation of the drug was complete.

Recovery of the gelled capsules offered considerable problems. Many different filter media were tried, using both gravity and suction filtration; but the gelatin capsules always clogged the pores of the filters. The only successful method of recovering the capsules was to allow them to settle from the surrounding liquid for several hours in a closed container. After the capsules had settled, the upper phase could be decanted.

When the recovered capsules were allowed to air

dry, a very hard, solid mass resulted. Many samples of capsules were washed with cold water, alcohol, formaldehyde, and various proportions and combinations of these materials, but with only occasional—and not necessarily reproducible—success. Treatment with a combination of formaldehyde and alcohol produced a desirable product. The best procedure was to add 1 ml. of formaldehyde to each milliliter of capsules and stir the mixture for 5 minutes. Two milliliters of ethanol were then added for each milliliter of formaldehyde which had been used, and the entire system was stirred for 5 more minutes. The large white floccules which had occurred upon the addition of the alcohol were next removed by gravity filtration through filter paper. Upon drying, the filter cake could be easily crumbled into a fine powder which was washed in a large volume of cold water and again filtered and dried.

The final phase of this study was concerned with actually coating pharmaceuticals and obtaining them as dry powders. The procedure consisted of preparing a solution containing 11 Gm. of sodium sulfate and 45 ml. of distilled water at 37° and another solution containing 4 Gm. of gelatin and 40 ml. of distilled water at 37°. The material to be coated was then thoroughly suspended or dispersed in the warm gelatin solution. Coacervation was effected by the addition of the sodium sulfate solution with stirring. After the coacervated system had been thoroughly mixed, it was cooled by immersion in an ice bath at 10° and stirring steadily until the temperature of the mixture was below 30°. The system was covered with aluminum foil, and the capsules were permitted to separate from the equilibrium phase by gravity. The gelled capsules were then recovered as a dry powder by the method previously described.

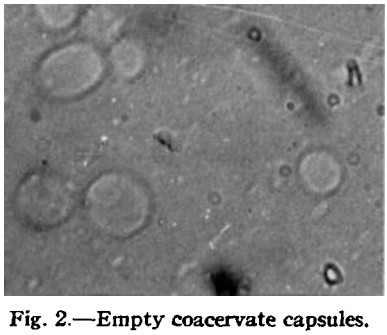


Fig. 2.—Empty coacervate capsules.

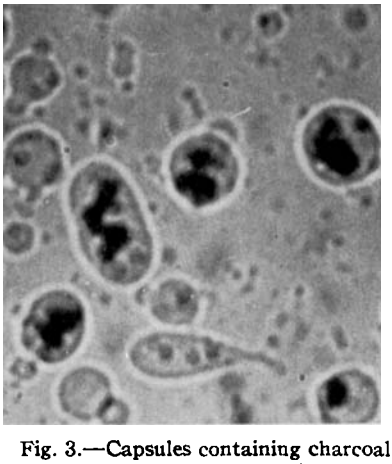


Fig. 3.—Capsules containing charcoal.

The following pharmaceuticals were coated and recovered as fine powders: riboflavin, cod liver oil, procaine penicillin G, carbon tetrachloride, charcoal, micro-ion-exchange resin, and castor oil. Aspirin and acetanilid could not be coated by this method because they were both incompatible with the system being used.

Aspirin was successfully coated and recovered as a fine powder, however, by using a modification of the above system. Two and five-tenths grams of aspirin were suspended in a solution containing 1 Gm. of gelatin and 50 ml. of distilled water at 37°. This system was then coacervated by the addition of 28.5 ml. of distilled water which contained 13 Gm. of sodium sulfate. The aspirin-containing coacervate drops which were produced were gelled and recovered as a powder by the method previously described.

Acetanilid was coated by suspending 1.5 Gm. in a solution containing 1 Gm. of gelatin and 25 ml. of distilled water at 37°. This system was then coacervated by adding 10 Gm. of sodium sulfate dissolved in 50 ml. of distilled water at 37°. The resulting coacervate drops, containing acetanilid, were gelled and recovered as previously described.

During the coating experiments it was noticed that the size of the encapsulated materials had an effect on the diameter of the gelled coacervate drops. By comparing Fig. 2 (which is a photomicrograph of empty coacervate capsules) with Fig. 3 (which shows coacervate capsules containing charcoal) and with Fig. 4 (which shows coacervate capsules containing riboflavin), the effect of "particle" size can be seen. Small coacervate drops like those in Fig. 2 are able to coalesce and form larger drops which will encapsulate large particles or cohering small particles. This phenomenon can be seen in Figs. 3 and 4. Whenever particles smaller than the coacervate drops are present, they are coated singly or as groups without any increase in coacervate drop size. This can be seen in the case of some charcoal particles in Fig. 3.

SUMMARY AND CONCLUSIONS

1. The objectives of the work were to investigate the phenomenon of coacervation as it is related to the coating of pharmaceuticals and to originate and develop procedures to be employed when coating pharmaceuticals by coacervation.

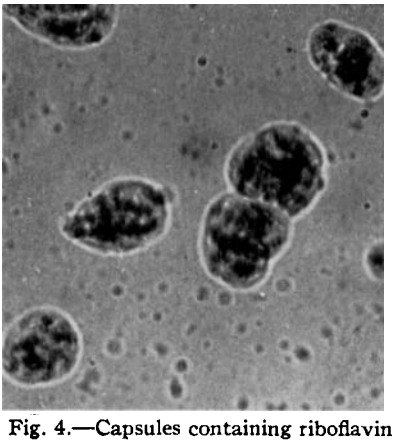


Fig. 4.—Capsules containing riboflavin.

2. A preliminary investigation revealed that many different compounds could be used to coacervate gelatin solutions.

3. By varying the proportion of the components of a coacervate system, the amount of a coacervate phase and/or the composition of a coacervate could be controlled.

4. Almost any insoluble particle can be coated with coacervate drops.

5. Of all the variables investigated, it appeared that only those that affected interfacial tension had an effect on coacervate drop size.

6. Pharmaceuticals can be coated by coacervation, but they may intensify the degree of coacervation.

7. A process was originated for obtaining coacervate-coated pharmaceuticals as fine, dry powders.

8. The following pharmaceuticals were coated and recovered as fine, dry powders: riboflavin, cod liver oil, procaine penicillin G, carbon tetrachloride, charcoal, a micronized ion-exchange resin, castor oil, aspirin, and acetanilid.

9. Coacervation not only seems to be an effective method of coating pharmaceuticals but also a superior method of coating many drugs.

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Preparation of a Phase Diagram for Coacervation

By RUSSELL E. PHARES, JR., and G. J. SPERANDIO*

The objective of this study was to develop a method of preparing a phase diagram of coacervated systems using physical measurements instead of chemical analyses. The per cent weight-in-weight concentration of three-component solutions of micro and macromolecules was related to the refractive indexes and specific gravities of the solutions by an algebraic equation. The composition of both phases of several different coacervated systems was determined by using the derived equations. By making accurate quantitative dilutions, it was also possible to determine the composition of solutions the specific gravity of which did not remain linear because of a high concentration of electrolyte. This study indicates that the points needed for plotting a phase diagram (including tie lines) of three-component systems can quickly and accurately be determined by using measurements of specific gravities and refractive indexes.

Most of the quantitative assay procedures currently available cannot be universally adapted to three-component systems without separating the components. The procedure of Leach and Lithgoe, as corrected by Williams (1, 2), for the determination of the percentage composition of a solution of water plus methanol plus ethanol could be very useful if it were not for the need of preparing extensive tables. If this technique, which uses specific gravity and refractive index, could be improved to eliminate the need of tables, it would be useful not only for the analysis of alcohol but also for obtaining the data necessary for plotting three-component phase diagrams. The role of phase diagrams in pharmacy will become increasingly important as coacervation, which was recently introduced as a pharmaceutical operation (3), is used as a means of obtaining many improved pharmaceuticals.

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The purpose of this work was to develop a simple method of preparing a three-component phase diagram which would eliminate the need for complicated chemical assays of the ingredients. It was felt that the per cent weight-in-weight concentration of a solution of three components could be calculated directly from the experimentally determined specific gravity and refractive index of the solution without using tables.

THEORY

The weight-in-weight composition of an ideal three-component solution in which there is no interaction may be represented by

$$m + n + p = 1 \qquad \text{(Eq. 1)}$$

where m, n, and p represent the grams of solvent, ingredients 1 and 2, respectively, per gram of solution. Therefore

$$m = 1 - n - p \qquad \text{(Eq. 2)}$$

The refractive index of the solution can be written

$$\text{R.I. of solution} = \text{R.I. of solvent} + Cn + Dp$$

or

$$p = (\Delta\text{R.I.} - Cn)/D \qquad \text{(Eq. 3)}$$

2. A preliminary investigation revealed that many different compounds could be used to coacervate gelatin solutions.

3. By varying the proportion of the components of a coacervate system, the amount of a coacervate phase and/or the composition of a coacervate could be controlled.

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Most of the quantitative assay procedures currently available cannot be universally adapted to three-component systems without separating the components. The procedure of Leach and Lithgoe, as corrected by Williams (1, 2), for the determination of the percentage composition of a solution of water plus methanol plus ethanol could be very useful if it were not for the need of preparing extensive tables. If this technique, which uses specific gravity and refractive index, could be improved to eliminate the need of tables, it would be useful not only for the analysis of alcohol but also for obtaining the data necessary for plotting three-component phase diagrams. The role of phase diagrams in pharmacy will become increasingly important as coacervation, which was recently introduced as a pharmaceutical operation (3), is used as a means of obtaining many improved pharmaceuticals.

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THEORY

The weight-in-weight composition of an ideal three-component solution in which there is no interaction may be represented by

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where m, n, and p represent the grams of solvent, ingredients 1 and 2, respectively, per gram of solution. Therefore

$$m = 1 - n - p \qquad \text{(Eq. 2)}$$

The refractive index of the solution can be written

$$\text{R.I. of solution} = \text{R.I. of solvent} + Cn + Dp$$

or

$$p = (\Delta\text{R.I.} - Cn)/D \qquad \text{(Eq. 3)}$$

where C and D are proportionality constants for ingredients 1 and 2, respectively. These constants must be determined experimentally on known samples.

The following equation, which is the third one needed to solve for the three unknowns, is true for dilute solutions and ideal solutions

$$\text{sp. gr. of solution} = \text{sp. gr. of solvent} + AN + BP \quad \text{(Eq. 4)}$$

A and B are proportionality constants for ingredients 1 and 2, respectively, while N and P are the grams of ingredients 1 and 2 per gram of solvent.

$$N = n/(1-n-p) \text{ and } P = p/(1-n-p) \quad \text{(Eq. 5)}$$

It follows from Eqs. 3, 4, and 5 that

$$n = \frac{(\Delta\text{sp. gr.})(C) - (\Delta\text{R.I.})(\Delta\text{sp. gr.} + A)}{(BC - AD) + (\Delta\text{sp. gr.})(C - D)} \quad \text{(Eq. 6)}$$

By using Eqs. 2, 3, and 6, one can find the relative weight-in-weight concentrations of the three components.

EXPERIMENTAL

The experimental work was divided into two parts. Part I entailed verification of Eqs. 3 and 4 and the determination of constants A, B, C, and D for sodium sulfate, gelatin, and water. Part II was the preparation of a phase diagram of sodium sulfate, gelatin, and water, using Eqs. 2, 3, and 6. This coacervate system was selected so that the resulting phase diagram could be compared with the one presented by Bungenberg de Jong (4).

Part I.—The refractive indexes and specific gravities of several sodium sulfate solutions and gelatin solutions of known concentrations were plotted against concentration on rectangular coordinate paper (Tables I–IV and Figs. 1, 2, and 3). As can be seen from the graphs, a straight-line relationship exists between refractive index and concentration of both sodium sulfate and gelatin solutions; a straight-line relationship also exists between specific gravity and concentration of gelatin solutions. It will be noticed, however, that a linear relationship exists between specific gravity and concentration of sodium sulfate solutions only at concentrations less than 10 Gm. of sodium sulfate per 100 ml. of water.

TABLE I.—COMPOSITION OF GELATIN SOLUTIONS FOR DETERMINATION OF CONSTANTS A AND C

Sample	Gelatin, Gm.	Water, Gm.
1	5.00	99.25
2	7.54	98.76
3	9.99	99.25
4	15.00	99.25
5	15.02	99.23

TABLE II.—SPECIFIC GRAVITIES AND REFRACTIVE INDEXES OF GELATIN SOLUTIONS FOR DETERMINATION OF CONSTANTS A AND C

Sample	Sp. Gr.	N^a	R. I.	n^b
1	1.01438	0.0504	1.339443	0.0479
2	1.02026	0.0764	1.343206	0.0710
3	1.02611	0.1007	1.346748	0.0915
4	1.03887	0.1511	1.353621	0.1312
5	1.03993	0.1514	1.353682	0.1315

[a] N = Gm. of gelatin per Gm. of water. [b] n = Gm. of gelatin per Gm. of solution.

TABLE III.—COMPOSITION OF SODIUM SULFATE SOLUTIONS FOR DETERMINATION OF CONSTANTS B AND D

Sample	Sodium Sulfate, Gm.	Water, Gm.
1	2.01	99.46
2	4.01	99.56
3	5.00	99.25
4	6.00	99.54
5	7.99	99.51
6	9.96	99.51
7	9.99	99.25
8	10.01	99.25
9	10.03	99.27
10	14.54	99.25
11	25.11	99.24

TABLE IV.—SPECIFIC GRAVITIES AND REFRACTIVE INDEXES OF SODIUM SULFATE SOLUTIONS FOR DETERMINATION OF CONSTANTS B AND D

Sample	Sp. Gr.	P^a	R. I.	p^b
1	1.01785	0.0202	...	...
2	1.03443	0.0403	...	...
3	1.04323	0.0504	1.338463	0.0480
4	1.05092	0.0603	...	...
5	1.06706	0.0804	...	...
6	1.08311	0.1001	...	...
7	1.08375	0.1006	...	...
8	1.08332	0.1008	1.344637	0.0916
9	1.08378	0.1010	1.344730	0.0917
10	1.12218	0.1465	1.350125	0.1278
11	1.19135	0.2530	1.359930	0.2019

[a] P = Gm. of sodium sulfate per Gm. of water. [b] p = Gm. of sodium sulfate per Gm. of solution.

The refractive indexes of all samples in this work were determined at 37° using an immersion refractometer. All specific gravities were determined at 37° using a 25-ml. pycnometer weighed on a Mettler type TB5 analytical balance. When the constants

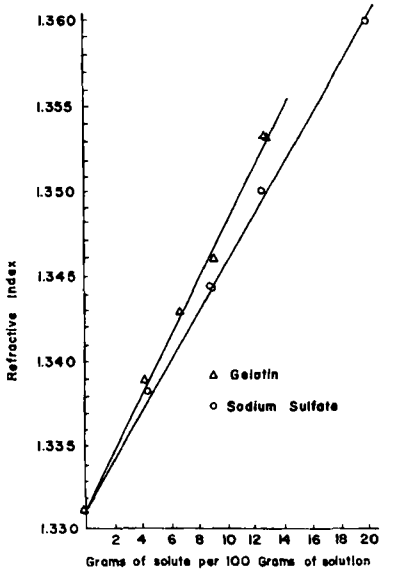


Fig. 1.—Concentration *vs.* refractive index of gelatin and sodium sulfate solutions.

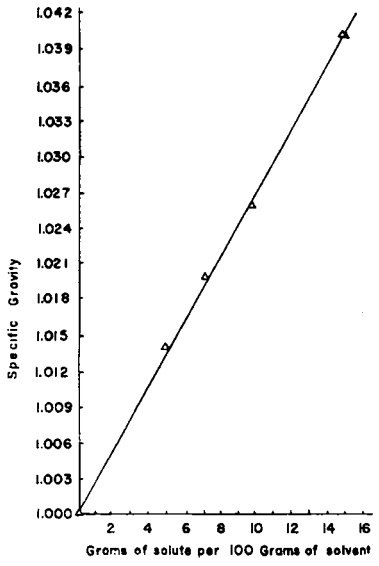


Fig. 2.—Concentration *vs.* specific gravity of gelatin solutions.

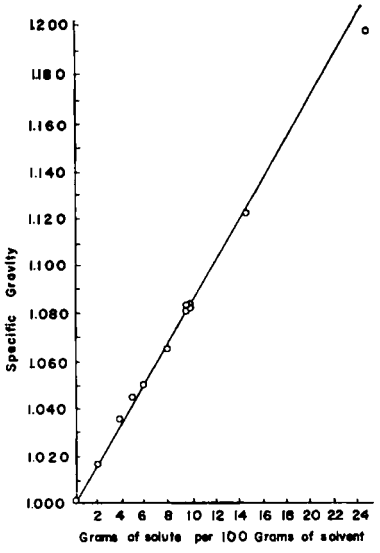


Fig. 3.—Concentration *vs.* specific gravity of sodium sulfate solutions.

A, *B*, *C*, and *D* (which are the slopes of the lines on Figs. 1, 2, and 3) were statistically calculated along with their 99% confidence interval estimates, the following values were obtained: $A = 0.250058 \pm 0.0298$ (gelatin), $B = 0.820425 \pm 0.0128$ (sodium sulfate), $C = 0.169258 \pm 0.0046$ (gelatin), and $D = 0.141455 \pm 0.0024$ (sodium sulfate).

Equations 3 and 6 were verified by using them to predict the concentrations of several dilute solutions, all of which contained known amounts of gelatin, sodium sulfate, and water (the concentrations of gelatin and sodium sulfate were never more than 10% w/w). In all samples tested, the true concentration and the calculated concentration based on Eqs. 3 and 6, using the experimentally determined refractive index and specific gravity of each solution, were within 0.5% of one another.

Part II.—Five samples containing known amounts of water, sodium sulfate, and gelatin (Table V) were prepared and stored at 37° for 5 days. Sample 1 was the only system that did not coacervate. After 5 days, the two phases of the coacervated samples were separated by centrifuging at 5000 r.p.m. for 10 minutes. Since both the equilibrium phase and the coacervate phase of each system was a clear solution, the weight-in-weight composition of each phase was determined in the following manner, using the previously verified equations. From each coacervate phase, a sample weighing approximately 15 Gm. was accurately weighed and then diluted with approximately 100 Gm. of distilled water; the weight of the water was then accurately determined. The specific gravities and refractive indexes of these dilutions were measured. From these measurements, the compositions of the undiluted coacervate phases were calculated using Eqs. 2, 3, and 6 (Table VI). It was necessary to dilute the coacervate phases because they were too viscous to pour into standard pycnometers and because they had specific gravities too high to be linearly related to possible sodium sulfate concentrations.

The composition of each equilibrium phase was calculated using Eqs. 7 and 8. Equation 7 is the equation of the tie line which connects the composition of the total system with the composition of the coacervate phase and the composition of the equilibrium phase. The equation of each tie line

Table V.—Composition of Coacervate Samples Used for Preparing Phase Diagram

Sample	Gelatin, Gm.	% w/w	Sodium Sulfate, Gm.	% w/w	Water, Gm.	% w/w
1	5.88	5.00	11.76	10.0	99.25	85.0
2	5.11	5.00	13.09	11.0	99.27	84.0
3	6.02	5.00	14.46	12.0	99.21	83.0
4	6.10	5.00	15.85	13.0	99.25	82.0
5	6.17	5.00	17.28	14.0	99.24	81.0

Table VI.—Percentage Composition of Each Phase of the Coacervated Systems[a]

	Coacervate Phases, Compn.			Equilibrium Phases, Compn.		
Sample	Gelatin[b]	Sodium Sulfate[b]	Water[b]	Gelatin[b]	Sodium Sulfate[b]	Water[b]
1	Did Not Coacervate					
2	17.00	9.50	73.50	3.00	11.25	85.75
3	22.00	9.50	68.50	2.00	12.25	85.75
4	25.00	9.50	65.50	1.50	13.50	85.00
5	27.00	9.50	63.50	1.00	15.00	84.00

[a] The per cent compositions are given to the nearest 0.25%. [b] The values represent the per cent weight-in-weight concentration of each ingredient.

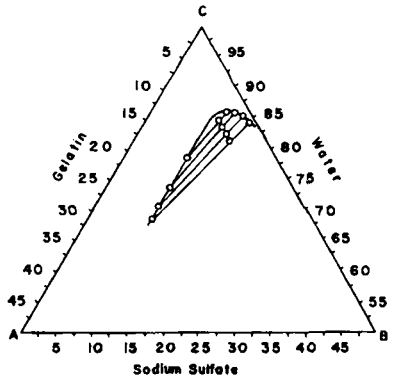


Fig. 4.—Phase diagram of gelatin, water, and sodium sulfate.

$p = \alpha + \delta n$, was determined as shown below by using the total known composition of each coacervated system and the calculated composition of the corresponding coacervate phase. $(p_C = \alpha + \delta n_C) - (p_T = \alpha + \delta n_T)$ can be rearranged to give

$$\frac{p_C - p_T}{n_C - n_T} = \delta$$

and therefore

$$\alpha = p_T - \delta n_T$$

$$p_E = \alpha + \delta n_E \qquad \text{(Eq. 7)}$$

The subscripts C, T, and E represent coacervate phase, total coacervate system, and equilibrium phase, respectively. By combining Eqs. 3 and 7

$$n_E = \frac{\Delta RI - \alpha_D}{\delta D + C} \qquad \text{(Eq. 8)}$$

The compositions of the equilibrium phases are presented in Table VI. The data in Table VI are plotted in Fig. 4.

SUMMARY AND CONCLUSIONS

1. The objective of this work was to develop a rapid method of determining the weight-in-weight concentration of a three-component solution using the experimentally determined specific gravity and refractive index of the solution.
2. Formulas were derived for calculating weight-in-weight concentrations without the need of tables.
3. The formulas are applicable to all three-component solutions in which there is no interaction of the ingredients.
4. In solutions in which there is interaction of the ingredients, such as aqueous solutions of ethanol, or methanol, or sodium sulfate, the weight-in-weight concentration can be determined by using a "diluting technique" which would minimize the effects of interaction.
5. The assay procedure is satisfactory for obtaining the data necessary for plotting three-component phase diagrams.

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Use of 3-Azabicyclo[3.2.2.]nonane in the Mannich Reaction III

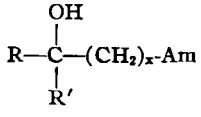
γ-Amino Tertiary Alcohols

By C. DEWITT BLANTON, JR., and W. LEWIS NOBLES

Syntheses of a group of γ-amino tertiary alcohols by application of the Grignard reaction to the corresponding Mannich bases are described. These alcohols are to be screened for possible pharmacological activity.

IN OUR EARLIER publications (1, 2), a number of substituted β-amino ketones and γ-amino secondary alcohols employing 3-azabicyclo[3.2.2]-nonane as the amine moiety were synthesized for pharmacological screening. Denton and his associates (3) have previously established that certain structural modification of β-amino ketones had an effect on physiological activity. The authors wish to report upon the extension of our earlier studies to include the preparation of γ-amino tertiary alcohols.

The general structure of the γ-amino tertiary alcohols prepared by Denton and co-workers (4–10), as well as those presented here, are represented as



Of the tertiary amino carbinols which have been reported in the literature, many have shown

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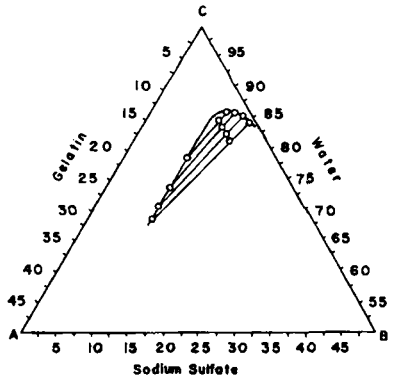


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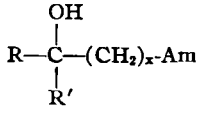
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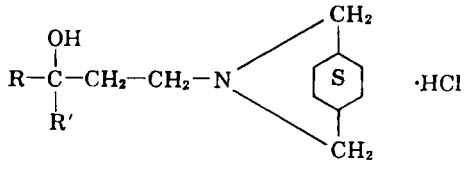
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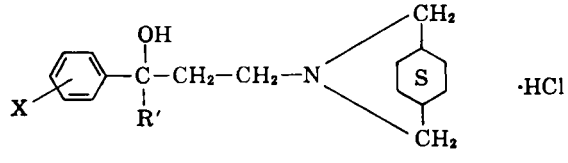
TABLE I.—γ-AMINO TERTIARY ALCOHOLS (HYDROCHLORIDE SALTS)



No.[a]	R	R′	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Methyl	Phenyl	41.5	234–234.5	$C_{18}H_{22}ClNO$	69.79	69.53	9.05	8.98	4.52	...
2	Phenyl	Benzyl	70.0	247–248	$C_{24}H_{27}ClNO$	74.71	74.47	8.31	8.20	3.63	3.75
3	Phenyl	Phenyl[d]	52.1	247–248	$C_{23}H_{25}ClNO$	73.38	73.61	8.17	8.47	3.72	3.79
4	Ethyl	Phenyl	64.8	225–227	$C_{19}H_{24}ClNO$	70.48	70.52	9.27	9.05	4.33	4.50
5	Phenyl	*p*-Methylphenyl	46.2	270–271	$C_{24}H_{27}ClNO$	74.71	74.69	8.31	7.91	3.63	3.55
6	Phenyl	*p*-Methoxyphenyl	40.0	244–245	$C_{24}H_{27}ClNO_2$	71.71	71.82	8.02	7.87	3.48	3.58
7	Phenyl	1-Naphthyl[d]	16.4	253–255	$C_{27}H_{27}ClNO$	75.97	76.10	7.62	7.56	3.28	3.32
8	Phenyl	*p*-Ethoxyphenyl	60.6	240–242	$C_{25}H_{29}ClNO_2$	72.18	72.03	8.24	8.03	3.37	3.55
9	Benzyl	*p*-Methoxyphenyl	39.0	231–232.5	$C_{25}H_{29}ClNO_2$	72.18	72.33	8.24	8.19	3.37	3.44
10	Benzyl	1-Naphthyl[d]	22.4	257–258	$C_{28}H_{29}ClNO$	76.28	76.57	7.83	7.60	3.18	3.17
11	Benzyl	*p*-Ethoxyphenyl	48.7	227–228	$C_{26}H_{29}ClNO_2$	72.62	73.39	8.44	8.67	3.26	3.22
12	Benzyl	2-Naphthyl	52.1	256–259	$C_{28}H_{28}ClNO$	77.12	77.24	7.86	7.78	3.21	3.30
13	Phenyl	*p*-Butoxyphenyl	28.8	214–215	$C_{27}H_{32}ClNO$	73.03	73.24	8.63	8.68	3.15	3.22
14	Benzyl	*p*-Methylphenyl	66.6	228–230	$C_{25}H_{28}ClNO$	75.03	74.90	8.57	8.66	3.50	3.42
15	Phenyl	Cyclohexyl	39.8	250–252	$C_{23}H_{30}ClNO$	73.08	72.65	9.60	9.12	3.71	4.11

[a] All γ-amino tertiary alcohols in this table were recrystallized from an ethanol-acetone or ethanol-ether solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa. [d] Calculated for 0.25 mole of water.

TABLE II.—γ-AMINO TERTIARY ALCOHOLS (HYDROCHLORIDE SALTS)



No.[a]	X	R	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	*p*-Fluoro	Phenyl[d]	36.2	270.5–271.5	$C_{23}H_{23}ClFNO$	70.05	70.02	7.49	7.43	3.55	3.62
2	*p*-Fluoro	Benzyl	70.6	233 –235	$C_{24}H_{21}ClFNO$	71.36	71.44	7.74	8.16	3.47	3.61
3	*p*-Chloro	Phenyl[e]	63.1	281 –283	$C_{23}H_{23}Cl_2NO$	66.03	65.94	7.25	7.08	3.35	3.37
4	*p*-Chloro	Benzyl	60.1	255 –260	$C_{24}H_{21}Cl_2NO$	68.57	68.29	7.38	7.56	3.33	3.31
5	*p*-Bromo	Phenyl	58.7	275 –277	$C_{23}H_{23}BrClNO$	61.26	61.02	6.44	6.22	3.11	3.05
6	*p*-Bromo	Benzyl	62.4	258 –260	$C_{24}H_{21}BrClNO$	62.01	62.08	6.72	6.66	3.01	2.88
7	*p*-Iodo	Phenyl	39.4	272 –277 dec.	$C_{23}H_{23}ClINO$	55.48	55.62	5.88	5.90	2.81	2.64
8	*p*-Iodo	Benzyl	36.6	244 –244.5	$C_{24}H_{21}ClINO$	56.31	56.56	6.10	5.93	2.74	2.69

[a] All γ-amino tertiary alcohols in this table were recrystallized from an ethanol-acetone or ethanol-ether solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa. [d] Calculated for one-fourth mole of water. [e] Calculated for two-thirds mole of water.

greater antispasmodic activity than the ketones from which they were derived (9–11). Some are suitable for the treatment of Parkinsonism and also for the treatment of similar tremors brought about as side-effects by certain tranquilizing drugs (12, 13). Numerous amino carbinols have been synthesized and reported (14–20) to exhibit pharmacological activity—such as antispasmodics, analgesics, antihistaminics, and local anesthetics. Some of the amino carbinols were not of interest, but they have been reported as useful intermediates for preparation of active agents. Extension of the present work to include such derived products will be presented in subsequent publications.

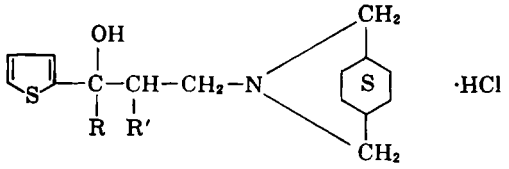
PHARMACOLOGICAL RESULTS

Only partial results from the preliminary biological screening are available.[1] Results of tests for activity as antispasmodics, analgesics, and local anesthetics are not available and will be the subject of future publications. None of the γ-amino tertiary alcohols submitted for screening have exhibited the antimicrobial activity or toxic properties observed for the corresponding Mannich bases.[2] Compound 2 (Table II) was considered in preliminary studies as a good enzyme inhibitor of cholesterol biosynthesis. Further tests revealed no significant ability to lower total plasma or liver cholesterol content. Compound 5 (Table II) was characterized as having marginal ability for enzyme inhibition of cholesterol biosynthesis. Interesting pharmacodynamic and/or chemotherapeutic properties were not reported in the limited amount of data available.

[1] The pharmacological results reported in this section were supplied by Dr. Paul N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

[2] Unpublished data.

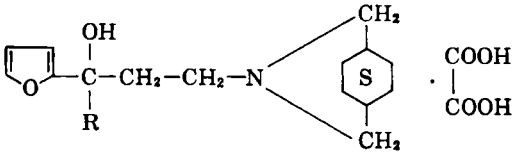
TABLE III.—γ-AMINO TERTIARY ALCOHOLS



No.[a]	R	R′	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Phenyl	Hydrogen	36.2	230–232 dec.	$C_{21}H_{23}ClNOS$	66.73	66.60	7.47	7.52	3.71	3.91
2	Benzyl	Hydrogen	82.3	227–228.5	$C_{22}H_{25}ClNOS$	67.41	68.17	7.71	7.89	3.57	3.41
3	*p*-Methylphenyl	Hydrogen	22.4	246–247	$C_{22}H_{25}ClNOS$	67.41	66.94	7.71	7.55	3.57	3.34
4	Phenyl	Methyl	10.4	244–245	$C_{22}H_{25}ClNOS$	67.41	67.34	7.71	7.57	3.57	3.28
5	Benzyl	Methyl	34.9	225–226	$C_{23}H_{27}ClNOS$	68.04	68.55	7.99	8.06	3.45	3.44

[a] All γ-amino tertiary alcohols in this table were recrystallized from an ethanol-acetone or ethanol-ether solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

TABLE IV.—γ-AMINO TERTIARY ALCOHOLS



No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Phenyl[d]	44.0	125–127	$C_{22}H_{29}NO_6$	65.79	65.91	7.03	7.09	3.35	3.38
2	Benzyl[e]	63.7	93– 95	$C_{24}H_{31}NO_6$	63.74	63.30	7.40	7.12	3.10	3.13

[a] All γ-amino tertiary alcohols in this table were recrystallized from an ethanol-acetone or ethanol-ether solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline & French Laboratories, Philadelphia, Pa. [d] Calculated for 1.25 moles of water. [e] Calculated for 1.25 moles of water.

EXPERIMENTAL

The preparation of γ-amino tertiary alcohols involves the reaction of a β-amino ketone with a Grignard reagent. Six different reagents were utilized in the preparation of this series of compounds: methylmagnesium bromide, ethylmagnesium bromide, phenylmagnesium bromide, benzylmagnesium chloride, *p*-methylphenylmagnesium bromide, and cyclohexylmagnesium bromide. Only the first three were obtained commercially. Higher yields were generally obtained when the reagent was prepared immediately before use. The procedure of Gilman and Catlin (21) was followed for the preparation of the Grignard reagents.

The general method of Pohland and Sullivan (19, 22) for the preparation of γ-amino tertiary alcohols was employed as described by Rogers and Nobles (23). The parent β-amino ketones used for the preparation of the γ-amino tertiary alcohols reported at this time were presented in paper I (1) of this series; three exceptions are the ketones used for preparation compounds 7 and 8 (Table II), 1 and 2 (Table IV), and 13 (Table I). For compound 3 (Table I), it was first necessary to prepare *p*-butoxyacetophenone. This was accomplished by the procedure of Allen and Gates (24). The preparation and characterization of *p*-butoxyacetophenone have been reported by Profft (25) and Bockstahler (26). For the preparation of compounds 7 and 8 (Table II), it was necessary to prepare *p*-iodoacetophenone. This was accomplished by the procedure of Adams and Noller (27).

β - [3 - (3 - Azabicyclo[3.2.2.]nonyl)] - 4 - (n - butoxy)propiophenone.—From the alkoxy ketone, the β-amino ketone (Mannich base) was prepared in a 69.6% yield by the procedure described earlier (1). A melting point of 193–195° was recorded.

Anal.—Calcd. for $C_{21}H_{32}ClNO_2$: C, 68.92; H, 8.86; N, 3.87. Found: C, 68.82; H, 8.76; N, 3.87.

β - [3 - (3 - Azabicyclo[3.2.2.]nonyl] - 1 - (furyl) - 1-propanone.—This β-amino ketone used for the preparation of tertiary alcohols 1 and 2 (Table IV) was prepared in a 59.7% yield by the procedure previously outlined (1). The compound melted with decomposition at 211–213°.

Anal.—Calcd. for $C_{15}H_{22}ClNO_2$: C, 63.48; H, 7.81; N, 4.94. Found: C, 63.58; H, 7.75; N, 5.07.

β - [3 - (3 - Azabicyclo[3.2.2.]nonyl] - 4 - iodopropiophenone.—This β-amino ketone used for the preparation of tertiary alcohols 7 and 8 (Table II) was prepared in a 38.2% yield by the procedure previously outlined (1). The compound melted with decomposition at 228–230°.

Anal.—Calcd. for $C_{17}H_{23}ClINO$: C, 48.63; H, 5.48; N, 3.34. Found: C, 48.34; H, 5.53; N, 3.53.

Tertiary alcohols of the three types described in paper I (1) are listed in Tables I–IV.

For the isolation of the γ-amino tertiary alcohols of the furan derivatives (Type II), the product of the Grignard reaction had to be treated with oxalic acid instead of hydrogen chloride. When attempts to isolate the product as the hydrochloride were made, only resinous and tarry products were observed. It appears that under such acid conditions the furan derivatives are readily decomposed. This is not an

unexpected characteristic for furans. The procedure for the isolation of the neutral oxalates was patterned after that of Adamson (16).

SUMMARY

A total of 31 γ-amino tertiary alcohols which are to be screened for possible pharmacodynamic or chemotherapeutic activity is presented. It is anticipated that further modifications of these structures and detailed pharmacological reports will be presented in later publications.

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By S. F. QUAN*, M. I. GOLDENBERG, and C. W. ABBOTT

Saline and antibiotic solutions, with and without ascorbic acid, were injected into 7-day-old chick embryos inoculated with *Leptospira icterohaemorrhagiae* 1 day after infection. All inoculations were made into the yolk sac. The treated embryos were observed for prolongation of life and for survival rates over a period of 12 to 14 days. Demethylchlortetracycline was about twice as active as tetracycline and oxytetracycline and about 10 times as active as chlortetracycline. Ascorbic acid alone did not influence the course of the infection and did not affect their therapeutic activity when given with antibiotics.

DEMETHYLCHLORTETRACYCLINE (DMCT), a recently introduced commercial antibiotic, is reported to have advantages over the older tetracycline antibiotics with respect to chemical stability, antibacterial activity, and efficiency of maintaining an effective serum level (1–4).

The chemical structure of DMCT is identical to that of chlortetracycline with the absence of a methyl group in the 6 position of the tetracycline molecule. While DMCT has not been reported to have been tested in the chemotherapy of leptospirosis, chlortetracycline (CT), oxytetracycline (OT), and tetracycline (TC) have been effective against experimental leptospiral infections in chick embryos, hamsters, guinea pigs, dogs, and cattle (5–10). Furthermore, Howarth (11) was successful in clearing swine carriers of *Leptospira pomona* with CT and OT, while Stoenner and his associates (12) eliminated *L. ballum* from a naturally infected colony of mice using CT.

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unexpected characteristic for furans. The procedure for the isolation of the neutral oxalates was patterned after that of Adamson (16).

SUMMARY

A total of 31 γ-amino tertiary alcohols which are to be screened for possible pharmacodynamic or chemotherapeutic activity is presented. It is anticipated that further modifications of these structures and detailed pharmacological reports will be presented in later publications.

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DEMETHYLCHLORTETRACYCLINE (DMCT), a recently introduced commercial antibiotic, is reported to have advantages over the older tetracycline antibiotics with respect to chemical stability, antibacterial activity, and efficiency of maintaining an effective serum level (1–4).

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Also, CT and OT have been used with success against leptospiroses in man (10, 13).

In our previous study (14) we found the activities of OT and TC about the same, but many times more active than CT. This finding was similar to that of Kiser, *et al.* (15), who reported TC more active than CT against *L. icterohaemorrhagiae* and *L. pomona* in chick embryos. However, in both of these studies the more active tetracycline antibiotics were administered in a solution containing ascorbic acid as a buffer, while the solutions of CT were given without buffer. It was not known whether ascorbic acid has an effect on the infection or the therapeutic quality of the drugs.

This report is concerned with (*a*) the effect of ascorbic acid on chick embryos inoculated with a suspension of *L. icterohaemorrhagiae*, (*b*) the efficacy of DMCT in solutions with and without ascorbic acid, and (*c*) a comparison of DMCT with CT, OT, and TC in dosage ranges capable of producing a significant prolongation of mean life and protecting 50% of the infected chick embryos.

MATERIALS AND METHODS

Ascorbic Acid.—Commercial packages of TC are available in vials containing 100 mg. of tetracycline hydrochloride with 250 mg. of ascorbic acid, 250 mg. TC HCl with 750 mg. ascorbic acid, and 500 mg. TC HCl with 1.5 Gm. of ascorbic acid. In this study ascorbic acid was given in the amount of 3 mg. for each milligram of tetracycline antibiotic per egg. When ascorbic acid was given alone, 4 mg. per egg was used. The latter solution was prepared from a 200 μm. stock solution which contained 1.76 Gm. of ascorbic acid, ACS grade, in 50 ml. of distilled water. The pH of this solution was 2.5. It was sterilized by filtration, stored in air-tight vials in the refrigerator, and used during a period of 2 months with good results. For use, 4.0 ml. of the stock solution was partly neutralized with 0.35 ml. of 2 *N* NaOH and diluted with 2.7 ml. of sterile physiological saline. Each embryo was given 0.2 ml. of this solution as the control treatment with ascorbic acid.

Infection.—The infectious agent, the test animals, and the methods of infecting and treating the embryos have been described in detail in a previous paper (14). Briefly, the method used to infect the eggs was as follows. A fresh culture of *L. icterohaemorrhagiae*, strain LT-331, was transplanted from Fletcher's to Stuart's medium and grown for 7 days at 28°, then diluted 1–10 with 1% peptone water for infecting eggs. Fertile White Leghorn eggs were purchased unincubated from a commercial hatchery. On arrival they were incubated for 7 days, then candled before they were inoculated in the yolk sac with 0.2 ml. of spirochetal suspension. On the following day the infected embryos were candled, randomized, and then treated. The treated eggs were incubated upright, and candled daily for the next 12–14 days to check their viability.

Antibiotics.—Sterile powders of the hydrochloride salts of the antibiotics were used in this study. The powders were kept free of moisture in the refrigerator. For each test, fresh solutions were prepared using sterile physiological saline.

Antibiotic solutions containing ascorbic acid were prepared by adding appropriate volumes of the ascorbic acid stock solution to weighed samples of the antibiotic, so that for each milligram of antibiotic hydrochloride there was 3 mg. of ascorbic acid.

Treatment of infected embryos was effected by inoculating 0.2 ml. of the appropriate dilutions of antibiotics, with or without ascorbic acid, into the yolk sac on the day following infection. Each embryo was treated only once.

Analysis of Results.—All of the results were analyzed on the basis of the mean life of groups of animals. For purposes of calculating mean life in groups having survivors at the end of the observation period (14 days), surviving animals were considered dead on the 18th day. Also, because deaths occurring before the 5th day have been shown to be due to trauma rather than leptospiral infection, survival measurements were based on the number of viable infected embryos observed on the 4th day. Additionally, the survival times of various treatment groups were analyzed for time-per cent effect by the method of Litchfield (16), and for dose-survival effects by the method of Litchfield and Wilcoxon (17). By the latter method, the survival percentages were based on numbers surviving on the 12th day, as we have found previously that deaths became irregular again beginning on the 12th day. These methods of analysis are identical to those used in our previous paper (14).

The statistical analysis of the effect of ascorbic acid was made on pairs of parameters in which one member of the pair was given ascorbic acid and the other was not. Differences in mean life were considered significant at the 5% level using the Student *t* test. Differences of the ET_{50}'s and of the slope functions were compared, again by the methods of Litchfield, and Litchfield and Wilcoxon (16, 17).

RESULTS

The results of treatments, with and without ascorbic acid, in 12 pairs of infected groups of embryos are shown in Table I. Four of the pairs were treatment controls in which the infected animals were treated with saline or a saline solution of ascorbic acid; the other eight pairs were treated with solutions of antibiotics with or without ascorbic acid.

Analysis of the average life of the controls, as separate pairs or as a total without regard to ascorbic acid, showed that ascorbic acid had no influence on the average life of the embryos inoculated with leptospires. The average life of the saline-treated controls was 6.55 days; of ascorbic acid-treated, 6.38 days; of all controls taken together, 6.46 days. There is no significant difference among these averages even at the 1% level. Furthermore, they do not differ significantly from a mean life of 6.44 days, the average of 25 similar tests conducted over a 3-year period reported previously (14).

The slope functions of the time-per cent effect curves of the control pairs were tested for parallelism by calculating their ratios (16). The slope function

TABLE I.—EFFECT OF ASCORBIC ACID ON ANTIBIOTIC THERAPY OF CHICK EMBRYOS EXPERIMENTALLY INFECTED WITH *L. icterohaemorrhagiae*

Test Date	Antibiotic	Dose/Egg μm.	Dose/Egg mg.	No. Eggs Each	Survival — Without Ascorbic Acid — Mean Life	ET$_s$	S[a]	Survival — With Ascorbic Acid — Mean Life	ET$_{50}$	S[a]	Sig. Dif.[b]
2-23	Control	...	...	20	6.6	6.1	1.11	6.1	5.5	1.16	+
3-16	(saline)	...	...	20	6.2	5.8	1.21	6.4	6.2	1.17	0
4-6		...	...	18	6.5	6.0	1.08	6.4	6.1	1.15	0
4-27		...	...	18	6.9	6.1	1.15	6.6	6.2	1 16	0
4-27	Demethylchlor-	0.05	0.03	14	8.2	7.7	1.15	7.3	7.0	1.18	0
4-27	tetracycline	0.5	0.3	14	14.4	15.6	1.64	14.8	14.4	1.35	0
4-6	Oxytetracycline	0.2	0.09	18	11.6	10.5	1.43	11.4	11.8	1.36	0
4-6		1.0	0.5	18	14.8	15.0	1.45	14.2	15.0	1.60	0
3-16	Tetracycline	0.2	0.08	14	10.8	10.5	1.48	12.0	11.6	1.27	0
3-16		1.0	0.5	14	13.5	13.5	1.60	14.2	14.9	1.27	0
2-23	Chlortetra-	1.0	0.5	19	10.0	8.7	1.22	10.2	9.8	1.16	0
2-23	cycline	2.5	1.3	19	13.6	14.6	1.47	14.2	14.6	1.30	0

[a] S = Slope function (an equivalent of the standard deviation). [b] Significant difference at 5% level; 0 = none of three parameters, + = of ET$_{50}$.

ratios showed that these curves were all parallel within experimental error, and therefore not different from one another. The ET$_{50}$'s were compared by computing the reaction time ratio, *i.e.*, the ratio of the ET$_{50}$'s. In these comparisons of the four control pairs, only one pair showed a significant difference. This difference was found only in the test of 2-23, the first test of this series. The comparative test used is especially sensitive to the accumulation of daily effects. When the 2-23 control pair was analyzed by a *t* test comparing the daily differences, these paired results were also significantly different. As these four pairs of controls were analyzed for differences by three parameters, 12 comparisons were made. Since the addition of ascorbic acid gave no other significant difference, the single exception in 12 observations was given little weight in the over-all evaluation of the action of ascorbic acid on the infection. The average life of none of these control pairs, as already indicated above, was significantly different.

Among the antibiotic-treated embryos, the differences in mean life of embryos with and without ascorbic acid varied from 0.2 days for the pair treated with 1.0 μm. CT to 1.2 days for the pair treated with 0.2 μm. TC. The difference between the ET$_{50}$'s of these pairs varied from zero days for 2.5 μm. CT and 1.0 μm. OT to 1.4 days for 1.0 μm. TC. The difference between the slope functions varied from 0.03 for the 0.05 μm. DMCT pair to 0.33 for the 1.0 μm. TC pair. The differences in this function were less among groups of animals treated with smaller dosages of each of the antibiotics (where less than 15% of the embryos survived the observation period of 14 days) than among those groups treated with larger doses (where about 50% of the embryos survived for 14 days). The range of the differences for the former groups varied from 0.03 for 0.5 μm. DMCT to 0.21 for 0.2 μm. TC, and for the latter groups, from 0.15 for 1.0 μm. OT to 0.33 for 1.0 μm. TC. The wide range of these parameters showed that the experimental error in these drug treatments was large. Nevertheless, within experimental error, statistical analyses showed that ascorbic acid did not have an effect on the therapeutic action of the drugs.

Table II shows the chemotherapeutic effect of various dosages of DMCT in chick embryos inoculated with leptospires, without regard to the effect of ascorbic acid. DMCT given in a dose of 0.05 μm. prolonged the average life of the embryos by 1.5 days and extended the ET$_{50}$ by 1.6 days. The baseline for comparing prolongation of life was established in our previous studies at from 3 to 5 days. In the present study, this level of prolongation of life was found at a dosage of 0.1 μm. of DMCT. With increasing dosages of this drug, not only was there an increase in life, but also the number and

TABLE II.—EFFECT OF TETRACYCLINE ANTIBIOTICS ON THE SURVIVAL OF CHICK EMBRYOS INOCULATED WITH *L. icterohaemorrhagiae*

Compd.	Dose/Egg μm.	Dose/Egg mg.	No. Tests	Survivors on Day 1	4	8	9	12	13	14	Survival — Mean Days	ET$_{50}$	S	Prolonged Life Mean Days	ET$_{50}$
Demethyl-	1.0	0.6	1	13	10	10	10	9	9	5	15.7	...	...	9.3	...
chlortetra-	0.5	0.3	3	37	29	25	24	20	16	14	14.0	...	...	7 6	...
cycline	0.25	0.15	3	41	36	32	31	27	19	13	13.9	16 0	1.68	7.5	10.2
	0.2	0.12	1	14	13	11	11	9	6	4	13.6	13.2	1.41	7.2	7.4
	0.1	0.06	5	63	44	35	28	9	5	0	10.3	10.1	1.34	3.9	5.3
	0.05	0.03	3	39	33	9	1	0	0	0	7.9	7.4	1.13	1.5	1.6
Controls	...	...	8	153	124	1	0	0	0	0	6.4	5.8	1.11	0	0
Tetracycline	1.0	0.5	2	28	20	19	17	15	11	6	13.9	14.1	1.48	7.6	8.1
	0.2	0.09	2	28	25	20	20	10	6	1	11.4	10.9	1.39	5.1	4.9
Controls	...	...	2	40	30	0	0	0	0	0	6.3	6.0	1.22	0	0
Oxytetra-	1.0	0.5	2	36	30	28	26	21	15	15	14.5	15.0	1.51	8.1	9.0
cycline	0.2	0.09	2	36	31	28	26	10	3	2	11.2	11.8	1.39	4.8	5.8
Controls	...	...	2	36	27	0	0	0	0	0	6.4	6.0	1.14	0	0
Chlortetra-	2 5	1.3	2	38	28	27	27	21	16	7	13.9	14.6	1.40	8.1	8.9
cycline	1.0	0.5	2	38	33	28	18	3	2	2	10.2	9.4	1.26	4.4	3.5
Controls	...	...	2	41	34	0	0	0	0	0	5 8	5.7	1 16	0	0

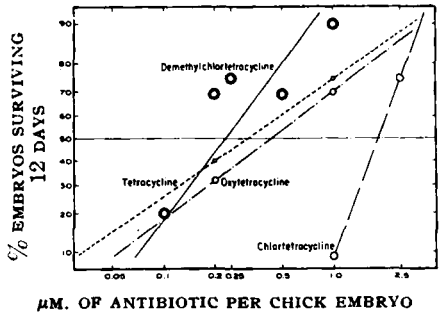


Fig. 1.—Dose-survival rates of chick embryos experimentally infected with *L. icterohaemorrhagiae* and treated with tetracyclines.

percentage of survivors were increased. At the 1.0-μm. per egg dose, 10 of the embryos survived 4 days; of these nine survived 12 days.

Also given in Table II are survival data for embryos treated with three other tetracyclines in doses which can be used to compare them with DMCT. At a dose of 0.2 μm. per egg, the prolongation of mean life and extension of the ET_{50} for DMCT were 7.2 and 7.4 days, respectively; for TC, 5.1 and 4.9 days, and for OT, 4.8 and 5.8 days. At 1.0 μm. the prolongation of mean life for DMCT, OT, and TC was 9.3, 8.1, and 7.6 days, respectively. The weakest activity was shown by CT. Chemotherapeutic activity by the other three antibiotics at levels of 0.2 μm. and 1.0 μm. was approximated by CT at dosages of 1.0 μm. and 2.5 μm., respectively. These data indicate that DMCT was slightly more active than OT and TC and several times more active than CT against *L. icterohaemorrhagiae* in chick embryos.

Figure 1 shows the percentages of embryos surviving on the 12th day after infection in relation to the dose of DMCT. For various reasons already discussed in the first paper of this series, the dose-survival effect data in these tests were heterogeneous. The dose-effect line is drawn by inspection in terms of best fit. Accordingly, the ED_{50} for DMCT is about 0.25 μm. On the other hand, only two doses of the other tetracyclines were studied. The dose-effect lines for these antibiotics were drawn through the two points marking the survival rates of the doses on the 12th day. These lines indicate ED_{50}'s of 0.30, 0.40, and 1.9 μm. for TC, OT, and CT, respectively. These lines and the ED_{50}'s show that DMCT was slightly more active than TC and OT, and all three were more active than CT by five to eightfold.

DISCUSSION AND CONCLUSIONS

Ascorbic acid given in 4-mg. quantities, or about 23 μm. per egg, had no apparent influence on the survival of chick embryos inoculated with *L. icterohaemorrhagiae*. In addition, the therapeutic action of the antibiotics DMCT, CT, OT, or TC was not affected by the incorporation of 3 mg. of ascorbic acid for each milligram of antibiotic used. The preparation of solutions of tetracyclines was facilitated by the addition of ascorbic acid. These observations conform to the known properties of ascorbic acid, which is primarily a vitamin and is often used as a buffer and an antioxidant.

Although the dose-effect curves of TC, CT, and OT were drawn from just two points for each drug in this study, the activities found are in good agreement with those reported previously for these drugs when they were studied in many dosages over a wide range of levels (14). In that study none of the embryos survived after treatment with 0.1 μm. of OT or TC; the ED_{50} of these two compounds was 0.35 μm. In the present study (Fig. 1) the curves for OT and TC predicted the survival of 20 and 25% of the embryos at a dose of 0.1 μm. and ED_{50}'s of 0.3 and 0.4 μm., respectively. When the results of past and present studies are considered together, the activities of OT and TC may be interpreted as the same, and one line can be drawn to represent their dose-effect. This line should be drawn through an ED_{50} of 0.35 μm. and parallel to the line of DMCT to reflect little or no survival at the 0.1-μm. dose. Similarly, the line for CT should be redrawn to encompass the previous findings. For the new CT line, the slope would again be that of the DMCT line, with the ED_{50} at about 3.0 μm. Drawing all of these dose-effect curves with the same slope as that of DMCT infers that all of these compounds act by the same mechanism.

Although there is yet no proof that the chemotherapeutic activity of these drugs is based on a common mechanism, the following factors make this thesis reasonable. (*a*) The same chemical structure, that of tetracycline, is basic for each of these drugs. (*b*) Sweeney, *et al.* (2), found DMCT and CT to have the same antibacterial activity, while other investigators found only minor differences between these tetracyclines (4, 18). (*c*) Eidus, *et al.* (19), found the distribution of all of these compounds to be similar in mouse organs. (*d*) The *in vivo* action of OT and CT on experimental leptospirosis in hamsters was similar, with but minor differences (7, 20). In chick embryos, we have observed in this study the action of DMCT, OT, and TC to be similar and, in the previous study, the similarity of the actions of OT and TC (14).

On the other hand, differences in the activity of the various tetracycline analogs have been reported, although in some cases the differences were minor. These differences in activity are expected, and may be due to the various chemical constituents present or absent on the basic tetracycline structure. In DMCT the presence of the chloro substituent may give the compound greater activity, and the absence of the methyl group may stabilize the molecule. Thus, DMCT has been reported as the most active and most stable of the tetracyclines, whereas CT has been the least stable.

The descending order of biological activity most often listed for these compounds is: DMCT, OT, TC, and CT. As stated above, some of these differences were minor both *in vitro* and *in vivo*. In studies where stability may not have been a factor, the activity of CT was equal to or better than that of DMCT. Sweeney, *et al.* (2), found this equality of action in their antibacterial studies, while Kunin, *et al.* (3), reported similarities in renal clearance and serum binding. Eidus and his associates (19) observed both orders of activity. These investigators reported that the *in vitro* antibacterial spectrum and effectiveness of DMCT and CT resembled one another. In a prophylactic protection test against intraperitoneal challenge of *Diplococcus pneumoniae*,

CT was slightly more effective than DMCT, and each was significantly more effective than OT, TC, and *N*-pyrrolidino-methyl-tetracycline. However, in their study with tubercle bacilli they rated DMCT four times as active as OT and 16 times as active as CT. These latter results approximate those of the present study; DMCT was about two times as active as OT and 10 times as active as CT.

From the above it is clear that the observed activity of DMCT does not only depend upon the chemical structure of the compound alone, but also on the pharmacodynamics of the compound as well as the experimental procedures. Again referring to the report of Eidus and his co-workers (19), it is noted that their results in *in vitro* and *in vivo* tests with *Diplococcus* were clearly supported by their finding that DMCT and CT were retained longer and in higher concentrations than OT, TC, and *N*-pyrrolidino-methyl-tetracycline. In addition, supporting evidence for the retention of DMCT and CT is given in the studies of renal clearance, urinary excretion, and protein-binding by Kunin, *et al.* (3), and Sweeney, *et al.* (2).

On the other hand, the results of Eidus' group with tubercle bacilli, and our results here with leptospires in chick embryos, are better aligned with half-life measurements of the tetracyclines in serum. Kunin and his associates (3) found DMCT to have the longest half-life in human serum, CT the shortest, with TC and OT intermediate. Serum half-life, however, has limited meaning. Many investigators have pointed to differences in tissue and serum concentrations (3, 19, 21). Both Kunin, *et al.* (3), and Eidus, *et al.* (19), reported the tendency of these drugs to sequestrate in body compartments, notably the liver; and Kuemmerle and Contzen (21) found high concentrations in tissues involved in inflammatory processes. Thus, when speaking of the action of these drugs, the activity and stability of the over-all process must be considered.

Some clinical studies reported DMCT in 150-mg. doses to have advantages over TC in 250-mg. doses (2, 22). At variance with these reports, however, are the observations of Roberts and his associates (23). This disagreement challenged some of the former investigators to re-examine the relative merits of these two antibiotics. The additional studies not only confirmed some of the advantages of DMCT, but also provided a better understanding of how these drugs may best be used (24, 25).

From the clinical and experimental reports discussed here, DMCT appears to be a superior tetracycline analog. Being a new drug, however, its real value cannot be determined without the cumulative data concerning possible side effects (26).

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2-Aminobenzenethiol Derivatives as Potential Psychotherapeutic Agents II

By KARL A. NIEFORTH and ROGER C. ROBICHAUD

A similarity between reserpine and the phenothiazine type psychotherapeutic agents is described and illustrated with Stuart-Briegleb molecular models. In an attempt to validate this similarity a series of 2-aminobenzenethiol derivatives was synthesized and screened, resulting in unusual pharmacological properties.

IN A PREVIOUS report (1), a similarity between reserpine and the phenothiazine-type psychotherapeutic agents was described. This relationship involves a five-point correlation which was illustrated by two-dimensional figures and will be further demonstrated here with Stuart-Briegleb molecular models. The correlation is shown by a template (Fig. 1) which is constructed using reserpine as a model. Reserpine was used since it is a relatively rigid structure of known configuration and facilitates the location of the various groups (2). The circles represent the location of the oxgyen on carbon number 11, the aromatic nitrogen, the aliphatic nitrogen, and the carbonyl group attached to position 16. The hash marks show an area of aromaticity. Figure 2 illustrates how reserpine appears when viewed under the template outlining the five areas of interest.

Chlorpromazine has the structural features characteristic of the more active phenothiazine tranquilizers, and when examined under the reserpine template, demonstrates the similarity mentioned before. Figure 3 shows that the sulfur is in the same area as the methoxy of reserpine; the aromatic nitrogen of chlorpromazine occupies the same area as the aromatic nitrogen of reserpine; the aliphatic nitrogens of both compounds are in the same area; the chlorine atom is in the same area as the carbonyl group of reserpine; and there is an area of aromaticity under the hash marks in both compounds. Several types of substituents are found in position two of marketed compounds, such as chloro (chlorpromazine), trifluoromethyl (trifluprom azine), methylmercapto (thioridazine), acetyl (acepromazine), and many others. Due to the possibility of free rotation around the single bonds of the alkyl chain, chlorpromazine could be placed under the template with all of the groups in position, except the chlorine atom, which could be attached to the benzene ring under the hash marks, presenting a situation similar to that existing with the unsubstituted phenothiazines.

The positioning of *trans*-chlorprothixine under the template is illustrated in Fig. 4. The presence of the double bond limits the amount of rotation in this compound, and if three of the groups are in position, the final groups must be in the correct area. This increased rigidity may be responsible for the fact that in some pharmacological tests, such as motor activity reduction upon intraperitoneal injection, *trans*-chlorprothixene is six times as active as chlorpromazine (3).

The *cis* isomer of chlorprothixene is only one-tenth as active as the *trans* isomer or on the order of activity of phenothiazine derivatives without

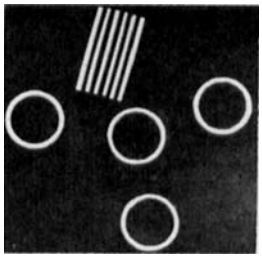


Fig. 1.—Template used to show relationship between reserpine, chlorpromazine, and chlorprothixene.

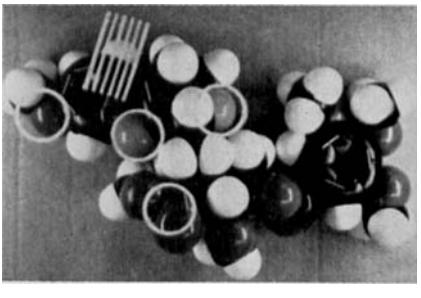


Fig. 2.—Reserpine viewed under the template.

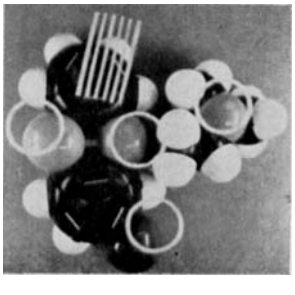


Fig. 3.—Chlorpromazine.

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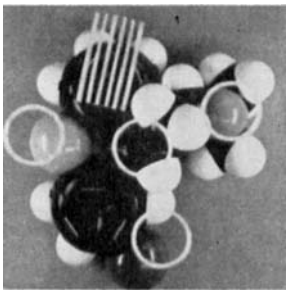


Fig. 4.—*trans*-Chlorprothixene.

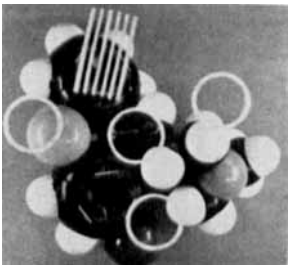


Fig. 5.—*cis*-Chlorprothixene.

substituents on the two position. The sulfur, ethylenic group, and the aliphatic nitrogen could be placed in position, but then the chlorine atom would be attached to the benzene ring under the hash marks. This is similar to the mentioned alternate arrangement of chlorpromazine. Also, as illustrated in Fig. 5, the sulfur, ethylenic group, and the chlorine could be in position and the aliphatic nitrogen would be out of its correct area.

This arrangement is not meant to be an absolute prerequisite for psychotherapeutic activity for compounds which do not have all of the features known to have activity. One such compound is tetrabenazine. Although most of the compounds with these features are tranquilizers, a few (such as imipramine) are stimulants.

A series of derivatives of 2-aminobenzenethiol was synthesized and screened in an attempt to determine the necessity of both aromatic rings of the phenothiazine nucleus. Due to the small number of compounds synthesized and the limited number of animals used in the screening, the interpretations of the pharmacological results must be very general.

EXPERIMENTAL

The synthetic procedures for all of these compounds are thoroughly described in a previous report (1). Nine additional compounds have been synthesized (Table I); the pharmacology of all 17 compounds will be discussed.

The following compounds were reported in the first paper of this series: KN15, methyl *N*-(*N'*-dimethylaminopropyl)-*N*-methylaminophenyl sulfide; KN16, ethyl *N*-(*N'*-dimethylaminopropyl)-*N*-methylaminophenyl sulfide; KN17, isopropyl *N*-(*N'*-dimethylaminopropyl)-*N*-methylaminophenyl sulfide; KN18, methyl *N*-(*N'*-diethylaminopropyl)-*N*-methylaminophenyl sulfide; KN19, ethyl *N*-(*N'*-diethylaminopropyl)-*N*-methylaminophenyl sulfide; KN20, isopropyl *N*-(*N'*-diethylaminopropyl)-*N*-methylaminophenyl sulfide; KN32, ethyl *N*-(*N'*-diethylaminoethyl)-*N*-methylaminophenyl sulfide; and KN33 isopropyl *N*-(*N'*-diethylaminopropyl)-aminophenyl sulfide.

The screening technique used was that of Malone and Robichaud (4), in which the compounds were injected intraperitoneally over a wide range of logarithmically spaced dosages into intact unanesthetized albino rats and the qualitative and semiquantitative symptomology carefully observed using a standardized work sheet.

RESULTS AND DISCUSSION

The dose–time-response profiles of compounds KN15, KN16, KN17, KN18, KN19, KN20, KN29, KN30, KN31, and KN32, although exhibiting slight quantitative differences, are qualitatively identical. Compound KN15 will be described to represent this group.

At 10 mg./Kg., there was a transient increase in motor activity accompanied by a pronounced exophthalmus, mydriasis, and a mild but persistent pilomotor erection. No mitigation of an aggressive personality or drop in body temperature was noticed at this dose.

At 32 mg./Kg., the animal exhibited a pronounced hyperemia of ears and feet, an increase in motor activity, body tremors, exophthalmus, mydriasis, a short burst of clonic convulsions with recovery, pilomotor erection, stereotypy consisting of head shaking, chewing motions, prancing of forelegs, and evidence of disorientation. The peak of stimulation was at approximately 15 minutes after injection. Biphasic activity is evidenced at 1 hour after injection by ataxia and a decrease in motor activity. During this period of decreased motor activity, some of the compounds other than KN15 brought about a perceptible drop in body temperature. Four hours after injection, the animal had returned to an essentially normal state.

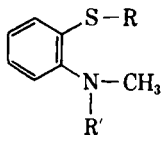
At 100 mg./Kg., an increase in motor activity, hyperemia, tremors, exophthalmus, Straub tail erection, mydriasis, clonic convulsions, apparent salivation, positive Robichaud test, loss of grip strength, and loss of righting reflex were noticed. The animal died of respiratory arrest 10 minutes after injection.

Compound KN33 follows a different dose–time-response pattern. At 32 mg./Kg., there was a general quieting of the animal with enophthalmus, decrease of motor activity, positive Robichaud test, personality change to passivity and a drop of 3.5° in body temperature 1 hour after dosage. The animal was grossly normal 4 hours after the injection.

At 100 mg./Kg., there was a profound blanching of the skin, analgesia, mydriasis, enophthalmus, anoxia-induced clonic convulsions, and death by respiratory arrest 7 minutes after injection.

The piperidine and piperazine compounds KN34 through KN39 were probably the most interesting. KN34 will be described as typical of this group, although slight qualitative and quantitative differences occurred.

TABLE I.—ALKYL 2-DIALKYLAMINOALKYLPHENYL SULFIDES



KN	R	R′	B.p./Pressure	Yield, %	Nitrogen, %—Calcd.	Nitrogen, %—Found
29	CH_3	$CH_2CH(CH_3)CH_2N(CH_3)_2$	144/2.3	56	11.09	10.51
36	CH_3	$CH_2CH_2CH_2$—N⟨ ⟩	174/1.0	68	10.06	10.23
38	CH_3	$CH_2CH_2CH_2$—N⟨ ⟩N—CH_3	156/0.2	44	14.28	14.41
30	C_2H_5	$CH_2CH(CH_3)CH_2N(CH_3)_2$	142/2.3	53	10.51	10.35
37	C_2H_5	$CH_2CH_2CH_2$—N⟨ ⟩	181/0.75	68	9.58	9.94
34	C_2H_5	$CH_2CH_2CH_2$—N⟨ ⟩N—CH_3	144/0.05	47	13.67	13.99
31	$CH(CH_3)_2$	$CH_2CH(CH_3)CH_2N(CH_3)_2$	154/4.0	70	9.91	9.58
35	$CH(CH_3)_2$	$CH_2CH_2CH_2$—N⟨ ⟩	151/0.25	70	9.14	9.47
39	$CH(CH_3)_2$	$CH_2CH_2CH_2$—N⟨ ⟩N—CH_3	156/0.05	70	13.07	13.21

Ten minutes after injection of 10 mg./Kg., there was a transient decrease in motor activity and a general change of attitude from a more or less passive state to a rather fearful one. Thirty minutes after injection, the rat had apparently recovered and was grossly normal. The next day, in addition to a slightly decreased motor activity, the animal displayed a loss of grip strength and pilomotor erection. During the following days, the animal became progressively more excitable and slightly hyperemic. At 7 days after injection the rat seemed unable to remain quiet, continually pranced about, washed itself, etc.

At 32 mg./Kg., there was an immediate decrease in motor activity followed by progressive motor activity depression which reached a peak 30 minutes after injection. At this time the animal lost its grip strength, had mydriasis, pseudoblepharoptosis, blanching, salivation, pilomotor erection, and a loss of body temperature of 4°. During this time the animal would alternate between brief periods of motor activity stimulation and motor activity depression. The following day, the animal had a strange behavioral profile in that it seemed impervious to the investigator's actions. The animal would walk around, bump into objects, fall off bench tops, then remain perfectly still for a short time and then repeat the entire performance. It was felt that this was strongly suggestive of hallucinations. The animal had either the inability or lack of desire to cling to an inverted metal screen.

KN35 had much the same profile as KN34, except the motor activity change was not so pronounced. KN36 was similar to KN34 with perhaps even more "hallucinogenic" activity and less of the hypothermia. KN37 had no "hallucinogenic" activity at 32 mg./Kg., but one animal that survived the 100 mg./Kg. dose was similarly disorientated. KN39 was slightly different from the other compounds in that the lethal dose was 316 mg./Kg., rather than 100 mg./Kg., and that death took place 1 hour after injection, instead of the usual 10 minutes. The animals at the nonlethal doses were very disorientated, had a strongly positive Robichaud test, and a generally emaciated appearance. All animals were sacrificed at the end of 7 days and showed signs of possible hepatotoxicity.

A different animal was used for each dose level. In equivocal cases a second animal was injected. Thus, three rats were used in KN15, three rats were also used in KN37.

While the small number of animals used should not lead one to believe that "statistically" significant results were obtained, it is sufficient, we feel, to point out definite trends of behavior.

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Effects of Interacting Colloids on Transport Rates

By W. I. HIGUCHI

A theoretical analysis along with the resulting equations pertinent to the qualitative and quantitative aspects of the dissolution and deposition of solid materials in colloidal solutions and transport of solutes across diffusional barriers is presented.

IN A NUMBER of situations involving diffusion of a solute, colloidal agents present may be expected to play important roles, such as dissolution and deposition of solid materials in colloidal solutions and transport of solutes across diffusional barriers, physiological or otherwise.

A survey of the literature has shown that the general problem has not received much theoretical consideration. Because of the frequent occurrence of systems involving colloids, an analysis of this problem appeared to be worthwhile. This report gives such an analysis and the resulting equations, which are relatively simple, should be useful both qualitatively and quantitatively in discussing all problems of transport in which colloids are suspected of being important.

NO COLLOID CASE

Figure 1 illustrates the problem of the diffusional transport of a single solute at a concentration $C=C_0$ in compartment I to compartment II where the solute concentration is $C=C_h$. We assume that the mixing rates in compartments I and II are sufficiently rapid, so that the rate will be controlled entirely by the barrier in the region $0 \leq X \leq h$, where X is the coordinate and h is the barrier thickness. Also convection in the barrier is assumed to be negligible.

From the biopharmaceutical standpoint, the barrier may be a membrane, or it may represent the diffusion layer controlling the deposition or dissolution rate of solid materials *in vitro* or *in vivo*. In the latter cases, one of the compartments will be a solid phase.

It is convenient to discuss problems of this type in two portions—the nonsteady state and the steady state.

For the particular case $C=C_0$, $C=C(x)=0$, and $C=C_h=0$ at time = 0, the Fick's law treatment gives (1)

$$\tau = \frac{h^2}{6D} \qquad \text{(Eq. 1)}$$

for the lag time, where D is the diffusion coefficient of the solute in the barrier. The lag time corresponds roughly to the initial nonsteady state period during which simultaneous transfer of the solute through the barrier and concentration buildup of the solute in the barrier is taking place. In this case τ defines the nonsteady state period. Equation 1 assumes that at all times the total amount of solute in the barrier phase is small compared to the amounts of solute in compartment I, and that the diffusion coefficient, D, is independent of concentration in the range of $C(x)$ encountered in the barrier. In most practical situations involving simple diffusion these assumptions are valid.

At times large compared to τ, $\partial C(x)/\partial t = 0$ throughout the barrier, and we have the steady state diffusion case. We may then write for the diffusional transport rate (1)

$$G = \frac{D(K_I C_0 - K_{II} C_h)}{h} \qquad \text{(Eq. 2)}$$

where K_I and K_{II} are the *equilibrium* partition coefficients, K_I = concentration of solute in barrier/concentration of solute in I and K_{II} = concentration of solute in barrier/concentration of solute in II.

When $K_I C_0 \gg K_{II} C_h$

$$G = \frac{D K_I C_0}{h} \qquad \text{(Eq. 3)}$$

Thus, according to Eq. 3 the transport rate of a drug across a lipid barrier separating two aqueous compartments would be directly proportional to the lipid-water partition coefficient, and the initial diffusion controlled dissolution rate of a solid in pure solvent would be proportional to the solubility, $K_I C_0$. As we shall see below, these conclusions may frequently be in serious error when applied to cases involving interacting colloids.

STEADY STATE DIFFUSION WITH COLLOIDS

In most solute dissolution and deposition problems and in many membrane transport problems the steady state considerations alone should explain the behavior of the system.[1] Therefore, while the nonsteady state case will be considered briefly later, let us first examine the steady state situation in detail.

The steady state treatment here differs from that (2) in which the interaction of the solute with reactant results in only one or a few new products. Here one must consider the possibility of a very large number of "species" resulting from the reactions

$$nS + \text{colloid} = [\text{colloid-}S_n]$$

where S is a solute molecule and n may go from unity to a very large number. For the moment, let us assume that there will be only a single species of free colloid. Also we assume that the barrier (or diffusion layer) is pure liquid, except for the solute and colloid.

In Fig. 2 we have broken down the barrier into

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[1] The criteria for this is that we are primarily interested in transport involving amounts of solute much larger than those present in the barrier at any time and that the times for appreciable changes in solute concentration in each of the compartments are large compared to the lag time of the barrier.

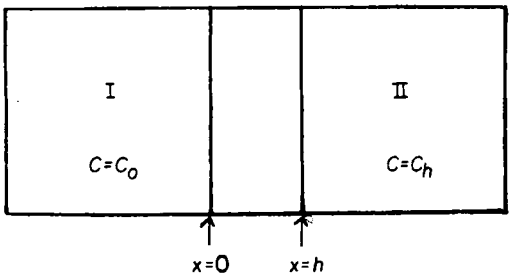


Fig. 1.—Illustration of the problem of diffusion across barrier in the region $0 < x < h$.

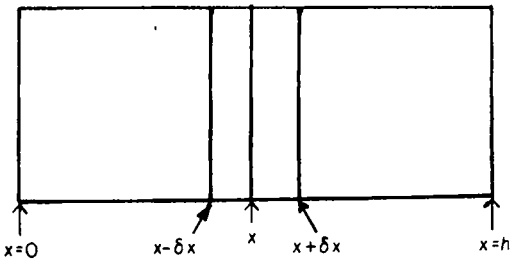


Fig. 2.—Consideration of barrier elements in the region $0 < x < h$. (See text for explanation.)

sublayers of thickness, δx, so that $\delta x \ll h$. Now assume that the interaction between the colloid and solute occurs rapidly enough so that in the element of volume δx there is equilibrium[2] between the bound and free solute. The amount of solute bound per mole of colloid particles, C_b, will then be a function only of the free solute concentration, C, at constant temperature and pressure. As is well known, C_b generally increases with increasing C and goes to zero when C goes to zero.

Steady state requires that no net changes in solute or colloid concentration occur in any element of volume of the barrier with time. This means that what comes into element from one side must leave at the same rate from the other side. Focusing our attention on the plane at x and the two adjacent elements of volume, we see that the rate of diffusion of the colloid across the plane x will be

$$G_c = \frac{D_c(x)\,[M(x - 1/2\delta x) - M(x + 1/2\delta x)]}{\delta x} \qquad \text{(Eq. 4)}$$

where $D_c(x)$ is the average diffusion coefficient for the colloid particle over the two elements of volume and $M(x - {}^1/_2\delta x)$ and $M(x + {}^1/_2\delta x)$ are the average concentrations of the colloid in the two elements.

Now the rate of diffusion of total solute across the plane at x will be

$$G_s = \frac{D_s(x)\,[C(x - 1/2\delta x) - C(x + 1/2\delta x)]}{\delta x} + \frac{D_c(x)\,[C_b(x - 1/2\delta x)M(x - 1/2\delta x) - C_b(x + 1/2\delta x)M(x + 1/2\delta x)]}{\delta x} \qquad \text{(Eq. 5)}$$

where D_s is the diffusion coefficient of the free solute in the medium, $C(x - 1/2\delta x)$ and $C(x + 1/2\delta x)$ are the average free solute concentrations in the two elements, and $C_b(x - 1/2\delta x)$ and $C_b(x + 1/2\delta x)$ are the average amounts of the bound solute per mole of colloid in the two elements. The first term in Eq. 5 is the diffusion rate of the free solute across plane x, and the second term[3] is the rate of solute transport by the "carrier" mechanism.

In the case $\delta x \to 0$ the above equations may be approximated by

$$G_c = D_c(x)\left(\frac{dM}{dx}\right)_x \qquad \text{(Eq. 6)}$$

and

$$G_s = D_s(x)\left(\frac{dC}{dx}\right)_x + D_c(x)\left(\frac{d[C_bM]}{dx}\right)_x \qquad \text{[Eq. 7)}$$

Equation 7 may also be

$$G_s = D_s(x)\left(\frac{dC}{dx}\right)_x + D_c(x)\left[C_b\frac{dM}{dx} + M\frac{dC_b}{dx}\right]_x \qquad \text{(Eq. 8)}$$

It is worthwhile to point out that Eq. 8 shows that even when $G_c = 0$, *i.e.*, no net diffusion of the colloid, there may be a contribution to solute diffusion by the colloid. This is an important special case discussed below.

Special Case of $G_c = 0$, D_c and D_s Independent of C.—This case would apply to the many situations in which there is no net transfer of the colloid itself. Thus, in the dissolution or deposition of a solid the colloid must not be a part of the solid, but acts only as a "carrier" in the solution phase. The assumption of constant D_s would be a good one for dilute solutions (no solute-solute interactions). For D_c to be constant, it would be necessary that the size (hydrodynamic radius) of the colloid be relatively insensitive both to the colloid concentration and to the amount of solute bound in the range of C encountered in the barrier (or diffusion layer). If the colloid is a flexible chain organic polyelectrolyte, if the organic solute is an ion with opposite charge, and if the ionic strength of the medium is low, this assumption may be very poor because the extent of expansion of the polyelectrolyte-solute complex may be very sensitive (3) to the degree of binding. On the other hand, if the colloid is relatively rigid or, if a micelle does not swell appreciably with increased

[2] Most interactions of interest to us—solubilization and physical adsorption—generally are expected to have small free energies of activation. For these cases, the rate of equilibration would be expected to be determined by how rapidly the free solute molecules will diffuse to or away from the colloid particle in question. The corresponding equilibration times then can be shown to be the order of Ka^2/D_s, where a is of the order of the dimensions of the colloid particle, D_s is the solute diffusion coefficient, and K is the colloid-solvent distribution coefficient, treating the colloid as a phase. For commonly occurring values of K and a, these times are indeed generally much smaller than the barrier lag times to be discussed later—unless the barrier thickness, h, is the order of the colloid particle size. This means that there may not be equilibrium for a colloid particle having traveled distances smaller than $L = a(KD_c/D_s)^{1/2}$, where D_c is the diffusion caefficient for the colloid particle; but if L is small compared to h, this should be negligible.

[3] This assumes that the bound solute is transported without loss or gain from one element to the next. Thus, δx must be taken small compared to $L = a\sqrt{KD_c/D_s}$ (see *Footnote* 2), but large compared to Brownian motion distances. This can be done because the latter are much smaller than L. However, C_b now must be interpreted as both a space average (over δx) and a number average (over different extents of equilibration of bound and free solute).

binding and if intercolloidal interactions are negligible, D_c may not vary much.

For this case Eq. 7 becomes

$$G_s = D_s \frac{dC}{dx} + D_c M \frac{dC_b}{dx} \qquad \text{(Eq. 9)}$$

Integration over the boundary conditions consistent with Fig. 1 then gives

$$G_s = \frac{D_s}{h} [K_1 C_0 - K_2 C_h] + \frac{D_c M}{h} [K_1 C_{b0} - K_2 C_{bh}] \qquad \text{(Eq. 10)}$$

where K_1, K_2, and D_s refer to the solvents[4] in the two compartments and the barrier as before. C_{b0} and C_{bh} are the values for C_b at $x = 0$ and $x = h$, respectively.

For two reasons Eq. 10 shows that the conclusions following Eq. 3 may not apply to situations involving colloids. First, D_c may be much smaller[5] than D_s. Second, the binding function C_b may not be linear with respect to C; therefore, $C_{b0} - C_{bh}$ may not be proportional to $C_0 - C_h$.

An extreme example of the first may be obtained from the studies of Wurster and Polli (6). From their experiments on the effect of Norite A on the dissolution rate of benzoic acid, these investigators pointed out that the adsorbent did not influence the dissolution rate as long as $C_h \ll C_0$; *i.e.*, only free solute contributed to the transport rate under these conpitions. Equation 10 is consistent with these conclusions if we note that D_c in this case was several orders of magnitude smaller than D_s. This more than offset the effect of the factor (MC_{b0}) which may have otherwise increased the dissolution rate significantly.

Recent studies (7) on the dissolution rate of dibutyl phthalate droplets in normal saline containing 0.10% polysorbate[6] 20 show that the rate is at least 1 order of magnitude slower than predicted by solubility data. These observations are consistent with the formation of highly swollen micelles or "microemulsions" that diffuse slowly.

It is of interest to note that the above point concerning nonlinearity of rates with respect to $C_0 - C_h$ leads to equations which have been able to describe toe phenomenon of "facilitated" diffusion (8) in biological membranes. If the function, C_b, is assumed to be of the Langmuir type, *i.e.*

$$C_b = \frac{k_1 C}{1 + k_2 C}$$

where k_1 and k_2 are the conventional constants, then Eq. 10 becomes

$$G_s = \frac{D_s}{h} (K_1 C_0 - K_2 C_h) + \frac{D_c M}{h} \left(\frac{K_1 k_1 C_0}{1 + k_2 C_0} - \frac{K_2 k_1 C_h}{1 + k_2 C_h} \right) \qquad \text{(Eq. 11)}$$

The expression in which only the second term of Eq. 11 is important has been employed to explain the transport of sugars through membranes (8).

Other Steady State Cases.—Let us briefly consider other possible situations of interest which follow from the general Eqs. 7 and 8. If in a solid deposition process the colloid is deposited to some extent along with the solute, then the dM/dx term will contribute toward a greater rate. Similarly, the dissolution rate of a solid material containing an interacting colloid will be greater by this term when other conditions (solute solubility, C_h, colloid concentration in solution) remain the same. In both of these instances, if the solid composition and solubilities are known, Eq. 8 may be integrated to give an equation analogous to Eq. 10.

As Eq. 7 applies to the single colloid species problem, it will be necessary to write the following equation for the general case

$$G_s = D_s \frac{dC}{dx} + \sum_i D_{ci} \frac{d[C_{bi} M_i]}{dx} \qquad \text{(Eq. 12)}$$

and if $(dM_i/dx) = 0$ for all i, *i.e.*, no net colloid transport, we obtain

$$G_s = D_s \frac{dC}{dx} + \sum_i D_{ci} M_i \left(\frac{dC_{bi}}{dx} \right) \qquad \text{(Eq. 13)}$$

Therefore, if a mixture of colloidal particles is present, Eq. 13 states that the importance of the particular species in transport is directly proportional to its concentration, diffusion coefficient, and the gradient of its solute binding. On the basis of Eq. 13 one would expect that, for example, deposition of cholesterol in the human body would not be greatly facilitated by high cholesterol levels if most of the cholesterol is distributed among large particles in the physiological fluids, but would be if the solute is bound to the smaller molecules present. Also the *in vivo* release of drugs by dissolution in the gastrointestinal tract may be facilitated by the smaller interacting agents in the intestinal fluids if sufficient binding is present, but to a much lesser extent if the important binding agents are large particles.

NONSTEADY STATE ASPECTS

Let us examine the lag time, τ, for solute diffusion involving colloids for the single colloid species case in which the colloid concentration is constant in the barrier with time and position and where the solute binding is proportional to the free solute concentration. We may write two expressions for the lag time. One is used when the free solute transport is more important, and the other when the "carrier" transport is more important.

When $D_s C_0 \gg D_c M C_{b0}$, the lag time problem would be the same as that for solute diffusion into and through an absorbing emulsion layer (4). Therefore, we may write

$$\tau \simeq \frac{h^2}{6 D_m} \qquad \text{(Eq. 14)}$$

for the same problem leading to Eq. 1, but with the

[4] The local heterogeneous nature of the medium due to the presence of the colloid may be ignored for present purposes if the colloid concentration is low (a few per cent or so). Actually the permeability constant $P = D_s K_1$ in Eq. 10 should be $D_m K_m$, where D_m is the effective diffusion coefficient of the solute in the *colloid solution*, and K_m is the partition coefficient for the compartment I phase and the *colloid solution*. However, $D_s K_1$ and $D_m K_m$ do not differ much for dilute solutions (4). For nonsteady state considerations, this matter must be taken into account because D_m may be much smaller than D_s, even though $D_s K_1 \simeq D_m K_m$.

[5] By about one order of magnitude for high molecular weight macromolecules (3) and micelles (5) and perhaps by two to three orders of magnitude when larger colloidal agents are involved. This follows from the Stokes-Einstein relation, $D_c = kT/6\pi v a$, where k is the Boltzmann constant, T is absolute temperature, a is the hydrodynamic radius of the colloid, and v is viscosity.

[6] Marketed as Tween 20 by the Atlas Powder Co., Wilmington, Del.

barrier containing an interacting colloid material. Now D_m is given by (4)

$$D_m = \frac{P_m}{K_m}$$

where P_m and K_m are the permeability coefficient and partition coefficient for the compartment I phase and the colloid solution barrier phase. It has been shown (4) that P_m and $P = D_s K_1$ would be about the same for dilute colloid solutions, *i.e.*, the steady state rates of the free solute contribution to transport and of the zero colloid case would be essentially the same. However, K_m would be greater than K_1 by the factor

$$\frac{C_0 + MC_{b0}}{C_0}$$

Therefore, τ in Eq. 14 would be greater than the τ given by Eq. 1 by approximately this factor.

When $D_s MC_{b0} \gg D_s C_0$, the lag time for the same problem will be determined by the transport rate of the colloid and we may write

$$\tau \simeq \frac{h^2}{3D_c} \qquad \text{(Eq. 15)}$$

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(1) Jost, W., "Diffusion in Solids, Liquids, and Gases," Academic Press Inc., New York, N. Y., 1952.
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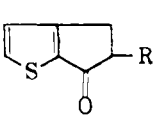
Thiaindanones II

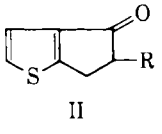
Nitration, Acetylation, and Mannich Reactions

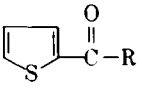
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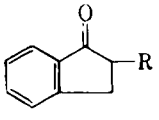
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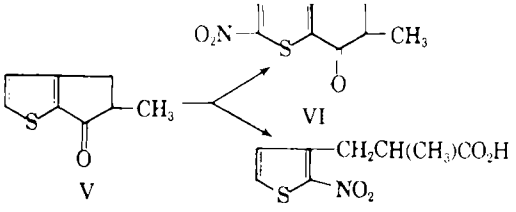

I


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The nitration of 5-methylthiaindanone (V) resulted in both ring substitution (VI) and in ring cleavage (VII). The ratio of nuclear substitution to carbonyl displacement was dependent upon the temperature at which the nitration was performed. At a temperature below −6° (−6 to −15°) only the nuclear substituted nitro derivative was isolated, whereas above 0° (0 to 10°) both carbonyl displacement and ring substitution occurred (Table I).


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Structures VI and VII were assigned to the nitration products on the basis of analysis, neutralization equivalent in the case of VII, and analogy to the nitration products of 2-acetylthiophene.

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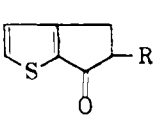
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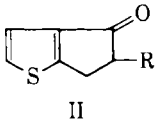
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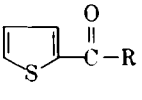
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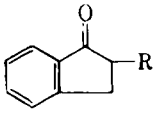
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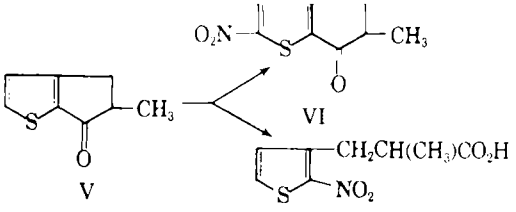

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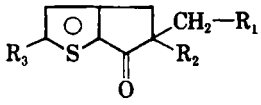
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TABLE II.—THIAINDAN-6-ONE MANNICH BASES

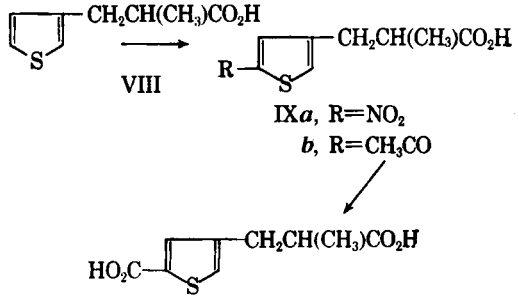


R_1	R_2	R_3	M.p., °C.	Yield, %	Formula	Analyses, % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
$(CH_3)_2N$	CH_3	H	184–186[a]	60	$C_{11}H_{16}ClNOS$[b]	53.81	6.52	5.70	53.69	6.56	5.67
C_4H_8NO[c]	CH_3	H	184–186[a]	30	$C_{12}H_{18}ClNO_2S$[b]	54.26	6.26	...	54.29	6.26	...
$C_5H_{10}N$[d]	CH_3	H	189–190[a]	56	$C_{14}H_{20}ClNOS$[b]	58.84	7.00	...	59.01	7.08	...
$(CH_3)_2N$	CH_3	NO_2	183–185[e]	54	$C_{11}H_{15}ClN_2O_3S$[b]	45.43	5.16	9.63	45.67	5.13	9.16
$(CH_3)_2N$	C_6H_5	H	103–104[a]	57	$C_{15}H_{17}NOS$	70.85	6.24	5.17	70.60	6.45	5.03
$(CH_3)_2N$	H	H	178–179[e]	80	$C_{10}H_{14}ClNOS$[b,f]	51.83	6.04	6.04	52.01	5.88	6.08
$(CH_3)_2N$	CH_3	Cl	215 dec.[a]	72	$C_{11}H_{15}Cl_2NOS$[b]	47.19	5.39	...	47.15	5.56	...
$(CH_3)_2N$	CH_3	H	186–188[e]	72	$C_{11}H_{16}ClNOS$[b,g]	53.81	6.52	...	53.85	6.43	...

[a] Recrystallized from an ethanol-ethyl acetate mixture. [b] Hydrochloride. [c] Morpholino. [d] Piperidino. [e] Recrystallized from ethanol. [f] Calcd.: Cl, 15.33; S, 13.87. Found: Cl, 15.15; S, 13.85. [g] 5-Methyl-5-dimethylaminomethylthiaindan-4-one.

place during the nitration of 2-thiophenecarboxylic acid and 2-acylthiophenes at low temperature (−6°), but is secondary to nuclear substitution in the 5-position.

The nitration of α-methyl-β-(3-thienyl)propanoic acid (VIII) yielded a mononitro product different than VII. The yield was low (25%); most of the starting material was recovered. None of the 2-substituted derivative (VII), however, was isolated. The acylation of VIII also gave a mono acetylated product. The latter was oxidized with sodium hypobromite to yield a diacid. On the basis of earlier work by Hartough and Kosak (2) on the acylation of 3-methylthiophene and the work of Rinkes (3) on the nitration of 3-methylthiophene, we have assigned structures IX*a* and IX*b*, respectively, to the products of the nitration and acylation of α-methyl-β-(3-thienyl)propanoic acid. Consequently, the diacid was assigned structure X.

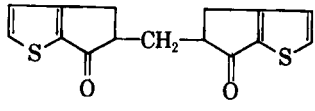


VIII

IX*a*, R=NO_2
b, R=CH_3CO

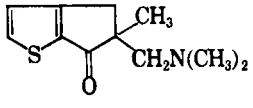
X

The applicability of the Mannich reaction to the thiaindanones was acertained by the preparation of several Mannich bases (Table II). The reactions in ethanol, with one exception, proceeded smoothly and resulted in good yields of the expected products. This procedure, however, with the unsubstituted thiaindan-6-one (I, R = H), resulted in the bis product (XI). The normal Mannich product was obtained when a mixture of benzene and nitrobenzene was used as a solvent.

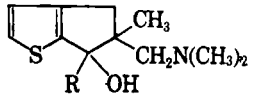


XI

The reduction of the Mannich ketone (XII) with sodium borohydride provided XIII*a*, whereas the reaction of XII with benzyl magnesium bromide yielded XIII*b*. Thus far, the esterification of XIII*a* and XIII*b* by the procedures described by Pohland and Sullivan (4, 5) and the procedure described by Burckhalter and Johnson (6) on related aminoalkanols has been unsuccessful.



XII



XIII *a*, R=H
b, R=$C_6H_5CH_2$

Preliminary studies indicated that the Mannich bases (Table II), in general, exhibit antibacterial activity. A detailed report will be published at a later date.

EXPERIMENTAL[1]

5-Methyl-2-nitro-thiaindan-6-one.—*Method A.*—The procedure employed was essentially that described by Steinkopf and Jaffe (7) for the nitration of 2-acetylthiophene. A cold acetyl nitrate solution (prepared by the careful addition of 40 Gm. of

[1] All melting points were taken on a Fisher-Johns apparatus and are corrected; boiling points are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 infracord spectrophotometer.

fuming nitric acid. sp. gr. 1.50 to 50 ml. of acetic anhydride cooled to −6°) was added slowly with vigorous stirring to a solution of 20 Gm. (0.13 mole) of 5-methylthiaindan-6-one in 75 ml. of acetic anhydride maintained at 10°. After the addition was complete, the reaction mixture was stirred for 30 minutes and then poured, with stirring, into 200 ml. of cold water. The resulting oily layer was extracted 10 times with 40-ml. portions of ether; the combined extracts were washed three times with 100-ml. portions of 10% sodium bicarbonate solution. The ether solution was evaporated to dryness, and the resulting solid was recrystallized fom ethanol to give 5 Gm. (21%) of product, m.p. 98–100°. The infrared spectrum in carbon tetrachloride showed sharp carbonyl absorption at 1740 cm.$^{-1}$, nitro absorption at 1340 cm.$^{-1}$, and the absence of nitro absorption at 1520 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_7NO_3S$: C, 48.76; H, 3.55; N, 7.17. Found: C, 49.06; H, 3.76; N, 7.37.

Method B.—The above procedure was followed, except that the reaction temperature was maintained at −6°. From 15.2 Gm. (0.1 mole) of 5-methylthiaindan-6-one, there was obtained 11.7 Gm. (66%) of product, m.p. 98–100°. A mixed melting point with the product obtained by *Method A* showed no depression.

α-Methyl-β-(2-nitro-3-thienyl)propanoic Acid.—The sodium carbonate wash solutions obtained in *Method A* were neutralized with 10% sulfuric acid and extracted five times with 100-ml. portions of ether. The ether was evaporated and the residual solid recrystallized from water to give 1 Gm. (4.5%) of product, m.p. 135–136°. The infrared spectrum in carbon tetrachloride showed carbonyl absorption at 1690 cm.$^{-1}$, broad hydroxyl absorption beginning at 3400 cm.$^{-1}$ and extending to 3000 cm.$^{-1}$, and sharp nitro absorption at 1520 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_9NO_4S$: C, 44.44; H, 4.18; neut. equiv., 215. Found: C, 44.38; H, 4.17; neut. equiv., 217.

The sodium carbonate solution from *Method B* did not yield acid materials.

α-Methyl-β-(5-nitro-3-thienyl)propanoic Acid.—The procedure followed was that employed by Rinkes (3) for the nitration of 3-methylthiophene. A mixture of 15 Gm. (0.09 mole) of α-methyl-β-(3-thienyl)propanoic acid in 30 Gm. of acetic anhydride was added dropwise to an acetyl nitrate solution (prepared by the addition of 15 Gm. of nitric acid, sp. gr. 1.5, to 25 Gm. of acetic anhydride at 0°). The resulting solution was stirred for an additional 20 minutes at a temperature maintained between 0° and −10°, then poured with stirring into ice water and extracted 15 times with 50-ml. portions of ether. The ether was evaporated and the residual solid recrystallized from water to give 5.0 Gm. (25%) of product, m.p. 95–96°. The infrared spectrum in carbon tetrachloride showed sharp hydroxyl absorption at 3500 cm.$^{-1}$, broad bonded hydroxyl absorption from 3300 to 3000 cm.$^{-1}$, carbonyl absorption at 1750 cm.$^{-1}$, and nitro absorption at 1380 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_9NO_4S$: C, 44.44; H, 4.18. Found: C, 44.68; H, 4.14.

α-Methyl-β-(5-acetyl-3-thienyl)propanoic Acid.—A solution of 34 Gm. (0.2 mole) of α-methyl-β-(3-thienyl)propanoic acid and 20.4 Gm. (0.2 mole) of acetic anhydride was stirred and heated to 60°. The source of heat was removed and 2 ml. of 85% orthophosphoric acid was added dropwise so that the temperature remained below 90°. Thereafter, the mixture was heated to 100° for 2 hours, then cooled to 50°, treated with 40 ml. of water and stirred rapidly for 30 minutes. The oil that separated was washed five times with water, taken up in 150 ml. of 10% sodium carbonate solution, reprecipitated with concentrated hydrochloric acid, and extracted with two 100-ml. portions of ether. The ether was evaporated and the residual oil distilled to give 16 Gm., b.p. 140–160°/0.5–2 mm. and 15 Gm. (35.4%), b.p. 160–180°/2 mm. Redistillation of the higher boiling fraction gave product, b.p. 182°/2.5 mm.; n_D^{28} 1.5548.

Anal.—Calcd. for $C_{10}H_{12}O_3S$: C, 56.60; H, 5.66; neut. equiv., 214. Found: C, 56.34; H, 5.83; neut. equiv., 213.

α-Methyl-β-(5-carboxy-3-thienyl)propanoic Acid.—The procedure employed was adapted from that described by Farrar and Levine (8) for the oxidation of ketones to acids. A sodium hypochlorite solution (prepared by passing 32.2 Gm. (0.45 mole) of chlorine into a solution of 44 Gm. (1.1 moles) of sodium hydroxide in 60 ml. of water) was heated on a steam bath to 55° and treated dropwise with 10 Gm. (0.07 mole) of crude α-methyl-β-(5-acetyl-3-thienyl)propanoic acid so that the temperature did not rise above 70°. Thereafter, the mixture was heated to 70° and maintained at this temperature for 15 minutes. The excess sodium hypochlorite was decomposed by the addition of a solution of 50 Gm. of sodium bisulfite in 40 ml. of water. The solution was made acid to litmus with concentrated hydrochloric acid and extracted twice with 200-ml. portions of ether. The ether was evaporated and the resulting solid recrystallized seven times from water to give 5 Gm. (33%) of white crystals, m.p. 158–160°. The infrared spectrum in carbon tetrachloride showed maxima at 1690 cm.$^{-1}$ and at 1710 cm.$^{-1}$, characteristic of aromatic and aliphatic carboxyl groups.

Anal.—Calcd. for $C_9H_{10}O_4S$: C, 50.47; H, 4.67; neut. equiv., 107. Found: C, 50.52; H, 4.92; neut. equiv., 107.

Thiaindanone Mannich Bases (Table II).—A mixture of 0.1 mole of the requisite thiaindanone, 0.1 mole of the amine hydrochloride, 0.1 mole of paraformaldehyde, 10 ml. of ethanol, and 1 ml. of concentrated hydrochloric acid was refluxed for 3 hours. The reaction mixture either was concentrated and treated with ether or poured, with stirring, into 100 ml. of acetone. The solid was removed by filtration and recrystallized from a suitable solvent.

Bis-(6-oxo-5-thiaindanyl)methane.—A solution of 10 Gm. (0.07 mole) of thiaindan-6-one, 3.0 Gm. (0.1 mole) of paraformaldehyde, 8.15 Gm. of dimethylamine hydrochloride, and 2 drops of concentrated hydrochloric acid in 30 ml. of ethanol was refluxed for 2 hours. The solid which precipitated from the solution was removed by filtration and washed with 10% sodium hydroxide solution and 5% hydrochloric acid. The solid was insoluble in the following hot solvents: ether, ethanol, water, benzene, toluene, dimethyl formamide, acetone, and dioxane. The material (15 Gm., 75%) was refluxed in toluene for 1 hour, filtered, and dried; m.p. 156–158°.

Anal.—Calcd. for $C_{15}H_{12}O_2S_2$: C, 62.47; H, 4.19. Found: C, 62.22; H, 4.37.

5-Methyl-5-dimethylaminomethylthiaindan-6-ol.—A modification of the procedure outlined by Chaikin and Brown (9) for the reduction of aldehydes and ketones was employed. To a solution of 18.9 Gm. (0.5 mole) of sodium borohydride in 400 ml. of 50% methanol, cooled to 20°, was added dropwise, with stirring, a solution of 5-methyl-5-dimethylaminomethylthiaindan-6-one (prepared from 56 Gm. (0.23 mole) of the hydrochloride) in 100 ml. of ethanol. The rate of addition of the ketone was controlled in order to maintain a reaction temperature of about 30°. The mixture, following the addition of the ketone, was heated for 2 hours at 50°. Thereafter, the mixture was concentrated *in vacuo* to a solid, treated with 200 ml. of water, and extracted with three 150-ml. portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate, separated from the drying agent by filtration, and concentrated *in vacuo*. The residual solid (23 Gm., 47.5%) was recrystallized from ethanol, to give product, m.p. 110–111°. The infrared spectrum of the product in carbon tetrachloride showed absorption at 3600 cm.$^{-1}$ characteristic of hydroxyl groups, and the absence of the carbonyl absorption at 1660 cm.$^{-1}$.

Anal.—Calcd. for $C_{11}H_{17}NOS$: C, 62.56; H, 7.49; S, 5.17. Found: C, 62.39; H, 7.75; S, 15.00.

5-Methyl-5-dimethylaminomethyl-6-benzylthiaindan-6-ol.—The method of Gilman and Catlin (10) was used in the preparation of benzylmagnesium chloride. In a 1-L. three-necked flask fitted with a stirrer, dropping funnel, and a condenser provided with a drying tube containing calcium chloride and soda lime, were placed 9.5 Gm. (0.4 Gm. At.) of magnesium turnings, 25 ml. of dry ether, 5 ml. of a solution of 49.34 Gm. (0.3 mole) of benzyl chloride in 200 ml. of dry ether, and a crystal of iodine. Stirring was begun, and the remaining ethereal solution of benzyl chloride was added over a period of 30 minutes and then refluxed for an additional 30 minutes.

The method of Pohland and Sullivan (5) was used for the Grignard reaction. To the Grignard reagent, prepared as described above, was added a solution of 16.1 Gm. (0.08 mole) of 5-methyl-5-dimethylaminomethylthiaindan-6-one in 200 ml. of dry ether over a period of 30 minutes. The reaction mixture was decomposed by the dropwise addition of a saturated solution of ammonium chloride. The ether was decanted from the solid and dried over anhydrous magnesium sulfate.

The ether solution was evaporated on a steam bath to give a red oil which partially crystallized on cooling in a dry ice–acetone bath. The solid substance (10 Gm., 44%) was removed by filtration and after several recrystallizations from ethanol melted at 113–115°. The infrared spectrum showed hydroxyl absorption at 3600 cm.$^{-1}$ and the absence of the characteristic carbonyl maximum between 1600 and 1700 cm.$^{-1}$.

Anal.—Calcd. for $C_{18}H_{23}NOS$: C, 71.76; H, 7.64. Found: C, 71.61; H, 7.71. A white methiodide salt was prepared by the usual procedure and recrystallized from ethanol, m.p. 228° dec., darkening at 220°.

Anal.—Calcd. for $C_{19}H_{26}INOS$: C, 51.46; H, 5.85. Found: C, 51.63; H, 5.95.

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The synthesis of a number of substituted 2-aminobenzoxazoles and benzoxazolinones is described. The majority of the compounds exhibit marked muscle relaxant activity, two of the most potent being 2-amino-5-chlorobenzoxazole[1] and 5-chlorobenzoxazolinone.[2]

As early as 1943 Goodman and associates (1) reported that benzimidazole produced a reversible flaccid paralysis in various species of laboratory animals. However, it was not until 1952 when Domino and co-workers (2) investigated a series of benzazoles that more than a passing interest was expressed in this class of compounds.

The interesting pharmacological properties of the benzazoles mentioned above and the marked central nervous system depressant activity of substituted benzoxazolinones (3, 4) prompted

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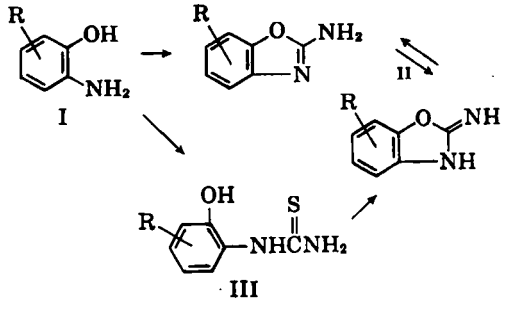
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us to investigate various substituted benzoxazoles further.

Initial efforts were directed toward the preparation of substituted 2-aminobenzoxazoles, inasmuch as few such derivatives were described. In addition, these compounds would serve as intermediates to more complex compounds. Other investigators (5–7) have described the preparation of similar compounds.

2-Aminobenzoxazoles (II) may be prepared conveniently from *o*-aminophenols (I) either by reaction with cyanogen bromide (7) to give the desired product directly or by first converting to the thiourea (III) with ammonium thiocyanate followed by cyclization (8).

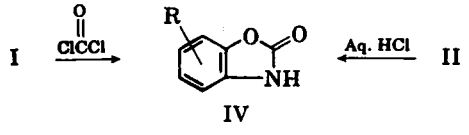


The conversion of thioureas to 2-aminobenzoxazoles is accelerated by maintaining an alkaline pH and by adding metallic oxide which results in the formation of the insoluble sulfide that is removed by filtration.

The use of ammonium thiocyanate for the preparation of thioureas unfortunately did not always yield crystalline or easily purified products. In the course of our work it was found convenient to convert the crude thiourea to the desired benzoxazole without loss in yield. The thioureas that were isolated and identified are listed in Table I.

The observation that 2-amino-5-chlorobenzoxazole (V) is a potent spinal cord depressant agent (9) stimulated our interest in other halogenated 2-aminobenzoxazoles. In addition to the thiourea and cyanogen bromide methods, halogenation of 2-aminobenzoxazole and 5-substituted-2-aminobenzoxazole was employed for the preparation of the corresponding 6-halo-2-aminobenzoxazoles. Halogenation proceeded to give the 6-halo derivatives. This was verified by comparison of the products with the 6-halo derivatives obtained by the unequivocal synthesis *via* the thiourea and cyanogen bromide methods. The results were in agreement with the observation made by Desai and associates (8) on the bromination of 2-aminobenzoxazole.

In the course of our studies it was observed that the 2-aminobenzoxazoles could be hydrolyzed readily to the corresponding benzoxazolinones (IV) which also exhibited marked muscle relaxant activity.



A number of benzoxazolinones was prepared (Table III) either by the hydrolysis of the corresponding 2-aminobenzoxazoles (II) or by treatment of the requisite 2-aminophenol (I) with phosgene (10).

As with 2-aminobenzoxazoles, halogenation of benzoxazolinone and substituted benzoxazolines proceeded to give the corresponding 6-halobenzoxazolinones.

The reaction of 2-amino-5-chlorobenzoxazole (V) with methyl iodide and ethyl β-bromopropionate gave 5-chloro-2-imino-3-methylbenzoxazoline (VI) and 5-chloro-3-(2-ethoxycarbonylethyl)-2-iminobenzoxazoline (VII), respectively. The methylation and proof of structure of all *N*-methylated derivatives is the subject of a previous publication (11).

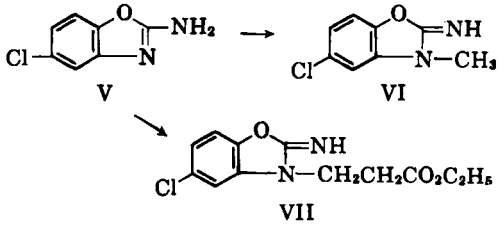
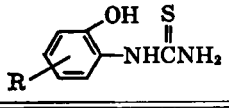


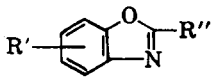
TABLE I.—SUBSTITUTED *o*-HYDROXYPHENYLTHIOUREAS



R	Recrystd. From	M.p., °C.	Formula	N Analyses, % Calcd.	N Analyses, % Found
4-Cl	Ethyl acetate-heptane	157 to 157.5 dec.	$C_7H_7ClN_2OS$	13.8	13.5
4-CH_3O	Methanol	174–175	$C_8H_{10}N_2O_2S$	14.1	13.8
5-Cl	Ethyl acetate	173–174	$C_7H_7ClN_2OS$[a]	13.8	13.6
4-CH_3	Ethyl acetate	156–157	$C_8H_{10}N_2OS$	15.4	15.0

[a] Calcd.: C, 41.5; H, 3.5. Found: C, 41.5; H, 3.6.

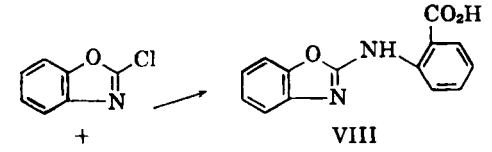
TABLE II.—BENZOXAZOLES



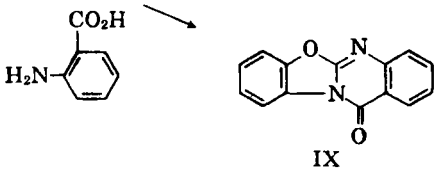
R′	R″	Recrystd. From	Method	M.p., °C.	Formula	N Analyses, % Calcd.	N Analyses, % Found
H	$NHCOCH_2Cl$	Benzene	...	180–180.5	$C_9H_7ClN_2O_2$	13.3	13.5
5-Cl	NH_2	Benzene	*A, B*	183–184	$C_7H_5ClN_2O$[a]	...	...
5-Cl	$NHCOCH(CH_3)_2$	Heptane	...	182–183	$C_{11}H_{11}ClN_2O_2$	11.7	11.6
5-CH_3O	NH_2	Benzene	*A, B*	165–166	$C_8H_8N_2O_2$	17.1	16.8
6-Cl	NH_2	Benzene	*E*	184–185	$C_7H_5ClN_2O$	16.6	16.4
5-CH_3	NH_2	Benzene	*A,B*	141–142	$C_8H_8N_2O$	18.9	18.6
H	*o*-$NHC_6H_4CO_2H$	Methanol	...	304–305	$C_{14}H_{10}N_2O_3$	11.0	10.9
5-Cl	H	Petroleum ether	...	37–37.5	C_7H_4ClNO	9.1	8.7
5-F	NH_2	Benzene	*A, B*	148–149	$C_7H_5FN_2O$	18.4	18.3
5-Br	NH_2	Acetone-water	*B*	177–178	$C_7H_5BrN_2O$	13.1	12.7
5-I	NH_2	Acetone-water	*B*	196–197	$C_7H_5IN_2O$	10.7	10.6
5-Cl	$NH(CH_2)_3N(CH_3)_2$	...	...	78–79	$C_{12}H_{16}ClN_3O$	16.6	16.5
5-Cl	SCH_3	Methanol-water	...	87–89	C_8H_6ClNOS	7.0	7.0
5-Cl	NC_5H_{10}[b]	Acetone-water	...	106–107	$C_{12}H_{13}ClN_2O$	11.8	12.1
5-Cl-6-Br	NH_2	Methanol-water	*E*	237	$C_7H_4BrClN_2O$	11.3	11.3
7-Cl	NH_2	Acetone-benzene	*B*	218–219	$C_7H_5ClN_2O$	16.6	16.7
5-Cl	$NHCH_2C_6H_5$	Acetone-water	...	136–139	$C_{14}H_{11}ClN_2O$	10.8	10.7
4-Cl	NH_2	Acetone-water	*B*	200–201	$C_7H_5ClN_2O$	16.6	16.6
5,6-diCl	NH_2	Acetone-water	*E*	239–240	$C_7H_4Cl_2N_2O$	13.8	13.6
5-Cl	$NHNH_2$	Methanol-water	...	196–197	$C_7H_6ClN_3O$[c]	22.9	22.8
5-Cl-7-SO_3H	NH_2	Water	*B*	300	$C_7H_5ClN_2O_4S$	11.3	11.3
5-Cl	OCH_3	Acetone-water	...	80.5–81.5	$C_8H_6ClNO_2$	7.6	7.6
5-NH_2	NH_2	Methanol-ether	...	300 dec.	$C_7H_8ClN_3O$[d]	22.6	22.1
5-NO_2	NH_2	Cellosolve-water	*B*	300	$C_7H_5N_3O_3$	22.4	23.0
5-Cl	$NHCOCH_3$	Methanol	...	220–221	$C_9H_7ClN_2O_2$	13.3	13.3
5,7-diBr	NH_2	Acetone-Benzene	*E*	253–254	$C_7H_4Br_2N_2O$	9.6	9.6
4,5,7-triCl	NH_2	2-Butanone	*B*	296–297	$C_7H_3Cl_3N_2O$	11.8	11.9

[a] *Reference 7.* [b] Piperidino. [c] Calcd.: C, 45.8; H, 3.3. Found: C, 45.9; H, 3.4. [d] Hydrochloride.

A representative arylaminobenzoxazole was prepared from 2-chlorobenzoxazole and anthranilic acid (6). The reaction, when performed in methanol, gave predominantly *N*-(2-benzoxazolyl) anthranilic acid (VIII) and some 12-benzo(d)quinazo(2,3-b)oxazole-12-one(IX). The same reaction when conducted in glacial acetic acid yielded predominantly the ring compound (IX). Neither of the compounds above exhibited significant muscle relaxant properties when tested in experimental animals.



VIII



IX

EXPERIMENTAL[3]

Syntheses

2-Aminophenol, 2-amino-4-chloro and 3-amino-5-chloro-2-hydroxybenzenesulfonic acid are available commercially. 2-Amino-6-chlorophenol (5), 2-amino-4-fluorophenol (12), 2-amino-4-methoxyphenol (10), 2-amino-4-methylphenol (10), 2-amino-4-nitrophenol (13), 2-amino-5-chlorophenol (14), 2-amino-4-bromophenol (15), 2-amino-4-iodophenol (16), 2-amino-4,6-dibromophenol (16), 2-amino-3-chlorophenol (17), and 2-amino-3,4,6-trichlorophenol (18) were prepared according to procedures described in the literature.

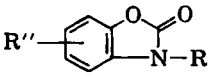
Thioureas (Table I).—To a solution of 0.3 mole of the 2-aminophenol in 300 ml. of water containing 34 ml. (14.6 Gm., 0.4 mole) of concentrated hydrochloric acid was added 30.4 Gm. (0.4 mole) of ammonium thiocyanate. The resulting solution was placed in a porcelain evaporating dish and heated on steam bath for 3 hours. Water (300 ml.) was added to the cooled mixture and the product removed by filtration. The filtrate was heated again on a steam bath for 3 hours and the additional product removed by filtration. Purification was accomplished by recrystallization from a suitable solvent (Table I). Yield, 50–60%.

2-Aminobenzoxazoles (Table II).—*Method A.*—A solution of 0.3 mole of the *o*-hydroxyphenylthiourea in 1 L. of methanol was treated with 100 Gm. (0.45 mole) of lead monoxide and the resulting mixture refluxed with stirring for 4 hours. The lead sulfide and excess lead monoxide were removed by filtration and washed thoroughly with hot methanol. Evaporation of the solvent and recrystallization of the residue, using charcoal as a clarifying agent, gave 70–90% of the 2-aminobenzoxazoles (Table II). In general, a crude thiourea was employed, the yield of product ranged from 45–60% based on the aminophenol.

Method B.—A stirred solution of 1.1 moles of cyanogen bromide (19) in 200 ml. of 70% methanol

[3] All melting and boiling points are uncorrected.

TABLE III.—BENZOXAZOLINONES



R′	R″	Recrystd. From	Method	M.p., °C.	Formula	N Analyses, % Calcd.	N Analyses, % Found
H	6-Cl	Methanol-water	*E*	195–196	$C_7H_4ClNO_2$[a]	...	...
H	5-Br	Methanol-water	*C*	214–216	$C_7H_4BrNO_2$[b]	...	...
H	5,7-diBr	Ethyl acetate	*E, D*	260–261	$C_7H_3Br_2NO_2$[c]	...	...
H	5-CH_3O	Ethyl acetate	*D*	169–170	$C_8H_7NO_3$[d]	...	...
H	5-Cl-6-Br	Methanol-water	*E, D*	204–205	$C_7H_3BrClNO_2$	5.6	5.7
H	5-NH_2	Water	*D*	227–229	$C_7H_6N_2O_2$	18.7	18.7
H	5,6-diCl	Methanol-water	*E, D*	205	$C_7H_3Cl_2NO_2$	6.9	6.8
C_2H_5	5-Cl	Heptane	*F, G*	73–75	$C_9H_8ClNO_2$	7.1	6.8
CH_3	5-Cl	Methanol	*F*	133–135	$C_8H_6ClNO_2$	7.6	7.7
CH_2=$CHCH_2$	5-Cl	Petroleum ether	*G*	62–64	$C_{10}H_8ClNO_2$	6.7	6.7

[a] *Reference 10.* [b] *Reference 20.* [c] *Reference 21.* [d] *Reference 22.*

was treated with a solution of 1 mole of the substituted *o*-aminophenol in the minimum amount of methanol at room temperature. The solution was allowed to stand for 40 minutes, then neutralized with concentrated sodium hydroxide solution. Most of the methanol was distilled *in vacuo*, and the residual material was treated with water. The solid was removed by filtration and recrystallized from a suitable solvent (Table II) after treatment with charcoal. Yields of 50–70%, based on the aminophenol, were obtained.

Benzoxazolinones (Table IV).—*Method C.*—The method of Close, *et al.* (10), was followed. A suspension of 0.1 mole of substituted *o*-aminophenol and 0.2 mole of sodium acetate in 300 ml. of ethyl acetate was stirred and treated with a solution of 0.11 mole of phosgene in 200 ml. of ethyl acetate. After refluxing briefly, the solution was cooled and washed with water and 5% hydrochloric acid, respectively. The ethyl acetate solution was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was recrystallized to give 60–90% of product (Table III).

Method D.—A solution of 0.1 mole of substituted 2-aminobenzoxazole in 200–300 ml. of 1*N* hydrochloric acid was refluxed for 3 to 5 hours. The mixture was cooled, and the solid was removed by filtration. Recrystallization gave 50–70% of product (Table III).

Halogenation of 2-Aminobenzoxazoles and Benzoxazolines.—*Method E.*—A solution of 1.4 moles of the 2-aminobenzoxazole or benzoxazolinone in 2 L. of a suitable solvent (chloroform, ethyl acetate, etc.) was cooled to 5° and treated with a solution of 1.6 moles of the halogen in the same solvent. The temperature was maintained at 10–15° during the addition and stirring was continued for 1 to 2 hours. The solid was removed by filtration, suspended or dissolved in water, and neutralized with ammonia. The solid was removed by filtration and recrystallized to give 60–80% of product (Tables II, III).

5-Chloro-3-substitutedbenzoxazolinones (Table III).—*Method F.*—A solution of 0.1 mole of 5-chlorobenzoxazolinone in 200 ml. of water containing 4.3 Gm. of sodium hydroxide was stirred and treated with 0.12 mole of dimethyl or diethyl sulfate. The mixture was warmed on a steam bath until it became neutral and then cooled. The solid was removed by filtration and recrystallized to give 65–70% of product.

Method G.—The procedure described by Close, *et al.* (10), (*Method B*), with minor modifications, was followed. A solution of 0.055 mole of potassium hydroxide and 0.05 mole of 5-chlorobenzoxazolinone in 120 ml. of cellosolve was stirred for 1 hour, then treated with 0.05 mole of alkyl bromide or iodide. After refluxing for 3 hours, the solvent was distilled and the residue recrystallized to give 70–80% of product.

5-Chloro-2-hydrazinobenzoxazole.—The method described by Sam, *et al.* (11), for the preparation of 5-chloro-2-dimethylaminobenzoxazole was followed using 9.4 Gm. (0.05 mole) of 2,5-dichlorobenzoxazole, 5 Gm. (0.1 mole) of hydrazine hydrate, and 100 ml. of water. Six grams (67%) of product was obtained after recrystallization from a methanol and water mixture, m.p. 196–197°.

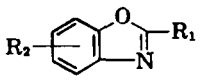
N,N-Bis [2-(5-chlorobenzoxazolyl)]-N′,N′-dimethyl-1,3-propanediamine and 5-Chloro-2-(3-dimethylaminopropylamino)benzoxazole.—A solution of 18.8 Gm. (0.1 mole) of 2,5-dichlorobenzoxazole(11) in 100 ml. of chloroform was treated with 10.2 Gm. (0.1 mole) of 3-dimethylaminopropylamine. After the initial reaction had subsided, the solution was refluxed briefly and cooled. A precipitate of 3-dimethylaminopropylamine hydrochloride was removed by filtration, and the chloroform was distilled *in vacuo*. The oily residue solidified and was recrystallized from dilute methanol to give 10 Gm. of solid, m.p. 140–144°. Repeated recrystallization of the solid from dilute methanol gave 5.5 Gm. (27%) of *N,N*-bis[2-(5-chlorobenzoxazolyl)]-*N′,N′*-dimethyl-1,3-propanediamine, m.p. 146–148°.

Anal.—Calcd. for $C_{19}H_{18}Cl_2N_4O_2$: N, 13.8. Found: N, 13.6.

The dilute methanol solutions were combined and concentrated *in vacuo*. The residual oil was extracted with chloroform and dried over anhydrous magnesium sulfate. Distillation of the chloroform solution gave 8 Gm. (32%) of 5-chloro-2-(3-dimethylaminopropylamino)benzoxazole, b.p. 168–170° (0.4 mm.), m.p. 78–79°.

5-Chloro-2-methylmercaptobenzoxazole.—A solution of 37 Gm. (0.2 mole) of 5-chloro-2-mercaptobenzoxazole (5) and 12 Gm. of sodium hydroxide in 200 ml. of water was treated with 25.2 Gm. (0.2 mole) of dimethylsulfate and stirred for 2 hours. The solid (32 Gm., 82%) was removed by filtration, washed with water, and recrystallized from dilute methanol, m.p. 87–89°.

TABLE IV.—PARALYZING AND LETHAL DOSES OF BENZOXAZOLES ON INTRAPERITONEAL ADMINISTRATION TO MICE



	R_1	R_2	PD_{50}[a]	LD_{50}[b]	Index[c]
(A)	CHLORINATED 2-AMINOBENZOXAZOLES				
	NH_2	4-Cl	36	54	1.5
	NH_2[d]	5-Cl	81	376	4.6
	NH_2	6-Cl	66	347	5.3
	NH_2	7-Cl	85	180	2.1
	NH_2[e]	6-Br[f]	103	294	2.9
(B)	5-SUBSTITUTED 2-AMINOBENZOXAZOLES				
	NH_2	F	115	450	3.9
	NH_2	Br	144	189	1.3
	NH_2	I	150	150	1.0
	NH_2	CH_3	103	360	3.5
	NH_2	CH_3O	220	432	2.0
	NH_2	NO_2	[g]	350	...
	NH_2	NH_2	1500	2000	1.3
	NH_2[h]	H	99	392	4.0
(C)	5-CHLORO-2-SUBSTITUTED BENZOXAZOLES				
	H	Cl	230	530	2.3
	$NHCOCH_3$	Cl	250	1500	6.0
	$NHCOCH_2Cl$	H[f]	400	450	1.0
	$NHCOCH(CH_3)_2$	Cl	350	1200	4.0
	$NHCH_3$[i]	Cl	180	230	1.3
	$N(CH_3)_2$[i]	Cl	280	280	1.0
	SCH_3	Cl	350[j]	350	1.0
	NC_5H_{10}[k]	Cl	1000[j]	1000	...
	$NHNH_2$	Cl	[g]	80	...
	OCH_3	Cl	750	1450	1.9
	$NH(CH_2)_2N(CH_3)_2$	Cl	[g]	230	...
	$NHCH_2C_6H_5$	Cl	[g]	1280	...
	SH[l]	Cl	100	120	1.2
(D)	DI AND TRI SUBSTITUTED 2-AMINOBENZOXAZOLES				
	NH_2	5-Cl-6-Br	105	240	2.3
	NH_2	5,6-diCl	132	300	2.3
	NH_2	5-Cl-7-SO_3H	1440[j]	1440	...
	NH_2	5,7-diBr	420	780	1.9
	NH_2	4,5,7-triCl	1750	2000	1.1

[a] Dose in mg./Kg. which causes a loss in righting reflex in 50% of the animals. [b] Dose in mg./Kg. which causes death in 50% of the animals. [c] Ratio of LD_{50} to PD_{50}. [d] *Reference 7.* [e] *Reference 8.* [f] Included here for convenience. [g] Stimulant. [h] *Reference 23.* [i] *Reference 11.* [j] No acute loss in righting reflex. [k] Piperidino. [l] *Reference 24.*

5-Chloro-2-piperidinobenzoxazole.—A solution of 8 Gm. (0.04 mole) of 5-chloro-2-methylmercaptobenzoxazole in 40 ml. of piperidine was refluxed for 22 hours. The solution was cooled and diluted with 250 ml. of water. The precipitate was collected and dissolved in dilute hydrochloric acid. A small amount of acid-insoluble material was removed by filtration and the product was precipitated with sodium hydroxide solution. After recrystallization from dilute acetone, there was obtained 8.7 Gm. (92%) of product, m.p. 106–107°.

2-Benzylamino-5-chlorobenzoxazole.—A solution of 23 Gm. (0.12 mole) of 5-chloro-2-mercaptobenzoxazole (5) and 26.6 Gm. (0.25 mole) of benzylamine in 100 ml. of xylene was refluxed for 6 hours. The product was removed by filtration and dissolved in hot methanol. The solution was made alkaline with sodium hydroxide, and the product was precipitated by dilution with water. The solid was collected and recrystallized from dilute acetone to give 20 Gm. (65%) of product, m.p. 136–139°.

5-Chloro-2-methoxybenzoxazole.—A cold, stirred solution of 2.1 Gm. of sodium in 50 ml. of anhydrous methanol was treated rapidly with a cold solution of 15 Gm. (0.08 mole) of 2,5-dichlorobenzoxazole (11) in 150 ml. of methanol. The reaction mixture was immediately poured into 1 L. of ice water. The white precipitate (14 Gm., 96%) was removed by filtration, washed with water, and recrystallized from dilute acetone, m.p. 80.5 to 81.5°.

N-(2-Benzoxazolyl)anthranilic Acid.—To a solution of 13.8 Gm. (0.1 mole) of anthranilic acid in 150 ml. of ethanol was added dropwise 15.4 Gm. (0.1 mole) of 2-chlorobenzoxazole. The resulting solution was refluxed on a steam bath for 3 hours and the precipitated 12-benzo(d)quinazo(2,3-b)oxazole-12-one (3 Gm.) removed by filtration. The filtrate was concentrated *in vacuo* and the residue extracted with 200 ml. of 10% sodium hydroxide solution. Neutralization of the solution with dilute hydrochloric acid gave 10 Gm. of product which was recrystallized from methanol, m.p. 304 to 305°.

12-Benzo(d)quinazo(2,3-b)oxazole-12-one.—To a warm solution of 13.8 Gm. (0.1 mole) anthranilic acid in 100 ml. of glacial acetic acid was added dropwise 15 Gm. (0.1 mole) of 2-chlorobenzoxazole. After the initial reaction subsided, the solution was refluxed for 30 minutes. The acetic acid was

distilled *in vacuo* from the precipitated product. After washing with water, 10% sodium hydroxide solution, and water, respectively, the product was recrystallized from 2 L. of isopropanol. Ten grams of product were obtained, m.p. 253 to 253.5°.

Anal.—Calcd. for $C_{14}H_8N_2O_2$: N, 11.86. Found: N, 11.80.

5-Chlorobenzoxazole.—A mixture of 28 Gm. (0.2 mole) of 2-amino-4-chlorophenol and 50 ml. of formamide was heated for 2 hours at 170–180°. The black solution was distilled *in vacuo* at 30 mm. The distillate was redistilled to give 13 Gm. of product, b.p. 112–114° (30 mm.), m.p. 37 to 37.5°.

N-(2-benzoxazolyl)-α-chloroacetamide.—To a solution of 13.4 Gm. (0.1 mole) of 2-aminobenzoxazole and 7.9 Gm. (0.1 mole) of pyridine in 300 ml. of dry benzene was added gradually with stirring at room temperature 9.3 Gm. (0.1 mole) of α-chloroacetyl chloride. The resulting mixture was stirred at room temperature for 2 hours. The benzene was distilled *in vacuo;* the residue was washed with water and recrystallized from methyl ethyl ketone and then benzene to give 7 Gm. of product, m.p. 180 to 180.5°.

N-[2-(5-Chlorobenzoxazolyl)]isobutyramide.—The above procedure was followed using 8.5 Gm. (0.05 mole) of 2-amino-5-chlorobenzoxazole, 4 Gm. (0.05 mole) of pyridine, and 5.3 Gm. (0.05 mole) of isobutyryl chloride. The mixture was refluxed with stirring for 3 hours. Water (300 ml.) was added to the mixture and the layers separated. The organic layer was washed with dilute hydrochloric acid and water. Evaporation of the solvent gave 8 Gm. of product which was recrystallized from heptane, m.p. 182–183°.

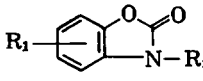
N-[2-(5-Chlorobenzoxazolyl)]acetamide.—The procedure for the preparation of the isobutyramide above was followed using 3.9 Gm. (0.05 mole) of acetyl chloride. After recrystallization from methanol, 8 Gm. of product, m.p. 220–221°, was obtained.

2,5-Diaminobenzoxazole Hydrochloride.—A suspension of 34 Gm. (0.19 mole) of 2-amino-5-nitrobenzoxazole in methanol was treated with Pd-C catalyst and hydrogenated at room temperature. After the required amount of hydrogen was absorbed (2 hours), the catalyst was removed by filtration and the solution diluted with 2 vol. of ether. Conversion of the resulting solid to the hydrochloride and subsequent recrystallization from a methanol-ether mixture gave 28 Gm. (79%) of product, m.p. 300° dec.

3-(2-Ethoxycarbonylethyl)-2-imino-benzoxazoline Hydrobromide.—A mixture of 15 Gm. (0.11 mole) of 2-aminobenzoxazole, 19 Gm. (0.11 mole) of ethyl β-bromopropionate, and 50 ml. of dry toluene was heated on a steam bath for 9 hours. The toluene was decanted from the semisolid residue. The latter was triturated with methyl ethyl ketone and the solid (10 Gm.) removed by filtration. Recrystallization from isopropanol gave product melting at 181 to 181.5°.

Anal.—Calcd. for $C_{12}H_{15}BrN_2O_3$: N, 8.9. Found: N, 8.6.

TABLE V.—PARALYZING AND LETHAL DOSES OF BENZOXAZOLINONES ON INTRAPERITONEAL ADMINISTRATION TO MICE



R_1	R_2	PD_{50}[a]	LD_{50}[b]	Index[c]
5-Cl[d]	H	86	210	2.4
6-Br[e]	H	237	445	1.9
6-Cl[f]	H	215	435	2.0
5-Br[g]	H	109	262	2.4
5,7-diBr[h]	H	420	392	0.8
4,5,7-triCl[i]	H	61	61	1.0
5-OCH_3[j]	H	250	650	2.6
5-Cl-6-Br	H	194	222	1.2
5-NH_2	H	420[k]	420[l]	2.6
5,6-diCl	H	150	263	1.8
5-Cl	C_2H_5	220	505	2.3
5-Cl	CH_3	800	2000	2.5
5-Cl	CH_2=$CHCH_2$	160	320	2.0

[a] Dose in mg./Kg. which causes a loss in righting reflex in 50% of the animals. [b] Dose in mg./Kg. which causes death in 50% of the animals. [c] Ratio of LD_{50} to PD_{50}. [d] *Reference 7, 25.* [e] *Reference 26.* [f] *Reference 10.* [g] *Reference 20.* [h] *Reference 21.* [i] *Reference 27.* [j] *Reference 22.* [k] No acute loss of righting reflex. [l] Delayed death.

TABLE VI.—COMPARATIVE LOSS OF RIGHTING REFLEX AND TOXICITY OF MUSCLE RELAXANTS ON INTRAPERITONEAL INJECTION TO MICE (28)

	PD_{50}[a]	LD_{50}[b]	Index[c]
Zoxazolamine	81	376	4.6
Chlorzoxazone	86	210	2.4
Mephenesin	130	471	3.6
Meprobamate	195	710	3.6

[a] Dose in mg./Kg. which causes a loss in righting reflex in 50% of the animals. [b] Dose in mg./Kg. which causes death in 50% of the animals. [c] Ratio of LD_{50} to PD_{50}.

Pharmacological Results[4]

The paralyzing action and lethal doses of the compounds described in this report are summarized briefly in Tables IV, V, and VI. A more extensive report of the pharmacological properties of the compounds will be the subject of another communication.

Of the various chloro-2-aminobenzoxazoles (Table IV), positions 5 and 6 provided compounds with similar activity. In the 2-amino-5-substituted benzoxazole series, the chloro, fluoro, and methyl substituted derivatives as well as the unsubstituted benzoxazole (R_2 = H) were the most active. Variation of the substituent in the 2-position of 5-chlorobenzoxazole by groups other than the primary amino, in general, results in a decrease in activity. Only the amides possess muscle relaxant activity comparable to 2-amino-5-chlorobenzoxazole (zoxazolamine). Muscle relaxant activity also is present in disubstituted and trisubstituted 2-aminobenzoxazoles; the activity, however, is less than that of the corresponding 2-amino-5-chlorobenzoxazole. Most of the benzoxazolinones that were evaluated possessed some degree of muscle relaxant activity (Table V). Several of the compounds listed in Table V possess muscle relaxant activity comparable to that of 5-chlorobenzoxazolinone (chlorzoxazone). Table VI is included for comparative purposes.

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[4] The pharmacological data was obtained by the staff of the Department of Pharmacology, McNeil Laboratories.

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Interactions Between Orange II and Selected Long Chain Quaternary Ammonium Salts

By GEORGE ZOGRAFI, PRAFUL R. PATEL*, and NORMAN D. WEINER

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IN RECENT YEARS the diverse properties of surface-active agents have led to an increased utilization of these substances in pharmaceutical preparations. Such uses include emulsification, wetting, solubilization, and disinfection. An important group of surface-active agents are the long chain quaternary ammonium compounds which have been shown to exhibit a high degree of activity against bacteria and fungi (1).

Because of the positive charge on the quaternary nitrogen, these compounds are extremely incompatible with substances such as soaps and anionic dyes, generally forming insoluble compounds. In view of this, many studies concerned with the introduction of quaternary ammonium compounds into pharmaceuticals and the subsequent incompatibilities, have been reported (2–4). Lachman, *et al.* (5), studied the interaction of 1-hexadecylpyridinium chloride, dodecyldimethyl(2-phenoxyethyl)ammonium bromide, and benzalkonium chloride with several anionic certified dyes. It was found that for equal quantities of the three quaternary ammonium salts, the amount of any dye which can be added without producing turbidity increases in the order: benzalkonium chloride, dodecyldimethyl (2-phenoxyethyl)ammonium bromide, and hexadecylpyridinium chloride. They concluded that this order of reactivity can be assigned to the electropositive center and the steric configuration of the molecule.

It was the purpose of this investigation to gain more insight into the nature of these interactions by isolating the compounds formed from each dye-detergent[1] combination, measuring their

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[1] The general term, detergent, is used throughout this paper to refer to the quaternary ammonium salts; it should not necessarily be implied that they are useful as detergents.

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solubility characteristics, and determining the thermodynamic quantities involved in the dissolution process.

EXPERIMENTAL

Materials.—The dye selected for this study was the sodium salt of *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid, commonly known as orange II. It was obtained, twice recrystallized from water, from Eastman Organic Chemicals and further purified by recrystallization from absolute alcohol. This dye is structurally similar to the commonly used certified dyes and has the advantage of being quite pure and free of isomers (6).

Four quaternary ammonium salts were selected: 1-hexadecylpyridinium chloride, commonly known as cetylpyridinium chloride (CPC); dodecyldimethyl(2-phenoxyethyl)ammonium bromide, commonly known as domiphen bromide (PDDB); (diisobutylphenoxyethoxyethyl)dimethylbenzylammonium chloride, commonly known as hyamine 1622 (DDBC); and dodecylquinolinium bromide (DQB), which was synthesized according to the method of Few, *et al.* (7). All compounds, recrystallized, had melting points which agreed with reported values (7, 8). All other chemicals used were of reagent grade.

Spectral Properties of Orange II.—The concentration of orange II in solution was measured spectrophotometrically using a Beckman model DU spectrophotometer. Maximum absorption occurred at a wavelength of 488 mμ in distilled water. The molar absorptivity index was 2.097×10^4. The wavelength of maximum absorbance and the absorptivity index were independent of ionic strength up to a value of 1.0.

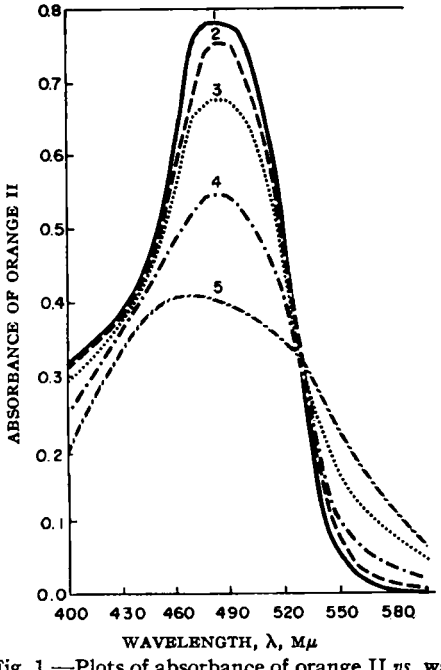


Fig. 1.—Plots of absorbance of orange II *vs.* wavelength in millimicrons at various pH values. Key: 1 ——, pH 0.73 to 8.05; 2 - - - -, pH 9.94; 3 . . . ., pH 11.34; 4 — · —, pH 11.98; and 5 — - - —, pH 12.34 to 12.91.

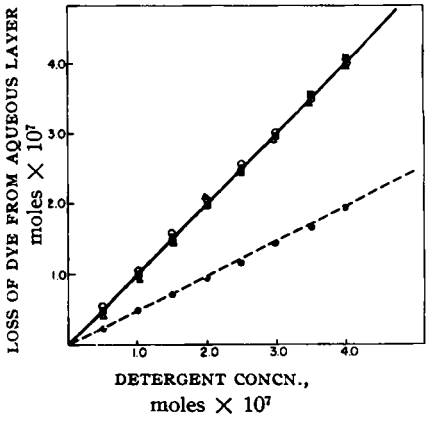


Fig. 2.—Dye loss from aqueous layer *vs.* detergent. Key: ■, CPC, pH 7.50; ●, CPC, pH 12.91; O, PDDB, pH 7.50; △, PDDB, pH 12.91.

In view of the acidic properties of the dye it was of interest to determine the absorption spectrum over a wide range of pH values. Therefore, a series of dye solutions, varying in pH from 0.73 to 12.91, were prepared. The concentration of the dye was kept constant and the ionic strength of all solutions, except that at pH 0.73, was brought up to 0.15 with sodium chloride. All pH measurements were made with a Beckman model G pH meter. The absorption spectrum of this dye, as shown in Fig. 1, remains unchanged from a pH of 0.73 to 8.05. Above pH 8.05, a decrease in absorbance can be noted, until at pH 12.34 or higher, the absorbance reaches a minimum. The constancy of the spectrum from pH 0.73 to 8.05 indicates that the chromophore is essentially unaltered over this range of pH, the sulfonic acid group being completely dissociated and the phenolic group undissociated. Above this pH the chromophore is altered due to dissociation of the phenolic auxochrome. This is reflected by the decrease in absorbance and the shift in the absorption maximum at pH 12.34 from 488 mμ to about 460 mμ.

Stoichiometry of Dye-Detergent Interactions.—It is generally expected that the stoichiometric relationship of a compound formed from monovalent ions of opposite charge will be 1:1. At high pH values (12.91), where the phenolic group of orange II is completely dissociated, the possibility of two detergent cations combining with one dye anion exists. Since for any consideration of the solubility characteristics of a slightly soluble salt, the number of ions involved must be known, it was decided to determine the stoichiometry of the various dye-detergent combinations.

The procedure used in this investigation was that of Few and Ottewill (9). It is based on the fact that a water-soluble dye is completely transferred to chloroform in the presence of an oppositely charged long-chain ion, due to the formation of a water-insoluble, chloroform-soluble "complex," while alone it is completely insoluble in chloroform. The method was modified in the following manner. Five milliliters of aqueous dye solution and 5 ml. of aqueous detergent solution were added to a 40-ml. glass-stoppered centrifuge tube. To this was added 10 ml. of chloroform. The tubes were then stoppered and shaken at least 50 times to insure complete

TABLE I.—ELEMENTAL ANALYSES[a] AND MELTING POINTS OF DYE-DETERGENT SALTS

Dye-Detergent Salts	pH at which Obtained	Analyses %—C— Calcd.	C— Found	—H— Calcd.	H— Found	—N— Calcd.	N— Found	M.p.
Dye-CPC-2 H_2O	7.50	68.40	68.26	8.00	7.93	6.46	6.72	140–141
Dye-2 CPC-4 H_2O	12.91	70.55	70.22	9.52	9.80	5.47	5.47	90–92
Dye-PDDB	7.50	69.02	68.85	7.75	7.78	6.35	6.69	118.5 to 119.5°
Dye-PDDB	12.91	69.02	69.39	7.75	8.23	6.35	6.75	118.5 to 119.5°
Dye-DDBC	7.50	69.90	69.43	7.23	7.39	5.69	5.31	121–122
Dye-DDBC	12.91	69.90	70.15	7.23	7.34	5.69	5.32	121–122
Dye-DQB	7.50	70.77	70.32	6.92	6.76	6.69	6.53	141–142
Dye-DQB	12.91	70.77	70.37	6.92	6.94	6.69	6.91	141–142

[a] Analyses performed by Dr. G. Weiler and Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, England.

extraction of the dye-detergent compound. The tubes were then centrifuged at 1500 r.p.m. for 10 minutes to obtain clear layers. Samples were removed from the aqueous layer and transferred directly to a 1-cm. spectrophotometric cell. Absorbance of the remaining dye was then measured at 488 mμ.

A solution containing a constant concentration of dye was equilibrated with one containing various concentrations of detergent, these concentrations all being less than the initial dye concentration. All solutions had an ionic strength of 0.15. The amount of dye-detergent complex transferred to chloroform in the presence of detergent was also determined at a pH of 12.91 (0.15 *M* NaOH), previously determined to be that pH where the dye is completely dissociated. Under these conditions, the pH of the aqueous layer, after extraction with chloroform, was adjusted with an equal quantity of 0.16 *M* HCl to allow the use of the molar absorptivity constant of the dye in distilled water.

To substantiate the stoichiometry of the various dye-detergent compounds, they were isolated in the following manner. Equal volumes of equimolar aqueous solutions of 0.1 *M* dye and detergent were mixed and allowed to stand for 12 hours. The same concentrations of dye and detergent, at a pH of 12.91, were also mixed and allowed to stand for the same length of time; an appropriate amount of NaOH was added to the dye solution before the addition of the detergent solutions. The combinations of dye and DDBC from distilled water and 0.15 *M* sodium hydroxide and dye-PDDB from 0.15 *M* sodium hydroxide yielded an oily layer which, when extracted with ethyl acetate, mixed with anhydrous sodium sulfate, filtered, and cooled, yielded a precipitate. These were recrystallized twice from ethyl acetate and dried under vacuum at room temperature. The combinations of dye plus PDDB, CPC, and DQB obtained from distilled water and DQB obtained from 0.15 *M* sodium hydroxide, yielded solids which were washed several times with distilled water and recrystallized twice from ethyl acetate. The CPC-dye combination obtained from 0.15 *M* sodium hydroxide required a water-acetone mixture (1:1) for recrystallization. All of these compounds were dried under vacuum at room temperature and submitted for elemental analysis.

Solubility of the Dye-Detergent Compounds.—Only the four salts obtained at neutral pH values were investigated, since the dye-CPC compound formed at high pH values is of little pharmaceutical interest. The general procedure for determining solubility was as follows. To about 30 ml. of distilled water in a 50-ml. container, 100 mg. of dye-detergent compound was added. The containers were sealed and placed into a constant temperature water bath, equipped with a rotating wheel, for 8 hours. This was the time required to insure equilibrium. The containers were then removed from the rotating wheel and allowed to sit in the bath to facilitate settling of the solid. To prevent removal of solid particles, the supernatant liquid was removed through glass wool plugs. Comparison with known solutions of dye and detergent alone showed no loss of dye or detergent due to adsorption on the glass wool or container in the range of concentrations used. The samples were added directly to a Beckman spectrophotometer absorbance cell and measured at 488 mμ. The absorbance of the dye at this wavelength was unaffected by the presence of dissolved detergent. This procedure was followed at four temperatures—25, 30, 35, and 40° ± 0.1°. To determine the effect of ionic strength, the solubility of the dye-DDBC compounds was measured at 25° in 0.01, 0.05, and 0.1 *M* sodium chloride.

Solubilization of Dye-Detergent Compounds in the Presence of Excess Detergent.—Precipitates formed from oppositely charged substances are known to be solubilized in the presence of surfactant micelles (10). Therefore, the solubility of each dye-detergent compound was measured in solutions containing excess detergent at concentrations below and above its critical micelle concentration (C.M.C.). The critical micelle concentrations of CPC, PDDB, and DDBC were measured by observing the characteristic break in the slope of a surface tension *versus* log concentration curve. Surface tension was measured using the Wilhelmy plate method (11); a thin roughened platinum plate was used to insure complete wetting of the plate. The critical micelle concentrations obtained are: CPC, 1.0×10^{-3} *M*; PDDB, 1.2×10^{-3} *M*; DDBC, 2.25×10^{-3} *M*. The value for CPC agrees with previously reported results (12), while the latter two values have not been previously reported. The critical micelle concentration of DQB is 4.8×10^{-3} *M*, determined by surface tension measurements (7). Following the procedure for measuring solubility, as outlined earlier, the appearance of dye in solution was measured at 488 mμ; the wavelength of maximum absorbance in the presence of micelles remaining unchanged from that of the dye in water.

RESULTS

Stoichiometry.—The number of moles of orange II transferred to chloroform at low pH was equal, in all cases, to the number of moles of detergent present, indicating a 1:1 stoichiometry. This was

TABLE II.—SOLUBILITY PRODUCTS (K'_{sp}) AND STANDARD THERMODYNAMIC FUNCTIONS FOR THE DISSOLUTION OF DYE-DETERGENT SALTS AT VARIOUS TEMPERATURES

Temp. Absolute	$K'_{sp} \times 10^{10}$	$\Delta F°$ Kcal. mole^{-1}	$\Delta H°$ Kcal. mole^{-1}	$\Delta S°$ e.u.
		Dye-CPC		
298	...	...	...	
303	0.0713	15.32	38.4	76.0
308	0.213	15.04	38.5	
313	0.689	14.56	38.4	
		Dye-PDDB		
298	0.582	13.96	11.8	
303	0.863	13.95	11.7	−7.3
308	1.15	14.01	11.8	
313	1.52	14.07	11.8	
		Dye-DDBC		
298	1.07	13.56	10.3	
303	1.42	13.65	10.3	−11.1
308	1.96	13.68	10.3	
313	2.60	13.73	10.3	
		Dye-DQB		
298	0.0548	15.35	18.0	
303	0.0906	15.31	18.0	9.0
308	0.146	15.27	18.0	
313	0.246	15.20	18.0	

also true over a pH range of 0.73 to 8.05 and at ionic strengths up to 1.0. At a pH of 12.91, 1 mole of orange II was transferred for every 2 moles of CPC, while a 1:1 relationship continued to exist for the other detergents. These results are graphically illustrated in Fig. 2. These observations are further substantiated by elemental analysis of the various dye-detergent compounds, as shown in Table I. Here it can be seen that, except for CPC, the same stoichiometry is obtained for each dye-detergent combination regardless of the pH.

Solubility.—The solubility of the various 1:1 compounds in distilled water, expressed as an apparent solubility product, K'_{sp}, is shown in Table II. The solubility product was obtained by using

$$K'_{sp} = (Q^+)(D^-) \qquad \text{(Eq. 1)}$$

where (Q^+) and (D^-) are the detergent and dye ion concentrations, respectively.

The effect of adding electrolytes can be seen in Table III. A significant increase in the apparent solubility product occurs as the ionic strength is increased. This may be explained on the basis of the following relationship for uni-univalent substances

$$K°_{sp} = (Q^+)(D^-)\,\gamma_\pm^2 \qquad \text{(Eq. 2)}$$

where $K°_{sp}$ is the thermodynamic solubility product, and $\gamma_\pm$ is the mean activity coefficient. Since the activity coefficient becomes less than unity as the ionic strength is increased, one would expect the apparent solubility product to increase, as is observed.

To determine whether the apparent solubility products obtained in distilled water are actually the thermodynamic solubility products, it is necessary to extrapolate the solubility products obtained at various ionic strengths to zero ionic strength and compare the two values. This was conveniently done by combining Eqs. 1 and 2 which gives

$$K°_{sp} = K'_{sp}\gamma_\pm^2 \qquad \text{(Eq. 3)}$$

Taking the logarithm of both sides of the equation and multiplying by −1 gives

$$pK°_{sp} = pK'_{sp} - 2\log\gamma_\pm \qquad \text{(Eq. 4)}$$

Using the extended form of the Debye-Hückel equation at 25° and rearranging Eq. 4

$$pK'_{sp} = pK°_{sp} - \frac{1.02\sqrt{\mu}}{1+\sqrt{\mu}} \qquad \text{(Eq. 5)}$$

where μ is the ionic strength. A plot of pK'_{sp} *versus* $\sqrt{\mu}/(1+\sqrt{\mu})$ should give a straight line with a slope equal to −1.02 and an intercept of $pK°_{sp}$. From the curves shown in Fig. 3, the thermodynamic solubility products at 25° are 0.601×10^{-10} for PDDB and 1.11×10^{-10} for DDBC, which is in good agreement with values obtained in distilled water, as shown in Table II. The slopes of these lines are −1.052 and −1.043 for PDDB and DDBC, respectively. Thus, it appears that the apparent solubility products determined in distilled water for these relatively insoluble compounds are the thermodynamic values.

The corresponding standard free energy, entropy, and enthalpy changes for the dissolution of the various compounds in distilled water are listed in Table II. The standard free energy change is obtained from

$$\Delta F° = -RT\ln K°_{sp} \qquad \text{(Eq. 6)}$$

where R is the gas constant in calories degree^{-1} mole^{-1}, and T is the absolute temperature. The standard entropy change is obtained from

$$\left(\frac{\partial\Delta F°}{\partial T}\right)_p = -\Delta S° \qquad \text{(Eq. 7)}$$

The standard enthalpy change at each temperature was determined from the Gibbs-Helmholtz equation

$$\Delta H° = \Delta F° + T\Delta S° \qquad \text{(Eq. 8)}$$

Solubilization in the Presence of Excess Detergent.—As seen in Figs. 4 and 5, the addition of a low concentration of detergent to a system containing the corresponding dye-detergent combination reduces solubility to zero. This may be attributed to the common ion effect for sparingly soluble substances. Further addition of detergent brings about a sudden increase in the solubility of the solid com-

TABLE III.—SOLUBILITY PRODUCTS (K'_{sp}) OF DYE-DETERGENT SALTS AT VARIOUS IONIC STRENGTHS (25°)

Ionic Strength	$K'_{sp} \times 10^{10}$ Dye-PDDB	Dye-DDBC
0.01	0.753	1.57
0.05	0.867	1.91
0.10	0.980	2.14

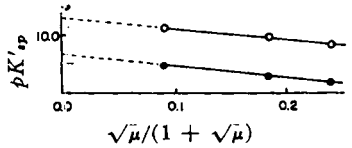


Fig. 3.—Plot of pK'_{sp} *vs.* $\sqrt{\mu}/(1+\sqrt{\mu})$. Key: ○, dye PDDB; ●, dye DDBC.

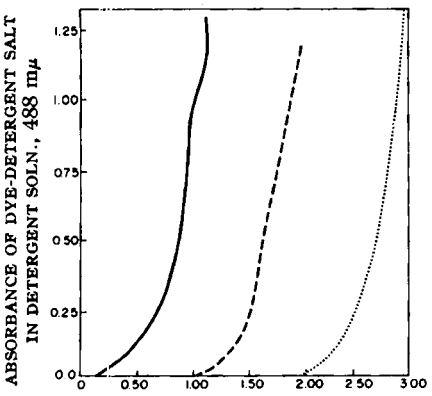


Fig. 4.—Plots of absorbance of dye-detergent salts in detergent solution at 488 mμ *vs.* detergent concentration. Key: ——, dye-CPC; - - - -, dye-PDDB; and . . . ., dye-DDBC.

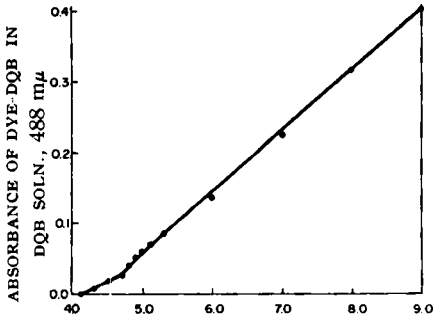


Fig. 5.—Plot of absorbance of dye-DQB in solution at 488 mμ *vs.* DQB concentration in solution.

pound. For PDDB and DDBC, this redissolution occurs at the critical micelle concentration. For DQB, solubility commences just below the critical micelle concentration, while for CPC, solubilization occurs significantly below the critical micelle concentration. It is also interesting to note, as shown in Fig. 5, that much less dye appears in solution for a given change of DQB concentration, compared to the other systems.

DISCUSSION

Stoichiometry and Solubility.—The formation of the various 1:1 dye-detergent compounds is apparently due to electrostatic bonding between the negatively charged sulfonate group of orange II and the cationic nitrogen of the various detergents. However, differences in the solubility of these compounds, and particularly differences in the temperature coefficients for solubility, indicate a marked dependence on the electronic and steric configuration of the cationic species. Such differences as reflected in the heat of solution are CPC ≫ DQB > PDDB ≃ DDBC.

The heat of solution of a salt is dependent on the difference between the energy required to overcome the attraction between oppositely charged ions and the energy gain due to the solvation of the ions. The positive values for $\Delta H°$ obtained in all cases would indicate that the energy required to overcome the attractive forces is greater than the energy of hydration. Since the attractive energy increases with the reciprocal of the distance between the ions, those ions capable of close approach should exhibit greater heats of solution, all other factors being equal. The greater heats of solution of the dye-CPC and dye-DQB compounds, compared to the dye-PDDB and dye-DDBC compounds, are therefore probably because CPC and DQB contain a planar, aromatic structure capable of close approach to the likewise planar and aromatic dye. On the other hand, PDDB and DDBC contain a positive nitrogen atom surrounded by large alkyl and alkoxyl groups in a tetrahedral arrangement. These systems, being bulky and sterically hindered, therefore, cannot approach the dye anion as close as the planar cations.

The large difference in the heat of solution of dye-CPC and dye-DQB appears to be due to more than steric considerations since both compounds are planar and aromatic. One would predict that ionic interactions should be stronger if the charge on each ion is localized to a greater extent. Both the pyridinium and quinolinium ring systems exhibit delocalization of their positive charge, the latter being capable of greater delocalization. On this basis, one would expect the pyridinium ion to be more tightly held to the anionic dye. Whether this difference can account for the observed differences in the heats of solution is difficult to say since the picture is further complicated by secondary attractive forces, energies of hydration, and steric hinderance. Whatever the major factors involved, they are probably also responsible for the fact that orange II can form a 1:2 salt with CPC when the naphthol group is ionized, while DQB and the other detergents cannot.

The large temperature coefficient for the solubility of dye-CPC indicates that the positive entropy changes associated with its dissolution are significant. This large increase of 76 entropy units may in part be accounted for on the basis of the two waters of crystallization, which are released upon dissolution of the salt. Since the standard entropy for water at 25° is 16.7 entropy units, the gain in entropy by this means should be about 33.4 entropy units. The small change in entropy exhibited by the other dye-detergent salts is generally in agreement with what is observed with most inorganic salts. For example, the standard entropy change for the dissolution of silver bromide, calculated from the solubility product data of Lewin (13), is about five entropy units. More accurate calorimetric data are necessary before the exact nature of the entropy changes can be understood, but it is apparent from these studies that entropy is a major factor in the interaction of orange II and CPC.

Solubilization in the Presence of Excess Detergent.—That detergent micelles are capable of solubilizing water-insoluble substances is well known and has been reviewed extensively by McBain (14) and Klevens (15). The solubilization of dye-detergent salts in excess detergent has been observed by Murkejee and Mysels (10). It was shown that for a number of dye-detergent combinations, solubilization occurred below the normal critical micelle concentration of the detergent. It was concluded that the presence of the insoluble dye-detergent salt induces the formation of mixed micelles consisting of the dye-detergent salt and the detergent. At

higher concentrations of detergent, they observed a transition from the dye-rich induced micelles to the more normal type of micelle, fewer dye ions per micelle being distributed uniformly among the great number of detergent micelles.

It can be seen in Figs. 4 and 5 that solubilization occurs very near or at the C.M.C. of all the detergents except CPC, although very slight solubilization below the C.M.C. of DQB may also be occurring. Thus, it would appear that dye-CPC, in particular, may be involved in a mixed micelle of the type shown by Mukejee and Mysels (10). It is also interesting to note the break in the slope of the solubilization curve (Fig. 4) for dye-CPC at 1.0×10^{-3} *M*, the critical micelle concentration of CPC. This may indicate a transition from induced to normal micelles as shown by Mukejee and Mysels.

The mechanism of micellization induced by a solubilizate has been discussed by Ekwall (16), who showed that fatty alcohols are solubilized by soaps significantly below the C.M.C. of the soap. He attributes this phenomenon to a lowering of the free energy of formation of the soap micelles by the formation of a mixed micelle of alcohol and soap. If the alcohol orients between the ionized soap molecules, the repulsive forces of the like-charged soap ions will be reduced, thus facilitating micelle formation.

Likewise, the combination of an unionized long chain dye-detergent salt and an ionized detergent could also result in a mixed micelle which forms below the C.M.C. of the detergent. The importance of having the proper chemical structure and steric configuration so that mixed micelles can form has been shown by Schulman and Cockbain (17) in their work with emulsions. Using long chain alcohols and soaps, they showed that the length of both alkyl chains, the size and polarity of the head groups, and even the presence of double bonds in the alkyl chains are important factors which govern the formation of stable films. Therefore, it is not surprising that all dye-detergent systems do not exhibit marked shifts in the concentration of detergent required for solubilization. The dye-CPC salt apparently is able to form such mixed films with CPC, perhaps because of the planar nature of all the head groups, while the other detergents are too large and bulky to be capable of forming a stable mixed film. For instance, even though DQB is planar, it has a high C.M.C. (4.8×10^{-3} *M*) and a small micellar weight (10,500) (18), which indicates an inability to pack many DQB molecules into a micelle due to the large size of the quinolinium head group. The presence of a 16 carbon chain CPC may also be important, since van der Waals attractions between the alkyl chains will contribute to the stability of a mixed film.

From a pharmaceutical point of view, the fact that these insoluble compounds can be solubilized by relatively low concentrations of surfactant should be useful in overcoming this common incompatibility. If systems capable of forming mixed micelles are used, even less detergent should be necessary. Of course, it must be kept in mind that the dye is now dissolved in a new phase, and that with some dyes a shift in the absorption spectrum of the chromophore may occur. Whether this is a practical problem will depend on the color desired in the final product; whether the antibacterial efficacy of the product is altered must also be considered.

SUMMARY AND CONCLUSIONS

1. The interactions between the dye, orange II, and quaternary ammonium compounds, 1-hexadecylpyridinium chloride (CPC), (2-phenoxyethyl)-dodecyldimethyl ammonium bromide (PDDB), (diisobutylphenoxyethoxyethyl)dimethylbenzylammonium chloride (DDBC), and dodecylquinolinium bromide (DQB) have been investigated.

2. The stoichiometry of these interactions has been determined by measuring the distribution of the dye between water and chloroform as a function of pH and ionic strength, in the presence of various detergent ion concentrations. This has been substantiated by isolation and elemental analysis of the various dye-detergent compounds. Below pH 8.05, where the dye has one ionized group, all detergents give compounds with 1:1 stoichiometry. Above pH 12.34, shown to be that region where the dye contains two ionized groups, dye-CPC shows 1:2 stoichiometry, while the other dye-detergent salts continue to show 1:1 stoichiometry.

3. The solubility products of the various dye-detergent salts have been determined at 25, 30, 35, and 40°. Thermodynamic functions calculated for the dissolution of the salts indicate marked differences in the strength of these interactions, with CPC in particular exhibiting a very strong interaction with orange II.

4. Micellar solubilization of the various dye-detergent compounds occurs in the presence of the excess common detergent. Solubilization of dye-CPC, significantly below the C.M.C. of CPC, is believed to be due to the formation of mixed micelles consisting of the nonionic dye-detergent compound and CPC.

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Synthesis of Amino Acids in the Catecholamine Series

By WALTER H. HARTUNG*, ALBERT N. MATTOCKS‡, and ROBERT I. ELLIN§

Tyrosine is generally proposed as the initial precursor in the biogenesis of epinephrine and norepinephrine. Methods are presented for the preparation of amino acids which could be included in alternate mechanisms or possibly interfere with the metabolic sequence. During the course of this study the following compounds were prepared: phenylserine and its ethyl ester, *p*-hydroxyphenylserine, *O*-methyl tyrosine, and ω-aminoacetylcatechol in an attempt to prepare dihydroxyphenylserine, DOPS. An effort was made to improve on the practicality of the methods previously used. In some cases a specific adaptation of known procedures was employed; in others new syntheses were developed.

RECENT STUDIES (1, 2) tend to advance Blaschko's original hypothesis (3) concerning the role of various amino acids in the biogenesis of catecholamines: tyrosine→3,4-dihydroxyphenylalanine (DOPA)→hydroxytyramine (Dopamine)→norepinephrine. Only a few steps in the classical sequence of reactions have been clearly demonstrated. The purpose of this investigation was to prepare, by convenient methods, amino acids related to those proposed in the biogenetic sequence. The amino acids may have functions of their own or serve as intermediates in alternate metabolic pathways leading to the formation of norepinephrine. Recently, Dickenson and Thompson (4) reported that phenylalanine was necessary for virus synthesis and that L-threophenylserine was the most active of a series of compounds tested against influenza A virus. Holtz, *et al.* (5), found that all organ extracts which decarboxylated DOPA also attacked the phenylserines. During the course of this study the following compounds were prepared: phenylserine and its ethyl ester, *p*-hydroxyphenylserine, *O*-methyl tyrosine, and ω-aminoacetylcatechol (in an attempt to prepare dihydroxyphenylserine, DOPS). An effort was made to improve on the practicality of the methods previously used. In some cases a specific adaptation of known procedures was employed, and in others new syntheses were developed.

EXPERIMENTAL

The melting points reported in the paper are uncorrected and were determined by the capillary tube method.

p-Hydroxyphenacyl Cyanide (II).—In 100 ml. of alcohol was dissolved 21.5 Gm. (0.125 mole) of *p*-hydroxyphenacyl chloride (6) (Ia), and the solution was warmed to 50°. To the warm solution was added, with stirring, 26 Gm. (0.46 mole) potassium cyanide in 80 ml. water. The mixture was kept warm and stirred for an additional 30 minutes, then allowed to cool to room temperature. The solution was acidified to litmus with hydrochloric acid and allowed to cool overnight in a refrigerator. The resulting crystals were filtered and redissolved in 50% alcohol to which 3 Gm. of decolorizing charcoal had been added. The mixture was warmed for 15–20 minutes on a hot plate and filtered while hot. Upon cooling, the clear filtrate yielded 10 Gm. (60%) of *p*-hydroxyphenacyl cyanide as tan crystals. Concentration of the filtrate produced no appreciable quantities of product. The dried cyanide melted with decomposition at 172°. No further purification was considered necessary for subsequent reactions.

Anal.—Calcd. for $C_9H_7NO_2$: N, 8.7. Found: N, 8.9.

Attempted Synthesis of α-Hydroximino-β-keto-β-(p-hydroxyphenyl)-propionic Acid Ethyl Ester from p-Hydroxyphenacyl Cyanide (II).—In 30 ml. of absolute alcohol which had been previously saturated with dry hydrogen chloride, 6 Gm. (0.037 mole) of *p*-hydroxyphenacyl cyanide (II) was dissolved. The solution was placed in a stoppered flask and allowed to stand at room temperature for 5 days. To this solution 100 ml. of distilled water was added, and the mixture was warmed to 30–60° and cooled overnight in a refrigerator. A small quantity of flocculent precipitate was observed in the beaker, and the mixture was extracted with ether to isolate all the reaction product. Evaporation of solvent from the ether extracts produced dark crystals which were dissolved in hot alcohol, charcoaled, and reprecipitated by cooling. A yield of 5.5 Gm. of yellow crystals was obtained. Two repetitions of this reaction produced only dark oily substances from which no crystalline material could be obtained.

Five grams of the yellow crystalline product of alcoholysis were dissolved in 100 ml. of ether, and the solution was saturated with hydrogen chloride. Butyl nitrite (2.4 Gm., 0.023 mole) was added to the solution over a period of 30 minutes. After an additional 30 minutes of stirring, the reaction mixture was transferred to a distilling flask, and the solvents were removed by heating. The residue was taken up in hot toluene and reprecipitated by cooling. Four grams of light gold crystals was obtained. The product melted at 153–155°. The calculated nitrogen content for α-hydroximino-β-keto-β-(*p*-hydroxyphenyl)-propionic acid ethyl ester is 5.91%, and the nitrogen content of the product as shown by Kjeldahl analysis was 11.0%. No

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study of the structure of the product was made, but it is interesting that the corresponding amide would contain 13.5% nitrogen and that the nitroso substituted derivative of the ester desired would contain 10.5% nitrogen. Should this compound actually have been α-hydroximino-β-keto-β-(3-nitroso-4-hydroxyphenyl)-propionic acid ethyl ester, the method might serve as a synthesis of compounds of this type. The possibilities of this reaction deserve further study.

p-Hydroxyphenylglyoxylohydroxamyl Cyanide (IVa).—To a solution of 5.5 Gm. (0.027 mole) of *p*-hydroxyphenylglyoxylohydroxamyl chloride (III*a*) in 100 ml. of alcohol was added 6.5 Gm. (0.1 mole) of potassium cyanide in 100 ml. of water. The solution was heated with occasional stirring for 1 hour on a steam bath. After allowing the mixture to cool, hydrochloric acid was added until the reaction was acid to litmus, and the mixture was cooled in the refrigerator. After 1 hour of cooling, the crystals that had precipitated were filtered, dried, and weighed. Filtration gave 1.5 Gm. of brown needles and ether extraction of the filtrate followed by evaporation of solvent produced 3.5 Gm. additional product, the total yield being 5 Gm. (95%). The crude nitrile was recrystallized from dilute alcohol, and the crystals melted at 160–161° with decomposition. Repeated reactions gave yields of 90–95%.

Anal.—Calcd. for $C_{10}H_8N_2O_3$: N, 14.7. Found: N, 14.1, 14.3.

α-Hydroximino-p-hydroxybenzoylacetic Acid (V).—In 50 ml. of 10% aqueous sodium hydroxide was dissolved 3.5 Gm. (0.018 mole) of *p*-hydroxyphenylglyoxylohydroxamyl cyanide (IV*a*), and the mixture was heated in an open beaker on a steam bath for 6 hours, evolution of ammonia being completed at the time. Crushed ice (100 Gm.) was added, and the cold mixture was acidified with dilute hydrochloric acid and extracted with ether. The ether was dried over anhydrous sodium sulfate and removed on a water bath. The residue did not require further purification since it was only slightly colored. A yield of 3.6 Gm. (96%) of crude acid was obtained as yellow crystals, melting with evolution of gas at 124–125°. Attempts at recrystallization of small amounts of the acid for analysis were unsuccessful; only amorphous products were obtained. A neutral equivalent of the compound was made by dissolving a portion in excess 0.02 *N* base and residually titrating with 0.02 *N* acid.

Anal.—Calcd. for N.E. $C_9H_9NO_6$: 225. Found: N.E. 219, 228.

p-Hydroxyphenylserine Hydrochloride (VI).—In 100 ml. of absolute alcohol containing 5 Gm. hydrogen chloride was dissolved 2.1 Gm. (0.01 mole) of the α-hydroximino acid (V); 3 Gm. of 10% palladium catalyst was added. The mixture was shaken in hydrogen at a pressure of 1 Atm., and the theoretical quantity of hydrogen was absorbed in 1 hour. The solution was filtered to remove the catalyst and the filtrate concentrated under reduced pressure to about 30 ml. Ether was added until a definite turbidity was apparent, and the mixture was then cooled overnight. The crystals which had formed were filtered, washed with ether, and dried. The product was recrystallized from hot alcohol. The crystals darkened to gold at 180–185° and melted to a red oil with evolution of gas at 224–225°. This melting point behavior is similar to that reported for the free base (7).

Anal.—Calcd. for $C_9H_{13}ClNO_4$: N, 6.01. Found: N, 5.96, 6.01.

3,4-Dihydroxyphenylglyoxylohydroxamyl Cyanide (IVb).—To 10.65 Gm. (0.05 mole) of 3,4-dihydroxyphenylglyoxylohydroxamyl chloride (6) (III*b*) dissolved in 100 ml. of alcohol was added 13 Gm. (0.3 mole) of potassium cyanide dissolved in 200 ml. of water. The mixture was heated, with occasional stirring, on a steam bath for 1 hour and allowed to cool. On acidification with dilute hydrochloric acid, a brownish mass formed which dissolved on addition of excess acid. The dark mixture was extracted with three 250-ml. portions of ether. The ether extract was dried over anhydrous sodium sulfate and evaporated to dryness on a steam bath. The resulting crude product was taken up in 50 ml. of ether, boiled with 1 Gm. of charcoal, and filtered. After the addition of 350 ml. of toluene, the solution became cloudy and was placed in a refrigerator for 2 hours. A yield of 5.5 Gm. (53%) of yellowish-green crystals was obtained, melting at 172 to 173.5°.

Anal.—Calcd. for $C_9H_9N_2O_4$: C, 52.43; H, 2.93; N, 13.6. Found: C, 52.43; H, 2.95; N, 13.5.

Attempted Preparation of α-Hydroximino-3,4-dihydroxybenzoylacetic Acid (VII).—A solution of 3,4-dihydroxyphenylglyoxylohydroxamyl cyanide (IV*b*) (0.02 mole) was dissolved in 100 ml. of 10% aqueous sodium hydroxide, and the mixture was heated on a steam bath until ammonia was no longer liberated. The reaction required 5–6 hours of heating. On cooling to room temperature, the solution was treated with 100 Gm. of crushed ice and acidified with dilute hydrochloric acid; an immediate evolution of gas was noted. The mixture was extracted with ether and the ether extract dried over anhydrous sodium sulfate, filtered, and evaporated to dryness on a steam bath. Very hygroscopic brown crystals were obtained. Further purification by recrystallization from ether produced yellow-brown crystals which were also hygroscopic. An accurate analysis could not be obtained.

Preparation of ω-Aminoacetylcatechol (VIII). (Attempted Synthesis of 3,4-Dihydroxyphenylserine).—In a hydrogenation flask were placed the product obtained from the hydrolysis of 0.02 mole of 3,4-dihydroxyphenylglyoxylohydroxamyl cyanide (IV*b*), 100 ml. of absolute alcohol containing 10 Gm. of dry hydrogen chloride, and 3 Gm. of a 10% palladium-on-charcoal catalyst. The mixture was shaken on a Parr hydrogenation apparatus until hydrogen was no longer absorbed, about 0.05 mole being taken up. The catalyst was filtered, and the filtrate concentrated *in vacuo* to 30 ml. Addition of 100 ml. of dry ether precipitated 4 Gm. of colorless crystals. Recrystallization from absolute alcohol gave 3.6 Gm. of a hydrochloride that melted to a red oil with evolution of a gas at 244–246°.

Anal.—Calcd. for 3,4-dihydroxyphenylserine hydrochloride, $C_8H_{11}NO_3 \cdot HCl$: C, 43.29; H, 4.85; N, 5.6. Calcd. for arterenol hydrochloride, $C_8H_{11}NO_3 \cdot HCl$: C, 46.72; H, 5.9; N, 6.8. Calcd. for ω-aminoacetylcatechol, $C_8H_9NO_3 \cdot HCl$: C, 47.18; H, 4.9; N, 6.8. Found: C, 46.65; H, 4.9; N, 6.9.

The analytical data were consistent with the values calculated for ω-aminoacetylcatechol or arterenol hydrochloride. The melting point of arterenol hydrochloride was reported by Simonoff (8) as 136° with decomposition. The melting point of the amino ketone hydrochloride reported by Barger and Dale (9) was 252° with decomposition. From the above data it may be assumed with reasonable certainty that the compound formed was ω-aminoacetylcatechol rather than arterenol or 3,4-dihydroxyphenylserine.

The apparent formation of ω-aminoacetylcatechol by this series of reactions, rather than 3,4-dihydroxyphenylserine, can be explained if the β-keto acid obtained by hydrolysis of 3,4-dihydroxyglyoxylohydroxamyl cyanide (IV*b*) underwent decarboxylation to form ω-hydroximinoacetylcatechol. The evolution of gas, presumably carbon dioxide, noted during the hydrolysis, is evidence of the possibility that decarboxylation occurred.

In an attempt to avoid decarboxylation, the mixture, resulting from the heating of 3 Gm. of 3,4-dihydroxyphenylglyoxylohydroxamyl cyanide (IV*b*) with sodium hydroxide was transferred without purification to a hydrogenation bottle. The flask was shaken with 0.5 Gm. of a 10% palladium catalyst in an effort to prevent poisoning of the 3 Gm. of additional catalyst which was later added. A negligible amount of hydrogen was taken up, indicating incomplete hydrogenation. The solution was acidified and extracted with ether. On evaporation of the ether, effervescence was again observed. The residue was dissolved in 100 Gm. of absolute alcohol containing 5 Gm. of dry hydrogen chloride and placed in a hydrogenation bottle with 3 Gm. of a 10% palladium catalyst. After hydrogenation, ω-aminoacetylcatechol, melting with decomposition at 244–246°, was obtained.

Phenylserine and Phenylserine Ethyl Ester Hydrochloride.—In a 500-ml. round-bottomed flask were placed 8.5 Gm. (0.08 mole) of benzaldehyde, 9.5 Gm. (0.04 mole) of carbobenzyloxyglycine ethyl ester, 2 Gm. of sodium wire, and 75 ml. of dry ether. The sodium wire was soon coated with a yellow layer from which it was freed by vigorous shaking. The sodium disappeared after standing 24 hours. The solution was filtered, and the resulting solid washed with dry ether. The crystals were dissolved in a minimum of water and acidified with dilute acetic acid. Upon evaporation of the solvent under reduced pressure, a viscous oil was obtained which solidified to a gel in the refrigerator. This was treated with 50 ml. of absolute alcohol containing 3 Gm. of dry hydrogen chloride and 2 Gm. of a 10% palladium-on-charcoal catalyst. On hydrogenation, 250 ml. of hydrogen was absorbed. The catalyst was filtered and the solution concentrated to 35 ml. under reduced pressure. Addition of dry ether produced 4 Gm. (41%) of phenylserine ether ester hydrochloride, melting at 157–161°. The melting point was in agreement with those already reported for phenylserine ethyl ester hydrochloride (10).

Anal.—Calcd. for $C_{11}H_{15}NO_5 \cdot HCl$: N, 5.70. Found: N, 5.78, 5.83.

N-Carbobenzyloxyphenylserine.—In a 500-ml. round-bottomed flask were placed 8.5 Gm. (0.08 mole) of benzaldehyde, 8.4 Gm. (0.04 mole) of carbobenzyloxyglycine, 2 Gm. (0.45 mole) of sodium wire, and 75 ml. of dry ether; reaction took place as previously described. Isolation of the product by the above procedure produced 8 Gm. of the sodium salt of *N*-carbobenzyloxyphenylserine. The crystals were dissolved in a minimum of water and acidified with dilute acetic acid. Evaporation of the solvent produced an oil which solidified on addition of dry ether and standing in the refrigerator; it melted at 83–85°.

Anal.—Calcd. for $C_{17}H_{17}NO_6$: N, 4.44. Found: N, 4.53.

Phenylserine.—*N*-Carbobenzyloxyphenylserine (1 Gm., 0.003 mole) was dissolved in 75 ml. of alcohol containing 7 Gm. of dry hydrogen chloride and 2 Gm. of a 10% palladium catalyst. About 100 ml. of hydrogen (a slight excess of the theoretical amount) was absorbed in 15 minutes. The catalyst was filtered and the filtrate concentrated under reduced pressure. Addition of an excess of dry

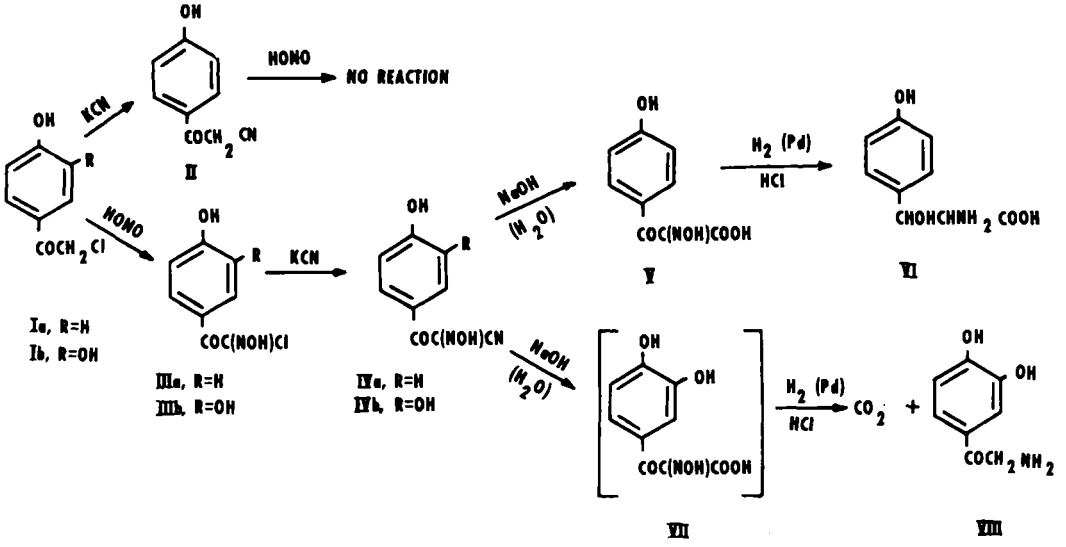


Figure 1.

ether produced 0.4 Gm. (57%) of crystals melting at 148–150°. Carrara and co-workers (11) reported the melting point of phenylserine hydrochloride to be 157°.

α - Hydroximino - β - (p - methoxyphenyl)-propionic Acid.—A 5.3 Gm. (0.024 mole) quantity of *p*-methoxybenzylmalonic acid was dissolved in 50 ml. of ether, and 4.85 Gm. (0.047 mole) of butyl nitrite was added. The mixture was placed in an ice bath and stirred while hydrogen chloride was passed into the beaker until none of the gas was absorbed. Stirring and cooling were continued for 20 minutes, and the reddish reaction mixture was warmed on a hot water bath until the ether had evaporated. A residue of slightly colored solid remained; this was dissolved in warm alcohol and reprecipitated by the addition of cold water to yield 4.95 Gm. (96%) of colorless crystals of α-hydroximino-β-(*p*-methoxyphenyl)-propionic acid. The recrystallized product melted at 156°. Hamlin and Hartung (12) reported a melting point of 156–157° for this compound. Subsequent reactions gave yields of 90–95%.

O-Methyltyrosine.—To a solution of 4 Gm. (0.019 mole) of the α-hydroximino-β-(*p*-methoxyphenyl)-propionic acid in 100 ml. ethyl alcohol was added 3 Gm. of a 10% palladium catalyst. To the mixture was then added 10 ml. of 36% hydrochloric acid; the mixture was shaken with hydrogen at a pressure of 10 Atm. The theoretical quantity of hydrogen was absorbed in 3.5 hours, and the mixture was filtered to remove the catalyst. The solvent was removed under reduced pressure on a hot water bath, and the residue was dissolved in 15 ml. of warm distilled water and titrated to neutral with 0.1 *N* sodium hydroxide using methyl red indicator. Cooling of the neutral solution precipitated 3.5 Gm. (93%) of *O*-methyltyrosine. The melting point, 295–296° with decomposition, agreed with the value reported (13, 14).

DISCUSSION

Phenylglyoxylohydroxamyl cyanide (II) was prepared by nitrosating phenacylchloride and reacting the resulting isonitroso compound with potassium cyanide. Both keto and cyano groups are known to promote nitrosation of adjacent aliphatic carbon atoms. Indeed, Pasquale (15) obtained good yields of II by nitrosating phenacyl cyanide. We were not, however, able to prepare *p*-hydroxyphenylglyoxylohydroxamyl cyanide (IV*a*) by nitrosating *p*-hydroxyphenacylcyanide, nor are reports available indicating that 3,4-dihydroxyphenylglyoxylohydroxamylcyanide (IV*b*) can be prepared by nitrosating dihydroxyphenacylcyanide. This would indicate that substituents adjacent to carbonyl groupings drastically affect nitrosation reactions.

The potential of these compounds as enzyme inhibitors or alternate precursors of norepinephrine were not investigated; however, a group of the National Institutes of Health (16, 17) reported recently on the activity of a number of related compounds as substrates and inhibitors of dopamine-β-oxidase.

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Assay Methods for Some *Vinca rosea* Alkaloids II

By IVAN M. JAKOVLJEVIC, L. DAVID SEAY, and REGINALD W. SHAFFER

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ether produced 0.4 Gm. (57%) of crystals melting at 148–150°. Carrara and co-workers (11) reported the melting point of phenylserine hydrochloride to be 157°.

α - Hydroximino - β - (p - methoxyphenyl)-propionic Acid.—A 5.3 Gm. (0.024 mole) quantity of *p*-methoxybenzylmalonic acid was dissolved in 50 ml. of ether, and 4.85 Gm. (0.047 mole) of butyl nitrite was added. The mixture was placed in an ice bath and stirred while hydrogen chloride was passed into the beaker until none of the gas was absorbed. Stirring and cooling were continued for 20 minutes, and the reddish reaction mixture was warmed on a hot water bath until the ether had evaporated. A residue of slightly colored solid remained; this was dissolved in warm alcohol and reprecipitated by the addition of cold water to yield 4.95 Gm. (96%) of colorless crystals of α-hydroximino-β-(*p*-methoxyphenyl)-propionic acid. The recrystallized product melted at 156°. Hamlin and Hartung (12) reported a melting point of 156–157° for this compound. Subsequent reactions gave yields of 90–95%.

O-Methyltyrosine.—To a solution of 4 Gm. (0.019 mole) of the α-hydroximino-β-(*p*-methoxyphenyl)-propionic acid in 100 ml. ethyl alcohol was added 3 Gm. of a 10% palladium catalyst. To the mixture was then added 10 ml. of 36% hydrochloric acid; the mixture was shaken with hydrogen at a pressure of 10 Atm. The theoretical quantity of hydrogen was absorbed in 3.5 hours, and the mixture was filtered to remove the catalyst. The solvent was removed under reduced pressure on a hot water bath, and the residue was dissolved in 15 ml. of warm distilled water and titrated to neutral with 0.1 *N* sodium hydroxide using methyl red indicator. Cooling of the neutral solution precipitated 3.5 Gm. (93%) of *O*-methyltyrosine. The melting point, 295–296° with decomposition, agreed with the value reported (13, 14).

DISCUSSION

Phenylglyoxylohydroxamyl cyanide (II) was prepared by nitrosating phenacylchloride and reacting the resulting isonitroso compound with potassium cyanide. Both keto and cyano groups are known to promote nitrosation of adjacent aliphatic carbon atoms. Indeed, Pasquale (15) obtained good yields of II by nitrosating phenacyl cyanide. We were not, however, able to prepare *p*-hydroxyphenylglyoxylohydroxamyl cyanide (IV*a*) by nitrosating *p*-hydroxyphenacylcyanide, nor are reports available indicating that 3,4-dihydroxyphenylglyoxylohydroxamylcyanide (IV*b*) can be prepared by nitrosating dihydroxyphenacylcyanide. This would indicate that substituents adjacent to carbonyl groupings drastically affect nitrosation reactions.

The potential of these compounds as enzyme inhibitors or alternate precursors of norepinephrine were not investigated; however, a group of the National Institutes of Health (16, 17) reported recently on the activity of a number of related compounds as substrates and inhibitors of dopamine-β-oxidase.

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TABLE I.—COLOR REACTIONS OF SOME *Vinca rosea* LINN. ALKALOIDS

Name	1% Ceric Ammonium Sulfate in 85% H_3PO_4, RT	1% Ferric Ammonium Sulfate in 85% H_3PO_4 After 10 Min. in Water Bath	1% Ferric Ammonium Sulfate in 75% H_2SO_4, RT
Vincaleukoblastine	Purple	Violet	Blue
Leurocristine	Blue violet	Pink	Blue → grey-blue
Leurosine	Pink	Pale yellow	Yellow → green
Leurosidine	Yellow → copper	Violet (strong)	Dark blue
Vindoline	Purple (strong)	Blue-grey	Purple (strong)
Catharanthine	Indigo → green	Yellow	Blue-violet
Virosine	Sky blue → yellow	Colorless	Pink → green
Perivine	Green → brown	Pale yellow	Umber
Reserpine	Sky blue → yellow	Lemon yellow	Blue → green
Vincarodine	Blue → green	Colorless	Blue → green → umber
Neoleurosidine	Violet → blue-violet	Magenta	Green → blue
Tetrahydroserpentine	Yellow → umber	Colorless	Violet → blue → green
Isoleurosine	Copper	Yellow → orange	Green → blue
(Indole)	Dark brown	Yellow	Yellow → green

vincaleukoblastine. However, at the maximum absorption peak, leurocristine has an extinction coefficient 25% lower than that of vincaleukoblastine.

There are still 35 alkaloids which have been isolated from *Vinca rosea* Linn. (*Catharanthus roseus* G. Don) in the Organic Chemical Development and Research Laboratories, Eli Lilly and Co. (11, 12); it is the purpose of this paper to give quantitative means for determining some of them.

It is very interesting that at room temperature a 1% solution of ceric ammonium sulfate in 85% phosphoric acid is an excellent reagent for distinguishing between most of these alkaloids.

A 1% solution of ferric ammonium sulfate in 85% phosphoric acid gives quite different colors from that of the ceric salt, but requires heating in a water bath.

Ferric ammonium sulfate, as a 1% solution in 75% sulfuric acid, offers at room temperature a third series of different colors—a great help in the identification of these alkaloids.

Table I gives the color reactions produced by these three reagents. Approximately 200–300 mcg. of the alkaloid dissolved in about 1 ml. of the corresponding reagent will give colors as shown in Table I.

EXPERIMENTAL

Leurosine

Analytical data for leurosine and vincaleukoblastine (3) show them as isomeric compounds: $C_{46}H_{56}N_4O_9$ having the dimeric indole-indoline group with a demonstrable oncolytic activity. The ultraviolet spectrum of leurosine has a maximum at 214 mμ in ethanol. Electrometric titration shows pKa′ at 7.5 in water (7).

The sulfate salt, m.p. 238–242° dec., has been used for the quantitative determination described below.

Materials.—Ferric ammonium sulfate, crystals, A.R.; phosphoric acid 85%, A.R.; and acetone, A.R., all from Mallinckrodt Chemical Works are utilized.

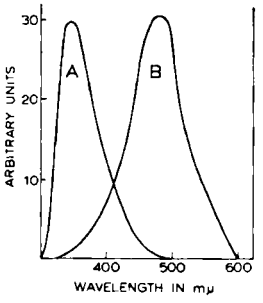


Fig. 1.—Excitation (A) and emission (B) spectra of leurosine sulfate fluorophor.

Color Reagent.—Five grams of ferric ammonium sulfate is dissolved in about 400 ml. of phosphoric acid in a 500-ml. volumetric flask by heating in a boiling water bath. The solution is cooled to room temperature and made up to volume with phosphoric acid. To prepare a reagent which is stable for several months, the larger crystals found in the reagent bottle should be pulverized and used.

Apparatus.—An Aminco-Bowman spectrophotometer (American Instrument Co., Inc., Silver Springs, Md.), equipped with 1-cm. square silica cells was used for the determination of excitation and emission spectra. A Turner model 110 fluorometer (G. K. Turner Associates) equipped with a set of matched borosilicate glass test tubes was employed for all fluorometric measurements. A filter 7–60 (transmitting light between 350 and 400 mμ) was employed as a primary filter, and a filter 2A (transmitting at 415 mμ and above) was employed as secondary filter.

Procedure.—One milligram of the standard leurosine sulfate and 1 mg. of the sample preparation are weighed to the nearest 0.01 mg. and transferred into separate 100-ml. volumetric flasks. The standard and the sample are dissolved and diluted to the mark with acetone. Two 1-ml. aliquots of the standard solution and two 1-ml. aliquots of the sample solution, equivalent to 10 mcg. of leurosine sulfate each, are transferred to separate 10-ml. volumetric flasks. The solvent is evaporated with a mild jet of air. To each of the four flasks, 5 ml. of the color reagent is added by a graduated cylinder. A reagent blank is prepared by adding 1 ml. of acetone and 5 ml. of the color reagent to a 10-ml. volumetric flask. The flasks

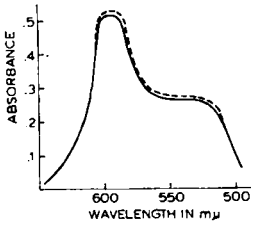


Fig. 2.—Cary recording spectrophotometric curve of the leurosidine chromophor. Key: ——, initially; - - - -, after 24 hours.

are placed simultaneously in an oil bath of 70 ± 3° and, after heating for 2 minutes, they are stoppered with glass stoppers. The heating is continued for a total of 40 minutes. The contents are cooled to room temperature and diluted to the mark with phosphoric acid.

In the example given, the concentration of the final solution is 1 mcg. of leurosine sulfate per milliliter, but as low as 0.02 mcg. can be easily estimated. The calibration curve passes through the origin and obeys Beer's law within this concentration range. It was noted also that the fluorophor formed is extremely stable for up to 8 hours after being diluted with phosphoric acid.

Figure 1 gives excitation and emission spectra of the fluorophor.

Leurosidine

Leurosidine is another dimeric indole-indoline compound having an ultraviolet spectrum essentially identical to that of leurosine. In chloroform as a solvent, even the infrared spectrum is similar to that of leurosine (10), m.p. 208–211° dec. pKa′ 5.5 and 8.8 in 33% dimethylformamide. $[\alpha]_D^{26} = +55.8°$ in chloroform (10, 11).

When leurosidine is treated with ceric ammonium sulfate color reagent, it exhibits a fluorescence which is approximately one-half that of leurosine; however, leurosidine exhibits a reddish color also which is not observed with leurosine. The same reddish color is given by vincaleukoblastine and leurocristine if treated with the same reagent. The fluorescence of vincaleukoblastine is about 30% of that of leurosine, and leurocristine has practically no fluorescence.

Materials.—Ferric ammonium sulfate, crystals, A.R.; phosphoric acid 85%, A.R., both from Mallinckrodt Chemical Works; and sulfuric acid concentrate, reagent, E. I. du Pont de Nemours & Company, Inc., are employed.

Color Reagent.—Five grams of ferric ammonium sulfate is dissolved in about 400 ml. of phosphoric acid in a 500-ml. volumetric flask by heating in a boiling water bath. The solution is cooled to room temperature, 10 ml. of sulfuric acid is added, and the volume made up to 500 ml. with phosphoric acid. As mentioned above, only the large crystals should be pulverized and used.

Apparatus.—A Beckman model DU spectrophotometer with 1-cm. glass cells is used.

Procedure.—Three milligrams each of the standard leurosidine sulfate and the sample is weighed to the nearest 0.01 mg., transferred to separate 10-ml. volumetric flasks, dissolved in water, and diluted up to volume. Two 1-ml. aliquots of both standard and sample solution are transferred into individual 10-ml. volumetric flasks; 2 ml. of water is added to each of the flasks. The solution is made to volume with the color reagent, and the flasks placed for 40 minutes in a water or oil bath at 100 ± 3°. After the heating period, the flasks are cooled to room temperature and, if necessary, the volume is corrected to 10 ml. using the color reagent. A reagent blank is prepared by transferring 3 ml. of water to a 10-ml. volumetric flask and diluting to volume with the color reagent. The reagent blank is heated simultaneously with the standard and the sample. The dark blue chromophor exhibits a peak at 605 mμ, where the calculations are made, and a well defined shoulder at 570 mμ. After 24 hours, both the peak and the shoulder will be found at the same place, except that their absorbances are slightly higher (Fig. 2).

The calibration curve made with 5–40 mcg./ml. of leurosidine sulfate in the final solution passes through the origin and obeys Beer's law.

Vindoline

Vindoline is considered the major alkaloid in the leaves of *Vinca rosea* Linn. On the basis of analytical and physical data, the empirical formula $C_{25}H_{32}N_2O_6$ has been found (4, 11). Vindoline is combined with the indole moiety in several dimeric *Vinca* alkaloids, m.p. 154–155°. pKa′ 5.5 in 66% dimethylformamide. $[\alpha]_D^{26} = -18°$ (chloroform). Ultraviolet absorption curve shows peaks at 212, 250, and 304 mμ in ethanol (2).

Vindoline dissolves in concentrated sulfuric acid and hydrochloric acid without color; in concentrated nitric acid or nitric acid diluted no more than 1:3, it dissolves giving a dark ruby red.

The strong red developed with ferric ammonium sulfate described below has been found specific for vindoline and until now has not been observed with any other alkaloid from *Vinca rosea* Linn., as seen in Table I.

Materials.—Ferric ammonium sulfate, crystals, A.R., Mallinckrodt Chemical Works; sulfuric acid concentrate, reagent, E. I. du Pont de Nemours & Company, Inc.; and acetic acid glacial, A.R., Mallinckrodt Chemical Works are utilized.

Color Reagent.—One gram of ferric ammonium sulfate is dissolved in 25 ml. of water and filtered if necessary. While swirling and cooling in ice water, 75 ml. of concentrated sulfuric acid is added in small increments. The reagent is stable for 2 days, after which time it becomes turbid and should be discarded.

Apparatus.—A Beckman model DU spectrophotometer with 1-cm. glass cells is employed.

Procedure.—One milligram each of the standard vindoline and the sample are weighed to the nearest 0.01 mg., transferred to separate 50-ml. volumetric flasks, and dissolved in and made up to volume with glacial acetic acid. Two 2-ml. aliquots of both standard and sample solutions, each equivalent to 40 mcg., are transferred into individual 10-ml. volumetric flasks. To each flask is pipeted 3 ml. of glacial acetic acid; the solution is made to volume with the color reagent. As soon as the reagent is added, a red (lavender) is observed. Although heat develops by mixing acetic acid with the color reagent, a contraction of the volume can be observed. The mixture is homogenized by inverting the flasks several times and is allowed to cool to room temperature. The final volume is made 15 minutes after the color reagent is added.

The reagent blank consists of 5 ml. glacial acetic acid and 5 ml. of the color reagent treated at room temperature as standard and sample.

The absorbances are measured at 538 mμ where the only peak appears. The color is stable for at least 4 hours, after which time the mixture becomes a little opalescent. The calibration curve is linear, passes the origin, and obeys Beer's law in concentrations from 1–10 mcg./ml.

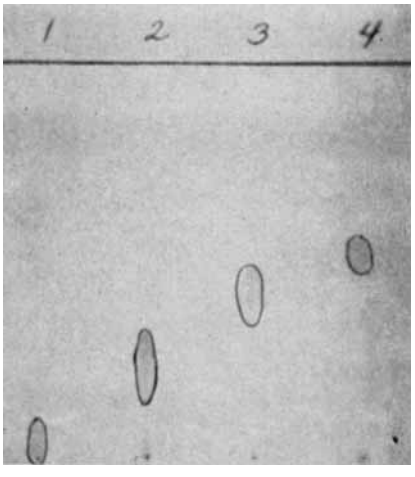


Fig. 3.—Solvent: 5% EtOH in acetonitrile; plate: 0.5*N* LiOH/alumina; sample: 50 mcg. Key: *1*, neo-leurosidine; *2*, leurosidine; *3*, vincathicine; *4*, leurocristine.

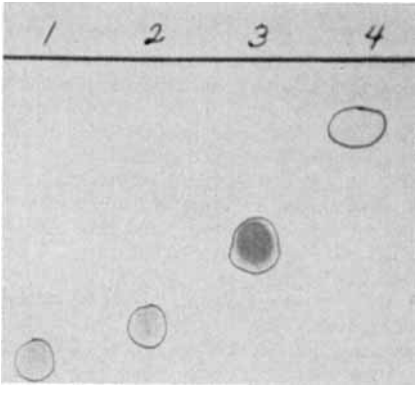


Fig. 4.—Solvent: 30% acetonitrile in benzene; plate: 0.5*N* LiOH/alumina; sample: 50 mcg. Key: *1*, leurosine; *2*, vincaleucoblastine; *3*, vindoline; *4*, catharathine.

THIN-LAYER CHROMATOGRAPHY

The chromatographic investigation of the *Vinca* alkaloids, as with most alkaloids, has been done mainly by employing paper chromatography (9). This technique has much to offer for identification purposes, but it suffers somewhat with respect to reproducibility and rigid control of conditions. There are also some limitations on the type of detection techniques employed. Thin-layer chromatography offers a better approach to the identification of most *Vinca* alkaloids (1, 10); the two systems discussed below are felt to have special advantages over paper chromatography as a means of identification. The methods are less time consuming, more reproducible, and more specific as a result of the use of a new color reagent.

The work discussed here was first performed by spotting the free alkaloids on silica gel plates prepared in the usual manner. Ethyl acetate, absolute ethanol, or a mixture of the two gave satisfactory results, except for tailing of several of the alkaloids.

Alumina plates (aluminum oxide Fluka for thin-layer chromatography) prepared in the usual manner eliminated some of the tailing, but not all separations were complete. Finally, the use of alumina plates prepared with 0.5 *N* lithium hydroxide worked well with a solvent containing 5% absolute ethanol in acetonitrile (v/v), except for some tailing of leurosine. The tailing of leurosine was eliminated with a 30% acetonitrile in benzene solution (v/v). The last system separates leurosine and vincaleukoblastine quite well, while other alkaloids travel too slowly. By making use of both systems, all the alkaloids mentioned below can be separated and identified readily (see Figs. 3 and 4).

All experiments were performed employing large chromatographic chambers (4 × 11 × 12 in.) which contained blotting paper wicks covering both narrow sides of the chamber and one of the large sides for a distance of approximately 9 in. from the bottom. The sulfate salts of the alkaloids studied thus far behave the same as their corresponding free alkaloids in a given system. The samples were prepared as 1% solutions in chloroform and have been stable with respect to chromatography for at least 24 hours if the solutions are stored in a refrigerator. Normally, 200 mcg. of the sample is spotted which permits the detection of as low as 1 to 3% of any other alkaloid which may be present.

The detection of the alkaloids may be accomplished by either of two methods. Most of them may be seen under a low wavelength ultraviolet light when the plate is viewed against a white background. All of the alkaloids react well with a

Table II.—Some Characteristics of Several *Vinca* Alkaloids

Name	Ultraviolet Fluorescence	Color	*Rf* Values 5% EtOH in Acetonitrile	*Rf* Values 30% Acetonitrile in Benzene
Neo-leurosidine	Purple	Brown → lavender	0.04	...
Leurosidine	Purple	Lavender	0.23	...
Vincathicine	Bluish-white	Pink → yellow	0.41	...
Leurocristine	Quenches	Lavender → pink	0.51	...
Leurosine	Blue	Bluish lavender	...	0.27
Vincaleukoblastine	Purple	Lavender	...	0.36
Vindoline	Quenches	Violet → pink	...	0.56
Catharanthine	Quenches	Yellow	...	0.85

mixture of 1% ceric ammonium sulfate in 85% phosphoric acid. If the color reagent is diluted with an equal volume of water, it is more conveniently sprayed; however, more than one spraying may be required with the diluted reagent to obtain sharp and stable colored zones. Table II shows the colors obtained, the ultraviolet characteristics, and the R_f values of the alkaloids in the two systems desiccatot discussed above.

Some precautions have been necessary to obtain the best results. The plates should be oven-dried at 105° for at least 30 minutes before using them. The dried plates may be stored for weeks in a suitable desiccator without noticeable effects on their performance. Color development is best if the plates are heated to 100° immediately after they are removed from the chamber and are kept warm for at least 5 minutes before spraying them. As mentioned above, the diluted color reagent is the best for the detection purposes in spite of the necessity of repeated sprayings. The colors are quite stable overnight if the plate is kept warm (90–100°) and no moisture is allowed to come into contact with the plate. The colors fade considerably if the plate is allowed to cool and absorb moisture from the air. The color development will be poor if the spraying is delayed more than 30 minutes after removing the plate from the solvent system, even though the plate is kept warm.

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Technical Articles

New Rheometer for Determining Rheological and Viscoelastic Properties of Fluids

By DUNCAN E. McVEAN* and JERE E. GOYAN‡

A rising cylinder rheometer is described which can be used to study the flow properties of fluids in the low shear regions similar to those caused by a falling particle. The rheometer may also be used to study viscoelastic properties of fluids. The rheometer was used to obtain flow curves in the low shear region of several pharmaceutical suspending agents. Bingham yield values were obtained by extrapolating methods from these flow curves. Dynamic viscosities of these materials were obtained by analysis of their relaxation behavior. The dynamic viscosities are compared with the plastic viscosities of the materials in the low shear range.

THE COMMONLY used suspension vehicles exhibit either pseudoplastic or plastic flow properties (1). Flow curves for these fluids over a wide range of shear rates may be obtained from viscometers currently in use (2).

However, the measurement of shearing forces at the very low shear rates produced by a falling particle is difficult to obtain with available viscometers. An example of the magnitude of desirable shear may be obtained as follows. Consider a 100-μ particle with a density of 2.00 falling in a Newtonian fluid having a density of 1.00 and a viscosity of 10 poise. The maximum shear rate (at the equator of the sphere) will be 0.16 reciprocal seconds. It was therefore decided to build a viscometer which could produce viscometric data at these low shear rates. An additional advantage to such an instrument would be the more precise determination of yield value obtainable because of the shorter extrapolation to zero shear rate.

The development of a new viscometer seemed desirable because of the difficulties that would be

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Technical Articles

New Rheometer for Determining Rheological and Viscoelastic Properties of Fluids

By DUNCAN E. McVEAN* and JERE E. GOYAN‡

A rising cylinder rheometer is described which can be used to study the flow properties of fluids in the low shear regions similar to those caused by a falling particle. The rheometer may also be used to study viscoelastic properties of fluids. The rheometer was used to obtain flow curves in the low shear region of several pharmaceutical suspending agents. Bingham yield values were obtained by extrapolating methods from these flow curves. Dynamic viscosities of these materials were obtained by analysis of their relaxation behavior. The dynamic viscosities are compared with the plastic viscosities of the materials in the low shear range.

THE COMMONLY used suspension vehicles exhibit either pseudoplastic or plastic flow properties (1). Flow curves for these fluids over a wide range of shear rates may be obtained from viscometers currently in use (2).

However, the measurement of shearing forces at the very low shear rates produced by a falling particle is difficult to obtain with available viscometers. An example of the magnitude of desirable shear may be obtained as follows. Consider a 100-μ particle with a density of 2.00 falling in a Newtonian fluid having a density of 1.00 and a viscosity of 10 poise. The maximum shear rate (at the equator of the sphere) will be 0.16 reciprocal seconds. It was therefore decided to build a viscometer which could produce viscometric data at these low shear rates. An additional advantage to such an instrument would be the more precise determination of yield value obtainable because of the shorter extrapolation to zero shear rate.

The development of a new viscometer seemed desirable because of the difficulties that would be

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encountered in modifying instruments now in use. Frictional forces encountered at low r.p.m. negate the use of coaxial cylinder rotational viscometers. Slattery (3) has shown in his study of the flow of non-Newtonian materials around a sphere that the use of falling sphere instruments would yield data requiring complex computations in order to extract viscometric data. Experiments involving capillary instruments are both time consuming and inefficient when used to study non-Newtonian materials.

An instrument which seemed ideally suited for low shear measurements of non-Newtonian materials is the translated cylinder (Pochettino) viscometer (4). Several authors have discussed and used this type of instrument (5–9). Bergen and Patterson (10) modified the instrument using a closed tube with a solid cylindrical bob. This enabled them to obtain very low shear rates. Unfortunately, the flow in such a closed system is complex; large correction factors were needed with their instrument.

Description of the Rheometer.—In general the instrument designed in this laboratory consists of a cylinder rising in a tube open at both ends; both tube and cylinder are immersed in the sample. A schematic diagram of the rheometer is presented in Fig. 1. The tube was selected by examining several lengths of tubing and choosing one which deviated from being round by no more than 0.005 cm. The supports for the tube consist of two sets of three stainless steel tapered fingers.

Since the tube is open at both ends, the complex flow patterns associated with closed-end tubes are avoided. The cylinder is raised at a uniform velocity through the liquid being studied. The resistance of the material to the motion of the cylinder through it is recorded with a strain gauge.

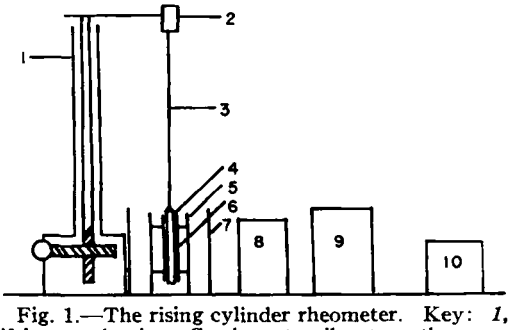


Fig. 1.—The rising cylinder rheometer. Key: *1*, lifting mechanism, Gardner tensile strength apparatus, Gardner Laboratory, Bethesda, Md.; *2*, Statham transducer, G1-1.5-350, Statham Instruments, Hato Rey, Puerto Rico; *3*, Coats and Clark's O.N.T. extra strong button and carpet cord, S. S. Kresge Co.; *4*, 12.70 × 0.4302-cm. stainless steel cylindrical rod or bob; *5*, 15.88 × 5.08-cm. stainless steel sample container; *6*, 10.16 × 0.4646-cm. hollow stainless steel tube or cup; *7*, temperature controlled water bath; *8*, motor controller, GT 21, G. K. Heller Co., Las Vegas, Nev.; *9*, transducer amplifier-indicator, 311, Sanborn Co., Waltham, Mass.; *10*, Varian recorder, G-10, Varian Associates, Instrument Div., Palo Alto, Calif.

The diameter of the sample container was large with respect to the diameter of the cup to minimize the error due to flow of the liquid through the annulus between the cup and the walls of the container. The water bath, tensile strength apparatus, and motor are all mounted on a stainless steel plate attached to a vibradamp.[1] The rates of shear were 0.166, 0.219, 0.304, and 0.434 seconds^{-1}. Nearly vibrationless stirring of the water bath was accomplished with a centrifugal pump.[2] Temperature was maintained at 30 ± 0.1°.

The equations necessary for the instrument may be derived as

$$\eta = \frac{F}{dS/dt} = (\text{dyne-sec. cm.}^{-2}) \quad \text{(Eq. 1)}$$

where η is viscosity, F is the force per unit area on the cylinder (shear stress), and dS/dt is the rate of

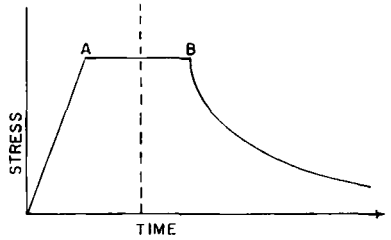


Fig. 2.—A typical upcurve for a non-Newtonian fluid is shown to the left of the dotted line. Point *A* represents the point where steady-state flow begins. Point *B* to the right of the dotted line represents the beginning of relaxation following cessation of steady-state flow. The dotted line signifies several minutes of elapsed time.

shear. F is computed from the following (11)

$$F = \left[\frac{2\pi h\eta}{\ln (R_T/R_C)}\right] V_{avg} \quad \text{(Eq. 2)}$$

where h = length of the cylinder, R_C = radius of the cylinder, R_T = radius of the tube, and V_{avg} = the average velocity of the fluid flow in the annulus. V_{avg} is related to the rate of rise of the cylinder through

$$V_{avg} = V\left\{\left[\frac{R_C \ln R_C - R_T \ln R_T - R_C + R_T}{[\ln (R_T/R_C)]\,(R_T - R_C)}\right] + \frac{\ln R_T}{\ln (R_T/R_C)}\right\} \quad \text{(Eq. 3)}$$

where V is the rate of rise of the cylinder in cm. seconds^{-1}. Further calculations show that the shear stress (dynes cm.$^{-2}$) for the rising cylinder rheometer is

$$F = \frac{Wg}{2\pi h R_C} = 71.204\ W \quad \text{(Eq. 4)}$$

where W is the recorded force in grams, and g is the gravitational acceleration with substitution of the dimensions of the instrument providing the final equality. The rate of shear strain (seconds^{-1}) is

$$\frac{dS}{dt} = \frac{V_{avg}}{\ln (R_T/R_C)} \frac{1}{R_{avg}} = 26.61\ V_{avg} \quad \text{(Eq. 5)}$$

[1] Fisher Scientific Co.
[2] Model No. 1612, Gorman-Rupp Industries, Inc., Belleville, Ohio.

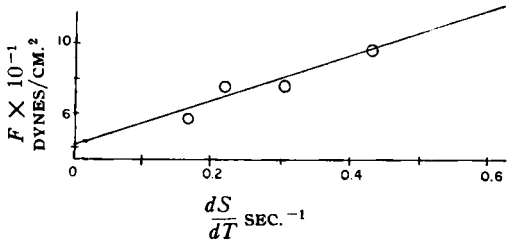


Fig. 3.—Viscosity curve of tragacanth initial mucilage determined by the rising cylinder rheometer with intercept being the Bingham yield value.

a mixer[5] for 1 hour. Then the solution was allowed to stand for 1 hour to release the entrapped air. The ammonia was added slowly with mixing to neutralize the Carbopol 934 and to convert it into its mucilagenous form. This mucilage was diluted to produce mucilages of concentrations 0.10, 0.15, 0.23, and 0.30% (w/v).

EXPERIMENTAL

The cylinder was uniformly raised 15 mm. through a sample producing an upcurve such as shown in Fig. 2. It should be noted that steady-state flow occurred almost immediately. The value of the force at which steady-state flow occurs is dependent upon the apparent viscosity of the sample and the particular rate of rise selected.

TABLE I.—YIELD VALUES (DYNES/CM.^{2}) FOR TRAGACANTH AND CARBOPOL 934 MUCILAGES

Tragacanth						Carbopol 934		
No. 1	No. 2	A	Initial	Super Initial	Supreme	0.10%	0.15%	0.23%
23.0	14.5	16.6	42.4	156.4	123.4	1.7	16.8	131.1

TABLE II.—VALUES OF THE APPARENT VISCOSITY IN POISE AT LOW RATES OF SHEAR FOR TRAGACANTH AND CARBOPOL 934 MUCILAGES

	Tragacanth						Carbopol 934		
Sec.$^{-1}$	No. 1	No. 2	A	Initial	Super Initial	Supreme	0.10%	0.15%	0.23%
0.166	211.4	126.5	206.6	380.1	1469.8	966.9	8.1	73.1	660
0.219	178.5	105.9	182.6	321.9	1232.9	785.8	7.8	70.9	595
0.304	149.7	83.9	161.2	267.8	1036.2	631.6	7.2	68.1	490
0.434	127.0	73.0	145.2	227.6	884.8	511.5	6.5	61.4	325

where R_{avg} is the radius of the midpoint of the annulus between the tube and the cylinder.

Solving Eq. 2 for η yields the equation for viscosity proposed by Fox and Flory (12) for a Pochettino type viscometer

$$\eta = \frac{(F/2\pi h)\ [\ln (R_T/R_C)]}{V_{avg}} \qquad \text{(Eq. 6)}$$

After substitution of the dimensions of the instrument, Eq. 6 becomes

$$\eta = 2.5761\ (W/V_{avg}) \qquad \text{(Eq. 7)}$$

Likewise, Eq. 3 becomes

$$V_{avg} = 0.4449\ V \qquad \text{(Eq. 8)}$$

Materials.—Six samples of powdered tragacanth gum U.S.P. were obtained from the S. B. Penick Co. The samples were identified as tragacanth No. 1, No. 2, A, Initial, Super Initial, and Supreme. Mucilages were prepared according to the following formula: tragacanth powder, 20 Gm.; benzoic acid, 1 Gm.; glycerin, 60 ml.; and purified water, 1000 ml.

The water and glycerin were mixed, heated to boiling, and the benzoic acid was dissolved in them. This was followed by sprinkling the powdered gum tragacanth on the boiling solution. After boiling for 5 minutes, the mixtures were agitated at intervals during the next 48 hours. After hydrating for 68 hours, the mucilages were homogenized.[3]

A stock solution of Carbopol 934[4] was also prepared according to the formula: ammonia (28% in water), 7 Gm.; Carbopol 934 powder, 10 Gm.; and distilled water, 983 ml. The Carbopol 934 powder and the distilled water were mixed with

Since the relaxation of stress within the sample at constant strain could easily be determined, it was decided to analyze these data to see if additional information could be obtained about the behavior of the samples. Examination of stress

To compare this instrument with another viscometer, the viscosity of Karo syrup was obtained using the M-2 viscometer (13); it was 11.218 poise. The viscosity of the syrup determined by the rising cylinder instrument was 11.517 poise. This was considered a satisfactory agreement.

Figure 2 also presents a representation of a typical downcurve or stress relaxation curve obtained from the rising cylinder rheometer after cessation of steady-state flow. The stress was allowed to relax at constant strain for 9380 seconds or until it became zero.

The resisting force upon the cylinder at each rate of shear was converted to dynes cm.$^{-2}$ with Eq. 4. Then these values were plotted *versus* the rate of shear strain to give a flow curve for each mucilage in the low shear range. Figure 3 is an example of one such curve.

The plotted points appeared to be linear and did not pass through the origin. These points were therefore fitted to a straight line by the method of least squares, the intercept of the line with the stress axis being taken as the classical Bingham yield value for the material. The yield values for each of these mucilages determined by the above methods are listed in Table I. The apparent viscosity of the mucilages at each rate of shear are found in Table II.

[3] Hand Homogenizer, catalog No. 63-102, K and L Scientific Co., Columbus, Ohio.
[4] B. F. Goodrich Chemical Co., Cleveland, Ohio.
[5] "Lightnin" model L mixer, Mixer Equipment Co., Inc., Rochester, N. Y.

TABLE III.—RELAXATION OF STRESS IN THE RISING CYLINDER RHEOMETER AND THE RELAXATION SPECTRA FOR 2% TRAGACANTH SUPER INITIAL MUCILAGE

Time, Sec.	0.166 Sec.$^{-1}$ σ dynes cm.^{2}	0.166 Sec.$^{-1}$ H dynes cm.^{2}	0.219 Sec.$^{-1}$ σ dynes cm.^{2}	0.219 Sec.$^{-1}$ H dynes cm.^{2}	0.304 Sec.$^{-1}$ σ dynes cm.^{2}	0.304 Sec.$^{-1}$ H dynes cm.^{2}	0.434 Sec.$^{-1}$ σ dynes cm.^{2}	0.434 Sec.$^{-1}$ H dynes cm.^{2}
0	223.65	...	271.56	...	335.51	...	459.44	...
0.38	215.32	177.43	261.89	163.50	326.54	1446.09	347.97	108.90
0.76	205.21	221.89	247.72	16.39	309.52	108.92	330.24	108.90
1.14	194.10	192.27	238.03	122.20	287.45	241.66	311.87	99.83
1.52	183.92	155.31	223.87	203.17	264.67	217.81	293.43	88.49
1.90	167.26	283.95	208.20	195.77	236.18	290.22	271.93	217.82
2.28	150.67	236.62	193.25	136.05	202.79	242.08	248.07	181.52
2.66	137.71	177.07	178.29	186.30	175.09	165.87	222.73	109.07
3.04	125.68	123.86	159.71	203.82	154.73	108.90	199.73	163.47
3.42	120.12	118.10	141.77	181.28	141.70	72.62	169.75	145.35
3.80	112.79	48.71	133.58	130.46	131.94	39.95	149.03	87.13
7.60	83.17	24.35	76.83	24.48	88.79	14.53	92.14	16.34
11.40	72.98	13.31	62.66	12.22	73.27	9.72	75.26	7.26
15.20	64.72	8.86	53.69	8.15	64.37	6.34	69.14	4.78
19.00	58.24	1.77	46.28	8.96	59.46	5.08	61.45	3.26
38.00	52.69	1.78	40.30	1.22	44.79	0.36	47.64	1.10
57.00	46.21	1.19	35.82	0.54	39.87	0.24	44.57	0.36
76.00	45.29	1.10	29.83	0.20	37.45	0.90	44.57	0.11
234.50	27.70	0.23	22.36	0.19	27.70	0.12	29.98	0.01
1172.50	9.26	0.08	14.95	0.03	24.42	0.01	26.92	0
2345.00	9.26	0	14.95	0.01	21.15	0	23.07	0
4690.00	0	...	14.95	0	16.31	...	22.29	...
7035.00	...	...	14.95	...	9.75	...	21.50	...
9380.00	...	...	14.95	...	8.12	...	0	...

relaxation is usually visualized in terms of a model consisting of a spring and dashpot in series (14). Under constant strain the force on the spring (at any time t) must be equal and opposite to the force on the dashpot. Since the element does not change length during the experiment

$$\frac{\dot{\sigma}}{G} = -\frac{\sigma}{\eta} \qquad \text{(Eq. 9)}$$

where σ = stress in the spring, $\dot{\sigma}$ = rate of change of stress in the spring, η = viscosity of dashpot liquid, and G = spring modulus.

Solving with the boundary condition $\sigma = \sigma_0$ at zero time

$$\sigma = \sigma_0 e^{-t/\tau} \qquad \text{(Eq. 10)}$$

where $\tau = \eta/G$

Any real liquid will appear to have many such models in parallel leading to

$$\sigma = l\sum_i G_i e^{-t/\tau i} \qquad \text{(Eq. 11)}$$

where $\tau_i = \eta_i/G_i$ and l is the elongation.

Now σ/l may be defined as $G(t)$, then

$$G(t) = \sum_i G_i e^{-t/\tau i} \qquad \text{(Eq. 12)}$$

Allowing i to increase without limit,

$$G(t) = \int_{-\infty}^{\infty} G(\tau) e^{-t/\tau} d\tau \qquad \text{(Eq. 13)}$$

or

$$G(t) = \int_{-\infty}^{\infty} \tau G(\tau) e^{-t/\tau}\, d \ln \tau \qquad \text{(Eq. 14)}$$

$$= \int_{-\infty}^{\infty} H(\tau)\, e^{-t/\tau}\, d \ln \tau \qquad \text{(Eq. 15)}$$

where $H(\tau) \equiv \tau G(\tau) \equiv H$ is called the relaxation spectrum.

When the rising cylinder is stopped abruptly during steady-state flow, the shear rate just prior to cessation of flow will be defined as $(\dot{\gamma})_0$. Solving Eq. 9 with this boundary condition

$$\sigma = \eta\dot{\gamma}_0 e^{-t/\tau} = \dot{\gamma}_0 \tau G e^{-t/\tau} \qquad \text{(Eq. 16)}$$

The same development as above gives

$$\sigma = \dot{\gamma}_0 \int_{-\infty}^{\infty} \tau H e^{-t/\tau}\, d \ln \tau \qquad \text{(Eq. 17)}$$

The exponential goes from zero to 1 as τ goes from 1 to infinity. This may be approximated as a step function going from zero to 1 as $t = \tau$, (Alfrey's rule (18)) producing

$$\sigma = \dot{\gamma}_0 \int_{\ln t}^{\infty} \tau\, H\, d \ln \tau \qquad \text{(Eq. 18)}$$

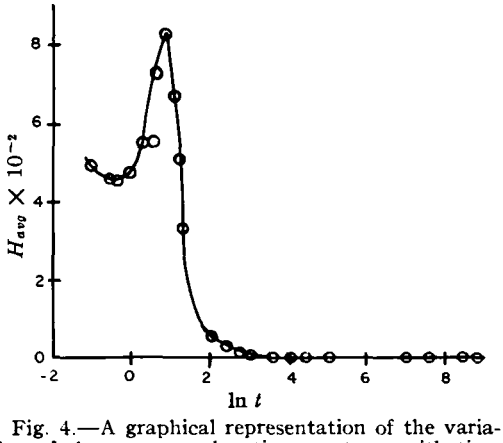


Fig. 4.—A graphical representation of the variation of the average relaxation spectrum with time for 0.30% Carbopol 934 mucilage determined from data obtained from the rising cylinder rheometer.

Differentiation of Eq. 18 leads to (15, 16)

$$-\frac{d\sigma}{d \ln t}\bigg|_{t=\tau} = \tau\dot{\gamma}_0 H \qquad \text{(Eq. 19)}$$

solving for $H(\tau)$

$$H(\tau = t) = -\frac{1}{\dot{\gamma}_0 t}\frac{d\sigma}{d \ln t} \qquad \text{(Eq. 20)}$$

In the analysis of the relaxation curves of the mucilages, the force in dynes cm.$^{-2}$ was plotted *versus* the natural logarithm of the experimental time and the curve graphically differentiated at each time interval. These differentials were substituted into Eq. 19 along with rate of shear and t to obtain H.

Since no trend was apparent in the value of H obtained for each rate of shear from the rising cylinder data, the values of H at each time interval were averaged to obtain H_{avg} for each mucilage. (An example of the scatter of values of H that go into an H_{avg} is presented in Table III.) H_{avg} was then plotted *versus* ln t to obtain the form of the relaxation spectrum. A sample plot of the function is found in Fig. 4.

In Eq. 18 $\sigma/\dot{\gamma}_0$ would have the dimensions of viscosity. The integral is designated the dynamic viscosity (17). It has been postulated that dynamic viscosity should be equivalent to plastic viscosity (consistency) for a given shear rate (18, 19). It was therefore decided to obtain dynamic viscosities to compare with previously determined plastic viscosities.

H_{avg} was multiplied by t to give tH_{avg}, and this function was plotted *versus* ln t.

Figure 5 is a sample plot of tH_{avg} *versus* ln t. Graphical integration of these plots of tH_{avg} *versus* ln t yielded values of dynamic viscosities for the different mucilages. The limits of the integration were from 0.38 seconds at the low end to the time in seconds when the experiment was terminated. In no case was ln t extrapolated to lower t values than experimentally determined. Values for the dynamic viscosities and plastic viscosities are given in Tables IV and V.

Discussion.—A new viscometer capable of obtaining viscometric data at shear rates comparable with those produced by suspension particles has been developed.

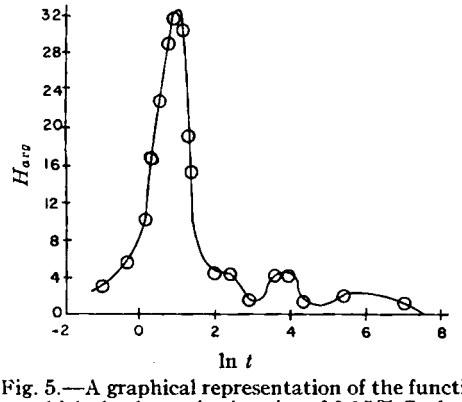


Fig. 5.—A graphical representation of the function from which the dynamic viscosity of 0.15% Carbopol 934 mucilage is obtained from data from the rising cylinder rheometer.

TABLE IV.—DYNAMIC VISCOSITIES OF 2% TRAGACANTH MUCILAGES

Mucilages	Plastic Viscosity, poise	Dynamic Viscosity, poise
No. 1	73.1	149.40
No. 2	39.3	84.00
A	107.1	184.80
Initial	127.7	266.80
Super Initial	501.8	1431.00
Supreme	232.5	719.00

TABLE V.—DYNAMIC VISCOSITIES OF CARBOPOL MUCILAGES

Mucilages, %	Plastic Viscosity, poise	Dynamic Viscosity, poise
0.10	3.5	1.47
0.15	30.2	52.56
0.23	121.3	417.60

The instrument was used for the determination of the viscosities (plastic and apparent), yield values, relaxation spectra, and dynamic viscosities of a series of pharmaceutical suspending agents.

The plastic viscosities and yield values were obtained by fitting the observed points to a straight line by the method of least squares since the points appeared linear. The apparent viscosities were obtained by dividing the shear stress obtained from the line by the corresponding shear rate.

The apparent linearity of the plots is probably misleading. If the dynamic viscosity should be equal to the plastic viscosity (19), the low value obtained from plastic viscosities (Tables IV and V) may be explained by assuming that the flow curves (Fig. 3) are actually concave downward. The need for more experimental work in the low shear region seems established. Accurate prediction of suspension stability must await such comprehensive studies.

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Compression of Pharmaceutical Powders I

Theory and Instrumentation

By STEPHEN SHLANTA, JR.†, and GEORGE MILOSOVICH

Considering that compression of a powder bed involves time dependent flow, it is reasonable to expect that this process would exhibit viscoelastic properties. To test this concept, an instrument was developed to measure stress relaxation of a powder bed under constant strain. Preliminary relaxation data obtained on several powders indicated correlation with physical properties of the compacts. Tests of factors known to influence tablet capping, hardness, and friability showed that these factors also had marked effect on relaxation data.

THE RECENT DEVELOPMENT of special feeding devices for tableting presses has made it possible to compress powders directly into uniform tablets without the usual granulation steps. The obvious savings in equipment, labor, and time effected by this processing shortcut, including better dose uniformity and smaller dosage size, make the ability to tablet all pharmaceutical powders directly highly desirable. However, many of these materials cannot be compressed into suitable tablets without prior treatment. At the present time the lack of basic knowledge of tableting mechanism has seriously hindered the elucidation and solution of this problem.

A great majority of the published work on pharmaceutical tableting has been directed toward the evaluation of excipients such as binders, lubricants, and disintegrants. Only recently have systematic efforts been made to define the mechanism of compression (1–9), but the majority of these studies have dealt with the behavior of granules under pressure. Considerable investigation into powder compression has been conducted in other applications, notably in the fields of powder metallurgy and ceramics. The types of materials studied, however, have few physical properties in common with most pharmaceutical powders.

Huffine (10), using the methods pioneered by Walker and Balshin, obtained correlations between quasi-equilibrium pressure-volume behavior and principle compression mechanisms for a number of powders, including some of pharmaceutical interest. He postulated a general theory for powder compaction based on the existing literature and his results. Assuming that this theory is valid, compaction may be visualized as a composite of several processes: particle diffusion into void space, fracture, elastic deformation, plastic deformation, and cohesion between particle surfaces. It is probable that these processes occur simultaneously, although not necessarily to the same degree, at any stage in the compression cycle. Also, in practice, tablets are formed by a process more closely related to impact loading, and the degree of air expulsion during initial pressure loading may have a pronounced effect on tablet quality. This effect was not considered by Huffine since it is not likely to be important in equilibrium studies, except in rare instances.

Considering that all of these processes depend on flow and thus are probably time dependent, it would be unrealistic to expect quantitative correlation between equilibrium studies and tablets produced under nonequilibrium conditions. Thus, the effect of one process may predominate in very short compression times, yet could be completely overshadowed when longer experimental times are used. In fact, it is probable that compression data can have physical meaning in terms of a tableting process only if they are obtained in equivalent time periods and with equivalent loading rates.

Nonequilibrium data are necessary to investigate these concepts. Such data on non-Newtonian flow systems are usually obtained through the measurement of stress relaxation under constant strain and creep under constant

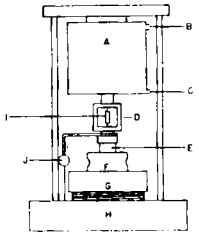


Fig. 1.—Apparatus for studying powder bed stress relaxation under constant strain. Key: A, cylinder and piston; B, compressed air; C, port used to raise piston; D, force transducer; E, tablet punch; F, tablet die, 0.5 in. i.d.; G, adjustable platen; H, base; I, signal from transducer; J, dial micrometer.

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† Parke, Davis and Co. Research Fellow.

stress (11). It was felt that such techniques could be employed advantageously for the study of powder compression. This communication reports the results obtained in the initial exploration of these considerations.

EXPERIMENTAL

Instrumentation.—A Carver press formed the nucleus of the apparatus shown schematically in Fig. 1. A piston driven by compressed air was used in place of the hydraulic loading system to obtain pressure loading rates approaching more closely those of high-speed rotary presses. The maximum rate obtained with this equipment using a $^1/_2$-in. punch was 10^4 p.s.i./second, giving a maximum pressure of 18000 p.s.i. in 1.8 seconds. Dimension was such that the piston was forced against the bottom of the cylinder when the force on the piston top exceeded the force on the powder bed. This insured a fixed movement of the upper punch so that data at constant strain could be obtained. Pressure on the powder bed as a function of time was obtained by installing a Daytronic model 140-5K force transducer between the piston ram and the upper punch. The demodulated signal (Daytronic model 200H) was recorded on a Varian G-14 recorder. Movement of the upper punch was measured by fastening a dial micrometer to the upper punch assembly. All studies were made using dies lubricated with stearic acid.

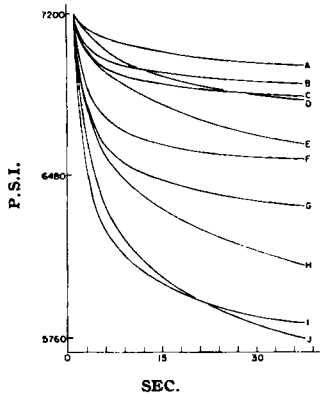


Fig. 2.—Relaxation curves for powder beds. Key: A, 70/100-mesh glass spheres; B, sulfathiazole I; C, crystalline lactose; D, aspirin crystals; E, sodium *p*-aminosalicylate; F, spray dried lactose; G, amorphous lactose; H, sodium chloride crystals; I, cornstarch; J, magnesium trisilicate.

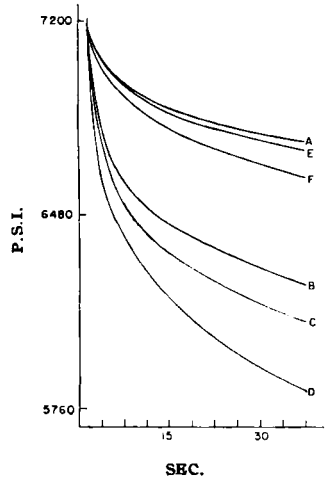


Fig. 3.—Effect of particle size and size distribution on relaxation curves. Key: A, 20/40 salicylamide; B, micronized salicylamide; C, 80/120 sodium chloride; D, <230 sodium chloride; E, 80/120 sodium PAS; F, 40/200 sodium PAS.

Stress Relaxation Results.—Several pharmaceutical powders were chosen on the basis of varied tableting properties for initial stress relaxation measurements. No attempt was made to control such known variables as particle size, size distribution, or moisture since only qualitative information was sought. Consequently, these powders were tested directly as received from respective suppliers, the only modification being the incorporation of 1% stearic acid. Preliminary compression tests were used to determine the weight of each material required to form a tablet or compressed bed of fixed thickness at 7200 p.s.i. Stress relaxation data for all of these materials were then obtained by suitable adjustment of the bottom platen to give a maximum recorded pressure of 7200 p.s.i. Figure 2 shows that the results of this series varied widely between the small relaxation of relatively incompressible 70/100-mesh glass spheres to the large relaxation of starch and magnesium trisilicate. In either of these two extremes, a suitable tablet was not formed. Except for aspirin, those materials forming good tablets by direct compression all showed medium relaxation. The relaxation of aspirin was in the same low range as that obtained for materials giving poor tablets. All of these materials except the glass sample were compressed at three pressures and three compression speeds on a Colton 216 rotary press equipped with a Colton force feed attachment.

Since particle size and size distribution are known to affect the tablet forming properties of powders, relaxation measurements were made on 80/120 and <230-mesh sodium chloride, 20/40-mesh and micronized salicylamide, and 80/120 and 40/200-mesh sodium PAS. Figure 3 shows that for each material the degree of relaxation was dependent upon the size or size distribution.

Sodium PAS samples were equilibrated at five relative humidities and relaxation data were obtained. Figure 4 shows the effect of humidity on relaxation for this series. Qualitative correlation with physical properties of the resulting compacts showed increasing hardness and decreasing friability with decrease in moisture.

Because strain could not be applied instantaneously, the effect of loading rate on relaxation was investigated. The equation describing the relationship between stress, P, strain, $\Delta V/V$, and time, t, for viscoelastic compression is

$$\frac{P_t}{(\Delta V/V)_t} = \sum_{i=1}^{n} \frac{P_i}{(\Delta V/V)_i} e^{-t/\tau_i} \quad \text{(Eq. 1)}$$

where the subscript i refers to the ith element relaxing from an initial stress and strain. Differentiating with respect to time gives

$$\frac{-dp}{dt} = \sum_{i=1}^{n} \frac{P_i}{\tau_i} e^{-t/\tau_i} \quad \text{(Eq. 2)}$$

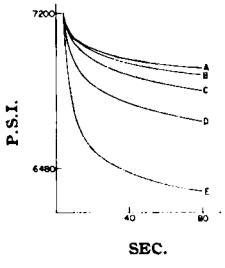


Fig. 4.—Effect of moisture on relaxation curves for sodium PAS stored at various relative humidities. Key: A, 85%; B, 75%; C, 33%; D, 22%; E, ~0%.

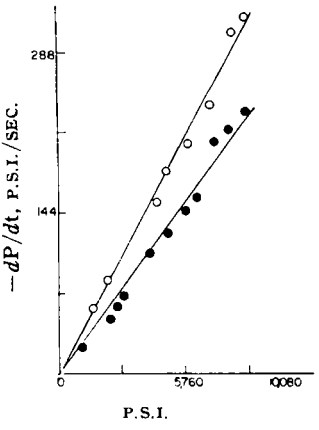


Fig. 5.—Effect of maximum pressure on initial relaxation slope for sodium chloride. Key: O, 1.8 seconds loading time; ●, 3.4 seconds loading time.

for the case $\Delta V/V$ constant. For a specific time period, a, the exponentials are constant and

$$\frac{-dp}{dt} = \sum_{i=1}^{n} \frac{e^{-a/\tau_i}}{\tau_i} P_i \qquad \text{(Eq. 3)}$$

Equation 3 reduces to

$$\frac{-dp}{dt} = k P \qquad \text{(Eq. 4)}$$

for all elements relaxing from an initial pressure P at fixed time. Accordingly, relaxation data for sodium chloride samples compressed at several pressures (weight and platen adjusted to keep $\Delta V/V$ constant) were obtained at two loading rates. Figure 5 is a plot of initial $-dp/dt$ against maximum pressure obtained at 1.8 seconds for 1.8-second loading time and 3.4 seconds for 3.4-second loading time.

DISCUSSION AND CONCLUSIONS

These preliminary studies were conducted to ascertain the feasibility of using stress relaxation data to investigate powder compaction. On the basis of the results obtained, the techniques appear to be valuable. Even though instrumentation was crude, evidence was obtained to show that factors known to affect tableting also had effects on stress relaxation data. The fact that seemingly anomalous behavior appears in some of these results is undoubtedly due to lack of experimental control or inadequate knowledge of the compression mechanism.

Certainly, correlation between tablet quality and bonding strength, surface area and porosity of the tablet, elastic compressibility, relaxation, particle size and shape, bulk density and flow properties of the powder may be expected. The technique reported in this communication is useful for studying elastic compressibility, relaxation, and flow under pressure. These important properties have received little attention in past studies on pharmaceutical powders; their measurement should help in understanding powder compression.

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Notes

Mode of Action of Endomycin-Neomycin Synergism

By W. T. SOKOLSKI and M. R. BURCH

The mode of action for endomycin-neomycin synergism against *Candida albicans* was postulated to be an action of endomycin which influences permeability of the cell membrane and facilitates the entry of neomycin in the cell. A neomycin-resistant strain was also found to be resistant to endomycin, but the combination of antibiotics was still synergistic. The permeability hypothesis of endomycin was strengthened by data showing that the cell membrane barrier was less resistant to increasing osmotic pressure in the presence of endomycin.

ENDOMYCIN is an antifungal and antibacterial complex of agents first described by Gottlieb, *et al.* (1). Endomycins A and B were reported to be synergistic with neomycin against *Candida albicans* UC1392 (2). Because endomycin lowers the surface tension of water and hence acts like a detergent, a cell wall permeability change has been postulated to be a factor in the synergism of endomycin and neomycin; presumably endomycin facilitates the passage of neomycin through the cell membrane. A precedent for this detergent type of synergistic activity was reported by Sokolski, *et al.* (3), and by Karaila (4).

One approach to show that endomycin affects the cell wall permeability was to use an organism which was resistant to neomycin. According to our hypothesis, the resistant organism should be sensi-

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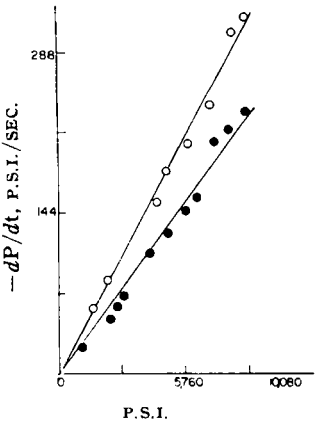


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TABLE I.—GROWTH OF *C. albicans* PARENTAL AND RESISTANT CULTURES IN THE PRESENCE OF NEOMYCIN, ENDOMYCIN, AND COMBINATIONS

C. albicans Strain	Antibiotic: Neomycin, mcg./ml.	Antibiotic: Endomycin, u./ml.	Absorbance, 650 mμ: Incubation, hrs. 20	44	120
Sensitive-parent	200	...	0.05	0.05	0.05
	50–100	...	0.05–0.06	0.05	0.25–0.33
	10–20	...	0.14–0.27	0.38–0.40	0.48–0.59
	5	...	0.42	0.47	0.71
	...	8–10	0.04	0.14–0.23	0.37–0.39
	...	1–4	0.23–0.34	0.37–0.39	0.38–0.45
	10	2	0.04	0.04	0.04
	5	2	0.04	0.04	0.27
	0	0	0.42	0.45	0.59
Neomycin-resistant	200	...	0.14	0.33	0.43
	50–100	...	0.23–0.34	0.47–0.51	0.52–0.61
	10–20	...	0.45	0.53–0.54	0.63–0.66
	5	...	0.50	0.53	0.63
	...	8–10	0.36–0.41	0.42–0.46	0.45–0.48
	...	1–4	0.42–0.43	0.48–0.51	0.52–0.54
	10	2	0.06	0.06	0.06
	5	2	0.06	0.06	0.06
	0	0	0.44	0.48	0.58
Endomycin-resistant	50–200	...	0.05	0.05	0.05–0.17
	10–20	...	0.05	0.04–0.40	0.39–0.60
	5	...	0.37	0.50	0.60
	...	10–20	0.13–0.39	0.25–0.41	0.29–0.60
	...	1–8	0.41–0.48	0.48	0.48–0.52
	5	2	0.03	0.03	0.04
	0	0	0.46	0.51	0.55

tive to neomycin in the presence of subinhibitory concentrations of endomycin. A second and possibly more convincing approach was to show that *C. albicans* was less resistant to increasing osmotic pressure in the presence of endomycin.

METHODS

The preparation of neomycin used in these studies was a commercial grade neomycin sulfate (Res. 9291) which was estimated (5) to contain 90% neomycin B and 10% neomycin C. Antibiotic potency was determined to be 650 mcg. neomycin B base equivalent per milligram. All concentrations used in these experiments were expressed in terms of neomycin B base equivalent.

The endomycin preparation was an amorphous sodium salt that was arbitrarily assigned a potency of 1000 units per milligram. The preparation was found to contain endomycins A and B, both of which were reported (2) as being synergistic with neomycin.

Neomycin-resistant and endomycin-resistant strains of *C. albicans* UC 1392 were developed by stepwise transfers to increasing concentrations of antibiotic in broth medium (yeast assay broth) consisting of 1% cerelose (glucose), 0.25% yeast extract, and 0.1% monopotassium phosphate. The neomycin-resistant strain was maintained in yeast assay broth containing 100 mcg. neomycin per milliliter, the endomycin-resistant strain in broth with 10 units endomycin per milliliter, and the parent strain in broth with no antibiotic.

The test organisms for inoculum in the determination for minimum inhibitory concentrations (MIC) were grown in shaken flasks with yeast assay broth and antibiotic for 24 hours at 37°. The cells from each culture were recovered by centrifugation, washed twice, and resuspended with the medium. The suspension was adjusted to an absorbance of 0.5 at 650 mμ.

The MIC tests were conducted in 18 × 150-mm. tubes which were optically matched for a Lumetron colorimeter. Sterile filtered antibiotic solutions were added to duplicate tubes which, when diluted to 10 ml., gave the concentrations indicated in Table I. The volumes in all tubes were brought to 2 ml. with sterile water. For each culture, 0.15 ml. of the inoculum suspension was added to 500 ml. of 10/8th strength yeast assay broth, after which 8 ml. of seeded broth were pipeted to each tube in the respective series. All tubes were momentarily agitated and then incubated in a 37° water bath.

Commercially available detergents were also tested for synergism with neomycin as was the combination of endomycin and detergents with potassium chloride. These tests were conducted against the parent *C. albicans* in the same manner as described above. Two anionic detergents, sodium lauryl sulfate and sodium heptadecyl sulfate, were also tested for synergism with neomycin. The concentrations used are indicated in Table II.

TABLE II.—SYNERGISM BETWEEN NEOMYCIN AND ANIONIC DETERGENTS AGAINST *C. albicans* PARENTAL

Detergent	Concn., %	Absorbance, 650 mμ[a]: Without neomycin	With neomycin[b]
Sodium lauryl sulfate (sodium dodecyl sulfate)	0.01	0.42	0.05
	0.005	0.40	0.05
Sodium heptadecyl sulfate	0.02	0.38	0.05
	0.01	0.41	0.05
None	...	0.45	0.48

[a] After 24 hours incubation at 32°C. [b] Ten micrograms neomycin B base equivalent.

RESULTS AND DISCUSSION

The results of the effect of endomycin, neomycin, and the combination against the parent and two antibiotic-resistant strains of *C. albicans* are shown in Table I. Absorbance at 650 mμ was used as an indication of the amount of growth. Microscopic wet-mount examinations were made of all tubes containing growth for the detection of bacterial contamination. No contaminated cultures were observed.

The response of the parent strain confirmed the synergistic activity with endomycin and neomycin previously reported (2). The results after 120 hours incubation show that 10 mcg. neomycin plus 2 units endomycin per milliliter inhibited growth and gave the same result as 200 mcg. neomycin per milliliter or >10 units endomycin per milliliter. Individually, 100 mcg. neomycin or 10 units endomycin per milliliter allowed some growth to occur.

The endomycin-resistant strain was more sensitive to neomycin than was the parent strain, as indicated by the lesser growth with neomycin alone at all reading periods. Synergism with the combination of antibiotics was apparent since no growth occurred with as little as 5 mcg. neomycin plus 2 units endomycin per milliliter, whereas growth was observed with 50 mcg. or units per milliliter, respectively.

The neomycin-resistant strain acquired resistance to endomycin since there was good growth with 10 units endomycin per milliliter after 20 hours. In the presence of the combination, however, synergism still existed. Five micrograms of neomycin plus 2 units per milliliter of endomycin was more effective in preventing growth than 500 units of neomycin or 10 units per milliliter of endomycin alone.

The fact that the neomycin-resistant strain was also resistant to endomycin may mean that the cell wall or membrane was altered during the development of resistance so that less antibiotic was absorbed and/or penetrated. The combination of the two agents was still synergistic, indicating that the action of endomycin was still effective in allowing more neomycin to enter the cell.

Table II shows that other anionic detergents were also synergistic with neomycin. These data are consistent with the concept that a chemical which can alter the permeability of the cell wall may be synergistic with antibiotics which are active in the cell. This detergent-antibiotic type of synergism has been shown to occur against the protozoa *Ochromonas danica* (3). A correlation between the surface tension-reducing activity on water and synergism with neomycin apparently was not present, but this is not surprising since the detergent action occurs between the cell and the surrounding liquid while the surface tension-depressing action is between the liquid and air.

Table III.—Influence of Endomycin on the Growth of *C. albicans* in Varying Concentrations of Potassium Chloride

Agents, Concn.		Absorbance, 650 mμ	
KCl, %	Endomycin, u./ml.	24 hr.	48 hr.
10	...	0.06	0.18
8	...	0.10	0.20
6	...	0.17	0.27
0.4 - 4.0	...	0.24–0.28	0.28
...	8	0.01	0.02
...	3–6	0.01–0.08	0.26–0.31
...	0.4–2	0.24–0.28	0.32
8	1	0	0.18
4–6	1	0.05 - 0.08	0.20–0.29
0.2 - 2	1	0.23 - 0.27	0.27–0.30
8	2	0	0
6	2	0	0.14
4	2	0.02	0.15
0.2 - 2	2	0.17–0.25	0.25–0.30
0	0	0.28	0.31

The possibility of a salt formation between neomycin and anionic detergents cannot be excluded. However, we previously could not show a third component with solutions containing neomycin and endomycin (3).

If the mode of action for endomycin-neomycin synergism is the action on the cell permeability by endomycin, then the cells should be less resistant to osmotic shock in the presence of endomycin; this was shown to be the case. Table III indicates that growth was inhibited with lower concentrations of potassium chloride in the presence of endomycin than without endomycin. Similar results were obtained with potassium chloride and sodium lauryl sulfate or sodium heptadecyl sulfate. However, detergents which were not synergistic with neomycin, polysorbate 80, and oxyethylene-oxypropylene polymer, did not affect the osmotic pressure resistance. Increased cell wall permeability in the presence of endomycin is believed to be a factor in endomycin-neomycin synergism.

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Evaluation of Caffeine and Ouabain as Permissive Agents in the Production of Myocardial Necrosis

By DUANE G. WENZEL, JOHN D. MACCARTHY, and CHARLES O. RUTLEDGE

Twelve groups of six rabbits per group were treated with nicotine, caffeine, ouabain, hypercholesterolemic diet, and various combinations of these for 24 weeks. Systolic blood pressures and coagulation times were obtained at 4-week intervals, and a histopathological examination of the hearts was made at death or sacrifice. Treatments including either nicotine or cholesterol significantly reduced the coagulation times. Most nicotine-treated groups demonstrated increases in systolic pressures. While myocarditis was observed in several of the animals receiving multiple treatments, such treatments did not increase the incidence or severity of coronary atherosclerosis.

THE CHRONIC oral ingestion of nicotine by hypercholesterolemic rabbits receiving periodic injections of ergonovine has been demonstrated to induce myocardial necrosis (1). Treatment with nicotine alone increases the systolic blood pressure and the rate of blood coagulation (2). Although it is generally accepted that hypertension and hypercoagulability of the blood may be related to myocardial infarction in the human, there has been no correlation between such nicotine-induced changes and myocardial necrosis in the experimental animal. The role of ergonovine in this cardiac-damaging regimen is still speculative. In contrast to other ergot alkaloids, ergonovine is not sympatholytic but actually enhances cardiovascular actions of the catecholamines (3). One might expect that it would similarly affect the catecholamine-mediated actions of nicotine. In fact, the combination of nicotine and ergonovine has been demonstrated to induce thickening and fibrosis of the small coronary branches (1). Such an effect suggests that a damaged vascular bed with the resultant alteration in tissue circulation may predispose the myocardium to pathological changes induced by atherosclerosis. The postulated requirement of pre- or concomitantly existent damage for the production of hypercholesterolemia-induced pathological changes has been suggested by others (4) and is consistent with the usual absence of coronary thrombosis or infarcts in animals receiving a hypercholesterolemic diet as their sole treatment.

In the present problem it was hypothesized that infarcts and/or myocardial necrosis may result when hypercholesterolemia is superimposed upon underlying conditions which damage the myocardium or its circulatory system. To test this hypothesis, caffeine and a cardiac glycoside were employed in an attempt to induce such an underlying condition. It has been demonstrated that the injection of several doses of caffeine to either guinea pigs or rats will induce degenerative changes of the myocardium (5). Similar responses in the rabbit have been reported to result from a single combined dose of caffeine and epinephrine (6), while strophanthin in conjunction with epinephrine has also induced this reaction (7). It was anticipated that the catecholamine-releasing action of nicotine could replace the administration of epinephrine *per se*.

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EXPERIMENTAL

Seventy-two female, albino, New Zealand strain rabbits were divided at 6 weeks of age into 12 groups of six per group. Each group received one of the following treatments: control, nicotine (N); hypercholesterolemic diet (HCD), N + HCD; caffeine (C), C + N, C + HCD, C + HCD + N; ouabain (O), O + N, O + HCD, O + HCD + N. Control treatment consisted of Purina rabbit chow and water *ad libitum*. Nicotine dosage was the equivalent of one pack of cigarettes daily on the basis of body weight, or 1.14 mg./Kg./day in the drinking water (8). The hypercholesterolemic treatment consisted of the basal diet to which 5% cottonseed oil and 1% cholesterol were added. Caffeine, in a dose of 5 mg./Kg. of the alkaloid, was injected intravenously as the sodium benzoate salt once each week; ouabain, 0.025 mg./Kg., was similarly administered. Each of these were given in one-half milliliter of isotonic saline.

Systolic pressures were determined with a Grant ear capsule at the initiation of treatment and at 4-week intervals thereafter. Coagulation times were obtained by the capillary tube method with blood from a stab wound in the tail. All measurements were performed on the same days that the caffeine and ouabain were administered and always preceded these injections. By this procedure the acute response to the administration of the drugs did not affect the blood pressure and coagulation

TABLE I.—EFFECTS OF NICOTINE, CAFFEINE, OUABAIN, AND HYPERCHOLESTEROLEMIA ON BLOOD PRESSURE AND COAGULATION TIME

Treatment	Difference from Initial Values at 16 Wks.	
	Systolic Pressure, mm. Hg	Coagulation Time, Sec.
Control	2	−1.8
Nicotine	32[a]	−7.9
Cholesterol	0	−4.9
Nicotine + cholesterol	21	−13.2
Caffeine	11	−10.4
Caffeine + nicotine	31[a]	−9.6
Caffeine + cholesterol	2	−10.6
Caffeine + nicotine + cholesterol	9	−12.4[b]
Ouabain	6	−6.2
Ouabain + nicotine	8	−8.9
Ouabain + cholesterol	−5	−10.5
Ouabain + nicotine + cholesterol	19[b]	−12.2[b]

[a] $P < 0.01$. [b] Statistically different from control ($P < 0.05$).

time determinations. Because of the loss of as many as four out of six animals in some groups after 16 weeks, blood pressure and coagulation time determinations were discontinued at this time. Treatments were continued for 24 weeks. The hearts of those animals which died during this period and the survivors which were sacrificed at 24 weeks were examined for microscopic evidence of pathological damage. Cross sections were made perpendicular to the basal apical axis at the level of the aortic ring valve and midway between the aortic ring and apex.

RESULTS AND DISCUSSION

Mean systolic blood pressures and blood coagulation times are presented in Table I. The Dunnett "t" test (9) was performed to determine the significance of differences between the control and test values at 16 weeks. As indicated in Table I, all nicotine-treated groups, except nicotine combined with ouabain or with caffeine and the hypercholesterolemic diet, induced significant ($p < 0.05$ and $p < 0.01$) increases in systolic pressure. There are no obvious reasons for the lack of a significant increase by these two groups. Coagulation times were consistently reduced by all treatments, although only nicotine or the cholesterol treatment—with or without caffeine or ouabain—caused a significant ($p < 0.05$) lowering.

Histopathological examination revealed minimal to advanced subintimal lipid deposition in the small to medium coronary arteries of all animals treated with the cholesterol diet. There was no indication that any of the treatments increased either the severity or the extent of the lipid deposition. Examination of the myocardium disclosed the presence of minimal focal areas of myocarditis in six of the treated animals. Two of these had received ouabain, nicotine, and cholesterol, while two were from the caffeine-nicotine-cholesterol group. One animal had received ouabain and another caffeine and nicotine. While the lack of more severe and consistent lesions does not allow any clear cut conclusions, it is apparent that those animals receiving the multiple treatment accounted for more than their anticipated share of the myocarditis. As there was no evidence of necrosis in the hearts, it is also apparent that the original hypothesis stating that myocarditis predisposes to atherosclerotic damage was not substantiated. It should be recognized that there was no real evidence that the injections of ouabain or caffeine even produced myocarditis. It is suggested that either the caffeine or ouabain treatments were not sufficiently effective to induce myocarditis or after repeated injections that the tissue reaction was reduced.

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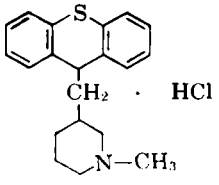
Antispasmodic Effects of 9-[(*N*-Methyl-3-piperidyl)methyl] Thioxanthene Hydrochloride

By H. LAUENER* and R. C. POGGE

A new compound, 9-[(*N*-methyl-3-piperidyl)methyl] thioxanthene hydrochloride (methixene hydrochloride)[1] has shown parasympatholytic properties, strong inhibition of gastrointestinal motility in rats, mice, and guinea pigs and less active inhibition of salivation and pupil dilation. Its oral toxicity is low.

CAVIEZEL, ET AL., have described the synthesis (1) and pharmacology (2) of a large series of thioxanthene derivatives. During early studies, interest was concentrated on the antitremorine properties which resulted in trial of one of these compounds—subsequently identified as methixene hydrochloride—in the symptomatic treatment of paralysis agitans for which relatively high dosage is required. The early work included mention of inhibition of gastrointestinal motility even by low doses of 9-[(*N*-methyl-3-piperidyl)methyl] thioxanthene hydrochloride. Later, clinical investigators explored the possibility that this compound might be useful in the symptomatic treatment of manifestations of gastrointestinal hypermotility and spasm. The clinical findings will be published elsewhere (3–5). The present study represents an expansion of the preliminary work to include motility studies in three species of experimental animals.

The chemical structure of methixene hydrochloride may be represented



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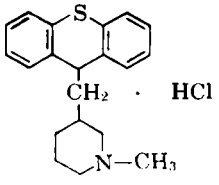
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rate of intestinal passage of charcoal in mice and rats was studied at the Wander Research Institute, Berne, Switzerland. The method employed was a modification of that reported originally by Macht, *et al.* (6). Specifically, the time during which a standard dose of atropine shows an optimum inhibitory effect was selected as the interval to be observed between administration of charcoal and sacrificing of the animals.

Male rats and mice of each sex were used. The animals were deprived of food on the previous evening. Five minutes after intravenous injection of either methixene hydrochloride or atropine, a charcoal suspension containing 1 Gm. of active charcoal (Norit A) and approximately 100 mg. of gum arabic in 7 ml. of distilled water were administered by stomach tube. Mice and rats were sacrificed by concussion 10 and 30 minutes later, respectively; the entire small intestine was quickly extirpated.

The distance traveled by the charcoal suspension and the total length of small intestine were measured with the intestine hanging freely.

In all animals, the distance traveled by the charcoal suspension was calculated in per cent of bowel length. Mean values of the treated groups were expressed in percentage of the control group. From the dose-effect straight line graphs, it was possible to read off the ED_{50}, *i.e.*, the dose with which the transport of charcoal would be delayed by 50%. Important data on the method are shown in Table I.

TABLE I.—METHOD USED IN DETERMINING THE ACTION OF METHIXENE HYDROCHLORIDE ON MICE AND RATS

	Mice	Rats
Animal weight	18–21 Gm.	140–180 Gm.
Animals per dose	10	6
Interval between administration of charcoal and sacrificing	10 min.	30 min.
Volume of charcoal suspension	0.05 ml.	0.2 ml.
Mean length of small intestine	45 cm.	100 cm.
Distance traveled by charcoal in per cent of bowel length in control animals	40–60%	55–75%

Action on Reflex Peristalsis in the Anesthetized Guinea Pig.—Guinea pigs (weighing 300–400 Gm.) which had been fasted for 24 hours were anesthetized with 1.2 Gm./Kg. of urethane administered subcutaneously. The intestine was ligated about 10 cm. from the cecal end of the ileum and a cannula tied about 6 cm. craniad. The cannula was connected to a pressure-recording instrument (pneumatic pressure-intensifier) and a water storage vessel which made possible a continuous raising of the pressure in the separated ileum segment. The peristaltic pressure-waves beginning with a constant pressure for each animal were measured. It has been found in our experiments that following injection of parasympatholytic agents, the critical pressure threshold does not rise, but the intensity and duration of the peristaltic waves decrease.

The peristaltic reflex was induced at intervals of 10 minutes by increasing the pressure for 3 minutes, then lowering it to normal. Thirty minutes after the operation, it was generally possible to reproduce the number and intensity of the pressure waves. To evaluate the action of the preparations, the wavelike increases of pressure for every 10 minutes of experimental period were added. The mean of three or four values before injection of the preparations was calculated; the values just after intravenous injection of the preparations were expressed in percentage of the control mean.

It was possible to read off the ED_{50}, *i.e.*, the dose with which reflex peristalsis would be inhibited by 50%, from the dose-effect curve. The preparations were injected intravenously in doses increasing threefold, the next higher dose being injected only when the peristaltic reflex could be induced in repeated experiments to the same extent.

Inhibition of Salivation in the Mouse and Rat.—Salivation was induced by intraperitoneal injection of 1 mg./Kg. of pilocarpine. At the same time the test preparation was injected intravenously (five rats or ten mice per dose). Salivation was observed 15 and 30 minutes after injection by wiping the mouth of the animal on a blotting paper. Only the presence or absence of saliva was noted. It was possible to draw a dose-effect curve with the number of animals without saliva in percentage of the number per group and to determine the ED_{50} from it.

Inhibition of Salivation in the Guinea Pig.—Salivation was induced by intraperitoneal injection of 3 mg./Kg. of pilocarpine. The test preparation

TABLE II.—PHARMACOLOGIC RESULTS OF METHIXENE HYDROCHLORIDE AND ATROPINE

	Methixene Hydrochloride	Atropine	Ratio Methixene Hydrochloride/ Atropine
Effect on Gastrointestinal Motility			
Mouse: ED_{50}, mg./Kg. i.v. for inhibition of charcoal passage	6.8	2.9	2.2:1
Rat: ED_{50}, mg./Kg. i.v. for inhibition of charcoal passage	1.4	0.33	4.2:1
Guinea Pig: ED_{50}, mg./Kg. i.v. for inhibition of peristaltic reflex	0.15	0.009	16.7:1
Effect on Salivation			
Mouse: ED_{50}, mg./Kg. i.v. (50% inhibition)	3.2	0.1	32:1
Rat: ED_{50}, mg./Kg. i.v. (50% inhibition)	3.5	0.04	87:1
Guinea Pig: ED_{50}, mg./Kg. i.v. (50% inhibition)	4.0	0.062	64.5:1
Mydriatic Effect			
Mouse: ED_{300}, mg./Kg. s.c.	1.3	0.065	20:1

was injected simultaneously and also intraperitoneally (eight animals per dose). Salivation was observed 30 minutes after injection by wiping the mouth of the animal on a blotting paper; the ED_{50} was determined by the same procedure employed in the experiment on mice and rats.

Action on Pupillary Width.—The pupillary width in unanesthetized mice was measured with a binocular lens with a magnification of 15 times 30 minutes after subcutaneous administration of the drugs (Pulewka, P., *Arch. Exptl. Pathol. Pharmakol.*, **168**, 307(1932)). The ED_{300} (dose which increases the pupil size to 300% of the control value) was determined.

RESULTS

Pharmacologic results of methixene hydrochloride and atropine are shown in Table II.

In three species tested, the relative potency of methixene hydrochloride to atropine reveals that with respect to inhibition of gastrointestinal motility, atropine is 2.2 to 16.7 times as potent, but with reference to inhibition of salivation, atropine is 32 to 87 times as potent. In the mouse, atropine is 20 times as potent as methixene hydrochloride with respect to mydriatic effect.

ACUTE TOXICITY

The values of the LD_{50} and their confidence limits have been calculated according to the Litchfield-Wilcoxon method (7). The intravenous LD_{50} for methixene hydrochloride is 18.0 (15.8 to 20.5) mg./Kg. in mice and 24.0 (21.0 to 27.4) mg./Kg. in rats. Orally, the LD_{50} is 430 (350–530) mg./Kg. in mice and over 1,500 mg./Kg. in rats.

CHRONIC TOXICITY

Initial studies on the tolerance of methixene hydrochloride during long term administration were carried out in rats. Three groups of ten animals each received 1, 4, and 15 mg./Kg. of the drug with the food for 9 months. An untreated group served as the controls. Weight gain in the treated animals and controls, hematological findings, and macroscopic and histologic examination of the organs gave no indication of toxic action of methixene hydrochloride in this dosage (2). Studies in rats for 18 months and in dogs for 12 months and suitable progeny studies are in progress and will be reported when completed.

CONCLUSION

It is evident that methixene hydrochloride is somewhat less active than atropine in the intestinal passage test and also with regard to inhibition of the peristaltic reflex. However, if the characteristic secondary actions of parasympatholytic agents, inhibition of salivation, and mydriatic action are considered, far higher doses of methixene hydrochloride are required to produce these effects than those of atropine.

In the three animal species studied, the therapeutic ratio of methixene hydrochloride (range between gastrointestinal effect and undesired side effects) is from four to 21 times higher than that of atropine.

Studies in experimental animals justify the therapeutic evaluation of methixene hydrochloride in the symptomatic management of conditions associated with gastrointestinal hypermotility or spasm.

Long term toxicity studies and progeny studies should be completed before methixene hydrochloride is released for general use.

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Analysis of Dosage Forms Containing Ephedrine and Barbiturate Combinations

By MARTIN I. BLAKE and DANIEL A. NONA

Procedures are presented for the determination of ephedrine salts in combination with barbiturates in tablets and capsules. An aliquot prepared from the dosage form is passed through a strong anion exchange resin. The ephedrine, contained in the eluate, is determined by titration with standard hydrochloric acid. The barbiturate is eluted from the column with acetic acid in ethanol and determined by nonaqueous titration. A modification of this procedure is proposed for formulations containing the sodium salt of the barbiturate. The methods are simple, accurate, and less time consuming than the official assay.

THE OFFICIAL ASSAY (1) for ephedrine sulfate and phenobarbital capsules involves ether extraction of the phenobarbital and a Kjeldahl distillation procedure for the estimation of the ephedrine content. The assay procedure has remained essentially unchanged since N.F. VIII when this dosage form first became official. The distillation technique was originally proposed by Hilty (2). The ephedrine sulfate solution is refluxed with hydrochloric acid and distilled in the presence of zinc dust and sodium hydroxide into a solution of standard acid. The ephedrine is determined by residual titration of the distillate. The method is tedious and time consuming.

Hilty and Wilson (3) analyzed tablets containing a combination of ephedrine sulfate and a barbiturate

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by ether extraction of the ephedrine and chloroform extraction of the barbiturate. Mattson (4) developed an ultraviolet spectrophotometric procedure for phenobarbital when in the presence of ephedrine sulfate. Comer and Bourne (5) analyzed capsules containing a combination of aminophylline, amobarbital, and ephedrine hydrochloride. The barbiturate and ephedrine were separated by solvent extraction and determined by infrared spectrophotometry.

Methods of analysis of ephedrine salts and barbiturates as individual components in various dosage forms were reviewed in earlier papers (6–8), and procedures based on ion exchange and nonaqueous titration were proposed for their determination.

In this paper procedures are presented for the analysis of capsules and tablets which contain combinations of ephedrine salts with barbiturates or barbiturate salts. The components are separated by passage through ion exchange resins and estimated by an aqueous or nonaqueous titration procedure.

EXPERIMENTAL

Preparation of Columns.—Two ion-exchange resins were employed in this study. The strongly basic anionic exchange resin Dowex 2-X8, 50–100 mesh, was prepared for use by adding about 10 Gm. of the resin in the form of an aqueous suspension to a chromatographic column, 1 × 30 cm. The resin column was washed with 100 ml. of distilled water, 100 ml. of 5% sodium hydroxide, and 200 ml. of distilled water. The strongly acidic cation exchange resin Dowex 50-X8, 200–400 mesh, was prepared by adding about 10 Gm. of resin to a column, 1 × 30 cm. When the resin settled, the column was washed with 200 ml. of distilled water, 100 ml. of 2 *N* hydrochloric acid, and finally with distilled water until the eluate was neutral. A solvent layer was always maintained above the resin column.

Table I.—Analysis of Ephedrine Salts and Phenobarbital Mixtures

Mixture	Weighed Amount, mg.	Recovery, %
Ephedrine sulfate	100.0	99.50 ± 1.14[a]
Phenobarbital	100.0	99.78 ± 0.26
Ephedrine HCl	100.0	99.48 ± 0.98
Phenobarbital	100.0	100.90 ± 0.09

[a] Average of at least six determinations ± standard deviation.

Analysis of Ephedrine and Phenobarbital Mixtures.—A sample containing 100 mg. of ephedrine sulfate or ephedrine hydrochloride and 100 mg. of phenobarbital, accurately weighed, was dissolved in 25 ml. of 50% ethanol. The solution was added to the column containing the strongly basic anion exchange resin. Additional 50% ethanol was passed through the column until 75 ml. of eluate was collected. The ephedrine, as free base, passed through the column and appeared in the eluate. The ephedrine was determined by visual titration with 0.1 *N* hydrochloric acid, using 2 drops of methyl red T.S. as the indicator.

The phenobarbital, retained by the column, was eluted with 50% acetic acid in 95% ethanol at a flow rate of 1 ml. per minute. Sixty milliliters of eluate was collected, and the solution was evaporated to dryness on a steam plate. Five milliliters of water was added, and the solution was again evaporated to dryness. This procedure was repeated once. The residue was dissolved in 20 ml. of dimethyl formamide, and the solution, magnetically stirred, was titrated with 0.1 *N* sodium methoxide in benzene-methanol, prepared and standardized as described previously (9). Titration was effected potentiometrically with a Fisher titrimeter equipped with a calomel and glass electrode system. The end point was determined from the break in the curve obtained by plotting millivolts *versus* volume of titrant.

The analysis of ephedrine and phenobarbital in synthetic mixtures is reported in Table I.

Analysis of Dosage Forms.—The proposed procedure was applied to solid dosage forms containing ephedrine salts combined with barbiturates.

Ephedrine sulfate and phenobarbital capsules N.F. XI and commercially available capsules of ephedrine sulfate and amobarbital were analyzed by transferring the contents of 20 capsules to a beaker. Forty milliliters of 50% ethanol was added, and the solution was mixed thoroughly. The solution was filtered into a 100-ml. volumetric flask, using washings of 50% ethanol to effect the transfer. Sufficient diluted alcohol was passed through the filter to fill

Table II.—Analysis of Dosage Forms Containing Ephedrine Salt and Barbiturate Mixtures

Dosage Form	Components	Labeled Amount Per Dosage Unit, mg.	Recovery, %
Ephedrine sulfate and phenobarbital capsules N.F.	Ephedrine sulfate	50.0	98.90 ± 0.46[a]
	Phenobarbital	50.0	98.85 ± 0.65
Ephedrine and Amytal pulvules	Ephedrine sulfate	25.0	99.50 ± 1.01
	Amobarbital	50.0	99.59 ± 0.34
Ephedrine and Amytal tablets	Ephedrine sulfate	8.0	101.50 ± 1.68
	Amobarbital	16.0	98.88 ± 1.02
Ephedrine and phenobarbital sodium capsules	Ephedrine	25.0	98.74 ± 0.98 99.51 ± 0.91[b]
	Sodium phenobarbital	50.0	99.42 ± 0.62
Ephedrine and Seconal sodium capsules	Ephedrine sulfate	25.0	98.94 ± 1.46 99.11 ± 0.32[b]
	Sodium secobarbital	50.0	99.22 ± 1.96
Ephedrine and Nembutal capsules	Ephedrine HCl	25.0	99.73 ± 1.37 99.42 ± 0.86[b]
	Sodium pentobarbital	25.0	99.46 ± 0.41

[a] Average of at least six determinations ± standard deviation. [b] By nonaqueous titration method.

the flask to the mark. An aliquot of this solution, containing 50 to 100 mg. of both ephedrine sulfate and barbiturate, was transferred by pipet to the column containing the strongly basic anion exchanger. The column was then eluted with 50% ethanol and 50% acetic acid in ethanol as described for ephedrine and phenobarbital mixtures. The data for these analyses are reported in Table II.

Ephedrine sulfate and amobarbital tablets were assayed by triturating 20 tablets in a mortar with approximately 25 ml. of 50% ethanol. The mixture was filtered into a 100-ml. volumetric flask with the aid of diluted alcohol rinsings. Sufficient diluted alcohol was passed through the filter to bring the volume to the mark. An aliquot containing 60 to 100 mg. of both ephedrine sulfate and amobarbital was treated as described in the previous paragraph for the capsule dosage forms. Results are reported in Table II.

A modified procedure was used for the analysis of ephedrine sulfate and phenobarbital sodium, ephedrine sulfate and secobarbital sodium, and ephedrine hydrochloride and pentobarbital sodium. A solution was prepared as described for the capsules, and an aliquot containing between 50 and 100 mg. of each active component was added to the column of the strongly acidic cation exchange resin. The column was eluted with 50% ethanol until 75 ml. of eluate was collected. This solution containing both the barbituric acid derivative and hydrochloric or sulfuric acid was passed through the column of strongly basic anionic exchange resin. The column was washed with water, and the barbiturate was eluted from the column with 50% acetic acid in ethanol. Sixty milliliters of eluate was collected, and the solution was evaporated to dryness on a steam plate and assayed nonaqueously as described earlier.

The strongly acidic exchange column which retained the ephedrine and sodium ion was eluted with a solution of 5% ammonia in 95% ethanol. A total of 60 ml. of eluate was collected in a 100-ml. beaker. The solution was gently aerated by passing a stream of air over the beaker until the ammonia was completely expelled. At least 3 hours was required for this step. During the aeration the liquid level in the beaker was maintained constant by frequent addition of alcohol. The solution was titrated visually with 0.1 *N* hydrochloric acid using 2 drops of methyl red T.S. as indicator. This solution containing ephedrine hydrochloride was evaporated to dryness on a steam plate, and the residue was analyzed for ephedrine content by nonaqueous titration. Ten milliliters of glacial acetic acid, 20 ml. dioxane, and 10 ml. of 6% mercuric acetate in glacial acetic acid were added to the beaker. The solution, magnetically stirred, was titrated potentiometrically using a Fisher titrimeter equipped with a calomel and glass electrode system. The titrant was 0.1 *N* perchloric acid in dioxane. Titration was also effected visually by adding 2 drops of methyl violet T.S. to the solution. The indicator color change was noted by using indicator solution in conjunction with a potentiometric titration. The color change corresponding to the graphic end point was from violet to an intense blue coloration.

DISCUSSION

Simple and accurate procedures are proposed for the analysis of combinations of ephedrine salts and barbiturates. They are less cumbersome and less time consuming than the official assay. Four or five assays may be conveniently conducted simultaneously.

When a solution of ephedrine sulfate or ephedrine hydrochloride and a barbiturate is passed through a strongly basic anion exchange resin, quaternary base type, the resin quantitatively splits the ephedrine salt; the free base appears in the eluate, and the acid component remains on the column. The barbiturate is also retained by the column. The ephedrine content of the eluate is readily determined by titration with standard acid. As reported in an earlier paper (7), 50% acetic acid in ethanol effectively removes the barbiturate from the column, but does not displace the hydrochloride or sulfate. The barbiturate content is determined by nonaqueous titration. Data are reported in Table I for the analysis of synthetic mixtures of ephedrine salts and phenobarbital. Application of the proposed procedure to the analysis of the official capsules and several commercially available capsules and tablets is reported in Table II.

A modification of the proposed method is required where the ephedrine salt is combined with a sodium barbiturate. When such a mixture is passed through a strong anionic exchange resin, sodium and ephedrine appear in the eluate. Potentiometric titration does not differentiate these bases. In the modified procedure the solution is first passed through the strong cation exchanger Dowex 50-X8. The ephedrine and sodium ion are retained by the column, and the barbiturate and hydrochloric acid or sulfuric acid appear in the eluate which is then passed through the strong anion exchanger Dowex 2-X8. By displacement elution with acetic acid, the barbiturate is removed from the column while the hydrochloride or sulfate remains on the column. The barbiturate is determined by nonaqueous titration. The strong cation exchanger which has retained the ephedrine and sodium is eluted with 5% ammonium hydroxide in alcohol. The ammonia displaces the weaker base ephedrine from the column; the sodium remains on the column. The ammonia is expelled from the eluate by gentle aeration, and the ephedrine content is determined by titration with standard hydrochloric acid and then by nonaqueous titration with perchloric acid in dioxane. This modification has been applied to three capsule dosage forms containing combinations of ephedrine an barbiturate salts. The data are recorded in Table II.

The modified procedure is applicable where sodium chloride and similar salts are present. Insoluble fillers, lubricants and diluents, and nonionic components in general, will not interfere with the assays. However, acids comparable in strength to the barbiturates and bases similar to ephedrine and their salts will interfere.

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Interfacial Properties of Phenothiazine Derivatives

Sir:

Recent publications have indicated that chlorpromazine and other phenothiazine derivatives accumulate at biological membranes (1–3), and that these membranes might be the site of pharmacological activity. This view is strengthened by recent work, which has demonstrated the active role of mitochondrial phospholipid membranes during oxidative phosphorylation (4), and by the fact that chlorpromazine has been shown to interfere with electron transport by uncoupling oxidative phosphorylation (5, 6). Since the accumulation of a drug at a membrane depends on its ability to be adsorbed at interfaces, we have initiated a study of the interfacial properties of various phenothiazine derivatives at the air-water interface and at interfaces covered with insoluble monomolecular films, which simulate the oriented structure of biological membranes.

We have measured the surface tension of chlorpromazine[1] (CPZ), chlorpromazine sulfoxide (CPZ-O), and trifluoperazine[2] (TFP) at 25°, using the Wilhelmy plate method (7). The solutions were prepared in a Sorenson buffer at pH 6.9 and at an ionic strength of 0.04. A plot of surface tension *versus* the logarithm of molar concentration is shown in Fig. 1. The differences in surface activity are quite large and the order appears to parallel that of accumulation at biological membranes (2). Since an increased fraction of undissociated molecules generally increases the surface activity of acidic and basic substances at liquid interfaces, the differences between TFP and the other substances may be attributed, in part, to differences in the degree of dissociation; the two pKa values for TFP are about 2.7 and 7.2 (8) while those of CPZ and CPZ-O are about 8.2 (8) and 8.5 (9), respectively. The importance of the undissociated species was observed at a pH of 2.3 where the drugs are dissociated to a great extent. Here, there is no surface activity below a concentration of 1.0×10^{-3} *M*. The almost complete lack of adsorption of CPZ-O is probably due to the sulfoxide group which is too polar to be oriented toward air at

[1] Marketed as Thorazine Hydrochloride by Smith Kline & French Laboratories.

[2] Marketed as Stelazine Dihydrochloride by Smith Kline & French Laboratories.

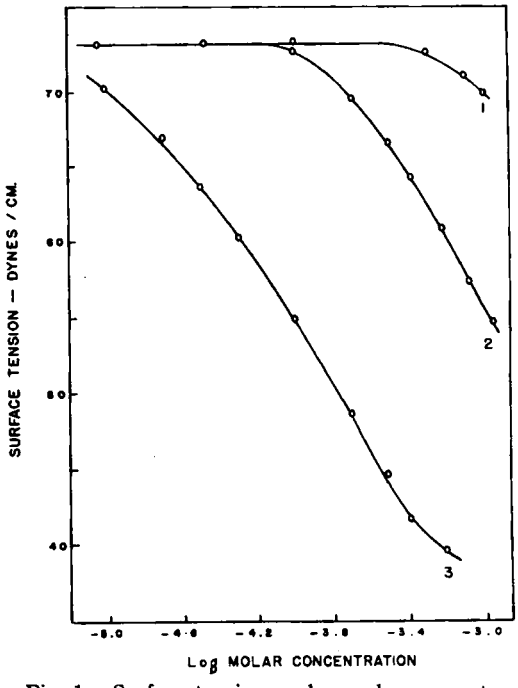


Fig. 1.—Surface tension *vs.* log molar concentration for: *1*, chlorpromazine sulfoxide; *2*, chlorpromazine; *3*, trifluoperazine in Sorenson buffer, pH 6.9 and ionic strength 0.04, at 25°C.

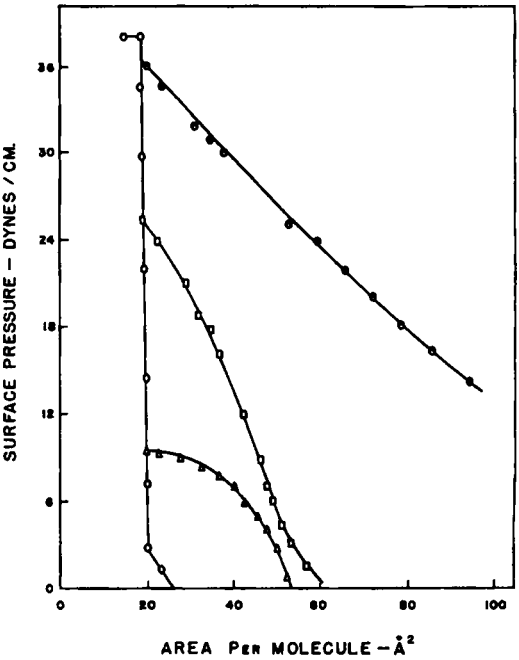


Fig. 2.—Surface pressure *vs.* area per molecule for stearic acid spread on: Sorensen buffer, pH 6.9 and ionic strength 0.04 (○); buffer plus 10^{-5} *M* chlorpromazine (□); buffer plus 10^{-5} *M* chlorpromazine sulfoxide (△); buffer plus 10^{-5} *M* trifluoperazine (●), at 25°C.

the interface. This lack of adsorption was also noted for trifluoperazine sulfoxide.

We have also measured the interfacial activity of these compounds in the presence of insoluble monomolecular films which were spread on a Langmuir-type balance (10). Figures 2 and 3 show the relationship between surface pressure (surface tension of solvent-surface tension of the film) and the area per molecule for stearic acid and L-α-dipalmitoyl lecithin spread on the same buffer solution as used previously, with and without dissolved drug. A concentration of 1.0×10^{-5} M was chosen since little, if any, surface activity was previously noted at the air-water

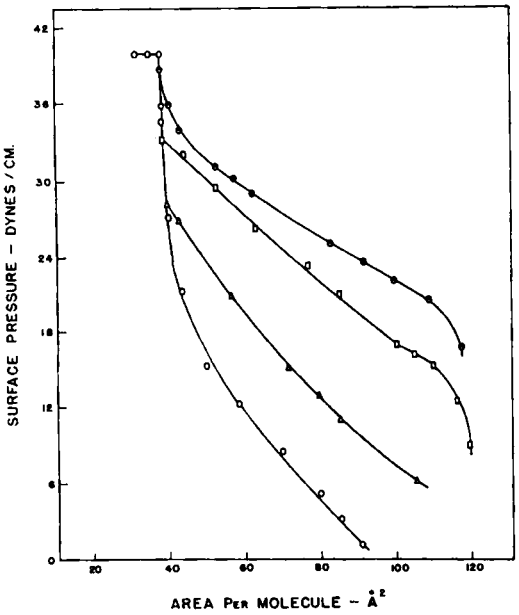


Fig. 3.—Surface pressure *vs.* area per molecule for L-α-dipalmitoyl lecithin spread on: Sorenson buffer, pH 6.9 and ionic strength 0.04 (○); buffer plus 10^{-5} M chlorpromazine (□); buffer plus 10^{-5} M chlorpromazine sulfoxide (△); buffer plus 10^{-5} M trifluoperazine (◓), at 25°C.

interface, whereas adsorption at membranes (2) and biological activity (5) have been reported.

The marked development of surface pressure at areas per molecule where stearic acid and lecithin exhibit none indicates penetration of the film by the dissolved drugs. Cockbain and Schulman (11) have studied the penetration of soluble surfactants into insoluble films and have indicated that the extent of the penetration and the strength of the resulting mixed film depends on such factors as, the chemical nature and the number of polar groups in the two molecular species, the van der Waals forces between nonpolar groups, the surface pressure of the insoluble film, the concentration of dissolved compound, the pH and ionic strength of the underlying solution, and the stereochemical configuration of the two molecular species in both polar and nonpolar groups. They concluded that in order to form a strong mixed film two strong points of contact must be made between polar and nonpolar portions of the molecules involved.

As can be seen in Figs. 2 and 3, penetration occurs to a great extent at higher areas per molecule but as the film is compressed to lower areas the drugs are ejected from the film. The relative surface pressure developed at the same area per molecule, and the ability to resist ejection, therefore, are measures of the interfacial activity of these drugs at a particular membrane under a given set of conditions. In view of this, it is interesting to note the differences between the three compounds studied and the correlation with earlier studies (2, 3).

Thus, it is apparent that at concentrations where biological activity is observed, a drug may be quite surface active at an insoluble monomolecular film, and yet, exhibit no activity at the air-water interface. It is also apparent that modification in the structure of the hydrophobic and hydrophilic portions of the drug can greatly alter its ability to accumulate at a particular interface.

Further studies, utilizing other films and drugs are being conducted. In addition, the effects of pH, ionic strength, and drug concentration are being considered.

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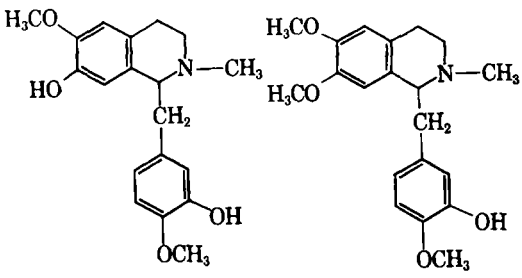
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New Opium Alkaloid

Sir:

Recent chromatographic studies have shown that opium may contain several alkaloids which have not been previously described (1, 2). Some of these are phenolic in nature and are of particular interest because of possible interference in the quantitative determination of morphine and because of the role they may play in the biogenetic pathways of the various opium alkaloids. One such phenolic alkaloid has now been isolated from crude opium and from the mother liquor produced during purification of morphine.[1] It was separated from morphine by extraction with chloroform in the presence of dichloroacetic acid (3). Further purification was achieved by extracting a strongly alkaline solution (about pH 13) with chloroform. This removed laudanine, laudanidine, and other monophenolic alkaloids, leaving the unknown compound in the aqueous phase. After adjustment of pH to about 8.5, the new alkaloid was extracted with chloroform and purified by crystallization from a mixture of ether and petroleum ether. Its purity was demonstrated by thin-layer and gas chromatography. Column acetylation (4) resulted in displacement of the gas chromatographic peak and eliminated tailing, indicating esterification of a phenol. This was confirmed by methylation with diazomethane which produced (±)-laudanosine, thus identifying the alkaloid as a benzyltetrahydroisoquinoline derivative. It became evident from its N.M.R. spectrum that the new compound must contain two phenolic hydroxyl groups and two methoxy groups. The positions of these groups were located by comparing the N.M.R. spectrum with those of laudanidine, pseudolaudanine, armepavine, and laudanosine. The stereochemistry of the new alkaloid was established by its relationship to (±)-laudanosine, thus making it possible to assign it the structure in formula I. The dextrorotatory isomer of this alkaloid was isolated from *Cocculus laurifolius* (*Menispermaceae*) by Kusuda (5), who named it coclanoline. Later Gopinath, *et al.* (6), found this isomer in *Anona reticulata* (*Anonaceae*) and called it reticuline. (±)-Reticuline [(±)-coclanoline] has been synthesized (7, 8), but has not previously been isolated from a natural source.

Consideration of the structure of (±)-reticuline indicates that it is very unlikely that racemization would occur during the isolation pro-



(±) Reticuline (I) Laudanidine (II)

cedure. This has been confirmed by Späth and Burger (9), who were unable to affect racemization of levorotatory laudanidine (II), a closely related alkaloid. They, therefore, concluded that laudanidine and laudanine [(±)-laudanidine], both of which have been isolated from opium, exist as separate, genuine alkaloids. Since laudanine is (±)-7-methylreticuline, it is reasonable to assume that the two alkaloids are closely related biogenetically. It is interesting to note that both have been postulated as biogenetic precursors for the morphine group of alkaloids (10–13). Recently, Battersby, *et al.* (14), and Barton, *et al.* (15), using radioactively labeled reticuline, have shown that this alkaloid is very efficiently converted to morphine in the opium poppy.

Details of the methods for isolation and characterization of (±)-reticuline will be published at a later date.

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REVIEWS

Rheology of Emulsions. Edited by P. Sherman. The Macmillan Co. (Pergamon Press), New York, N. Y., 1963. vii + 146 pp. 15 × 23 cm. Price $7.50.

The book is a compilation of the papers presented at a symposium held by the British Society of Rheology at Harrogate in October, 1962. The papers report the most recent work at the time on the flow properties of emulsions. The topics considered in the papers include the formation of emulsions, the effect of concentration, flocculation, and particle aggregation on the rheology of disperse systems, the behavior of liquid drops under laminar flow, stabilization of emulsions, and dielectric behavior of emulsions. Since the discussions involving the relationship of particle aggregation to rheological properties stress the "secondary minimum" in flocculation, one paper was devoted exclusively to a study of the diffuse double layer following the Derjaguin–Landau and the Verwey–Overbeek theories for the stability of colloidal systems. The editor apologized for the omission of a promised contribution on the rheological properties of the emulsifier film at the interface. It is unfortunate that a paper on this subject could not be included, because of the often cited importance by various authors of resiliency and toughness of an emulsifier film in contributing to emulsion stability.

The earlier symposium on the rheology of suspensions, entitled "Rheology of Disperse Systems" (Pergamon Press, 1959), together with the present book, make a valuable contribution to the rheology of suspensions and emulsions. Anyone teaching or doing research which involves the consideration of dispersed solids and liquids will want to have these companion works in his library.

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Organic Reactions. Vol. 13. Edited by A. C. Cope. John Wiley & Sons, Inc., 605 Third Ave., New York 16, N. Y., 1963. vii + 382 pp. 15 × 23 cm.

Each chapter of this latest volume treats an important synthetic reaction. The discussions are authoritative and critical covering the reaction and the methods of preparation and utilization of reagents. The organic reactions presented are covered completely and representative experimental procedures are given. Subjects covered in this volume are hydration of olefins, dienes, and acetylenes *via* hydroboration, halocyclopropanes from halocarbenes, free radical additions to olefins to form carbon–carbon bonds, and formation of carbon–hetero atom bonds by free radical chain additions to carbon–carbon multiple bonds.

Quantum Biochemistry. By Bernard Pullman and Alberte Pullman. Interscience Publishers, 605 Third Ave., New York 16, N. Y., 1963. xvi + 867 pp. 15.5 × 23 cm. Price $27.50.

The two main objectives of this book are to show biochemists how quantum mechanics can yield answers to the problems of the structure and mode of action of the constituents of living matter and to provide the quantum chemists with a general outline of the aspects of biochemistry in which their contribution may be useful. The authors note that topics of the same apparent importance are often given unequal treatment in their presentation but explain that the very early stage of development of quantum biochemistry is responsible. The work is divided into three major sections dealing with molecular orbitals for biochemists, electronic structure of fundamental biochemical substances, and electronic aspects of enzymic reactions.

NOTICES

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XXI° Congresso di Scienze Farmaceutiche. Conferenze e Comunicazioni, Pisa, 1961. Federazione Ordini Farmacisti Italiani, Rome, 1962. vii + 943 pp. 17 × 24 cm. Paperbound.

Outline of Pharmacology and Therapeutics. By Sister M. Mariel, C.S.A. Charles C Thomas, 301–327 E. Lawrence Ave., Springfield, Ill., 1963. xvi + 297 pp. 16 × 23.5 cm. Price $12.50.

Advances in Pharmacology. Vol. 2. Edited by Silvio Garattini and Parkhurst A. Shore. Academic Press, Inc., 111 Fifth Ave., New York 3, N. Y., 1963. vii + 392 pp. 15.5 × 23.5 cm. Price $12.

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Reviewed by Alfred N. Martin
Professor of Physical Pharmacy
Purdue University
School of Pharmacy and Pharmacal Sciences

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JANUARY TO DECEMBER, 1964

JOURNAL OF Pharmaceutical Sciences

June 1964 volume 53, number 6

Review Article

1,4-Benzodiazepines

By SCOTT J. CHILDRESS and M. I. GLUCKMAN

THE DISCOVERY of chlordiazepoxide,[1] 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide (I) (1), which prescription surveys indicate is the most frequently prescribed new drug in the United States, has brought about a considerable amount of work on the chemistry and biological activity of 1,4-benzodiazepines. A second benzodiazepine, 7-chloro-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (II) (2, 3), has been marketed as diazepam.[2] Two other benzodiazepines have been given extensive clinical study: 7-nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (III) (LA-1) (4) and 7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (IV), oxazepam (5).

These compounds have a wide spectrum of powerful effects on the central nervous system. Chlordiazepoxide and diazepam find their principal use as antianxiety agents with diazepam also recommended as a centrally acting muscle relaxant. Oxazepam is comparable to chlordiazepoxide with diminished side effects. LA-1 has been studied primarily as an anticonvulsant agent (6).

It is the purpose of this review to summarize the chemistry of the 1,4-benzodiazepines and report the structure-activity relationships that can be drawn. The literature has been covered through 1963. No attempt has been made to list clinical material. Patents have not been cited in the presence of a comparable scientific article.

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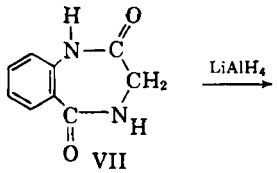
The authors are grateful to Drs. P. B. Russell, S. C. Bell, and T. S. Osdene for their comments on the manuscript.

[1] Formerly methaminodiazepoxide. Marketed as Librium by Roche Laboratories, Nutley, N. J.

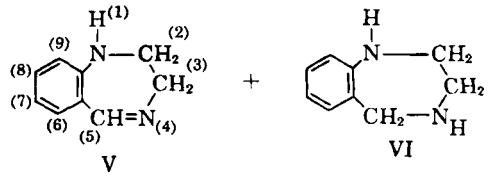
[2] Marketed as Valium by Roche Laboratories, Nutley, N. J.

1,4-BENZODIAZEPINES

Although 1,4-benzodiazepine itself has not yet been prepared, both 2,3-dihydro (V) and 2,3,-4,5-tetrahydro-1*H*-1,4-benzodiazepine (VI) have been obtained by reduction of 3,4-dihydro-2*H*-1,4-benzodiazepine-2,5-(1*H*)-dione (VII) with lithium aluminum hydride (7).



VII

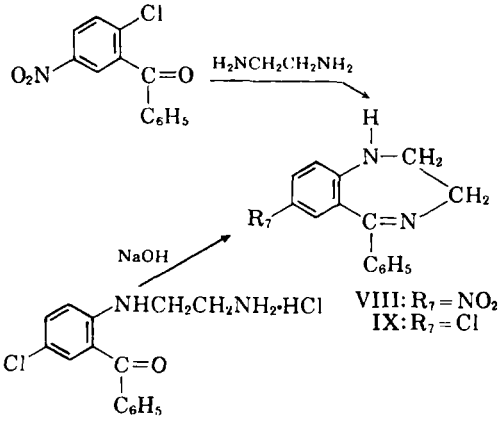


V VI

Similar reduction of 5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-ones gives compounds carrying the corresponding 5-phenyl groups (8, 9). Reduction of the analogous 7-chloro-2-phenyl-3,-4-dihydro-5*H*-1,4-benzodiazepin-5-one affords 7-chloro-2-phenyl-2,3,4,5-tetrahydro-1*H*-1,4-benzodiazepine, the 1-double bond being reduced before the carbonyl group (10). This preparative method is obviously limited by

availability of the requisite starting materials, but this limitation is not serious in view of the many routes to benzodiazepinones.

A reductive method is not suitable for nitro substituted compounds. 7-Nitro-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine (VIII) is obtained by treatment of 2-chloro-5-nitrobenzophenone with ethylenediamine (11). Success with this method has been reported with 2,5-dichlorobenzophenone, but greater activation of the halogen to be displaced would seem to be desirable (12).

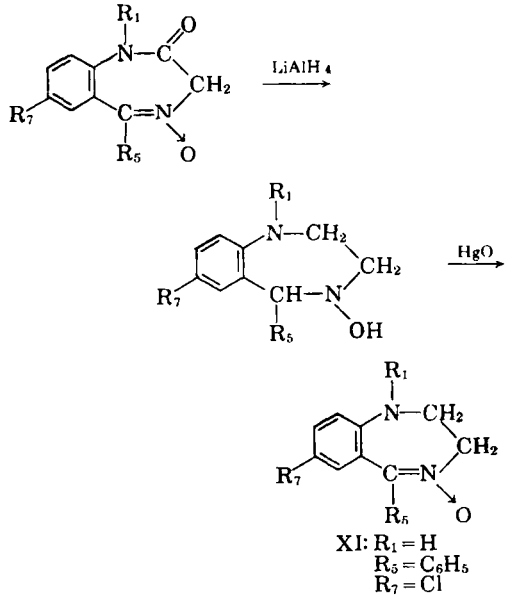


Desulfurization by Raney nickel of 1,3-dihydro-2*H*-1,4-benzodiazepine-2-thiones has also been reported to yield 2,3-dihydro-1*H*-1,4-benzodiazepines (13).

2-Aminoethylamino-5-chlorobenzophenone, which can be prepared in a number of conventional ways, is stable only as a salt; 7-chloro-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine (IX) results upon alkalinization (9, 14).

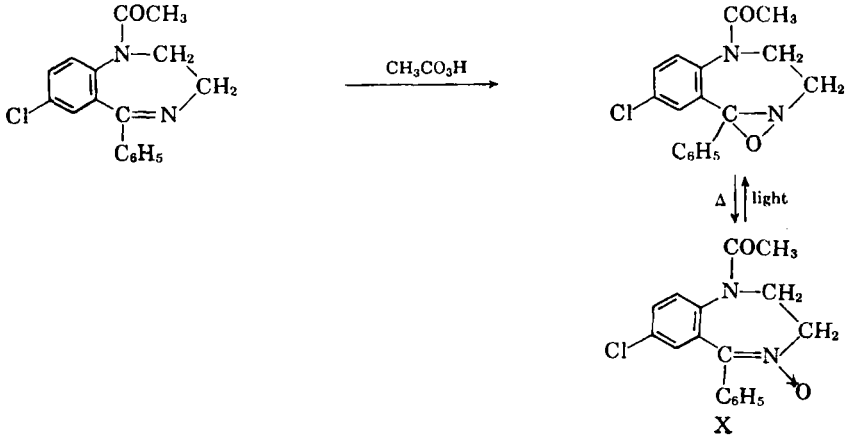
Compound IX has been acylated and alkylated in the 1-position. The 1-alkyl compounds are obtainable directly by applying the reductive method to the appropriate 1-alkyl benzodiazepin-2-one (9).

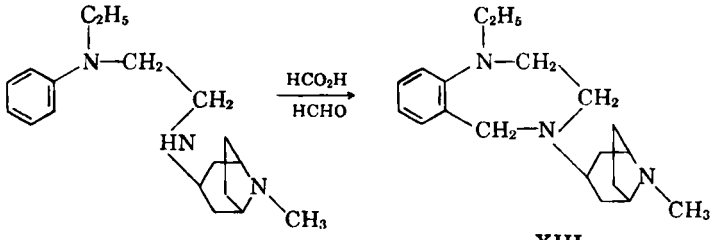
1-Acyl-2,3-dihydro-1*H*-1,4-benzodiazepines have been oxidized to the corresponding 4-oxides by peracetic acid (15). An intermediate 4,5-epoxy derivative rearranges with heat to the 4-oxide. The reverse transformation takes place upon exposure of the 4-oxide to light. An alternative source of 2,3-dihydro-1*H*-1,4-benzodiazepine 4-oxides is the oxidation with mercuric oxide of the corresponding 4-hydroxy-2,3,4,5-tetrahydro compounds. These latter compounds



are accessible by hydride reduction of 1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxides (8, 15).

In attempting to methylate *N*-ethyl-*N*-phenyl-*N'*-(3-tropanyl)ethylenediamine (XII) by the Eschweiler-Clarke method, Archer, *et al.* (16), obtained a viscous oil that proved to be 1-ethyl-4-(3-tropanyl)-2,3,4,5-tetrahydro-1*H*-1,4-benzodiazepine (XIII). It was shown that a *para* substituent on the phenyl group





XII XIII

leads to increased yields of benzodiazepine by reducing polymer formation. Although this type of ring closure would appear to have general applicability, it has not been studied extensively. Treatment of the products with methyl iodide causes quaternization on the two nitrogen atoms in the benzodiazepine ring.

Biological Activity.—An insufficient number of 5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine 4-oxides has been studied to permit conclusions about structure-activity relationships within the group. However, comparisons of the data in Table I with those of Table III (benzodiazepinones) show the corresponding compounds in the latter to be rather more active.

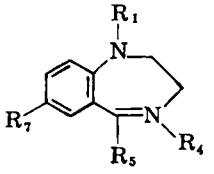
1 - Ethyl - 4 - (3 - tropanyl) - 2,3,4,5 - tetrahydro-1*H*-1,4-benzodiazepine (XIII) in the form of a bis quaternary salt is reported to have ganglionic blocking activity (16).

AMINO-1,4-BENZODIAZEPINES

In attempting to prepare 2-aminomethyl-6-chloro-4-phenylquinazoline 3-oxides by treating 6 - chloro - 2 - chloromethyl - 4 - phenylquinazoline 3-oxide (XV) (17) with the appropriate amines, Sternbach and Reeder (1) discovered the ring enlargement reaction that led to chlordiazepoxide. Although a normal reaction occurs with secondary amines to afford the expected 2-substituted aminomethyl products, with methylamine a rearrangement takes place to afford 7 - chloro - 2 - methylamino - 5 - phenyl - 3*H*-1,4-benzodiazepine 4-oxide (I) (chlordiazepoxide). The structure of I was established by spectral data and the hydrolysis of its reduction product (XVIII) to 2-amino-5-chlorobenzophenone, methylamine, and glycine. The exact tautomeric form was indicated by its acetylation to the 2-(*N*-methylacetamido) derivative. Cyanoethylation also takes place on the exocyclic nitrogen (18). Nuclear magnetic resonance data confirm the tautomeric structure—the signal from the *N*-methyl group is split into a doublet by spin-spin coupling with the proton on the nitrogen atom.

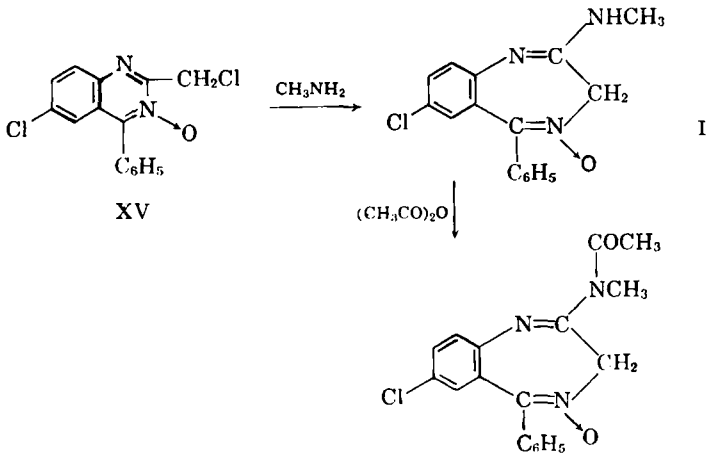
The rearrangement reaction proved to be general with ammonia and primary amines except for weak bases such as aniline which give the aminomethylquinazoline 3-oxides (19). With some amines, *e.g.*, allylamine, both the 6- and 7-membered ring products are formed (1). Electron releasing substituents in the 6- and 8-posi-

TABLE I.—STRUCTURE-ACTIVITY RELATIONSHIPS OF 2,3-DIHYDRO-1*H*-1,4-BENZODIAZEPINES[a]



Compd.	Structure R1	R5	R4	R7	Biological Activity,[b] Mice, mg./Kg., p.o. Motor Activity Decrease, MED	Anti-pentylene-tetrazol, ED50	Anti-Max. El. Shock, ED50	Anti-morphine, ED50	Ataxia at 1 hr., ED50
IX	H	C_6H_5	..	Cl	0.4	0.9	9.8	6.8	6.8
VIII	H	C_6H_5	..	NO_2	4	1.4	...	40	12
XI	H	C_6H_5	O	Cl	12.7	4	12.7	13	117
XIV	H	*o*-ClC_6H_4	O	Cl	4	4	9	7	51
X	CH_3CO	C_6H_5	O	Cl	40	78	150	250	78

[a] The test data reported in this article are those observed at Wyeth Laboratories and are used throughout to maintain consistency. Data from other laboratories are cited by reference. [b] The D.M.A. test is a subjective measure of reduction in spontaneous motor activity, and the values quoted are the minimum amount of drug required to produce such an effect. The estimate of the ataxic effect at 1 hour is based on the method of Dunham, N. W., and Miya, T. S., THIS JOURNAL, **46**, 208 (1957). The antagonism studies are modifications of those reported by Everett, G. M., and Richards, R. K., *J. Pharmacol. Exptl. Therap.*, **81**, 402(1944); Swinyard, E. A., Brown, W. C., and Goodman, L. S., *ibid.*, **106**, 319(1952); and Holten, C. H. *Acta Pharmacol. Toxicol.*, **13**, 113(1957).

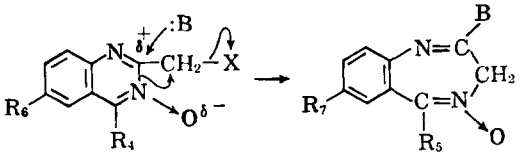


XV I

tions of the quinazoline ring are said to favor unrearranged products (20). Indeed, with 2-chloromethyl-6,8-dimethyl-4-phenylquinazoline 3-oxide no rearrangement takes place with methylamine. Once formed, aminomethylquinazoline 3-oxides appear to be stable. No report of their conversion into 7-membered ring compounds has appeared.

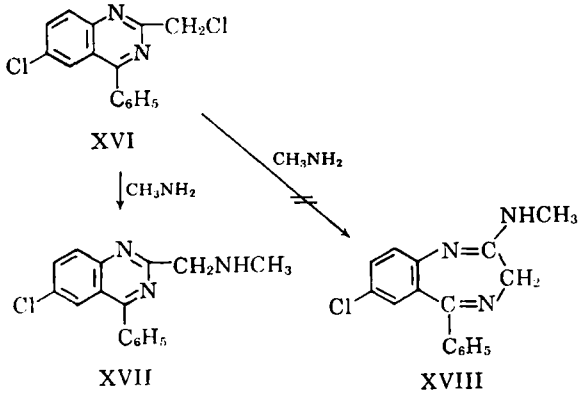
The 3-oxide function is necessary to permit rearrangement. 6-Chloro-2-chloromethyl-4-phenylquinazoline (XVI) and methylamine give only 6-chloro-2-methylaminomethyl-4-phenylquinazoline (XVII) (19).

benzodiazepine-2-thiones or their *S*-methyl derivatives has been developed (13). This reaction



Scheme I

is valuable since it makes available compounds having 2-dialkylamino substitution. (See Scheme II.) Compounds substituted in the benzo ring by an amino group have been prepared by reduction of nitro compounds (21).

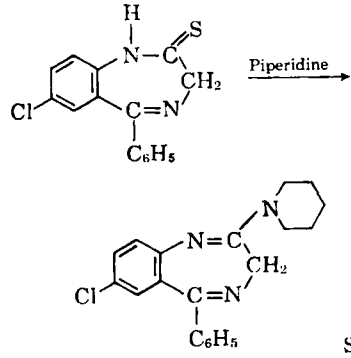


XVI XVII XVIII

A possible mechanism for the rearrangement involves the induction by the *N*-oxide function of a partial positive charge at C-2 which is reinforced by an electron attracting atom at C-6. Under nucleophilic attack, a concerted displacement of halide takes place through simultaneous attack of the 2,3-bonding electrons on the methylene carbon. Such a mechanism must rely on steric hindrance to explain the failure of strong secondary amines to effect rearrangement.[3] (See Scheme I.)

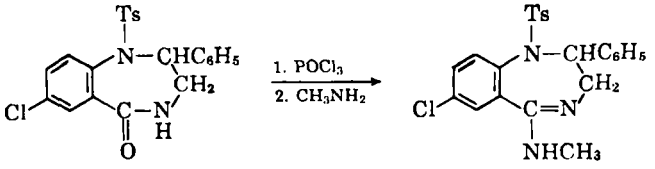
An alternative synthesis of 2-amino-5-phenyl-3*H*-1,4-benzodiazepines from the corresponding

In the synthesis of analogs of I a variety of substituents has been employed on the benzo ring



Scheme II

[3] Note added in proof—*Cf.* Farber, S., Wuest, H. M., and Meltzer, R. I., *J. Med. Chem.*, **7**, 235(1964).

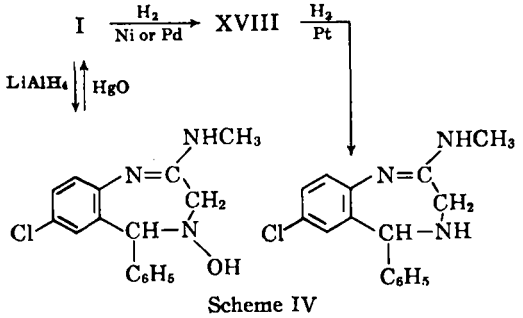


Scheme III XIX

and at the 3- and 5-positions (19, 20). These substituents largely parallel those of the benzodiazepinones (Scheme III). Compounds having the 2-amino group substituted by alkenyl, acyl, aralkyl, hydroxyalkyl, alkoxyalkyl, aminoalkyl, heteroalkyl, cyanoalkyl, and iminocarbamoyl groups have been prepared.

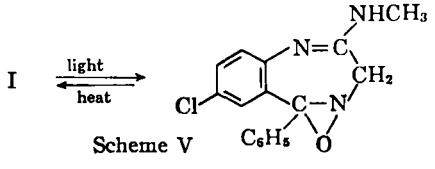
An isomer (XIX) of chlordiazepoxide has been prepared as a tosylate derivative from a related benzodiazepinone (10). Apparently the tosyl group could not be removed.

Catalytic reduction of the 2-aminobenzodiazepine 4-oxides removes the 4-oxide function (1, 19). Treatment with phosphorus trichloride brings about the same result. With platinum as catalyst, saturation of the 4,5-double bond occurs in addition to removal of the 4-oxide function. Reduction by lithium aluminum hydride gives the corresponding 4-hydroxy compound. (See Scheme IV.)



Scheme IV

Chlordiazepoxide undergoes the typical rearrangement of a nitrone in daylight, forming an oxaziridine (22). This process is reversed by heat (Scheme V.)



Scheme V

Biological Activity.—Chlordiazepoxide, the first of the useful benzodiazepines, is characterized by activity in a number of conventional pharmacological screens: antipentylenetetrazol, antielectroshock, antiemetric, antistrychnine, and antimorphine tests as well as a host of other more subjective measurements such as animal taming and muscle relaxation (ataxia) (23). Possibly the best single indicator of utility is the antipentylenetetrazol test.

Table II gives the results of certain of these pharmacological screens. Using the antipentylenetetrazol test as a guide, it is apparent that an electronegative substituent in the 7-position is of great importance. In order of decreasing potency: Cl > $CH_3 \cong H$. Other data indicate that NO_2 > Cl (4) and that CF_3 is a potent substituent (24, 25). Alkyl substitution on the 2-amino group reduces potency with the possible exception of CH_3. The effect of the *N*-oxide function is variable in this series. Its removal (XVIII) reduces the activity of chlordiazepoxide but increases the activity of the 2-amino analog (XX).

A phenyl group is the most satisfactory substituent in position 5. A 2-thienyl group reduces potency. Cycloalkyl groups are still less effective (19). It seems likely that *ortho* substitution of the 5-phenyl group would increase potency as it does in the benzodiazepinone series.

Chlordiazepoxide is rapidly absorbed from the intestines and is extensively metabolized in the rat and in man. 7-Chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide (XXXI), an active compound, has been identified among the metabolites (26).

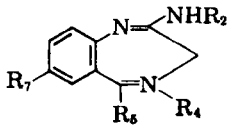
1,4-BENZODIAZEPINONES[4]

Benzodiazepin-2-ones.—Possibly the most important structures having the characteristic central nervous-system activity of chlordiazepoxide are the 5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-ones. Because of this, more attention has been paid to their synthesis than to the preparation of other benzodiazepines. A versatile group of reactions has been assembled for preparing these compounds.

One of the simplest methods and certainly the most straightforward is the treatment of 2-(α-haloacylamido)benzophenones with ammonia (3, 27). The bromo compounds are most often used. In liquid ammonia the intermediate 2-(α-aminoacylamido)benzophenones are isolated and then cyclized by heat. In alcoholic ammonia the cyclization occurs *in situ*. The yield in the

[4] A benzodiazepin-3-one is discussed under *Hydroxybenzodiazepines*.

TABLE II.—STRUCTURE-ACTIVITY RELATIONSHIPS OF 2-AMINO-3*H*-1,4-BENZODIAZEPINES[a]



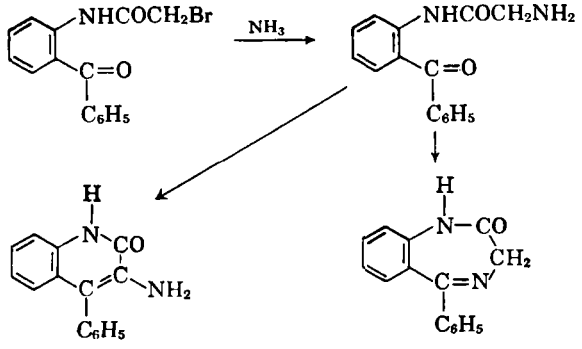
Compd.	Structure R_2	R_4	R_5	R_7	Biological Activity, Mice, mg./Kg., p.o. Motor Activity Decrease, MED	Anti-pentylene-tetrazol, ED_{50}	Anti-Max. El. Shock, ED_{50}	Anti-morphine, ED_{50}	Ataxia at 1 hr., ED_{50}
XX	H	..	C_6H_5	Cl	3.1	1.6	..	3.9	12.7
XXI	H	O	C_6H_5	Cl	50	3.6	..	6.7	28
XXII	CH_3	..	C_6H_5	H	12.5	>50	..	50	...
XXIII	CH_3	O	C_6H_5	H	25	>50	..	>50	...
XVIII	CH_3	..	C_6H_5	Cl	4	5.5	..	41	38
I[b]	CH_3	O	C_6H_5	Cl	4	3.7	14	14	22
XXIV	$C_6H_5CH_2$	O	C_6H_5	Cl	25	8	..	40	38
XXV	CH_3	..	C_6H_5	CH_3	50	>50	..	>50	...
XXVI	CH_3	O	C_4H_3S	Cl	50	23	..	47	...

[a] See footnotes to Table I. [b] Chlordiazepoxide.

case of 2-α-bromoacetamidobenzophenone is 85.5%. A possible competing reaction, the formation of the corresponding 2-aminomethylquinazoline, does not appear to be important, although the corresponding 3-amino-2(1*H*)-quinolones (XXVIII) have been obtained. In this method the amino group of the aminobenzophenone can be substituted by an alkyl group so that a 1-alkyl product is obtained. Secondary halides can be employed to afford 3-substituted benzodiazepin-2-ones.

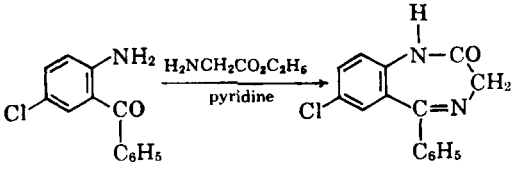
Because of its generality, the foregoing method is usually the first to be tried for making new benzodiazepin-2-ones.

The reaction of a 2-aminobenzophenone with an α-amino acid derivative yields 5-phenyl-1,3-dihydro - 2*H*- 1,4 - benzodiazepin - 2 - ones directly (27, 28). Typically, 2-amino-5-chlorobenzophenone and ethyl glycinate are heated in pyridine solution to afford 7-chloro-5-phenyl-1,3 - dihydro - 2*H* - 1,4 - benzodiazepin - 2 - one (XXIX) in a yield of about 50%. When there is steric hindrance from *ortho* substitution of the benzophenone, yields are lower. Both amino acids themselves, and amino acid chlorides (3) have been used in modifications of this method. In using the amino acid chloride it is clear that acylation is the first step and the intermediate aminoacylamidobenzophenone hydrochloride can be isolated. 3-Amino-2(1*H*)-quinolones (XXVIII) are formed as by-products. Whether acylation or imine formation takes place first when the ester is used has not been established. Both methods can be applied to *N*-substituted compounds. In addition to esters of glycine, esters of methionine and tyrosine have been used (27). Even a spiro compound has been made from the acid chloride prepared from 1-aminocyclopentane carboxylic acid (3).



XXVIII XXVII

In further variations of this method, the



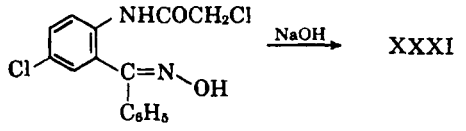
XXIX

amino acid derivative carries a protective group on the amine function. Carbobenzoxyglycine has been condensed with 2-amino-5-chlorobenzophenone by use of dicyclohexylcarbodiimide (29). Carbobenzoxyglycyl chloride reacts with the amine to form the same product (3). Since catalytic hydrogenolysis is not satisfactory, the protective group is removed by hydrogen bromide in acetic acid. The resulting intermediate is cyclized as before.

Another protective device that has been used is the trifluoroacetyl group. For example, 2-trifluoroacetamidoisobutyroyl chloride reacts with 2-amino-5-chlorobenzophenone to afford the expected intermediate, 2-(2-trifluoroacetamidoisobutyramido)-5-chlorobenzophenone (30). Mild alkaline hydrolysis and cyclization leads to 7 - chloro - 3,3 - dimethyl - 5 - phenyl - 1,3-dihydro-2*H*-1,4-benzodiazepin-2-one. The use of a protected amino acid is to be recommended for preparing 3,3-disubstituted benzodiazepinones.

Mild acid hydrolysis (see Scheme VI) of 2-amino- or 2-acetamidobenzodiazepine 4-oxides represents yet another process leading to benzodiazepin-2-ones (2). Since there is at present only one route to the 2-aminobenzodiazepine 4-oxides that is independent of the benzodiazepin-2-ones—namely, the rearrangement by amines of the 2-chloromethylquinazoline 3-oxides (see Scheme VII)—the hydrolysis method is of limited value. This is particularly so since 2-chloromethylquinazoline 3-oxides rearrange analogously with sodium hydroxide directly to benzodiazepin-2-one 4-oxides (2, 3). A host of such reactions has been carried out and this is probably the preferred synthesis when the 4-oxides are wanted. The method can be employed with almost any substituents on the benzo ring and no limits are known for the 4-substituent. By use of a compound having a 2-(1-chloroethyl) side chain, 7 - chloro - 3 - methyl - 5 - phenyl - 1,3 - dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide (XXXVI) has been made in ethanolic solution (3). A by-product in this reaction is 7-chloro-2-ethoxy-3-methyl-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide. Benzodiazepin-2-ones prepared in this fashion will always have hydrogen in the 1-position. This is not a serious limitation, for alkylation at this position is easy. Diazepam (II) can be made in this way.

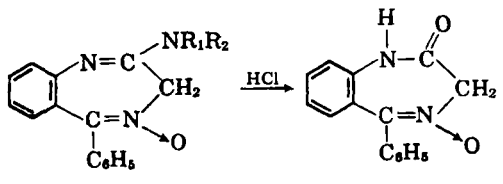
The oxime of 2-chloroacetamido-5-chlorobenzophenone having its hydroxyl group *anti* to the substituted phenyl ring undergoes self alkylation in base to yield the benzodiazepin-2-one 4-oxide, XXXI (2). The reaction is of academic interest



only in view of the several more useful syntheses.

The synthesis of benzodiazepin-2-ones by these methods is limited only by the availability of the required amino benzophenones. Considerable work has been done on methods for the preparation of these intermediates (31). The variations have resulted in benzodiazepinones having hydrogen (3, 27), halogen (2, 3), alkyl (3, 27), alkoxy (27), nitro (4), amino (32), trifluoromethyl (24, 25), carboalkoxy (33), carbamoyl, and cyano groups on the benzo moiety. There is mention in patent literature (32) of alkylthio, alkylsulfinyl, alkylsulfonyl, acylamido, and hydroxylalkylthio groups on this ring.

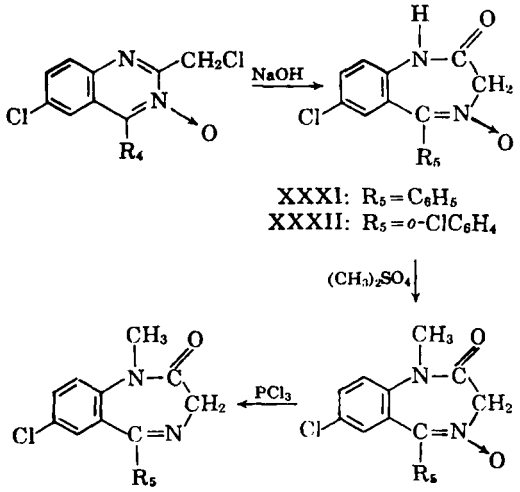
A phenyl group or a substituted phenyl group is most common at position 5, but compounds having methyl, cyclohexyl, thienyl (3), pyrryl, furyl (34), and pyridyl (35) groups have been prepared. Many of these compounds have been alkylated (2, 3) in the 1-position by alkyl, alkenyl (2), alkynyl (36), aralkyl, and cyanoalkyl (37) groups.



R_1 = H, alkyl
R_2 = H, acetyl

XXX

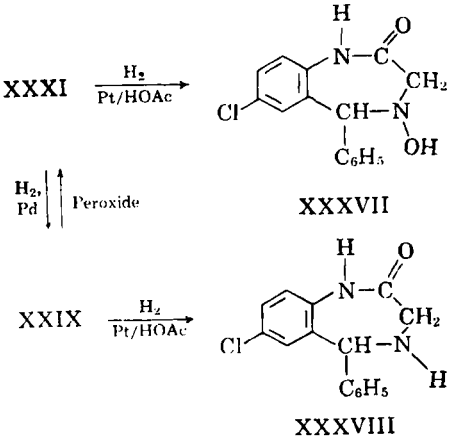
Scheme VI



Scheme VII

Groups placed in the 3-position include hydrogen, alkyl, alkoxyalkyl, alkylthioalkyl, aryl, and hydroxyaralkyl (2, 3, 27).

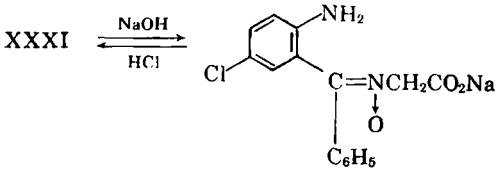
Catalytic hydrogenation of 1,4-benzodiazepin-2-one 4-oxides with palladium removes the 4-oxide function and may also remove a 7-chloro substituent (3). Hydrogenation using platinum in acetic acid is said to effect the conversion of benzodiazepin-2-one 4-oxides to the corresponding 4-hydroxy-4,5-dihydro compounds (XXXVII). The same treatment applied to a benzodiazepinone having no *N*-oxide leads to saturation of the 4,5-double bond (2). Phosphorus trichloride is the most selective reagent



for deoxygenating the 4-oxides. Peroxides serve to convert 1,4-benzodiazepin-2-ones into their 4-oxides; this reaction can be a useful alternative synthesis.

Oxaziridine formation from XXXI under ultraviolet irradiation has been observed as in the case of chlordiazepoxide (38).

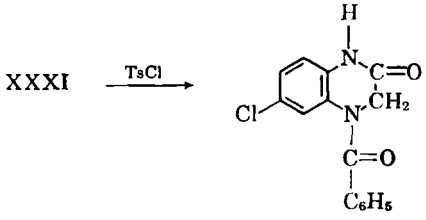
1,4-Benzodiazepin-2-ones and their 4-oxides are unstable in alkali, cleavage occurring between the 1- and 2-positions to afford benzophenone imines or nitrones, respectively. With the nitrones recyclization takes place in acid. With



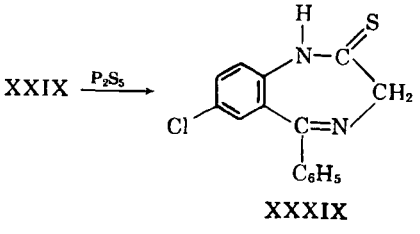
the imines regeneration of the benzodiazepinones is much less ready; hydrolysis of the imine bond predominates.

The nitration of 5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-ones by means of potassium nitrate and sulfuric acid has been shown to lead to 7-nitro derivatives (4). The entry of a second nitro group takes place in the *meta* position of the 5-phenyl ring (39). An earlier report (40) had placed the second nitro group in the 9-position.

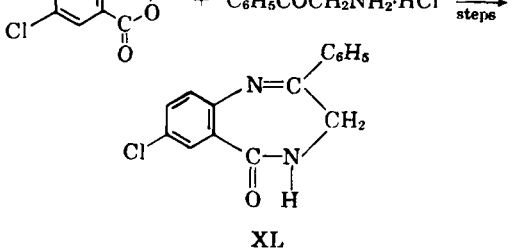
Beckmann rearrangement occurs when benzodiazepin-2-one 4-oxides are allowed to react with toluenesulfonyl chloride (41).



The carbonyl groups of benzodiazepin-2-ones (and benzodiazepin-5-ones) are readily converted to thione groups by phosphorus pentasulfide (10, 13, 42).



Benzodiazepin-5-ones.—Some isomers of the pharmacologically active benzodiazepin-2-ones have been made by different procedures (10). 7 - Chloro - 2 - phenyl - 3,4 - dihydro - 5*H*-1,4-benzodiazepin-5-one (XL) is obtained from the reaction of α-aminoacetophenone hydrochloride with 5-chloroisatoic anhydride by way of 2-amino-5-chloro-*N*-phenacylbenzamide as an intermediate. Reduction with a limited amount

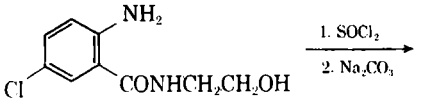


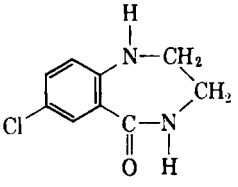
of lithium aluminum hydride gives the corresponding 1,2,3,4-tetrahydro compound. The carbonyl group is attacked only by more strenuous treatment with hydride.

Tosylation of 2-amino-*N*-(2-hydroxyethyl)-benzamides and subsequent heating of the *N,O*-

ditosyl compounds so formed leads to 1-tosyl-1,2,3,4 - tetrahydro - 5*H* - 1,4 - benzodiazepin-5-ones. The tosyl group can be removed by acid hydrolysis. In most cases, the intermediate ditosyl derivatives cannot be obtained pure since they tend to cyclize spontaneously. (See Scheme VIII.)

A modified approach avoids the necessity for removing the tosyl group. 2-Amino-5-chloro-*N*-hydroxyethylbenzamide treated with thionyl chloride and then with sodium carbonate solution affords 7-chloro-1,2,3,4-tetrahydro-5*H*-1,4-

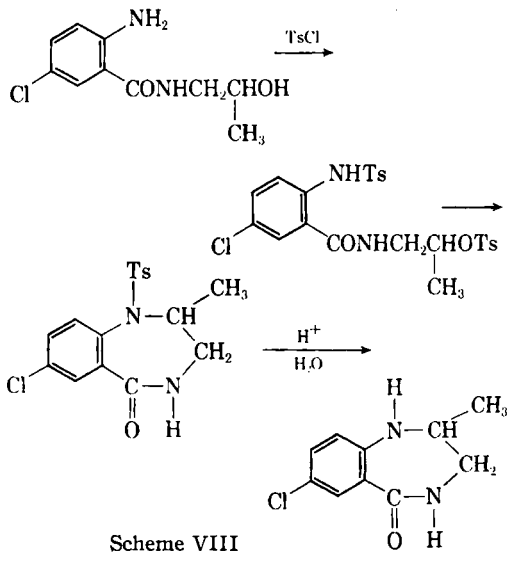




XLI

benzodiazepin-5-one (XLI). Reaction of 2-anilino - *N* - (2 - hydroxyethyl)benzamide with methanesulfonyl chloride affords 1-phenyl-1,2,3,4-tetrahydro-5*H*-1,4-benzodiazepin-5-one.

Biological Activity.—Benzodiazepin-2-ones are in general more potent than the corresponding 2-amino analogs. Again, for high potency; an electronegative substituent at position 7

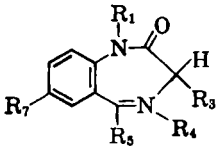


Scheme VIII

is required. Most of the data of Table III are for compounds having 7-Cl substitution, but other data (4) indicate that NO_2 > Cl and that CF_3 (24) is an effective replacement for Cl. Some excitation in the mouse has been observed with nitro compounds (43).

Alkylation at the 1-position is possible in this series and methylation of XXIX, giving II, results in an increase in the ataxic effect. Ataxia is a crude measure of muscle relaxation, an effect for which diazepam (II) is recommended. A decrease in potency is observed with methylation at position 3. The effect of an *N*-oxide function

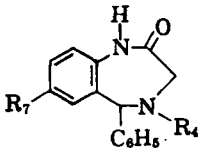
TABLE III.—STRUCTURE-ACTIVITY RELATIONSHIPS OF 1,3-DIHYDRO-2*H*-1,4-BENZODIAZEPIN-2-ONES[a]



	Structure					Biological Activity, Mice, mg./Kg., p.o.				
Compd.	R_1	R_3	R_4	R_5	R_7	Motor Activity Decrease, MED	Anti-pentylene-tetrazol, ED_{50}	Anti-Max. El. Shock, ED_{50}	Anti-morphine, ED_{50}	Ataxia at 1 hr., ED_{50}
XXVII	H	H	..	C_6H_5	H	50	36	...	>50	92[b]
XXX	H	H	O	C_6H_5	H	400	>50	...	>50	>100
XLII	H	H	..	C_6H_5	CH_3	50	>50	...	>50	>100
XLIII	H	H	O	C_6H_5	CH_3	50	38	...	>50	>100
XXIX	H	H	..	C_6H_5	Cl	6.2	0.9	...	2.3	11
XXXI	H	H	O	C_6H_5	Cl	6.2	2.0	...	12.8	48
II[c]	CH_3	H	..	C_6H_5	Cl	0.4	0.9	3.4	4.1	4.6
XLIV	C_2H_5	H	..	C_6H_5	Cl	3.1	1.0	9.4	4.2	19
XXXIII	CH_3	H	O	C_6H_5	Cl	25	2.2	...	26	48
XLV	H	CH_3	..	C_6H_5	Cl	25	6.5	...	15	54[b]
XXXVI	H	CH_3	O	C_6H_5	Cl	25	3.1	...	20	...
XLVI	H	H	..	*o*-ClC_6H_4	Cl	0.4	0.1	4.7	1.2	7.4
XXXII	H	H	O	*o*-ClC_6H_4	Cl	1.3	2.0	>40	6	>40
XXXIV	CH_3	H	..	*o*-ClC_6H_4	Cl	0.4	0.1	4	2.8	7
XXXV	CH_3	H	O	*o*-ClC_6H_4	Cl	4	0.65	15	5.2	3.2
XLVII	H	H	O	2-C_4H_3S	Cl	25	20	...	29	>100

[a] See footnotes to Table I. [b] At 1/2 hr. [c] Diazepam.

TABLE IV.—STRUCTURE-ACTIVITY RELATIONSHIPS OF 1,3,4,5-TETRAHYDRO-2*H*-1,4-BENZODIAZEPIN-2-ONES[a]



Compd.	Structure R_4	Structure R_7	Biological Activity, Mice, mg./Kg., p.o. Motor Activity Decrease, MED	Anti-pentylene-tetrazole, ED_{50}	Anti-Max. El. Shock ED_{50}	Anti-morphine, ED_{50}	Ataxia at 1 hr., ED_{50}
XLVIII	H	H	100	29	..	>50	>100
XXXVIII	H	Cl	...	5.2	13	44	29
XLIX	CH_3	Cl	100	8	..	18	>100

[a] See footnotes to Table I.

is variable, but in most of the cases its presence decreases potency.

The most active compounds of Table III contain a 5-*o*-chlorophenyl group. Other negative substituents at this location also increase activity. Placement at the *meta* and *para* positions is less effective in this respect. In Table IV, one can see that saturation of the 4,5-bond of XXIX (Table III), affording XXXVIII, leads to a reduction in activity.

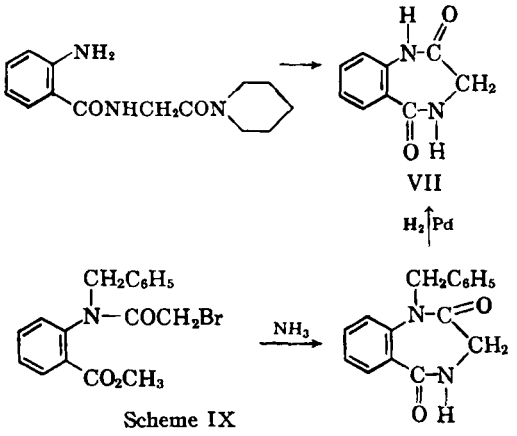
Diazepam has been studied extensively, and its pharmacology has been reported (44). Direct comparisons of diazepam and chlordiazepoxide have been made that show diazepam five to ten times as potent as chlordiazepoxide. These two compounds have also been compared with LA-1 (III) and XXXI in a Sidman avoidance situation (45). The nitro compound (III) was found to be the most potent of the four and to have the best ratio of activity to side effects. Strangely, XXXI was inactive.

The benzodiazepin-5-ones do not have activity of the chlordiazepoxide type.

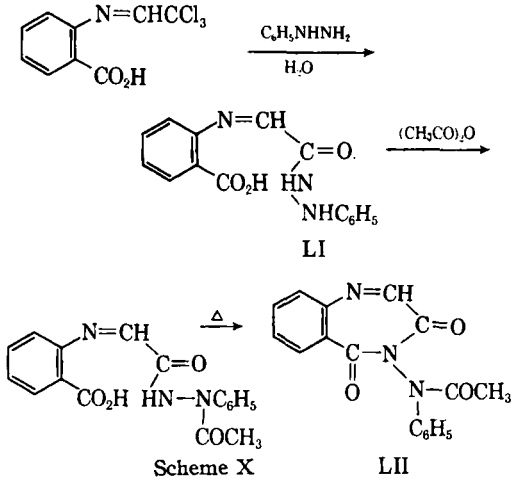
1,4-BENZODIAZEPINEDIONES[6]

3,4 - Dihydro - 2*H* - 1,4 - benzodiazepine-2,5-(1*H*)-dione (VII) can be made from *o*-aminohippurylpiperidine by heating in basic solution (7). The same compound is prepared by catalytic debenzylation of the 1-benzyl derivative (L), itself obtainable from 2-(*N*-benzyl-α-bromoacetamido)benzoic acid methyl ester by treatment with ammonia. (See Scheme IX.) Heating in alcoholic methylamine converts VII into the *N*-methylamide of *o*-aminohippuric acid.

The chloral derivative of anthranilic acid reacts with phenylhydrazine to give LI, which, in turn, is acetylated and cyclized by heating to afford 4 - (*N* - phenylacetamido) - 3*H* - 1,4-benzodiazepine - 3,5 - (4*H*) - dione (LII). (See Scheme X.) Compound LII is the earliest known 1,4-benzodiazepine, having been prepared in 1904 by Gärtner (46).



Scheme IX

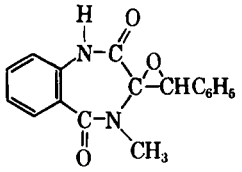


Scheme X

LII

Mohammed and Luckner, in a preliminary communication (47), have proposed structure LIII for the mold metabolite, cyclopenin. The evidence so far given for this structure is not completely convincing.

[6] Benzodiazepine-2,3-diones are discussed under *Hydroxybenzodiazepines*.

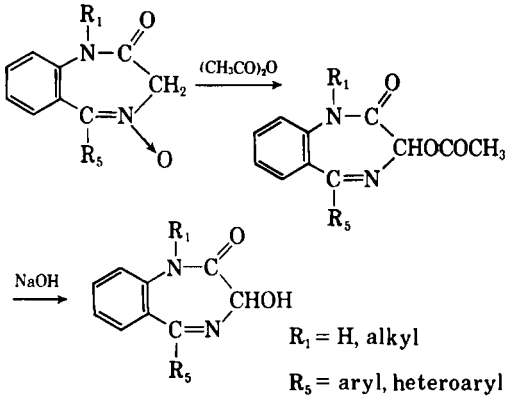


LIII

No biological activity has been reported for the benzodiazepinediones.

HYDROXY-1,4-BENZODIAZEPINES[6]

3 - Hydroxybenzodiazepin - 2 - ones.—5-Aryl - 1,3 - dihydro - 2*H* - 1,4-benzodiazepin-2-one 4-oxides, upon treatment with acylating agents such as acetic anhydride, undergo Polonovski-type rearrangements to afford 3-acyloxy-5-aryl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-ones (5). The acyl groups are easily hydrolyzed to yield the corresponding 3-hydroxy compounds. (See Scheme XI.) The structure of the rearranged compounds has been confirmed by replacement

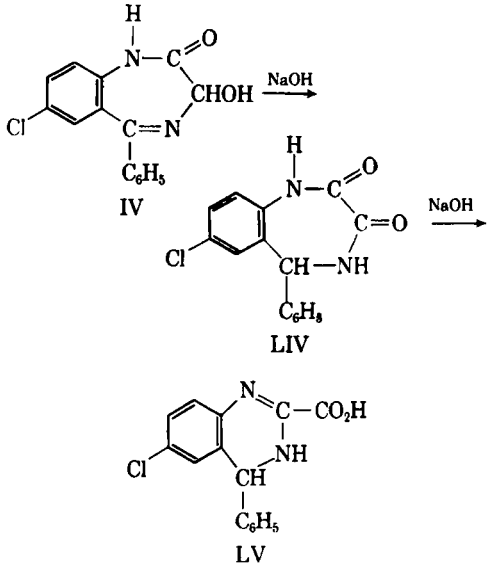


Scheme XI

of the hydroxy group by chloride followed by catalytic removal of the chlorine, giving compounds of known structure. N.M.R. data further indicate the 3-position for the hydroxy group by the spin-spin coupling of the hydroxy proton with the 3-hydrogen.

Although 3-hydroxybenzodiazepin-2-ones are stable as solids or in neutral solution, the iminocarbinol structure leads to a number of rearrangements on treatment with acid or base. For example, 7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (IV) is changed into 7-chloro-5-phenyl-4,5-dihydro-2*H*-1,4-benzodiazepin-2,3(1*H*)-dione (LIV) with warm sodium hydroxide. More vigorous treatment results in 6 - chloro - 4 - phenyl - 3,4 - dihydroquinazoline-2-carboxylic acid (LV). (See Scheme XII.) Parallel rearrangements take place with the 1-methyl analog (LVI), except that the final

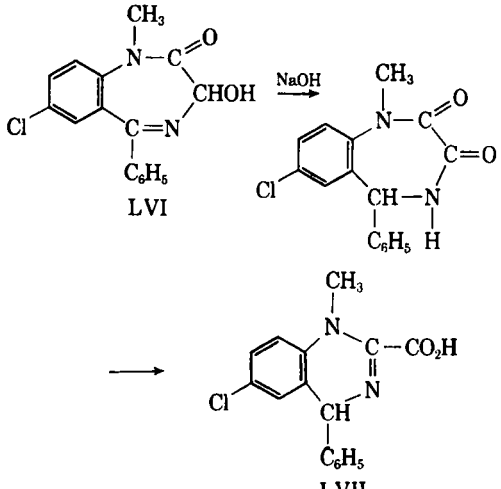
[6] 4-Hydroxybenzodiazepines are treated along with the corresponding oxidation products, the 4-oxides.



IV LIV LV

Scheme XII

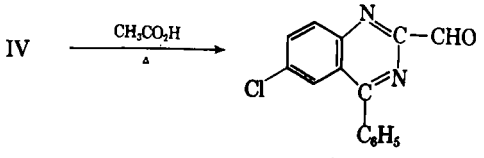
quinazoline (LVII) is a 1,4-dihydro rather than a 3,4-dihydro form (38). (See Scheme XIII.)



LVI LVII

Scheme XIII

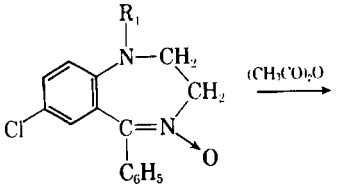
Compound IV rearranges in hot acetic acid, resulting in 6-chloro-4-phenylquinazoline-2-carboxaldehyde (LVIII) (41). Hydrazine causes the same rearrangement, the final product being the corresponding hydrazone. Primary amines give aldimines under similar conditions. Com-



LVIII

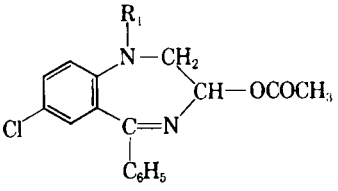
pound IV is easily converted into 7-chloro-3-ethoxy - 5 - phenyl - 1,3 - dihydro - 2*H* - 1,4-benzodiazepin-2-one (LIX) by way of the 3-chloro derivative (5).

3 - Hydroxybenzodiazepines.—The 2-oxo function is not required to permit the Polonovski rearrangements of the 4-oxides (48). 1-Acetyl-7-chloro - 5 - phenyl - 2,3 - dihydro - 1*H* - 1,4-benzodiazepine 4-oxide (LX) or its 1-methyl analog (LXI) rearranges as does IV, giving the 3-acetoxy-(LXII) and (LXIII) and, subsequently, the 3-hydroxy compounds (LXIV and LXV).



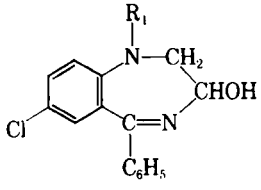
LX: $R_1 = CH_3CO$

LXI: $R_1 = CH_3$



LXII: $R_1 = CH_3CO$

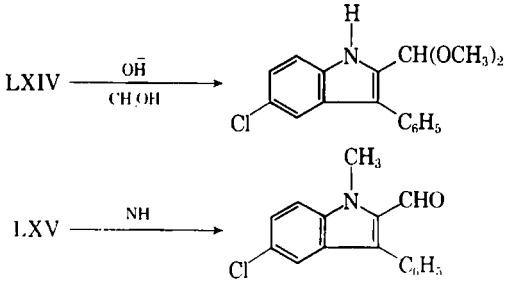
LXIII: $R_1 = CH_3$



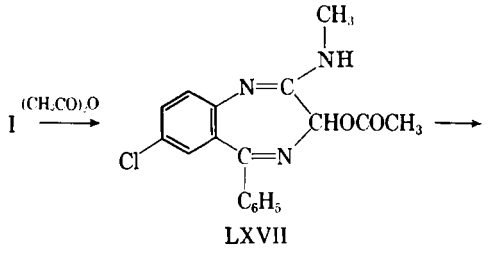
LXIV: $R_1 = CH_3CO$

LXV: $R_1 = CH_3$

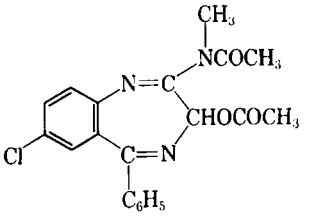
Alkaline conditions cause compounds LXIV and LXV to undergo ring contraction, furnishing indole derivatives.



2 - Amino - 3 - hydroxybenzodiazepines.—Acetylation of 2-amino-5-aryl-3*H*-1,4-benzodiazepine 4-oxides in pyridine solution under mild conditions to yield the corresponding 2-acetamido compounds has already been discussed. More vigorous acetylation in the absence of pyridine brings about a Polonovski-type rearrangement (49). For example, I is converted into 3-acetoxy - 2 - (*N* - methylacetamido) - 7 - chloro-5-phenyl-3*H*-1,4-benzodiazepine (LXVI) with acetic anhydride on the steam bath. The isolation of LXVII indicates that the rearrangement takes place before acetylation of the methylamino group.

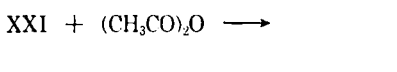


LXVII

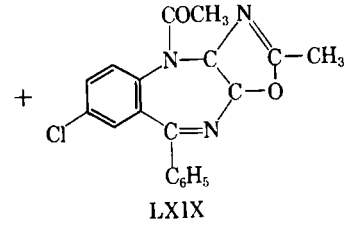


LXVI

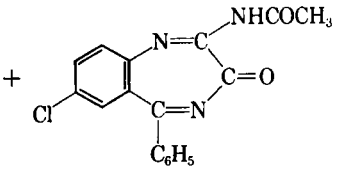
With an unsubstituted 2-amino group, diacetylation (LXVIII) occurs and two by-products (LXIX and LXX) are formed. The formation of 2-acetamido-7-chloro-5-phenyl-3*H*-



LXVIII



LXIX



LXX

1,4-benzodiazepin-3-one (LXX) is especially

difficult to account for. With LXX, a ring contraction takes place in hot acetic acid to afford *N* - acetyl - 6 - chloro - 4 - phenylquinazoline-2-carboxamide.

Both acetyl groups of LXVIII are removed in base yielding the corresponding 2-amino-3-hydroxy product (LXXI). Mild acid hydrolysis converts the diacetyl compound into 3-acetoxy-7-chloro - 5 - phenyl - 1,3 - dihydro - 2*H*- 1,4-benzodiazepin-2-one (LXXII).

Other Hydroxy Compounds.—7-Hydroxy-5-phenyl - 1,3 - dihydro - 2*H* - 1,4 - benzodiazepin-2-one has been prepared in the standard way by reaction of 2-amino-4-hydroxybenzophenone with glycine ethyl ester (27).

Biological Activity.—A group of compounds containing a 3-hydroxy group or a functional derivative thereof is listed in Table V. The general pattern of influences of the 1,5, and 7-position substituents seen in Tables II and III exists here.

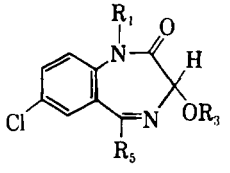
The 3-hydroxy group is a more effective substituent than an acetoxy or an ethoxy group. Particularly interesting compounds are LXXIII and LXXIV, whose ataxic doses are high in relation to their antipentylenetetrazol activity.

2-Amino-3-hydroxy compounds (Table VI) are much less potent than the corresponding 2-oxo-3-hydroxy products.

Oxazepam has been compared directly with chlordiazepoxide and diazepam in an induced conflict situation (50) which measures anti-anxiety effects. Diazepam was the most potent of the three compounds, with oxazepam and chlordiazepoxide approximately equipotent. Oxazepam had the most favorable ratio of activity to side effects.

The 3-hydroxy group is effective in reducing toxicity. The acute LD_{50} values in mice (p.o.) for oxazepam, diazepam, and chlordiazepoxide are >5000, 720, and 620 mg./Kg., respectively (44, 51). Possibly this reduced toxicity results from the ease of conjugation and subsequent elimination of oxazepam as a glucuronide. This metabolic product has been found in the dog (52).

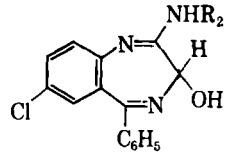
TABLE V.—STRUCTURE-ACTIVITY RELATIONSHIPS OF 3-OXY-1,3-DIHYDRO-2*H*-1,4-BENZODIAZEPIN-2-ONES[a]



Compd.	Structure R_1	Structure R_3	Structure R_5	Biological Activity, Mice, mg./Kg., p.o. Motor Activity Decrease, MED	Anti-pentylene-tetrazol, ED_{50}	Anti-Max. El. Shock, ED_{50}	Anti-morphine, ED_{50}	Ataxia at 1 hr., ED_{50}
IV[b]	H	H	C_6H_5	1.6	0.9	1.3	3.1	5.0
LVI	CH_3	H	C_6H_5	1.3	0.7	2.6	1.5	5.0
LXXII	H	CH_3CO	C_6H_5	1.6	2.6	...	7.5	7.1
LIX	H	C_2H_5	C_6H_5	25	5.7	...	21	56
LXXIII	H	H	*o*-ClC_6H_4	0.1	0.07	2.0	0.24	16
LXXIV	CH_3	H	*o*-ClC_6H_4	0.4	0.09	2.4	0.25	33
LXXV	H	CH_3CO	*o*-ClC_6H_4	1.0	0.26	3.7	0.12	30

[a] See footnotes to Table I. [b] Oxazepam.

TABLE VI.—STRUCTURE-ACTIVITY RELATIONSHIPS OF 2-AMINO-3-HYDROXY-3*H*-1,4-BENZODIAZEPINES[a]

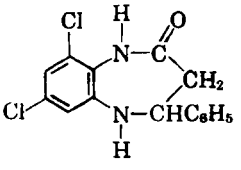


Compd.	Structure R_3	Biological Activity, Mice, mg./Kg., p.o. Motor Activity Decrease, MED	Anti-pentylene-tetrazol, ED_{50}	Anti-Max. El. shock, ED_{50}	Anti-morphine, ED_{50}	Ataxia at 1 hr., ED_{50}
LXXI	H	4	25	40	120	>40
LXXVI	CH_3	127	31	28	>40	>40

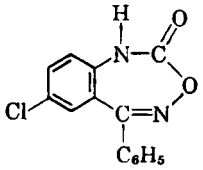
[a] See footnotes to Table I.

MISCELLANEOUS COMPOUNDS

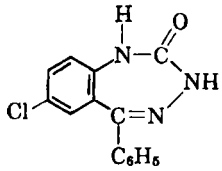
Several compounds that are not 1,4-benzodiazepines but that are analogs of active 1,4-benzodiazepines have been synthesized and are helpful in establishing the structural requirements for activity. Included are 7,9-dichloro-4-phenyl - 1,3,4,5 - tetrahydro - 2*H* - 1,5 - benzodiazepin-2-one (LXXVII) (53), 7-chloro-5-phenyl 3,1,4-benzoxadiazepin-2(1*H*)-one (LXXVIII) (54), 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,3,4-benzotriazepin-2-one (LXXIX) (55), and 7-chloro - 1 - methyl - 5 - phenyl - 3,5 - dihydro-4,1-benzoxazepin-2(1*H*)-one (LXXX) (56). No activity results are reported for the first of these compounds. The last three do not have activity of the same type as chlordiazepoxide.



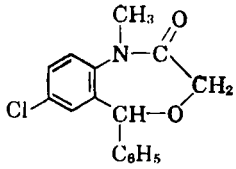
LXXVII



LXXVIII



LXXIX



LXXX

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Drug Partitioning I

Nonemulsifying Method for Measuring Distribution Coefficients

By D. R. REESE, G. M. IRWIN, L. W. DITTERT, C. W. CHONG, and J. V. SWINTOSKY

A simple rocking apparatus has been constructed for routine determination of distribution coefficients. With this apparatus, up to 36 two-phase samples in cylindrical tubes are equilibrated by rocking the horizontal tubes at 1 c.p.m. (cycle per minute) through an arc of 45°. This rocking causes the interface between the two immiscible phases to expand and contract slowly; it also causes the shape of each phase to vary constantly. These two actions facilitate uniform distribution of solute within each phase and facilitate drug transfer from one phase to the other. Emulsion formation is negligible since little turbulence is created. Considerable man hours are saved with some pharmaceutical systems if the rocking method, rather than the usual shake-out method, is used. The experimental method is simple and, where shake-out methods can be employed, gives results which are in agreement with results from shake-out methods. Data for the distribution of ephedrine, chlorpromazine and its salts, several benzoic acid derivatives, hexachlorophene, and bithionol are presented. Pharmaceutical applications of partitioning are discussed.

DETERMINING WITH convenience the partition coefficient or distribution character of drugs and related substances is becoming increasingly important in pharmacy and medicine. Beginning with Meyer (1) and Overton (2), in 1899, early workers (3–7) showed that the *degree* of biological action of many drugs is influenced by their oil/water partition coefficients. More recent workers (8–10) have shown that the *onset* and *duration* of action may also be affected by this property.

Brodie and co-workers (9, 11–13) and Nogami and co-workers (14–16) have shown that many compounds are absorbed from the gastrointestinal tract in their undissociated form. They have further shown that absorption may be related to the partition coefficient of the drug. Drug absorption from the oral cavity (17, 18) or through the skin (19, 20) is also influenced by the partition coefficient. Several authors (9, 21–23) have shown that the drug partitioning which occurs between various tissues and fluids is influenced by the drug's partition coefficient. Finally, it has been shown that metabolism in the liver (24) and renal tubule transfer (25) are influenced by the drug's pKa and partition coefficient.

While there are these numerous references to the role of partitioning in the biological action of drugs, the references to partitioning in the pharmaceutical literature are mostly limited to drug analysis (26–29). Pharmacists should, however, be aware of not only the biological role of drug partitioning, but also of drug partitioning within dosage forms and between dosage forms and body fluids. Partitioning data have been used in the design of pharmaceutical dosage forms by Garrett and Woods (30) and Hibbott and Monks (31) in their studies of preservatives for emulsions, and by Lachman, *et al.* (32), in their studies of preservatives for multidose parenterals. A relationship exists between the pharmacologic action and partition coefficients of many drugs (33–37), but apparently few, if any, deliberate attempts have been made to alter a drug's partition coefficient in a predictable manner. In 1944, Walton (18) did suggest the synthesis of salts and other derivatives with partition coefficients favorable to sublingual absorption. More recently, Collander (38) has made a thorough attempt to correlate partition coefficient, chemical structure, and solvent nature. It will be shown in the present study, for example, that two salt forms may have different apparent partition coefficients—a fact which may be important in formulation.

Emulsion taste, drug chemical stability, and drug

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release from emulsions, ointments, or suppositories (39) are often dependent on the amount of drug in the aqueous phase or on the ability of the drug to partition from a nonaqueous phase. In preparing emulsions of amines, for example, use of the amine base and a slightly alkaline aqueous phase should keep virtually all of the amine in the nonaqueous phase and thereby improve the taste of the emulsion. Diffusion from ointment to the skin may depend on a favorable aqueous phase pH so that the drug can partition from the nonaqueous phase.

Most two-phase pharmaceutical products are complex, *i.e.*, they contain several components in each phase. Many opportunities, therefore, exist for one component to influence the partition coefficient of another component. Drugs, antioxidants (40), flavoring agents, and preservatives can influence the apparent partition coefficients of one another. The partition coefficients of these substances can also be influenced by more inert components, such as electrolytes and macromolecules. For this reason, it is important to study partitioning in the complete formulation, even if data from simple formulations predict satisfactory results.

From such studies, it is clear that simple uncomplicated procedures for the routine determination of the solubilities and partition coefficients of compounds being screened for therapeutic activity would be useful.

Although a number of methods have been reported for determining partition coefficients (41–46), the one in common use is the shake-out method using separators. A simple rocking apparatus developed for two-phase systems, described in this report, overcomes or reduces the emulsification and foaming problems of the shake-out method and also avoids the possibility of anomalous equilibria as reported by Allen and McDowell (46). The second paper in this series (47) discusses the use of an inverted Y-tube apparatus to study partitioning of drugs from one aqueous phase through a nonaqueous phase into a second aqueous phase (*i.e.*, partitioning under simulated absorption conditions).

In this paper we will (*a*) provide a brief review of partition coefficient theory, (*b*) describe an apparatus devised for routine measurement of drug distribution between liquid phases, (*c*) report some of the parameters studied, and (*d*) describe some of the applications evaluated.

THEORETICAL

If a solute is introduced into a system containing two immiscible or slightly miscible liquids, it will distribute itself between the two liquids until a definite equilibrium concentration ratio is attained. The distribution law requires that this ratio, at constant temperature, be a constant, regardless of the total quantity of solute present, *i.e.*,

$$\frac{C_1}{C_2} = K \qquad \text{(Eq. 1)}$$

where C_1 and C_2 are the solute concentrations in the two liquid phases and K is the distribution or partition coefficient.[1]

This simple distribution law is exact only for ideal solutions. It is most closely approximated (*a*) when the liquid phases are completely immiscible, (*b*) when the solute neither associates nor dissociates in either phase, (*c*) when solute concentrations are relatively dilute, and (*d*) when the solute is only slightly soluble in either phase. When molecular association or other deviations from ideality occur, suitable modifications often can be applied to the distribution law to maintain a coefficient which is constant. Thus, for example, if a solute exists as undissociated single molecules in phase 2 and as a bimolecular species in phase 1, an equation may be written

$$\frac{\sqrt{C_1}}{C_2} = K' \qquad \text{(Eq. 2)}$$

When a solute partially dissociates in phase 2 and not in the other, then

$$\frac{C_1}{C_2(1-\alpha)} = K'' \qquad \text{(Eq. 3)}$$

where α is the degree of dissociation.

Equations can be derived for other cases which depart from ideality. The subject is covered adequately in accessible references (48). When systems being dealt with are nonideal, and corrections are not made for this, $\frac{C_1}{C_2}$ is sometimes designated as K_{apparent}.[2]

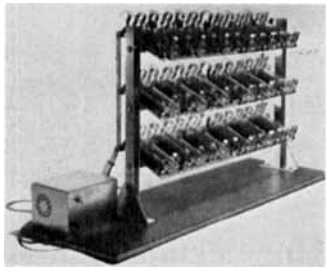


Fig. 1.—Photograph of the rocking device.

EXPERIMENTAL

Apparatus.—The rocking device is shown in Fig. 1. Its design, size, and construction materials are not critical. Our design has three interconnected shelves, each equipped with clips for holding 12 sample tubes. These shelves are connected to a synchronous motor which moves them from horizontal to 45° and back to horizontal with each revolution. Although the synchronous motor[3] is available in various speeds and our design permits rapid interchange from one motor to another, a rate of 1 c.p.m. (cycle per minute) was used in all routine studies. The Pyrex sample tubes (Fig. 2) have a volume of 100 ml. and are designed to hold a sample of 50 ml.

[1] Although the distribution law does not refer to the composition of the phases, the less polar phase is usually assigned the designation C_1 and placed in the numerator. This convention will be followed in this report.

[2] It is vital that the reader distinguish between the true (intrinsic) partition coefficient and the apparent (observed) partition coefficient. The true partition coefficient (TPC) for a drug is the one defined by the partition law and is, therefore, a measure of *only* the molecular species common to both phases. The apparent partition coefficient (APC) expresses the ratio of total drug observed in nonideal systems, regardless of the molecular species in which the drug exists. Since most drugs have the potential to ionize, associate, or complex, the partition coefficients reported for pharmaceutical systems are usually the apparent partition coefficients, which are thus valid only for the conditions under which they were measured. In pharmaceutical systems, when association or complexing occurs in the nonaqueous phase, APC is usually greater than TPC. When dissociation or complexing occurs in the aqueous phase, TPC is always greater than APC.

[3] Merkle-Korff Gear Co., Chicago, Ill.

(normally, 25 ml. of each phase). Their inner surfaces are smooth to minimize turbulence. Cork stoppers, with and without aluminum foil liners, were used to seal the ports.

Materials.—Ephedrine (hydrous) N.F.; benzoic acid U.S.P.; salicylic acid U.S.P.; *o*-chlorobenzoic acid and *o*-methylbenzoic acid, Matheson Coleman and Bell; *o*-nitrobenzoic acid, Eastman Organic Chemicals; bithionol, Winthrop-Stearns; hexachlorophene U.S.P.; chlorpromazine base, hydrobromide, hydrochloride, hydroiodide, maleate, succinate, hemisulfate, sulfate, and sulfanilate, Smith Kline and French Laboratories; and *N,N*-dimethyl oleamide, The C. P. Hall Co., were utilized.

Skellysolve B (petroleum naphtha), Skelly Oil Co.; spectroanalyzed cyclohexane, Fisher Scientific Co.; cottonseed oil (winterized), E. F. Drew and Co.; peanut oil, Planters Co.; isopropyl myristate, Kessler Chemical Co.; glyceryl tripelargonate (redistilled), Emery Industries, Inc.; castor oil U.S.P.; and mineral oil U.S.P. were also employed.

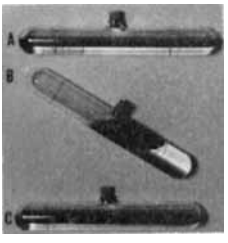


Fig. 2.—Pyrex sample tubes for the rocking device. Assuming a rocking rate of 1 c.p.m., then *A*, *B*, and *C* indicate the position of the sample tubes at, 0, 1/2, and 1 minute, respectively. The change in interfacial area is easily observed. Outside dimensions of the tubes are $1^1/_8 \times 8$ in., and they will conveniently hold 50 ml. of sample when tilted as in position *B*.

Analyses.—Concentrations of ephedrine in the aqueous and nonaqueous phases were determined volumetrically. Aqueous phase concentrations were also determined spectrophotometrically. Volumetric analysis of the aqueous phases followed the procedure described in N.F. XI. Volumetric analysis of the nonaqueous phases was accomplished by titration with 0.1 *N* perchloric acid in glacial acetic acid to the α-naphtholbenzein end point. The aqueous phases were analyzed spectrophotometrically by diluting aliquots in 0.1 *N* hydrochloric acid and reading the absorbance at 256.5 mμ. The nonaqueous phases were not analyzed spectrophotometrically, but the concentrations were calculated from the initial and final aqueous phase concentrations by difference. Volumetric analyses were used in the studies with ephedrine-cyclohexane-water systems as a check on the method and the results. Spectrophotometric analyses were used in the studies with ephedrine–fixed-oil–water systems.

Aqueous concentrations of benzoic acid and its derivatives were determined by titration with freshly standardized sodium hydroxide to the phenolphthalein end point. Nonaqueous concentrations were determined by titration with freshly standardized alcoholic sodium hydroxide to the α-naphtholbenzein end point.

Aqueous concentrations of bithionol were determined spectrophotometrically in 0.1 *N* sodium hydroxide using the absorbance maximum at 307 mμ. Aqueous concentrations of hexachlorophene were determined spectrophotometrically in 0.1 *N* sodium hydroxide using the absorbance maximum at 320 mμ.

Aqueous concentrations of chlorpromazine base and its salts were determined spectrophotometrically in 0.1 *N* hydrochloric acid using the absorbance maximum at 254.5 mμ. Concentrations of these materials in cyclohexane were determined spectrophotometrically using the absorbance maximum at 258 mμ.

Determination of Partition Coefficients by the Rocking Method.—With the rocker device in the 45° position (Fig. 2*B*), the desired volume of the aqueous phase was pipeted into each of the tubes. The less dense water immiscible phase was then carefully pipeted into the tubes with the stream directed against the tube wall just above the level of the aqueous phase and with slow draining to minimize mixing and emulsification.

The ports were stoppered with corks, and the rocking was generally carried out overnight. Normally, aliquots of each phase were analyzed after removing samples *via* pipets. The apparent oil/water (o/w) partition coefficient (*K*) was then calculated. True partition coefficients were obtained by buffering the aqueous phase to a pH at which virtually no dissociation of the drug could occur. The TPC's of ephedrine, chlorpromazine, and *o*-hydroxybenzoic acid were also calculated by the method of Butler (49) which utilizes two APC values obtained by altering the pH of the aqueous phase.

Determination of Partition Coefficients by the Shake-Out Method.—To avoid emulsion formation and the possibility of anomalous equilibria, the separators were gently inverted rather than vigorously shaken. A study of the systems employed showed

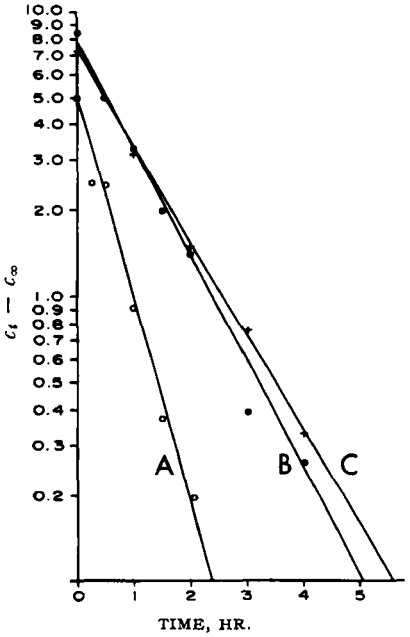


Fig. 3.—Rate of approach to equilibrium for systems containing ephedrine, water, and various nonaqueous phases, using the rocking device of Fig. 1 at 25°C. and rocking at 1 c.p.m. The ephedrine was initially all in the aqueous phase and its disappearance from this phase was followed. From the slopes of the curves, the half-lives for the approach to equilibrium for ephedrine in various nonaqueous phases were: *A*, cyclohexane, $t_{1/2}$ 0.4 hours; *B*, isopropyl myristate, $t_{1/2}$ 0.8 hours; *C*, peanut oil, $t_{1/2}$ 0.9 hours.

that 50 inversions were adequate to reach equilibrium.

RESULTS AND DISCUSSION

Rocking Method.—Our original partitioning studies utilized the separator shake-out technique. The frequent occurrence of emulsification led to a search for a better method for routine studies. The Craig countercurrent apparatus (44) and one modification of the Gershberg-Stoll disintegration apparatus (43) employ a rocking action. The rocking apparatus used in this study (Figs. 1 and 2) was constructed to facilitate solute transfer from one phase to the other and to facilitate uniform distribution of solute within each phase. These two actions are achieved, respectively, by (*a*) slow expansion and contraction of the interface and (*b*) constant variation in the shape of each phase.

In cylindrical tubes, rocking from horizontal to 45° causes almost as much change in interfacial area and phase shape as does rocking from horizontal to 90° (vertical). Preliminary studies showed that rocking rates faster than 1 or 2 c.p.m. often produced emulsification. Thus, the useful angle and rate of rocking were found to be 45° and 1 c.p.m. The port hole on the sample tube was placed in the center rather than on one end to avoid surface irregularities which might produce emulsification. This position of the port hole limits the volume the tube will hold, but this limitation has not been a problem.

All experiments were conducted in an air-conditioned room (25 ± 1°). The results, therefore, may have some variation, but are estimated to be not more than ±2–3%.

Equilibration Time.—After establishing that the rocking apparatus had little tendency to produce emulsions, it was necessary to establish the minimum time of rocking which would produce equilibrium in the systems under study. Nine tubes, containing cyclohexane and 0.1 *M* aqueous ephedrine as the two phases, were rocked; periodically, one of the tubes was removed, and the aqueous phase was analyzed for ephedrine. The final tube was allowed to rock for 24 hours. Thus, nine values were obtained at nine different time intervals. To plot the data as a first-order approach to equilibrium, the concentration (C_∞) in the 24-hour sample, considered to be the equilibrium concentration, was subtracted from the concentration at each time interval (C_t), and the difference plotted on a log scale against time. When, however, the study was conducted starting with the drug in cyclohexane, the plot was made by subtracting the concentration at each time from the equilibrium concentration and plotting the log of the difference against time.

The same procedure was used to obtain the log ($C_t - C_\infty$ or $C_\infty - C_t$) *versus* time plots for the ephedrine–isopropyl myristate–water and the ephedrine–peanut oil–water systems (see Fig. 3). Since the plots in Fig. 3 are straight lines, the approach to the equilibrium is first order and the half-lives for the three systems containing cyclohexane, isopropyl myristate, and peanut oil are 0.4, 0.8, and 0.9 hours, respectively. (The half-lives were approximately the same regardless of the direction from which equilibrium was approached.) From these data, it appears that viscosity has some influence on the rate of partitioning. After seven half-lives, systems following first-order kinetics are within 1% of true equilibrium. Thus, for the systems mentioned above, partition coefficients could be determined within 1% after approximately 6 hours of rocking.

Table I.—Influence of the Nonaqueous Phase on the Apparent Partition Coefficient of Ephedrine

Nonaqueous Phase	APC
Mineral oil	0.18
Skellysolve B	0.28
Cyclohexane	0.34
Isopropyl myristate	0.98
Cottonseed oil	1.03
Glyceryl tripelargonate	1.07
Peanut oil	1.32
Castor oil	3.76

Precision.—The precision of the rocking method was determined by comparing data obtained with the rocking method and the separator shake-out method using a system which does not readily emulsify. Thus, the two Skellysolve/water partition coefficients of ephedrine were determined by using 25 ml. of each phase in the rocking apparatus (16 hours) and in a 125-ml. separator (50 inversions), respectively. The partition coefficients determined by the two methods are the same within experimental error, *i.e.*, both values were 0.28. As expected, it was also found that the direction from which equilibrium is approached (oil to water and water to oil) had no effect on the observed partition coefficient.

Drug Concentration and Phase Volume.—Macy (50) and Collander (38) have shown that the only compounds which change appreciably in their partition coefficient with change in concentration are those in which a change in concentration also produces significant change in degree of association. In other words, the apparent partition coefficient may change, but not the true partition coefficient. The main effect of varying phase volume, other than this concentration effect, is mechanical, *i.e.*, sampling is made more difficult. With the system containing ephedrine, cyclohexane, and pH 11 aqueous buffer (to prevent dissociation), altering the ratio of the phase volumes from 20:80 to 80:20 had no effect on the ephedrine true partition coefficient. Using the same system with equal phase volumes, but varying the ephedrine concentrations from 0.01 to 0.1 moles/L., also did not affect the ephedrine true partition coefficient. It should be kept in mind, however, that higher concentrations may influence the activity—and thus the apparent partition coefficient—of a drug.

Nature of the Nonaqueous Phase.—In pharmaceutical partitioning studies the nonaqueous phase is usually lipoidal in nature, although the exact lipoidal composition of the various membranes affecting drug absorption is not known. For convenience, organic solvents are often used in *in vitro* partitioning studies to simulate these lipoidal materials; this is a practice which cannot always be justified. For this reason, oil/water partition coefficients of ephedrine were determined using both organic solvents and fixed oils as the nonaqueous phase. In this study, the samples were rocked for 48 hours, and the results are shown in Table I.

As might be expected, the partition coefficients

tend to parallel the ephedrine solubility in the nonaqueous phase. It is of interest that the castor oil partition coefficient is about four times higher than the partition coefficients obtained with the other fixed oils. Probably the hydroxyl group in the fatty acid chain of castor oil permits additional association between castor oil and ephedrine and thus increases the ephedrine solubility. The free fatty acid content of the oils may also be important.

Partition Coefficients of Various Drugs.—Both apparent and true partition coefficients for a number of substances have been obtained and representative results are shown in Table II. Partition coefficients over the range 0.03 to 5000 have been determined with the rocking device. No difficulty from the technique or apparatus was experienced in obtaining these values. The magnitude and precision of the values are limited only by the sensitivity of the analytical procedures. True partition coefficient

TABLE II.—PARTITION COEFFICIENTS OF VARIOUS DRUGS AND CHEMICALS

Drug or Chemical	Cyclohexane/Water Partition Coefficient APC	TPC
Ephedrine	0.34	0.41
Chlorpromazine	300	15,000
Benzoic acid	1.05	1.18
o-Nitrobenzoic acid	0.05	0.13
o-Hydroxybenzoic acid	0.16	0.32
o-Chlorobenzoic acid	0.31	0.46
o-Methylbenzoic acid	3.54	4.45
Hexachlorophene	350[a]	Not done
Bithionol	>80,000[b]	Not done

[a] Peanut oil/water partition coefficient. [b] Chloroform/water partition coefficient. No detectable bithionol in the aqueous phase.

(TPC) values up to 15,000 have been calculated from the APC data by the method of Butler (49). It should be remembered that the APC may vary considerably with a change in the pH of the aqueous phase.

Ephedrine was used in all the studies previously discussed and was selected as a model drug primarily because it is an amine and, in most of the systems employed, has a TPC near 1.[4] Ephedrine N.F. is commercially available as the hemihydrate, and it was frequently observed that some water of hydration is stripped during dissolution of ephedrine in nonaqueous solvents. Rosin, *et al.* (51), reported a difference in the liquid petrolatum solubility of hydrous and anhydrous ephedrine. Anhydrous ephedrine was prepared in our laboratory and had the same cyclohexane/water partition coefficient as the hydrated form. This result was anticipated, despite the data of Rosin, since the partition coefficients were equilibrium values obtained in the presence of a large quantity of water.

Chlorpromazine base has a very high partition coefficient compared with ephedrine, even when the aqueous phase is unbuffered. Under optimum conditions, as much as 15,000 times more chlorpromazine can be placed in the nonaqueous phase as in the aqueous phase.

Benzoic acid dimerizes in some organic solvents (48, 52); therefore, the APC of the monomer was found by dividing the square root of the concentration in cyclohexane by the concentration in water.

[4] A partition coefficient near 1 is desirable because it enables one to assay either phase conveniently.

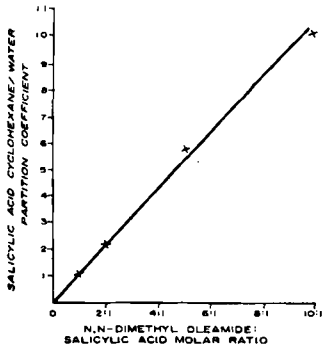


Fig. 4.—Influence of *N,N*-dimethyl oleamide on the cyclohexane/ water partition coefficient of salicylic acid. The initial aqueous phase concentration of salicylic acid was 0.01 *M* in all of the samples.

The value for the APC is 1.05. The TPC for the monomeric form of benzoic acid is 1.18 and was obtained in a similar manner, but using an acidified aqueous phase, so that no dissociation occurred.[5] The similarity of the APC and TPC indicates that very little dissociation occurs in the aqueous phase under normal conditions.

The ability to predict the effect of structure modification on the TPC of a drug could be very useful. For this reason, several *benzoic acid derivatives*, substituted in the *ortho* position, were studied to determine the effect of various substituent groups on the TPC of benzoic acid. No association of these derivatives was apparent in the cyclohexane phase at the concentrations used. The results are shown in Table II. As with benzoic acid, the TPC's were obtained by using acidified aqueous phases. The three polar substituent groups caused a shift in the cyclohexane/water partition coefficient of benzoic acid in favor of the aqueous phase (nitro > hydroxyl > chloro). In contrast, the nonpolar *o*-methyl group caused a shift in favor of the nonaqueous phase. The relative order of these results is not surprising if one considers (*a*) the electronic effects of the substituents (53), (*b*) the ability of adjacent hydroxyl and carboxyl groups to hydrogen-bond, (*c*) the over-all steric effects, and (*d*) the polarity of the two phases. Collander (38) further indicates that not only the nature of the groups but also their position and size are important.

In 1962, Higuchi and co-workers (54) reported that dimethyl amides complex with organic acids such as salicylic acid and thereby increase the solubility of the acid in organic solvents, such as cyclohexane and olive oil. In our laboratory, the effects of oil-soluble dialkyl amides on the partition coefficients of acidic drugs were studied. For example, the influence of *N,N*-dimethyl oleamide on the cyclohexane/water partition coefficient of salicylic acid was determined with the rocking device (Fig. 4). As expected, the partition coefficient increases as the amide-salicylic acid molar ratio increases. For example, a 10:1 amide:acid molar ratio shifts the salicylic acid partition coefficient between cyclohexane and water from 0.16 to 10.0. Thus, salicylic acid can be shifted from the aqueous phase to the nonaqueous phase by the use of complexing principles.

High partition coefficients would seem to be advantageous in germicides, since most microorganisms are enclosed by a lipid-like membrane. *Hexachlorophene* and *bithionol* are used for their germicidal action in many pharmaceutical products.

[5] Both the APC and TPC for benzoic acid were calculated by Eq. 2.

TABLE III.—CYCLOHEXANE/WATER PARTITION COEFFICIENTS AND CYCLOHEXANE SOLUBILITIES OF SEVERAL CHLORPROMAZINE SALTS AT 25°C.

	1	*2*	*3*	*4*	*5*
Salt	APC	pH Aqueous Phase	Concn. in Cyclohexane Phase,[a] mcg./ml.	Solubility in Cyclohexane,[b] mcg./ml.	Concn. in Cyclohexane Phase / Solubility in Cyclohexane
Hydrobromide	0.46	3.8	99	0.9	110
Hydrochloride	0.47	3.8	97	9.5	10
Hydriodide	0.47	3.8	103	2.7	40
Maleate	0.44	3.8	94	3.2	30
Succinate	1.71	4.2	205	72.0	3
Sulfanilate	0.67	3.9	129	0.7	180
Base	300	6.5	318	>5000	≪1

[a] From partitioning data; values expressed in terms of the base. The initial aqueous phase concentration of each salt was 1×10^{-3} *M* or 319 mcg./ml. in terms of the base. Therefore, if all the drug partitioned into the cyclohexane phase, the value in this column would be 319. [b] From separate solubility determinations in pure cyclohexane. The values are expressed in terms of the base.

From their low aqueous solubilities, it might be expected that the partition coefficients of these substances highly favor the nonaqueous phase. In Table II, the high apparent partition coefficients of hexachlorophene and bithionol are illustrated.

In studying *chlorpromazine* and *other phenothiazine salts* in our laboratories, it became readily apparent that some of the salts had unusual solubilities in lipids, *e.g.*, glyceryl monostearate. This lipid solubility may have a significant effect on formulation with phenothiazines and on their *in vivo* absorption. The cyclohexane/water partition coefficients for these phenothiazine salts were determined and representative results are shown in column *1* of Table III. The ratios in column *5* show that the chlorpromazine present in the cyclohexane phase (column *3*) greatly exceeded the solubilities of the salts (column *4*) in cyclohexane. This indicates that in each case virtually all of the chlorpromazine in the cyclohexane phase was the free base, not the salt.

From the data in column *1*, it would appear that the succinate and sulfanilate salts have higher APC's than the other salts. Actually, the slight differences in aqueous phase pH (column *2*) are enough to produce the observed differences in partition coefficient, again indicating that the base, (not the salt) is partitioning. This can be verified mathematically by using the observed pH's and the TPC of chlorpromazine.

Because some chlorpromazine salts are lipid soluble, cyclohexane may not properly simulate an *in vivo* absorption barrier. Studies are currently underway in our laboratory to investigate the influence of salt forms on the partitioning of phenothiazine type drugs, to investigate the influence of the lipid phase composition on the solubility and partition coefficient of the salts, and to determine the significance of these variables as they relate to the absorption properties of the drugs.

SUMMARY

1. A rocking apparatus, holding up to 36 samples, has been developed for studying drug partitioning. Illustrations of the application of this apparatus are given with data. The paper is introductory and is to be followed by more detailed studies of some of the areas of application described.

2. The rocking method, using an ephedrine-Skellysolve B-water system, was as reliable as the separator shake-out method. The rocking method described in this study does not tend to produce emulsions.

3. Phase volume and drug concentration did not influence the partition coefficient as long as these parameters were kept within reasonable limits, and association or dissociation of the drug was considered.

4. The influence of the nonaqueous phase on the partition coefficient of ephedrine was shown. With ephedrine, castor oil/water produced the highest observed partition coefficient and mineral oil/water the lowest.

5. The following cyclohexane/water TPC's were reported: ephedrine (0.41), chlorpromazine (15,000), benzoic acid (1.18), *o*-nitrobenzoic acid (0.13), *o*-hydroxybenzoic acid (salicylic acid) (0.32), *o*-chlorobenzoic acid (0.46), and *o*-methylbenzoic acid (4.45).

6. The cyclohexane/water partition coefficients of several salts of chlorpromazine varied fourfold. This variation was due to slight differences in the pH of the aqueous phase. It was shown that in the cyclohexane/water system, chlorpromazine base (but not its salts) partitions into the cyclohexane phase. Since some chlorpromazine salts are known to have appreciable solubility in selected lipids, cyclohexane may not be a suitable simulated *in vivo* absorption barrier for these salts.

7. Pharmaceutical applications of drug partitioning were discussed, and the advantages of more extensive studies of partition coefficients of new and old drugs were pointed out. In addition to the usual analytical and absorption applications, these were (*a*) deliberately attempting to alter the partition coefficients of drugs in a predictable manner, (*b*) improving emulsion taste, (*c*) improving drug chemical stability, (*d*) influencing drug release from emulsions, ointments, suppositories or other dosage forms, and (*e*) studying the influence of aqueous phase components, such as electrolytes, surfactants and macromolecules, on the partitioning of drugs, antioxidants, flavors, sweetening agents, and preservatives.

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and for an acid,

$$R = \frac{C_{gut}}{C_{plasma}} = \frac{1 + 10^{(pH_{gut} - pKa)}}{1 + 10^{(pH_{plasma} - pKa)}} \quad \text{(Eq. 2)}$$

In situ experiments (1–5) have shown that the distribution of some drugs approximates these equations. However, it is possible that an ionized

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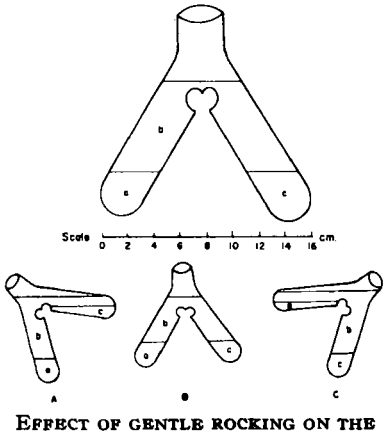
drug moiety might be sufficiently lipid-soluble to penetrate the gastrointestinal barrier, or that an unionized drug moiety might not be sufficiently lipid-soluble to penetrate the gastrointestinal barrier, or that an active transport process is involved. In these cases the above equations may be inadequate to predict gastrointestinal absorption.

It is suggested that the model depicted in Fig. 1 might be employed to simulate some factors involved in the absorption process—namely, the partitioning of drug between the gastrointestinal fluid and lipoidal phase and between lipoidal phase and plasma. Aqueous buffer systems of pH 2.0, 3.0, 5.0, and 7.4, cyclohexane, and an aqueous pH 7.4 buffer may be utilized to represent the various acidities of the gastrointestinal tract, the lipoidal barrier, and the acidity of the plasma, respectively. By use of this *in vitro* model the "absorption" process, *i.e.*, the passage of drug from one aqueous phase to the other, can be studied kinetically and as an equilibrium process.

EXPERIMENTAL

Apparatus and Reagents.—All chemicals were reagent grade unless specified otherwise. Salicylic acid, barbital N.F., antipyrine N.F., aminopyrine N.F.X, tetracycline HCl (Lederle Laboratories), potassium chloride, monobasic sodium phosphate, potassium acid phthalate, boric acid, sodium hydroxide, hydrochloric acid, cyclohexane, *n*-octyl alcohol, Leeds and Northrup model 7401 pH meter, and a Beckman model DB spectrophotometer were employed.

To prevent the aqueous phases from mixing, a glass tube and rocking apparatus were designed and constructed. (See Figs. 1 and 2.) The gentle rocking of the tubes accelerated drug transfer by causing the interfaces to expand and contract as shown in Fig. 1. The phases were sampled as necessary by inserting a pipet through the neck of the tube. The rocking apparatus[1] is of aluminum construction and possesses two pivot bars mounted on ball bearings. The pivot bars are tilted in one direction and then the other through attachment to a constant speed electric motor of 2 r.p.m., as pictured in Fig. 2. The degrees of tilt are controlled by a variable adjustment connecting-link attached to the motor. Spring clips are fastened to the framework of the pivot bars to accommodate the special glass tubes. The rocking apparatus is somewhat similar in design and application to one used in two-phase partition coefficient studies (6). The special tubes for these studies bear some similarity to tubes used for other purposes and previously reported (7).



EFFECT OF GENTLE ROCKING ON THE INTERFACES

Fig. 1.—Model used to simulate some factors involved in the absorption process. Phase *a* represents the various acidities of the gastrointestinal tract, phase *b* the lipoidal barrier, and phase *c* the pH of the plasma.

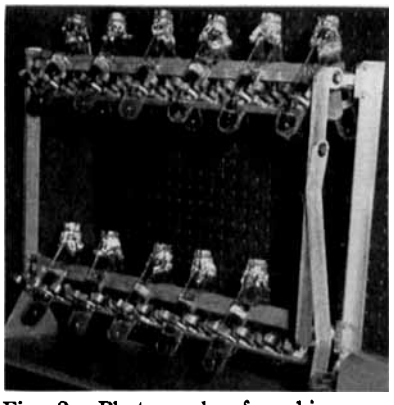


Fig. 2.—Photograph of rocking apparatus and glass tubes.

Procedure.—Aqueous buffered solutions of drug (15.0 ml.) were placed in one arm of the tube, 15.0 ml. of a pH 7.4 phosphate buffer solution was placed in the remaining arm, and 80.0 ml. of cyclohexane added. The system was gently rocked and the drug concentration determined at various time intervals. Salicylic acid, antipyrine, aminopyrine, and tetracycline concentrations were determined spectrophotometrically. Barbital solutions were adjusted to pH 9.4 with a borate buffer, then determined spectrophotometrically.

The pH 2.0 buffer was prepared by dissolving 3.0 Gm. of KCl in 1 L. of distilled water and adjusting the pH with concentrated HCl. The pH 3.0 buffer was prepared by dissolving 8.0 Gm. of potassium acid phthalate in 1 L. of distilled water and adjusting the pH with concentrated HCl. The pH 5.0 and pH 7.4 buffers were prepared by dissolving 5.7 Gm. of monobasic sodium phosphate in 1 L. of distilled water and adjusting the pH with 5 *N* NaOH. The pH 9.4 borate buffer was prepared by dissolving 6.0 Gm. of boric acid and 7.5 Gm. of KCl in 1 L. of distilled water and adjusting the pH with 5 *N* NaOH. All solutions used in the tetracycline experiments were prepared with deionized distilled water having a specific conductance of 2.5×10^{-6} ohm^{-1} cm.$^{-1}$ or less.

RESULTS AND DISCUSSION

Results are summarized in Table I. The initial disappearance of drug from phase *a* was approxi-

[1] The authors appreciate the assistance of Mr. Edward Bates and Mr. Allen Cook, R & D Mechanical Shop, and Mr. Andrew Airey, R & D Glass Shop, Smith Kline & French Laboratories, for assistance in constructing the equipment shown in Fig. 2.

TABLE I.—RESULTS OBTAINED FOR THE TRANSFER OF VARIOUS DRUGS IN THE *In Vitro* ABSORPTION MODEL

Drug	pH Phase *a* When Phase *c* is 7.4	$\frac{U}{U+I}k$, (hr.$^{-1}$)	k, (hr.$^{-1}$)	R, Theoret.	R, Exptl.
Salicylic acid	2.0	0.13	0.14	0	0
	3.0	0.12	0.24	0	0
	5.0	0.0046	0.46	0	0
	7.4	[a]	[a]	1.0	[a]
Barbital	2.0	0.0022	0.0022	0.72	0.74
	5.2	0.0022	0.0022	0.72	0.74
	7.4	0.0016	0.0021	1.0	1.2
Antipyrine	2.0	0.0059	0.0074	1.3	1.4
	5.0	0.0084	0.0084	1.0	1.1
	7.4	0.0090	0.0090	1.0	1.0
Aminopyrine	2.0	[a]	[a]	1000	[a]
	5.1	0.14	0.25	1.8	1.8
	7.4	0.26	0.26	1.0	1.0
Tetracycline HCl	2.0	[a]	[a]	...	[a]
	5.1	[a]	[a]	...	[a]
	7.4	[a]	[a]	...	[a]

[a] No drug transfer evident in 80 hours.

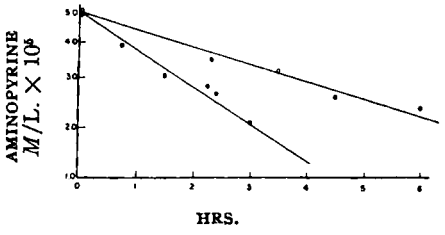


Fig. 3.—The effect of phase *a* pH on the disappearance of aminopyrine from an aqueous phase, through a cyclohexane phase, and into an aqueous pH 7.4 phase. Key: O, pH 5.1 phase *a*; ●, pH 7.4 phase *a*.

mately first order. Figure 3 illustrates that a change of pH in phase *a* affects the slope depicting disappearance of drug. This change in slope may be explained by the following equations. When the pH of phase *a* is such that the drug is completely unionized, transfer is represented by

$$-\frac{dC}{dt} = kC \qquad \text{(Eq. 3)}$$

and

$$\ln C = -kt + \ln C_0 \qquad \text{(Eq. 4)}$$

where C is drug concentration, t is time, and k is a constant. However, when the pH is such that the drug is partly ionized, transfer is represented by

$$-\frac{dC}{dt} = \frac{U}{U+I}kC \qquad \text{(Eq. 5)}$$

and

$$\ln C = -\frac{U}{U+I}kt + \ln C_0 \qquad \text{(Eq. 6)}$$

where U and I are unionized and ionized drug, and where $U/(U+I)$ is the fraction of drug unionized. According to these equations the "true" rate constant, k, is independent of pH. However, the slopes obtained by plotting $\ln C$ *versus* t yield apparent rate constants, *i.e.*, $[U/(U+I)]k$, which are a function of pH.

From the data in Table I, k is observed to be a constant at the different pH's at which barbital and aminopyrine were studied. In the case of antipyrine, some variability was observed for k. For salicylic acid k varied two to threefold. This greater variability in the value of k for salicylic acid is perhaps to be expected, since for a given concentration of this drug the concentrations of U and I vary approximately ninetyfold from pH 2 to 5. In this wide range of concentrations of U, the substantial change in I may influence the transfer rate of U between the immiscible phases.

In general, the results obtained are consistent with the hypothesis that for these drugs transfer is dependent upon the migration of the unionized drug moiety. The equilibrium distribution of drug between the two aqueous phases as indicated by R in Table I obeyed either Eqs. 1 or 2, depending on whether the drug was a base or an acid.

Table II lists the pKa and the fraction of drug unionized at various acidities for each drug studied.

Salicylic Acid.—Figure 4 illustrates the transfer of salicylic acid from a pH 2.0 aqueous phase to a pH 7.4 aqueous phase. In this case, as in most cases, the concentrations of drug in the cyclohexane phase were so low that they could not be accurately determined. Figure 5 is a first-order plot of the disappearance of salicylic acid from the pH 2.0

TABLE II.—FRACTION OF DRUG UNIONIZED AT VARIOUS ACIDITIES

Drug	pKa	pH	$\frac{U}{U+I}$
Salicylic acid	3.0	2.0	0.91
		3.0	0.50
		5.0	0.01
		7.4	[a]
Barbital	7.9	2.0	1.0
		5.2	1.0
		7.4	0.76
Antipyrine	1.4	2.0	0.80
		5.0	1.0
		7.4	1.0
Aminopyrine	5.0	2.0	0.00099
		5.1	0.56
		7.4	1.0
Tetracycline HCl	3.3,7.7,9.7	2.0	[a]
		5.1	[a]
		7.4	[a]

[a] Values less than 0.0001.

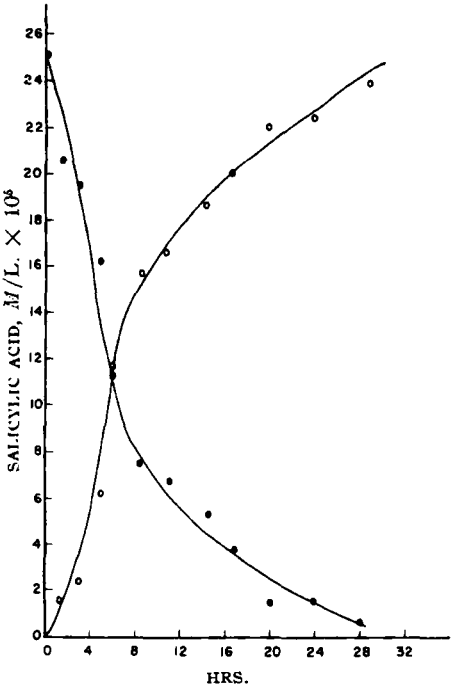


Fig. 4.—The transfer of salicylic acid from an aqueous pH 2.0 phase through a cyclohexane barrier to an aqueous pH 7.4 phase. The concentrations of salicylic acid in cyclohexane were low and could not be determined accurately (*e.g.*, less than 4×10^{-6} *M*/L.). Key; ●, pH 2.0 phase *a*; O, pH 7.4 phase *c*.

aqueous phase. It is evident from these data that at the pH of the stomach there is excellent drug transfer. As the pH of phase *a* rises and simulates that of the intestines, drug transfer falls off markedly. This is consistent with the statement that the absorption of weak acids occurs primarily from the stomach (2). It appears from Table I that at the pH of 7.4 in phase *a*, Eq. 2 is not obeyed. However, as shown in Table II, less than 0.01% of salicylic acid is unionized at pH 7.4. Since such a minimal amount of the migrating species is present, failure to detect drug transfer after 80 hours is perhaps a reasonable result.

Barbital.—The rates of barbital transfer were much slower than those of salicylic acid. It is also evident from the *R* values that salicylic acid should be absorbed more efficiently from the stomach than barbital. Barbital, which is largely undissociated in the pH range 2 to 7, should be absorbed throughout the entire gastrointestinal tract; whereas the rate of salicylic acid absorption by a passive mechanism would be expected to fall off markedly once the drug has reached regions of higher pH in the intestine.

Antipyrine.—The results illustrate that in the case of weak bases the higher the pH of phase *a* the better the drug transfer to phase *c*. From the data it would seem that antipyrine should be absorbed to some extent throughout the entire gastrointestinal tract. It is also evident that drug transfer should be identical when phase *a* is either pH 5.0 or 7.4. As shown in Table II, at these pH values antipyrine is essentially 100% unionized.

Aminopyrine.—Aminopyrine showed no transfer when the acidity of phase *a* simulated that of the stomach. At this pH aminopyrine is essentially completely ionized. At higher pH values the transfer increased and obeyed Eq. 1.

Tetracycline.—In the experiments involving tetracycline no transfer was evident when cyclohexane was employed as the lipoidal barrier. Pindell, *et al.* (8), have shown that tetracycline is absorbed from the stomach, duodenum, and ileum of the dog and have suggested that the absorption can be explained by the pH-partition hypothesis. It is interesting to note from their work that only 3% of an administered dose of tetracycline was absorbed in 1.5 hours. From the work of Stephens, *et al.* (9), it appears that at the pH of the stomach tetracycline would be cationic—as the pH rises the molecule would become a zwitterion—and then, at high pH values—an anion. In light of these findings it was then thought that perhaps cyclohexane was too nonpolar to simulate the intestinal barrier. In the case of tetracycline, for example, a more polar lipoidal barrier might allow transfer of a lipid-soluble charged species. It was found that *n*-octyl alcohol did not allow tetracycline transfer at pH 2.0 but did allow tetracycline transfer when the pH of phase *a* was 5.2 or 7.4. This transfer was also evident at these pH values in mixtures of *n*-octyl alcohol and cyclohexane up to a mixture of 80% cyclohexane. These studies, however, were complicated because buffer electrolytes also were able to pass very slowly through the *n*-octyl alcohol barrier, and to a lesser extent, through *n*-octyl alcohol–cyclohexane mixtures. It is of interest that a drug such as tetracycline which is ionized throughout the pH range of these studies may be soluble in lipid solvents. This suggests that some ionized drugs may probably show sufficient lipid solubility to be absorbed passively *in vivo* or that active transport processes may be involved. This idea and others will be subjects for some of our future studies of drug transfer and absorption.

The results of this investigation suggest that this apparatus and experimental method may have application in studying effects of additives on the transfer rates of absorptive processes. Also the methodology may be useful for studying drug binding. The substitution of a liquid membrane for a solid one in drug binding or other dialysis studies may overcome some of the experimental problems

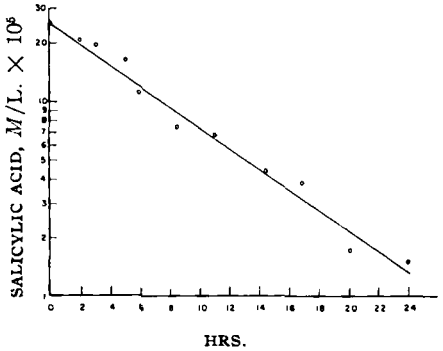


Fig. 5.—First-order plot of the disappearance of salicylic acid from an aqueous pH 2.0 phase, through a cyclohexane phase, and into an aqueous pH 7.4 phase.

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Distribution of Tritiated Derivatives of Fluorene in the Rat

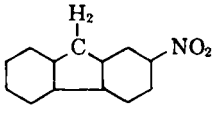
By F. E. RAY and O. O. WEJEBE

By the study of tritiated derivatives we have found that the liver carcinogen, 2-acetylaminofluorene, is concentrated to a greater extent in the liver of the rat than 2-acetylaminofluorenone, which produces only an occasional liver tumor. 2-Nitrofluorene, which also produces few, if any, liver tumors, gives a concentration that is intermediate. 2-Nitrofluorenone, which has not been tested for carcinogenicity, gives a concentration in the liver similar to 2-nitrofluorene. The highest concentrations in the liver result when the compounds are given by intraperitoneal injection.

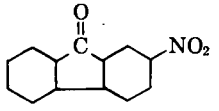
IN 1940, THE U. S. Department of Agriculture proposed the use of 2-acetylaminofluorene (AAF) as an insecticide; but before releasing it for field tests, it was sent to the Regional Laboratory in California for the determination of toxicity. After an extensive series of tests, Wilson, De Eds, and Cox (1) reported it to be carcinogenic to rats and mice. While this ended its career as an insecticide, it became of fundamental importance in the study of the etiology of cancer.

Whereas the carcinogenic hydrocarbons produce tumors almost exclusively at the site of application, acetylaminofluorene produces a wide variety of tumors distant from the site of application. The principal organ of attack, however, is the liver. It has been shown to be carcinogenic to mice, rats, rabbits, hamsters, dogs, and fowl. It is indeed fortunate that this compound was thoroughly tested *before* being released for general use.

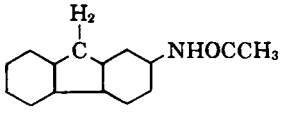
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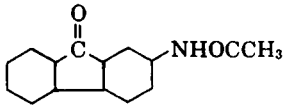
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(2-NF)
I



2-Nitrofluorenone
(2-NFone)
II



2-Acetylaminofluorene
(AAF)
III



2-Acetylaminofluorenone
(AAFone)
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Recent work by Ray, Cromer, Aycock, and Pitzer (2) has shown that it is practical to pre-

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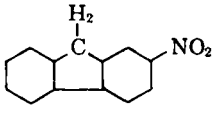
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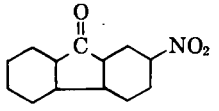
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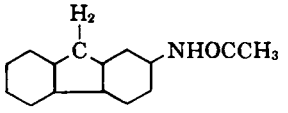
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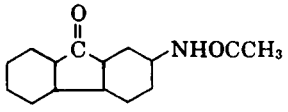
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pare radioactive 2-nitrofluorene and 2,7-dinitrofluorene by exposing the compounds to the action of tritium(H_2^3) for a number of days, and that isotopically pure amines are conveniently prepared from the corresponding nitro compounds. The reduction, catalytically with hydrogen and platinum, acetylation, and subsequent purification readily eliminate radioactive impurities produced during the irradiation with tritium. This simplifies, to a considerable extent, radiotracer studies with these compounds and their derivatives. It seemed worthwhile, therefore, to use the tritiation technique to compare 2-nitrofluorene (2-NF) (I) and 2-nitrofluorenone (2-NFone) (II). This was followed by a comparison of 2-acetylaminofluorene (AAF) (III) and 2-acetylaminofluorenone (AAFone) (IV).

EXPERIMENTAL

The nitro compounds, I and II, were tritiated by exposing 1 Gm. of each to 15 curies of tritium for 2 weeks at 27° and 666 mm. in 45 ml. of methyl chloride and 15 ml. of methanol. In two separate experiments the specific activity of 2-nitrofluorene after recrystallization from glacial acetic acid to constant radio-solubility was 173 μc./mg. and 194 μc./mg., respectively: 2-nitrofluorenone gave 74 μc./mg. and 84 μc./mg. It will be noted (formulas I and II) that the fluorenone has seven-ninths (78%) as many hydrogens to be replaced by tritium; however, the two aliphatic hydrogens replaced by oxygen are considerably more active than the remaining seven aromatic hydrogens and, therefore, more easily tritiated, giving a ratio of about one to four. The nitro compounds were administered as a basis of comparison with the amines.

Emulsions were made that contained equimolar amounts of the substances—0.24 mc. per cubic centimeter for 2-nitrofluorene (m.p. 156°) and 0.09 mc. per cubic centimeter for 2-nitrofluorenone (m.p. 218°). One milliliter dose was administered by stomach tube or by intraperitoneal injection. On reduction with platinum catalyst and hydrogen, followed by acetylation and purification to constant radio-solubility, yielded 2-acetylaminofluorene-H^3 (m.p. 192°) of specific activity, 26.2 μc./mg. Similarly, 2-acetylaminofluorenone-H^3 (m.p. 225°) gave 2.69 μc./mg., a ratio of about one to ten. The lower activity resulted because base acetylaminofluorenone was added in the purification process.

TABLE I.—RADIOACTIVITY PRESENT AT THE END OF 6 HOURS[a] (2-NITROFLUORENONE AND 2-NITROFLUORENE)

Tissue	2-Nitrofluorenone, %	2-Nitrofluorene, %
Liver	1.14	1.19
Kidney	0.21	0.18
Urine	4.98	2.09
Blood	0.61	1.05
Stomach	2.76	2.29
Stom. cont.	16.36	17.98
Spleen	0.03	0.04

[a] Oral administration.

Procedure.—Young adult Wistar rats weighing 170 Gm. each were used. Equimolar amounts of the two compounds (0.045 *M*) were heated with 1 ml. of 95% ethyl alcohol and made up to 10 ml. and homogenized with 1% aqueous methylcellulose solution. One milliliter of each was then fed *via* stomach tube. Another milliliter of each was diluted to 500 ml. with 95% ethyl alcohol to be used as a standard. The animals were housed in metabolism cages permitting the separate collection of urine and feces. At the end of the designated time, they were killed with ether and their organs removed. The tissues were treated with 10x portions of 1% sodium hydroxide, allowed to stand overnight in the cold room, homogenized, and made up to volume. Aliquots were then counted three or more times to 100,000 or 30 minutes in a dioxane-naphthalene-DPO-POPOP scintillator. Samples of the standard were included in every count. Corrections were made for background and quenching, and the results expressed as per cent of administered dose (68×10^6 to 155×10^6 counts/minute). The efficiency of the Tri-Carb scintillation counter used was 33.21 to 34% (737,000–755,000 counts/minute/microcurie).

RESULTS AND DISCUSSION

A comparison of the amount of radioactive material found at 6 hours after oral administration in the liver shows no substantial difference between the nitrofluorene and nitrofluorenone compounds (Table I, 1.14 and 1.19%). Little difference is found in the spleen, kidneys, and in the stomach and contents. The percentage of fluorenone derivative excreted in the urine is over twice that of the fluorene, indicating more rapid elimination of the former. This is in harmony with the lesser amount of fluorenone in the blood.

Table II gives the results of the 24-hour experiment in which the same compounds were administered intraperitoneally. Here again there is no real difference in the localization of the two compounds in the liver (7.04 and 6.72). Again, somewhat more of the fluorenone derivative seems to be excreted in the urine (13.82 to 9.80); this may thus indicate a real difference. Now the small difference in the percentages in stomach tissue has become substantial in favor of 2-nitrofluorene. While the amount of the two compounds in the blood is almost equal, a real difference seems to exist in favor of the fluorene derivative in the spleen. The somewhat slower elimination of 2-nitrofluorene and consequent greater concentration in blood, spleen, and stomach tissue may indicate that some hydrogen

TABLE II.—RADIOACTIVITY PRESENT AT THE END OF 24 HOURS[a] (2-NITROFLUORENONE AND 2-NITROFLUORENE)

Tissue	2-NFone, %	2-NF, %
Liver	7.04	6.72
Urine	13.82	9.80
Stomach	0.32	1.17
Stom. cont.	1.60	0.60
Intestine	16.47	18.07
Kidney	0.68	1.00
Spleen	0.50	1.70
Blood	1.36	1.53

[a] Intraperitoneal injection.

TABLE III.—2-ACETYLAMINOFLUORENE AND 2-ACETYLAMINOFLUORENONE[a]

	Oral 12 Hr.		Oral 24 Hr.		i.p. 24 Hr.	
	AAFone	AAF	AAFone	AAF	AAFone	AAF
Liver	1.63	1.80	0.68	1.16	3.75	13.46
Urine	16.61	13.06	15.33	19.63	22.43	24.91
Stomach	0.31	1.31	1.66	0.09	0.15	0.45
Stom. cont.	16.03	18.74	19.18	0.05	5.86	3.97
Intestine	36.86	48.64	16.41	57.67	20.81	25.85
Kidney	0.24	0.22	0.15	0.10	0.91	1.72
Spleen	0.03	0.02	0.03	0.001	0.08	0.43
Blood	0.39	0.59	0.57	1.27	0.46	0.51
Body fluids	0.88	0.76	0.32	0.15	4.30	1.83
Carcass	7.98	8.94	5.29	6.62	15.16	15.71

[a] Values are per cent of administered dose.

binding of fluorene occurs at the 9-position (=CH_2 group), although the effect may be purely a negative electrostatic influence of the C:O group. Later in this paper we will attempt to decide which effect is dominant.

The data for AAF and AAFone are given in Table III. At 12 hours following oral administration there seems to be only a slight difference in the percentages in the liver. Twenty-four hours after oral administration, however, there is almost twice as much AAF as AAFone in the liver (1.16/0.68). This is more dramatically shown in the 24-hour experiment after intraperitoneal injection (6) of the two compounds. Here the ratio in the liver is 13.46/3.75.

We find that AAF is eliminated to a somewhat greater extent in the urine than is AAFone, but this difference is not great enough to be significant. There are, however, much less of the nitro compounds eliminated in the 24-hour i.p. group, which is directly comparable to the 24-hour i.p.-AAF-AAFone group. It has been shown by Morris, Dubnik, and Johnson (4) and by Miller, *et al.* (5), that 2-nitrofluorene is carcinogenic but much less so than AAF, both in the number of tumors produced and the per cent of rats developing tumors. 2-Nitrofluorene, like 2-aminofluorenone, produces few, if any, liver tumors. It seems, therefore, that liver tumorigenesis is favored by the simultaneous presence of the amino and methylene groups. On this basis we predict that 2-nitrofluorenone would be still less carcinogenic than 2-nitrofluorene.

A comparison of the amounts of 2-nitrofluorene and 2-acetylaminofluorene excreted in the urine at the end of 24 hours after i.p. injection (Tables II and III) shows that 25% of the AAF is eliminated *versus* 8% of NF. Using N^{15}-labeled 2-NF (7) and the oral route of administration, Dyer (8) compared 2-NF with AAF and found that a total of 68% of NF material was excreted in urine *versus* 81% of AAF. We thus find the same order of elimination. This indicates that the amine is more readily metabolized than the nitro compound and suggests that reduction of the nitro group is a prerequisite to carcinogenesis (9).

Although a minimum concentration in a tissue such as liver seems necessary for activity, this is not, of course, the sole requisite. This is illustrated by the fact that the 24-hour i.p. animals given the nitro compounds showed a considerable concentration in the liver. This was in excess of the 24-hour i.p. animals with AAFone, but only about half that of AAF itself (Table III). Whether this indicates that the nitro compounds *would* produce liver tumors if administered *via* the intraperitoneal route remains to be demonstrated. That the i.p. route is effective in carcinogenesis of related compounds has been demonstrated by Morris, Wagner, Ray, Stewart, and Snell (6).

CONCLUSION

These experiments offer evidence in favor of our thesis that a carcinogen that attacks a particular organ has a certain affinity for that organ compared to a carcinogen that seldom if ever produces tumors in that organ. We find that 2-acetylaminofluorene has a greater affinity for the liver than 2-acetylaminofluorenone or 2-nitrofluorene, neither of which produces more than an occasional tumor in the liver.

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Inactivation of Chloramphenicol by Chloramphenicol-Resistant Bacteria

By SADAO MIYAMURA

Virtually all (94.5 per cent of 110) chloramphenicol-resistant strains of *Shigella, Escherichia,* and *Staphylococcus* isolated from clinical cases caused significant inactivation of the antibiotic, but none of the 29 resistant pseudomonads did so. None of the 235 clinically isolated sensitive strains, representing five genera, inactivated the drug. Resistant organisms derived *in vitro* from initially sensitive ones by repeated subculture in increasing concentrations of chloramphenicol had only limited ability to inactivate the antibiotic, but those produced *via* transformation inactivated it almost completely. Such transferred resistance was lost following treatment with acriflavine. Simultaneously, ability to inactivate chloramphenicol was lost.

INACTIVATION OF penicillin by the bacterial enzyme penicillinase has been studied by several workers, but similar observations on other antibiotics are limited. Microbial degradation of chloramphenicol was first described in 1949 by Smith and Worrel (1). Later a direct correlation between chloramphenicol resistance and ability of *Escherichia coli* to destroy the antibiotic was reported (2). However, the degradative activity of the organism did not seem to account for the full degree of resistance that was developed. Subsequently, Chabbert and Debruge (3) noted that resistant strains of *Staphylococcus*, cultured in conventional media, produce a substance that is diffusible in agar and that inhibits the action of chloramphenicol. More recently, Miyamura (4) showed that resistant dysentery bacilli also have the ability to inactivate chloramphenicol, and he described some of the properties of the inactivating substance.

The present paper demonstrates a relationship between resistance to chloramphenicol and ability to inactivate the compound in *Shigella, Salmonella, Escherichia, Pseudomonas,* and *Staphylococcus* isolated from clinical cases in Niigata Prefecture, Japan.

EXPERIMENTAL

Strains Tested.—The isolates tested included 105 strains of several types of *Shigella* (*Sh. flexneri* 1*b*, 5; 2*a*, 21; 2*b*, 10; 3, 45; 6, 8; var. X, 4; var. Y, 1; *Sh. sonnei*, 11), six strains of *Salmonella* (*S. typhi*, 4; *S. paratyphi* B, 2), 107 strains of *E. coli*, 31 strains of *Ps. aeruginosa*, and 125 strains of *Staph. aureus*. Each strain was isolated from a different patient between 1958 and 1961. In addition, *E. coli*, strains B and K-12, were used as representative sensitive parent strains from which resistant strains were developed in the laboratory.

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Test for Chloramphenicol Sensitivity.—The chloramphenicol sensitivity of each strain was determined by the plate-dilution method, using 10 ml. of heart infusion agar per plate. The final concentrations of the antibiotic ranged from 100 to 0.195 mcg./ml. in twofold dilutions. Each plate was inoculated aseptically by streaking with an 18-hour broth culture of the appropriate organism, the seed culture having been prepared previously by inoculating 10 ml. of broth with 0.1 ml. of a 20-hour culture. Plates were observed after incubation for 48 hours at 37°. The minimal inhibitory concentration was taken as the lowest concentration (mcg./ml.) of chloramphenicol required to inhibit visible growth completely.

Organisms were designated sensitive if there was no visible growth on plates containing 50 mcg. or less chloramphenicol per milliliter and were considered resistant if there was no inhibition at a concentration of 50 mcg. or more per milliliter.

Determination of Ability to Inactivate Chloramphenicol.—Organisms to be tested for ability to inactivate the antibiotic were inoculated into 10 ml. of heart infusion broth and incubated at 37° for 18 hours. Then to each culture was added an equal volume of 0.01 *M* phosphate buffer solution (pH 7.0) containing 100 mcg. of chloramphenicol per milliliter, and the tubes were placed in a constant temperature water bath at 43°, at which bacterial inactivation of the drug was maximum. After 20 hours of incubation, the tubes were heated to 60° for 30 minutes, the majority of cells were spun down in a centrifuge (5000 r.p.m. for 20 minutes), and the potency of the residual unreduced chloramphenicol remaining in the supernatants was determined by cylinder-plate assays, using *Bacillus subtilis* PCI 219 as the test organism.

Development of In Vitro Resistance to Chloramphenicol.—Chloramphenicol-resistant strains of bacteria were obtained by two methods.

Increasing Concentration Method.—Bacterial strains highly resistant to the antibiotic were produced from initially sensitive cultures by successively subculturing in progressively increasing concentrations of the drug, as reported by Coffey, *et al.* (5), Cavalli, *et al.* (6), Merkel and Steers (2), and others. In the present study, *Sh. flexneri* 2*a* strain 3195, *Sh. flexneri* 3*a* strain 11, and *E. coli* strains 60 and K-12 were rendered resistant by this technique, subcultures being made at intervals of 2 to 3 days over a period of 3 to 5 months.

Mixed Culture Method.—Drug resistance is easily transferred from *E. coli* to *Shigella* and vice versa following mixed cultivation of an appropriate donor and an appropriate recipient (7, 8). In this study two resistant donor strains and six sensitive recipient strains were used as follows:

Resistant Donor	Sensitive Recipients
E. coli strain H (MIC = 400 mcg./ml.)	(*a*) *Sh. flexneri* 2*a* strain 3195
	(*b*) *S. typhi* strain 375
	(*c*) *S. paratyphi* B strain 63
Sh. flexneri 3*a* strain 1871 (MIC = 200 mcg./ml.)	(*a*) *E. coli* strain 60
	(*b*) *E. coli* K-12
	(*c*) *E. coli* B

Each donor strain was naturally resistant, *i.e.*, it was isolated directly from patients.

One-tenth milliliter from a 20-hour broth culture of the donor and a like amount from a similar culture of the recipient organism were inoculated into a tube containing 5 ml. of heart infusion broth. The mixture was incubated (37°) for 20 hours. Then a loopful of the culture was streaked on a Drigalski agar plate containing 20 mcg. of chloramphenicol per milliliter for selection of resistant organisms. Colonies characteristic of the recipient bacteria were isolated, and the chloramphenicol resistance of the isolates was determined.

Reversal of Acquired Resistance.—Since it has been reported that acriflavine effectively eliminates F factor from *E. coli* K-12 (9) and is applied for elimination of drug-resistance factor in *Escherichia* and *Shigella* (10), the agent was tested.

An overnight broth culture of chloramphenicol-resistant organisms was inoculated into a broth containing a sublethal concentration of acriflavine (5–40 mcg./ml.) and incubated at 37°. After 20 hours, a loopful of the culture was streaked on a nutrient agar plate and then chloramphenicol-sensitive colonies were selected by means of the replica plating method (11).

RESULTS AND DISCUSSION

Naturally Resistant Strains.—The relation between susceptibility and resistance to chloramphenicol of all the strains isolated and their ability to inactivate the drug are shown in Table I. Marked inactivation was observed with virtually all resistant strains of *Shigella*, *Escherichia*, and *Staphylococcus*. Of the 110 strains of these genera studied, 104 (or 94.5%) caused more than 50% reduction of the inhibitory activity of the drug during a 20-hour period of contact. Only members of the genus *Pseudomonas* failed to exhibit a correlation between resistance and ability to impair the antibacterial activity of the antibiotic substantially. None of the 29 resistant strains of *Pseudomonas* tested caused so much as 50% loss of antibiotic activity. Among the 235 sensitive strains (representing five genera and including two pseudomonads) that were studied, only two strains of *Escherichia* (0.85% of the total of all sensitive strains) caused more than 50% reduction in the inhibitory activity of chloramphenicol. Therefore, exempting only the 29 resistant pseudomonads (7.75% of the total 374 strains tested), there appears to be a clear correlation in the organisms studied between resistance to chloramphenicol and ability to inactivate it.

Strains with Resistance Acquired In Vitro.—Resistant organisms obtained *in vitro* by isolation from cultures serially transferred in broth with increasing concentrations of chloramphenicol were tested for their ability to inactivate the drug. The data in Table II show that the ability of organisms to impair the antibacterial activity of chloramphenicol did, indeed, increase with acquisition of resistance as reported by others (2, 5). However, the capacity of such bacteria to reduce the potency of the drug was not so great as that of naturally resistant strains of the same species (compare with Table I).

In other experiments, organisms with "transferred resistance," *i.e.*, rendered resistant *via* the mixed culture method described under *Experimental*, were examined for their ability to reduce the potency of chloramphenicol. The data are shown in Table III. It is noteworthy that in all instances chloramphenicol solutions exposed to the transformed organisms, *i.e.*, those carrying "transferred resistance," lost more than 90% of their antibacterial potency. Comparison of relevant data in Tables II and III suggests that, with respect to the basis for resistance, there is a significant difference between organisms rendered resistant *via* the serial transfer method and those acquiring resistance *via* transformation (mixed culture method). Three examples can be noted. *Sh. flexneri* 2*a*-3195 with initial MIC of 0.39 mcg./ml. had no destructive effect on chloramphenicol, but when rendered resistant it was capable of inactivating the drug. However, the degree to which it did so depended on the method used to induce resistance. Organisms rendered resistant *via* transformation (Table III) did not acquire a degree of resistance so high as those

TABLE I.—RELATION BETWEEN SUSCEPTIBILITY AND RESISTANCE TO CHLORAMPHENICOL AND ABILITY OF BACTERIA ISOLATED FROM PATIENTS TO INACTIVATE THE ANTIBIOTIC

Organism	Susceptibility[a]		Number of Strains — Chloramphenicol Inactivation,[b] % 0–25 %	26–50%	51–75%	76–100%
Shigella	Resistant	45	0	3	10	32
	Sensitive	60	57	3	0	0
Salmonella	Sensitive	6	6	0	0	0
Escherichia	Resistant	44	0	2	8	34
	Sensitive	63	39	22	2	0
Pseudomonas	Resistant	29	23	6	0	0
	Sensitive	2	2	0	0	0
Staphylococcus	Resistant	21	0	1	6	14
	Sensitive	104	93	11	0	0

[a] Resistant: no inhibition of growth at chloramphenicol concentration of 50 mcg. or more per milliliter. Sensitive: inhibition of growth at 50 mcg. or less per milliliter. [b] Per cent reduction of chloramphenicol in broth culture of the microorganism. Initial concentration of antibiotic = 50 mcg./ml.

developing resistance during serial transfer (Table II), but they developed a far greater capacity for inactivating the drug. An analogous phenomenon can be seen in *E. coli* 60. *E. coli* K-12 developed the same degree of resistance regardless of the method used to induce it, but strains with transferred resistance (Table III) exhibited greater increase in ability to inactivate chloramphenicol than did strains that developed resistance during serial transfer. Resistance acquired *via* transformation in the absence of drug (mixed culture method) we designate "transferred" to distinguish it from the "selective" type of resistance developed by continuing exposure to increasing concentrations of the antibiotic during a long series of serial transfers.

Reversal of Acquired Resistance.—It seemed desirable to ascertain whether resistance acquired *via* transformation could be reversed and, if so, whether ability of the organisms to inactivate chloramphenicol would simultaneously be lost. When chloramphenicol-resistant *E. coli* 60 derived from the sensitive strain *via* transformation (Table III) was treated with acriflavine, there was substantial loss of resistance and complete loss of ability to inactivate the drug (Table IV).

The data presented above suggest that there are at least two different mechanisms involved in bacterial resistance to chloramphenicol. It seems clear that the resistance of some organisms can be attributed largely, if not entirely, to an inherent (or transferred) ability to inactivate the antibiotic, whereas in other organisms, *e.g.*, naturally resistant *Pseudomonas*, inactivation is not a significant factor in resistance. It may be suggested that in the latter instance resistance depends on the biochemical resourcefulness or versatility of the species.

When the normal or usual pathway from substrate to synthesized metabolites essential for growth and reproduction is blocked, some strains which can circumvent the roadblock by developing an adequate alternative pathway will be resistant. Figuratively speaking, the organisms will simply go around the obstruction without destroying it; if it is unable to develop a satisfactory biochemical detour and cannot destroy the roadblock, it will be sensitive. On the other hand, a species or strain that cannot develop such a detour route may still be resistant, if it can remove (or smash its way through) the roadblock or, in terms of bacteria and antibiotics, can destroy or inactivate the antibiotic molecule. Of course, it is possible that in some organisms both mechanisms of resistance may operate simultaneously.

From the present studies it seems that the naturally resistant strains of *Pseudomonas* we isolated from patients and the resistant strains of *Sh. flexneri* and *E. coli* developed from initially sensitive populations by the serial transfer technique have the same type of resistance, which stems primarily from what was referred to above as resistance due to biochemical versatility. It is, of course, possible that direct enzymatic attack on the antibiotic molecule may also contribute to the resistance of some of these strains, but such contribution, if any, appears to have minor significance. On the other hand, naturally resistant strains of *Shigella*, *Escherichia*, and *Staphylococcus* isolated from patients and resistant strains of *Sh. flexneri*, *S. typhi*, *S. paratyphi*, and *E. coli* developed *via* the transformation technique seem to owe their resistance primarily to native or transferred ability to destroy the chloramphenicol molecule or otherwise block its antibacterial action. These observations regarding bacterial resistance to chloramphenicol are reminiscent of the patterns of resistance to penicillin found in appropriate organisms.

It is proposed that some strains of chloramphenicol-resistant bacteria produce an enzyme that is analogous to penicillinase. Support for the enzymatic nature of the chloramphenicol-inhibiting factor has been published (4). Briefly, the substance was shown to be active in the pH range 5 to 8 at 40–45° but to be destroyed by heating at 60° for 30 minutes.

TABLE II.—RELATION BETWEEN INCREASE IN CHLORAMPHENICOL RESISTANCE (INDUCED BY SERIAL TRANSFER IN INCREASING CONCENTRATIONS) AND ABILITY TO INACTIVATE THE DRUG

Strain	Original MIC[a]	Original % Red[b]	Resistant MIC[a]	Resistant % Red[b]
Sh. flexneri 2a-3195	0.39	0	200	24
Sh. flexneri 3a-11	1.56	10	100	20
E. coli 60	0.78	7	400	35
E. coli K-12	3.12	5	200	23

[a] Minimal inhibitory concentration of chloramphenicol, mcg./ml. [b] Per cent reduction of chloramphenicol in broth culture. Initial concentration of antibiotic = 50 mcg./ml.

TABLE III.—RELATION BETWEEN INCREASED CHLORAMPHENICOL RESISTANCE (INDUCED BY TRANSFORMATION *via* MIXED CULTURE METHOD) AND CHLORAMPHENICOL INACTIVATION

Strain	Original MIC[a]	Original % Red[b]	Resistant MIC[a]	Resistant % Red[b]
Sh. flexneri 2a-3195[c]	0.39	0	100	>90
S. typhi 375[c]	1.56	5	200	>90
S. paratyphi B 63[c]	1.56	0	400	>90
E. coli 60[d]	0.78	7	50	>90
E. coli K-12[d]	3.12	5	200	>90
E. coli B[d]	0.39	10	50	>90

[a] Minimal inhibitory concentration of chloramphenicol, mcg./ml. [b] Per cent reduction of chloramphenicol activity in broth culture. Initial concentration of antibiotic = 50 mcg./ml. [c] Donor: *E. coli* H (MIC:400 mcg./ml.; % red: >90). [d] Donor: *Sh. flexneri* 3a-1871 (MIC:200 mcg./ml.; % red: >90).

TABLE IV.—LOSS OF CHLORAMPHENICOL RESISTANCE AND ABILITY TO INACTIVATE CHLORAMPHENICOL IN RESISTANT *E. coli* 60 TREATED WITH ACRIFLAVINE

Strain	MIC[a]	% Red[b]
Original	0.78	7
Resistant (produced by mixed culture)	50	>90
Resistant (after acriflavine treatment)	3.12	0

[a] Minimal inhibitory concentration of chloramphenicol, mcg./ml. [b] Per cent reduction of chloramphenicol activity in broth culture. Initial concentration of antibiotic = 50 mcg./ml.

SUMMARY

Three-hundred and seventy-four strains of *Shigella*, *Escherichia*, *Salmonella*, *Pseudomonas*, and *Staphylococcus* isolated from patients in Niigata Prefecture were examined for their ability to inactivate chloramphenicol. Most chloramphenicol-

col resistant strains of *Shigella*, *Escherichia*, and *Staphylococcus* exhibited such action, but the sensitive strains and all strains of *Pseudomonas*, whether resistant or sensitive, did not.

In the experiments on resistance developed *in vitro* and the inactivation of chloramphenicol, organisms with resistance transferred by the mixed culture method were far more effective inactivators of chloramphenicol than the resistant organisms developed by growth in progressively increasing concentrations of the drug. A direct correlation was observed between the gain and loss of resistance *in vitro* and the increase and decrease of chloramphenicol inactivation in *E. coli* strain 60.

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Determination of Thickness of Walls of Hard Gelatin Capsules by Radioisotopic Means

By GARNET E. PECK*, JOHN E. CHRISTIAN, and GILBERT S. BANKER

A procedure has been developed for the determination of the thickness of the wall sections of hard gelatin capsules. A chlorine-36 source was mounted in the tip of a stainless steel capsule dipping pin and was used to observe the differences in thickness of the wall sections. Twelve series (replications) of 25 determinations each were performed. The measurements taken included the weight, area, calculated weight per unit area, thickness in inches using a micrometer, and beta count rate. The count rates were plotted against the corresponding weight per unit area (mg./cm.^{2}) and thickness (in.). Regression curves were prepared using least squares calculations. A *t* test was used to compare the individual slopes of the regression curves with the mean slope. The applicability of this method of determining the thickness of wall sections of hard gelatin capsules is discussed.

THE FORMATION of hard gelatin capsules involves the use of stainless steel pins which are dipped into a liquid gelatin medium, after which the formed capsules are slowly dried. Not until the drying process has been completed is gauging of the wall sections currently possible. If the thickness of the drying capsules could be gauged early in the drying process, it would be possible to adjust the process more quickly should it produce capsules which were not within control limits. This would result in a saving of time and less loss of product. Furthermore, an isotopic method of evaluating capsule thickness as an in-process control could conceivably result in an automated line operation.

Some of the more important reported uses of radioisotopes in industry apply the principles of penetration and absorption of the isotopic radiations (1). These principles are generally applied to the area of thickness gauging. Those radioisotopes which emit beta particles are most frequently used. Since they are less penetrating than gamma rays, they may be used for measuring the thickness of thin films, paper, and plastic materials (2). One type of gauging system frequently employed is based on the principle of the change of transmission of beta particles through the medium (3). To measure very thin, low density materials—such as paper—carbon-14, a weak beta emitter, has been used (4). Other beta sources that have been used include chlorine-36, krypton-85, strontium-90, and thallium-204 (5–7). Iodine-131 was used to measure the thickness of thin films in the vicinity of 1 μ (8).

The purpose of this study was to select and evaluate a radioisotope to be used for the thickness gauging of wall sections of hard gelatin capsules based on the principle of beta particle absorption.

EXPERIMENTAL

The usual method of expressing the thickness of thin materials by radioisotopic means is in terms of weight per unit area (mg./cm.^{2}). From a study of No. 000 hard gelatin capsules, it was found that the average weight per unit area of capsule sections was 14.12 mg./cm.^{2}. Small pinpoint sources of two pure

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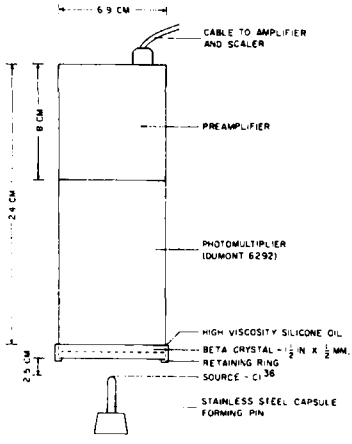


Fig. 1.—Beta detection system and chlorine-36 source.

beta particle sources (phosphorus-32 and chlorine-36) were observed using a series of aluminum absorbers of varying thicknesses expressed in mg./cm.^{2}. An aluminum absorber with a thickness of 13.3 mg./cm.^{2} was the closest available to the thickness of the gelatin sections measured. This aluminum absorber reduced the count rate of the chlorine-36 sample by about 30% compared to the sample counted with no absorber. Since the change in count rate was 19,000 c.p.m., it was thought that adequate sensitivity of detection of small differences in thickness might be achieved with the isotope. The densities of aluminum and gelatin are approximately equal; the change in count rate with gelatin sections with thickness was expected to be of a similar magnitude. The count rate of the phosphorus-32 sample was not appreciably diminished by the same aluminum absorber.

Preparation of Radioactive Pin.—A stainless steel capsule dipping pin, obtained from a producer of hard gelatin capsules, was used to contain the source. A cavity was drilled into the tip of a pin $^7/_{32}$ in. in diameter and $^1/_8$ in. deep. Since chlorine-36 is normally supplied as radioactive HCl, it was necessary to prepare a suitable insoluble salt of the acid. A 4.0% aqueous solution of mercurous nitrate containing 1.0% nitric acid was prepared. The labeled HCl was added to this solution. The mercurous chloride precipitate that formed was heated on a steam bath for 1 hour. The supernatant liquid was then tested for completeness of precipitation, and the volume was reduced to about 1.0 ml. The remainder of the liquid was allowed to evaporate at room temperature, resulting in large crystals of labeled mercurous chloride. The radioactive material was mounted in the tip cavity of the pin and was sealed in place with label glaze.[1]

Gauging System.—The normal thickness gauging system consists of a source, a detector, and an amplifying and recording system (4). The system used in this study may be divided into these parts as: source, chlorine-36 with an average count rate of 73,000 c.p.m. with the detector 2.5 cm. from the source; detector, anthracene crystal with a suitable photomultiplier and preamplifier (see Fig. 1); and amplifying and recording system, (*a*) RIDL model 115 pulse height analyzer,[2] and (*b*) RIDL model 200T scaler.[2]

Since the model 200T scaler was not equipped with a built-in amplifier, it was necessary to use the amplifier section of a pulse height analyzer. This recording system was used for all measurements. In order to observe the measurements visually, an auxiliary system was also used which consisted of a RIDL model 39-1 spectrum scanner-ratemeter[2] and a Minneapolis-Honeywell 0 to 50-mv. recorder.[3] Both recording systems could be operated simultaneously.

Sample Preparation and Measurement.—The gelatin sections used in this study were taken from No. 000 hard gelatin capsules manufactured by Eli Lilly and Co. This size was chosen because of the large sections that were obtained when the capsules were cut into rectangular sections. The capsules were handled with rubber gloves during the cutting and trimming operation. After the sections were cut from the walls of the capsules, they were trimmed so that the corners were right angles. The sections were then weighed and measured with vernier calipers. The weight per unit area of each section was calculated from these data. The sections were placed over the radioactive source and a 3-minute count taken. A 1-minute count was also taken to observe differences in counting time *versus* count rate. After these measurements were made, a hand micrometer was used to measure the thickness of the sections to the nearest 0.0001 in. Twenty-five sections were measured and represented one series. The capsules used in this study contained 7.001% moisture as determined by Karl Fischer titration of 1-Gm. samples.

Table I.—Thickness and Weight per Unit Area Measurements of Gelatin Sections, Series 10

Sample	Wt., mg.	Area, cm.^{2}	Wt./Unit Area, mg./cm.^{2}	Thickness, in.	Beta Count,[a] c.p.m.
1	38.7	2.654	14.243	0.0043	54,500
2	35.8	2.624	13.643	0.0039	56,091
3	34.1	2.448	13.930	0.0042	55,061
4	42.7	2.752	15.516	0.0047	52,612
5	38.0	2.614	14.537	0.0045	53,093
6	37.8	2.639	14.324	0.0043	54,122
7	39.1	2.827	13.831	0.0045	53,260
8	39.1	2.862	13.662	0.0040	56,124
9	39.8	2.995	13.289	0.0042	54,682
10	36.0	2.576	13.975	0.0043	54,070
11	37.5	2.719	13.792	0.0043	54,528
12	39.1	2.690	14.535	0.0043	54,290
13	37.8	2.738	13.806	0.0040	55,416
14	40.7	2.794	14.567	0.0042	54,782
15	46.3	3.181	14.555	0.0045	53,843
16	38.6	2.608	14.801	0.0044	54,166
17	40.2	2.907	13.829	0.0040	55,242
18	37.9	2.924	12.962	0.0039	56,373
19	38.0	2.723	13.955	0.0043	54,605
20	39.1	2.608	14.992	0.0043	53,757
21	36.0	2.693	13.368	0.0041	54,522
22	34.2	2.500	13.680	0.0040	55,548
23	39.1	2.818	13.875	0.0044	54,125
24	38.7	2.854	13.560	0.0040	55,171
25	39.6	2.884	13.731	0.0040	55,108

[a] One 3-minute count was taken for each.

[1] Fisher Scientific Co., Chicago, Ill.

[2] Radiation Instrument Development Lab., Northlake, Ill.

[3] Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.

TABLE II.—EVALUATION OF GELATIN THICKNESS MEASUREMENTS (COUNTS PER MINUTE *versus* WEIGHT PER UNIT AREA)

Series	$\bar{X}$, mg./cm.²	$\bar{Y}$, c.p.m.	Slope, c.p.m./mg./cm.²	Intercept, c.p.m.	σ_x[a]	s[b]	t test[c] for Slopes
1	14.3982	53,759.84	1,177.452	70,712.95	0.90427	319.957	−0.65253
2	14.2453	53,810.92	1,149.247	70,182.02	0.72794	454.216	−0.14401
3	14.3576	53,827.40	1,497.256	75,324.60	0.91856	362.708	−4.63426
4	14.2525	53,999.36	1,117.174	69,922.08	0.72984	392.863	0.13098
5	14.2006	55,250.00	1,066.583	70,396.12	0.80076	549.981	0.47095
6	14.1034	54,601.88	1,224.573	71,872.52	0.90114	489.658	−0.85850
7	14.2530	54,798.08	1,129.886	70,902.35	0.80935	751.861	0.00748
8	14.3250	54,653.40	1,004.431	69,041.87	0.59880	401.806	0.94516
9	14.0901	55,087.00	1,007.208	69,278.68	0.64103	521.677	0.76226
10	14.0383	54,603.64	1,126.230	70,414.00	0.56601	674.186	0.02118
11	14.0035	55,000.68	1,091.325	70,283.05	0.77976	451.454	0.34501
12	13.9992	55,152.84	983.939	68,927.20	0.80347	325.252	1.81982

[a] Standard deviation of X. [b] Standard error of estimate for the universe. [c] Critical region for t test: ± 2.069 for $\alpha = 0.05$ and $n - 2$ degrees of freedom.

RESULTS AND ANALYSIS

Using gelatin sections cut from the walls of the No. 000 hard gelatin capsules, 12 series (replications) of determinations were made, each series involving measurements of 25 different gelatin sections. The measurements taken on each of the 300 sections included weight of the section, area, calculated weight per unit area (mg./cm.²), thickness using a micrometer, and a 3-minute count of the section over the radioactive source. The average count rate for the beta source was 73,288 c.p.m. in air. Representative data as exemplified by series 10 are shown in Table I. With the least squares method, two separate regression curves were determined for each of the 12 series, one in which X (measured thickness) was in mg. per cm.², and the other in which X was in inches (Y in both cases was the corresponding beta count of the section). The necessary calculated values are summarized in Tables II and III. The regression curves of Series 1, 2, 3, and 4 are shown in Figs. 2 and 3. It should be noted that the individual points are not shown because of the great degree of overlapping that would result in showing 100 close packed points. For this reason Tables II and III also include the calculated intercepts for verification of the regression curves.

Nuclear Method Based on Weight/Unit Area.— In Table II the slopes of the various regression curves were summed; the mean slope was determined to be 1,131.275 c.p.m./mg./cm.². The standard deviation of the slopes was calculated using the equation (9)

$$\sigma_x = \sqrt{\frac{n\Sigma X^2 - (\Sigma X)^2}{n}} \qquad \text{(Eq. 1)}$$

and was 130.480 c.p.m./mg./cm.². Only the slopes of Series 3 and 12 did not fall within one standard deviation of the mean slope. A hypothesis was proposed to test the calculated slopes against the mean of these slopes. To test the hypothesis that each slope was either equal or not equal to the mean slope, the following equations were used along with the standard deviation of the X parameter of each series calculated using Eq. 1 (9)

Standard error estimate for the universe

$$s_{yx} = \sqrt{\frac{Y^2 - a\Sigma Y - b\Sigma XY}{n - 2}} \qquad \text{(Eq. 2)}$$

Hypothesis test for the slope

$$t_{n-2} = \frac{b - b_{\text{mean}}}{\sigma_x \sqrt{n}} \qquad \text{(Eq. 3)}$$

Only the slope for Series 3 could reject the hypothesis that the mean slope was not the true slope for an alpha error of 0.05.

Because of the number of measurements involved, it was felt that a composite curve could indicate the ability of the method to detect differences in gelatin thickness. Figure 4 represents the average slope and intercept for the 12 series. Using the mean slope, it would indicate that a change of 1 mg./cm.² would result in a change of 1,131 c.p.m. It must be remembered that these sections were not absolute standards, but samples prepared from market pack-

TABLE III.—EVALUATION OF GELATIN THICKNESS MEASUREMENTS (COUNTS PER MINUTE *versus* MICROMETER)

Series	$\bar{X}$, in.	$\bar{Y}$, c.p.m.	Slope, c.p.m./in.	Intercept, c.p.m.	σ_x[a]	s[b]	t test[c] for Slopes
1	0.004352	53,759.84	334.391[d]	68,312.68	0.000344	290.401	0.00703
2	0.004256	53,810.92	344.068	68,454.53	0.000255	334.217	1.22316
3	0.004300	53,827.40	447.773	73,079.07	0.000309	381.407	−2.89425
4	0.004252	53,000.36	335.684	68,265.55	0.000252	726.896	0.70087
5	0.004180	55,250.00	434.167	73,398.16	0.000219	350.185	−1.81397
6	0.004192	54,601.88	451.118	73,512.73	0.000261	303.279	−3.21956
7	0.004276	54,798.08	356.267	70,032.04	0.000257	354.829	0.71996
8	0.004304	54,653.40	326.981	68,726.68	0.000207	243.802	2.08703
9	0.004152	55,087.00	371.160	70,497.56	0.000200	354.962	0.14162
10	0.004224	54,603.64	409.651	71,907.04	0.000208	357.236	−0.97618
11	0.004176	55,000.68	361.472	70,095.74	0.000252	293.540	0.63119
12	0.004156	55,152.84	341.440	69,343.10	0.000232	312.637	1.28712

[a] Standard deviation of X. [b] Standard error of estimate for the universe. [c] Critical region for t test: ± 2.069 for $\alpha = 0.05$ and $n - 2$ degrees of freedom. [d] Inches $\times 10^{-4}$.

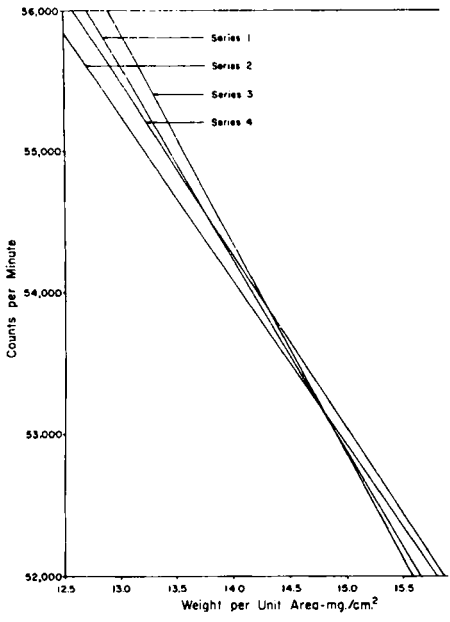


Fig. 2.—Beta thickness measurements of gelatin sections, X in mg./cm.^{2}; Series, 1, 2, 3, and 4.

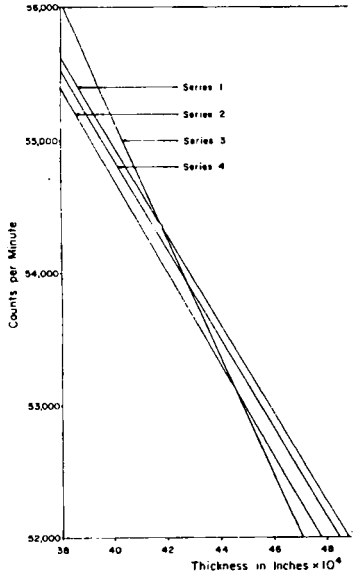


Fig. 3.—Beta thickness measurements of gelatin sections, X in inches; Series 1, 2, 3, and 4.

ages of gelatin capsules, and some degree of variation in thickness from one end of the section to the other end was periodically observed. This would affect the weight per unit area determinations, micrometer readings, and correlation to beta activity. Also, all measurements were not made under a controlled environment. At least a degree of the variation seen in the data may simply be attributed to sample variation as it exists in capsules marketed today.

Nuclear Method Based on Micrometer Thickness.—From Table III the slopes of the various regression curves were summed and the mean slope was 376.181 × 10^4 c.p.m./in. (376.181 c.p.m./1 × 10^{-4} in.). The standard deviation was calculated using Eq. 1 and was 44.635 × 10^4 c.p.m./in. It may be stated that the slopes from Series 3, 5, 6, and 8 did not fall within one standard deviation of the mean slope, but all slopes did fall within two standard deviations. The slopes were tested against the mean slope using the same t test mentioned in the previous section. The slopes that rejected the hypothesis that the mean slope was not the true slope were from Series 3, 6, and 8 for an alpha error of 0.05.

A composite curve was also prepared for this set of 12 series. Figure 5 is the result of the average of the

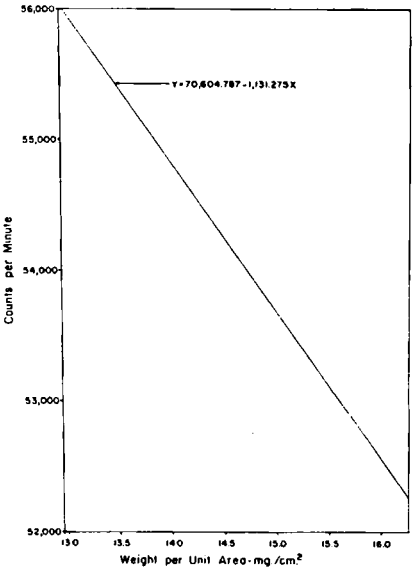


Fig. 4.—Beta thickness measurements of gelatin sections: mean of all series of count rate *versus* weight per unit area.

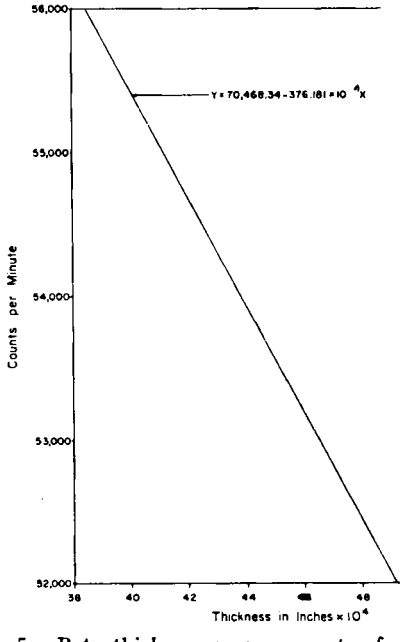


Fig. 5.—Beta thickness measurements of gelatin sections: mean of all series of count rate *versus* micrometer reading.

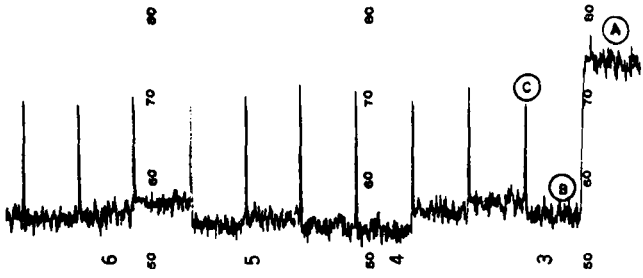


Fig. 6.— Visual recording of beta thickness measurements of gelatin sections, Series, 10. Key: A, source count rate with air; B, count rate with gelatin section over source; and C, sample being changed.

slopes and intercepts. One difficulty in the use of a hand micrometer is that the degree of pressure applied to the gelatin section may vary from measurement to measurement and from day to day. In addition, the micrometer reflects the thickness at only one point on a place surface, which would almost certainly not be the area on the surface measured by the collimated beta beam. However, only Series 6 shown in Table III fell far outside the critical region of the t test.

Visual Observation of Beta Thickness Measurements.—During the normal operation of the radioisotope counting equipment measurements were also recorded on chart paper using a 50-mv. recorder. Figure 6 is an example of a simultaneous measurement obtained with the auxiliary recording equipment of capsule thicknesses of Series 10. This clearly demonstrates the ability of the system to detect differences in thickness of the gelatin sections. Figure 6 also illustrates the possible variation in slopes from one series to the next because of the wide band of counting fluctuation. Although the counting times shown are 4 minutes per sample, a faster recorder speed could be used which would plot the thicknesses easily for shorter counting times. The speed of the recorder was 45 in. per hour.

DISCUSSION

The difficulties in obtaining the standard curves for the beta thickness gauging include the degree of scattering observed within each series of measurements and the lack of perfect reproduction of the slopes of each regression curve between series. The sources of error that may have contributed to the scattering are: (*a*) the small size of the sample which could cause errors in weighing and area determination of the section; (*b*) the variation in pressure when using the hand micrometer from one series to another; (*c*) that all measurements were not conducted in the same working area; and (*d*) slight variations in thickness from one end of the gelatin section to the other. Even though these sources of error were present, it is felt that once a system is in continuous operation many more observations could be made in a short period of time to prove the effectiveness of the method. The visual observation of the measurements through the use of a recorder clearly indicated that differences in the thickness of the gelatin sections could be observed.

The advantages of the thickness gauging of gelatin sections by the use of a beta source of radioactivity are: (*a*) nondestructive testing of a hard gelatin capsule; (*b*) in-process measurement of gelatin capsules or gelatin sheets when it is not possible by other means, resulting in quicker process adjustment should the process be out of control; (*c*) more frequent measurement of a sample with less importance placed on human manipulations of instruments such as a hand micrometer; and (*d*) the in-process beta gauging thickness testing technique might be incorporated into a system of automatic process control. The radioactive source containing pin used in this study could also be used in the routine laboratory testing of thickness of hard gelatin capsules as well as for in-process control. This pin method would be ideal for the thickness measurement of the capsule tips and the capsule walls.

The procedures outlined in this paper could also be applied to the development of isotope methods (isotope selection and procedures design) for thickness determinations of other pharmaceutical materials, such as bandage materials and assorted pharmaceutical films.

CONCLUSIONS

It was possible to detect differences in the thickness of wall sections of hard gelatin capsules by beta absorption measurements from 12 series of 25 measurements each when the measurements are based on weight per unit area or thickness in inches *versus* beta count.

In the measurements based on the weight per unit area, a change of 1 mg./cm.^{2} was indicated by an average change in beta count of 1,131 c.p.m.

A change of 0.0001 inch was indicated by an average change in beta count rate of 376 c.p.m. in the measurements based on thickness in inches.

The ability to detect differences in thickness of the wall sections of hard gelatin capsules was clearly shown by a simultaneous visual observation with a recorder.

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Effect of Deuterium Oxide on the Growth of Peppermint II

Histological Effects

By F. A. CRANE, M. I. BLAKE, R. A. UPHAUS, and J. J. KATZ

Representative sections of leaf, stem, and root of peppermint plants grown in 70 per cent D_2O nutrient were examined microscopically; comparison was made of the significant histological features with sections from plants grown in an aqueous nutrient. The major effect of deuterium on the growth of peppermint appears to be inhibition of cell divisions. Parenchyma cells are enlarged and vascular tissue appears to be reduced in the deuteriated plants. In general, the effects were more pronounced in growing tissues than in tissue exposed to deuterium after differentiation had occurred.

IN A PREVIOUS paper (1) the morphological effects of deuterium oxide on the growth of peppermint were reported. Peppermint plants were conveniently grown in 250-ml. Erlenmeyer flasks by liquid culture technique. The concentration of D_2O in the nutrient solution was varied from 0 to 100%. When the fully grown plants were harvested, samples of each plant part were killed in Randolph's modification of the Navashin fixing solution (2) and reserved for histological studies. While the histological effects were noted in all plants grown with D_2O concentrations in the nutrient up to 100%, comparison was made here only between the controls and the plants grown in 70% D_2O nutrient. Plants grown on intermediate D_2O concentrations showed effects in the same direction as the 70% plants, but to a proportionately lesser extent. Consequently, we compared the plants grown in the highest D_2O concentrations to the controls. Significant growth was not observed in plants grown in D_2O concentrations above 70% as reported earlier (1), and these plants were therefore not subjected to histological examination. It should also be noted that plants grown in nutrient containing 70% D_2O showed an uptake of 35% deuterium when the water obtained from combustion of the dried plants was analyzed for deuterium content. This represents organically bound exchangeable and nonexchangeable deuterium. The maximum histological effects described here thus result from the replacement by deuterium of about one-third of the hydrogen normally present in the tissue.

In this study representative sections of leaf, stem, and root of peppermint plants grown in 70% D_2O nutrient were examined microscopically and comparison of the significant histological features was made with comparable sections from plants grown in an aqueous nutrient.

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EXPERIMENTAL

Five peppermint plants (*Mentha piperita*) were grown on a nutrient containing 70% D_2O by the technique reported in the previous paper in this series. Three plants were grown in an aqueous medium and served as the controls. All plants were harvested after 50 days, at which time the controls went into the flowering stage. The plant parts studied for their histological response to D_2O in the nutrient included immature leaf (the smallest leaf

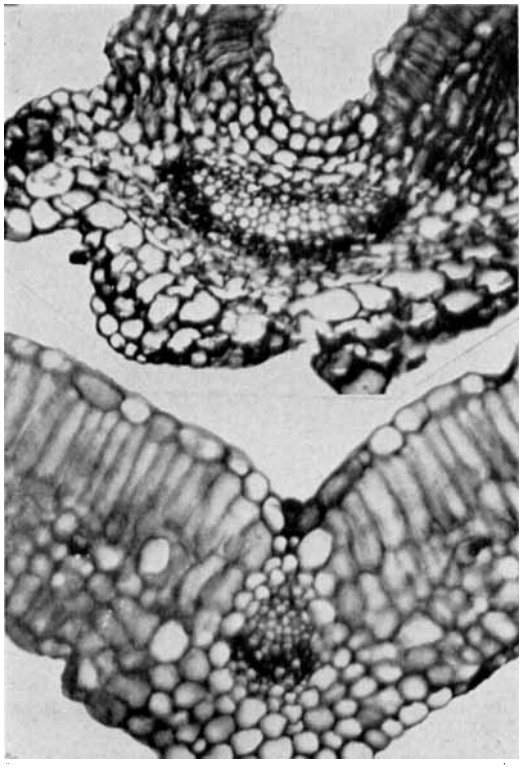


Fig. 1.—Transverse sections of immature leaves taken near apex of plants growing on (top) H_2O nutrient solution and (bottom) 70% D_2O nutrient solution. Both sections are taken from the midrib of the leaf. The magnification (× 100) is the same in both cases.

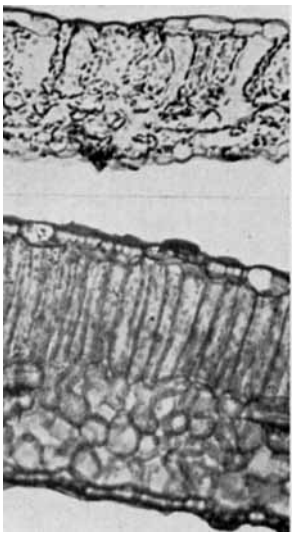


Fig. 2.—Transverse sections of mature leaves taken near the base of the primary stem of plants growing on (top) H_2O nutrient solution and (bottom) 70% D_2O nutrient solution. Magnification is the same in both cases (× 100).

at the apex discernible to the naked eye), mature leaf (near the base of the primary shoot), immature stem (directly below the pair of immature leaves under study), mature stem (directly above the mature leaf under study), and root (a section taken 2 cm. below the origin of the root on the stem base).

The tissues mentioned in the previous paragraph were dehydrated through an ethanol-xylol series and were imbedded in Tissuemat (55°) paraffin. They were sectioned at 20 μ thickness with a Spencer No. 820 rotary microtome. The sections were mounted on slides by Mayer's albumin fixative and were stained with Safranin O and Fast Green. They were permanently mounted in Canada balsam. The different histological features were measured in microns using an ocular micrometer for both the control and the 70% D_2O plant tissues. Photomicrographs of the sections of the plant parts were made with a Leitz Orthomat microscope camera and are shown in Figs. 1–5.

RESULTS AND DISCUSSION

Leaf.—Figures 1 and 2 show a photographic comparison of leaf sections of the control and the 70% D_2O plant. Table I summarizes measurements of the major histological features of representative sections of both young and mature leaves.

The young blade of the deuteriated plant is about twice as thick as the control. In the mature leaf this effect is much less pronounced, though still significant. The midrib shows little difference in thickness in response to deuteriation. However, there is a drastic reduction in midrib width and in the number of vascular elements, mainly xylem vessels and tracheids, which differentiated in the presence of D_2O. The dimensions of the tissues comprising the bulk of the leaf blade are greater in the deuteriated leaf—apparently the result of larger cells. Differences in all cases are more marked in the young leaf than in the mature leaf. Since the mature leaves had already gone through the earlier phase of their elongation cycle before the effect of D_2O was exerted on them, they do not exhibit differences so striking as those of the young leaf.

Deuterium oxide affects the kinetics of reactions involved in metabolism and thus interferes with the normal biochemistry of the plant. Of major significance is interference with cell division. Gross and Spindel (3) have recently treated at length the subject of inhibition of mitosis by deuterium. Stein and Forrester (4) studied the recovery of corn root cells after a 48-hour exposure to 80% D_2O solution and concluded that D_2O blocks cell division at interphase and metaphase. Flaumenhaft, *et al.* (5), Chorney, *et al.* (6), have observed that certain algae grown in a fully deuteriated medium are substantially larger than cells grown in water. Crespi, *et al.* (7), reported that algae transferred from an H_2O culture to 99.6% D_2O nutrient solution continued to grow, but cell division appeared to be strongly inhibited. Henderson (8) points out that one of the effects of D_2O on nucleic acids *in vitro* is impaired DNA strand separation which implies possible interference with DNA synthesis and cell division *in vivo*. We have observed that the growth of peppermint plants under the stress of D_2O is continuous, although it proceeds at a reduced rate compared to the controls. It appears that cell division is indeed severely impaired. The tissue cells tend to become greatly enlarged as a result of the inhibi-

TABLE I.—COMPARISON OF HISTOLOGICAL MEASUREMENTS IN LEAF FROM PEPPERMINT GROWN IN 70% D_2O NUTRIENT AND IN AQUEOUS NUTRIENT[a]

Feature	Young Leaf		Mature Leaf	
	H_2O	70% D_2O	H_2O	70% D_2O
Blade thickness	101.7 ± 0.1[b]	195.6 ± 0.4	161.0 ± 0.2	223.6 ± 0.6
Midrib thickness	235.7 ± 0.9	229.4 ± 0.3	367.3 ± 0.8	377.6 ± 0.1
Epidermal thickness				
Upper	12.9 ± 0.2	24.0 ± 0.3	14.4 ± 0.9	18.9 ± 0.4
Lower	10.2 ± 0.7	17.5 ± 0.6	8.8 ± 0.7	13.5 ± 0.6
Palisade layer				
Cell height	37.7 ± 0.4	69.0 ± 0.4	73.6 ± 0.4	85.8 ± 0.6
Cell width	8.9 ± 0.1	17.6 ± 0.8	15.5 ± 0.3	14.9 ± 0.4
Spongy mesophyll				
Cell diameter	44.3 ± 0.2	90.8 ± 0.2	57.0 ± 0.4	80.5 ± 0.5
Midrib vascular bundle				
Height	89.2 ± 0.9	82.1 ± 0.7	122.0 ± 0.9	127.9 ± 0.3
Width	207.4 ± 0.8	57.1 ± 0.6	218.0 ± 0.6	198.7 ± 0.4
No. of oil glands on surface[c]				
One-celled	13.0 ± 0.2	10.0 ± 0.1	3.2 ± 0.1	3.5 ± 0.1
Eight-celled	6.9 ± 0.2	5.5 ± 0.1	1.0 ± 0.1	5.0 ± 0.2

[a] All measurements are in microns, and each represents the average of at least 10 samples taken from not less than three different sections. [b] Standard deviation. [c] Based on a surface area of 6.60 × 0.02 mm. in all sections. Data are averages of at least 10 samples.

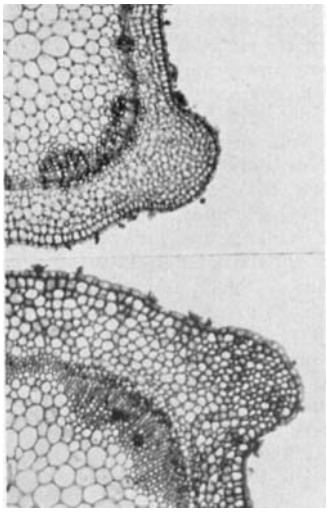


Fig. 3.—Transverse sections of immature stems taken near apex of plants growing on (top) H_2O nutrient solution, and (bottom) 70% D_2O nutrient solution. Magnification is the same in both cases (× 100).

tion of cell division. Impaired cell division and cell enlargement probably (but not necessarily) are directly related, since osmotic effects may also be important in cell enlargement.

The data in Table I indicate a marked increase in the size of palisade parenchyma and spongy mesophyll cells. This increased cellular volume has resulted in a decrease in the volume of intercellular atmosphere in the leaf. To compensate, the leaf has produced a greater number of stomata per unit area of leaf surface. Microscopic examination of the leaf surface indicated that in the deuteriated plant there were about 25% more stomata per unit area, thus permitting more rapid gas exchange at the epidermal surface.

The reduced rate of growth in the plant grown in D_2O nutrient was reflected in a greatly reduced rate of transpiration, evidenced by the smaller amounts of nutrient solution taken up by the plant as well as a lower requirement for tissue water to maintain the turgidity of the cells. A reduction in the development of vascular bundles in the deuteriated plant accompanied decreased transpiration of nutrient fluid.

It is apparent from Table I that in the young leaf there are fewer one-celled and eight-celled glandular trichomes in the deuteriated leaf. The number of trichomes is based on a count in an equal surface area in all sections. The glandular trichomes are produced at the apical meristem and develop while the leaves are still meristematic. Since it appears that D_2O interferes with cell division, a reduced number of presumptive glandular trichomes are initiated in the meristematic leaf. The effect is accentuated because the deuteriated leaves are substantially smaller than the corresponding control leaves. The effect on the glandular trichomes in the mature leaf is quite different. Since the mature leaves were meristematic prior to the exposure of the plant to D_2O, the number of presumptive glandular trichomes should have been the same in both the deuteriated and control mature leaf. Since the deuteriated leaves are smaller than the corresponding leaves of the control, the larger number of glands per unit surface area is to be expected. However, it is difficult to account for a fivefold increase in the number of eight-celled trichomes noted in the comparison of mature leaves.

Stem.—Figures 3 and 4 show typical sections of stem from the controls and plants grown in 70% D_2O nutrient. Both young and mature stems were compared. The histological measurements are shown in Table II.

The data in Table II indicate that the effect of D_2O in the young stem is essentially opposite to that found in the mature stem. In the young deuteriated stem, all of the features measured were larger than comparable measurements of the control stem. All growth occurred in the young stem after the plant was exposed to D_2O in the nutrient, whereas in the mature stem, most of the development took place prior to the exposure to D_2O.

The young stem section (cut a few millimeters below the shoot apex) from the plant grown in 70% D_2O nutrient was about twice the diameter of the control section measured in both concave and convex directions. The epidermis had more than twice the thickness, and large colenchyma bundles developed on all corners of the young deuteriated stem. A similar effect is evident with the large corner vascular bundles. Width of the cortex was doubled in the D_2O stem, and the width of the pith was much greater than in the control. Elongation of individual cells of pith and cortex occurred to a greater extent in the deuteriated stem.

The mature stem sections, obtained near the base of the erect stem, reflect growth responses to stimuli that exerted their influence mainly before the effect of D_2O could have operated to change the pattern of development. All cell divisions were complete, and the tissue was largely differentiated, except for that which occurred by way of further growth of the cambium in the vascular bundles. The cambium, a

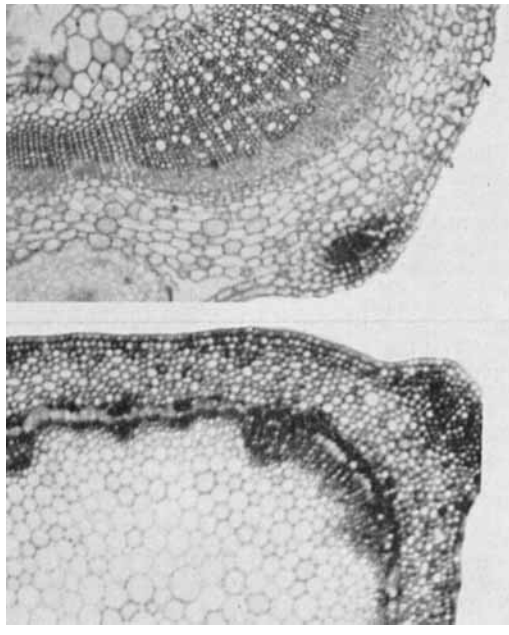


Fig. 4.—Transverse sections of mature stems taken near the base of plants growing on (top) H_2O nutrient solution and (bottom) 70% D_2O nutrient solution. Magnification is the same in both cases (× 100).

TABLE II.—COMPARISON OF HISTOLOGICAL MEASUREMENTS IN STEM FROM PEPPERMINT GROWN IN 70% D_2O NUTRIENT AND IN AQUEOUS NUTRIENT[a]

Feature	Young Stem		Mature Stem	
	H_2O	70% D_2O	H_2O	70% D_2O
Stem width				
Convex to convex	807.0 ± 5.2[b]	1437.5 ± 10.7	2728.7 ± 3.4	2050.0 ± 26.8
Concave to concave	587.0 ± 8.0	1247.3 ± 23.2	2699.0 ± 2.8	1782.0 ± 16.8
Epidermal thickness	9.3 ± 0.2	20.8 ± 0.2	20.9 ± 0.2	17.3 ± 0.3
Collenchyma ridge				
Length	242.0 ± 3.0	272.0 ± 3.5	313.0 ± 2.2	354.0 ± 4.0
Width	119.0 ± 2.7	207.6 ± 3.1	159.0 ± 1.6	177.4 ± 1.8
Corner vascular bundle thickness	83.1 ± 0.9	115.0 ± 0.9	496.2 ± 1.4	176.2 ± 1.7
Xylem thickness	53.7 ± 0.8	68.4 ± 0.6	367.5 ± 3.0	134.6 ± 1.5
Phloem thickness	29.4 ± 0.4	46.6 ± 1.2	128.7 ± 2.2	41.6 ± 0.4
Cortex width, convex side	112.6 ± 2.0	223.8 ± 2.0	261.8 ± 2.4	223.0 ± 2.4
Pith width	682.3 ± 2.5	890.0 ± 1.0	1758.0 ± 0.3	1397.2 ± 0.6
Single cortical cell				
Length	33.6 ± 0.4	35.7 ± 0.3	43.5 ± 0.7	42.0 ± 0.4
Width	27.7 ± 0.3	29.9 ± 0.4	66.0 ± 1.2	47.9 ± 1.1
Single pith cell				
Length	72.8 ± 1.0	86.4 ± 0.6	153.4 ± 4.7	124.0 ± 1.7
Width	62.8 ± 1.2	72.7 ± 0.8	122.4 ± 2.9	93.2 ± 1.6
No. oil glands on surface[c]				
One-celled	27.3 ± 0.6	21.6 ± 0.4	2.3 ± 0.1	6.0 ± 0.3
Eight-celled	16.0 ± 0.6	11.9 ± 0.3	1.5 ± 0.1	3.6 ± 0.2

[a] All measurements are in microns, and each represents the average of at least 10 samples taken from not less than three different sections. [b] Standard deviation. [c] Based on a surface area of 3.76 × 0.02 mm. in all sections. Data are averages of at least 10 samples.

layer of meristematic cells, continued to give rise to new vascular tissue in the control, while D_2O inhibited divisions in this lateral meristem as it did with primary meristematic tissue at the shoot apex. The corner vascular bundles in the mature D_2O stem were about one-third the size of that in the control. In fact, the control stem enlarged to greater dimensions than the D_2O stem in all features except the collenchyma ridge. The pith and cortical parenchyma of the D_2O mature stem were over 80% the dimensions of the control. Individual cells of the pith and cortex of the mature stem were also larger in the control than in deuteriated stems—more significantly in the pith.

Tissue sections selected for study from the control and deuteriated plants were taken at the same levels in relation to the apex and base of the stem. However, it should be noted that while on the average the control plants elongated about sixfold over a 50-day period, the D_2O stems at most doubled in size. Thus, the cells near the apex of the D_2O stem were further along in their pattern of differentiation than the comparable cells near the apex of the control stem. The lack of elongation of the stem in the deuteriated plant gives the impression that the control stem has been compressed to the length observed in the D_2O stem. This would, in effect, account for the larger dimensions reported in Table II for the young deuteriated stem and would also explain the greater amount of growth noted in the mature control stem compared with the mature D_2O stem. The young deuteriated stem section appears biologically older than the control section under study. The mature stem sections, however, are biologically similar in age, and this study demonstrates clearly the inhibitory effects of D_2O in the environment.

The collenchyma ridge in the D_2O mature stem was 10% greater in both dimensions than the corresponding control mature stem. Collenchyma serves a supporting function in the plant and develops by elongation from primary cells of the shoot apex along the corners of the square stem. Elongation of these cells is apparently not inhibited so much as other tissues by the presence of D_2O, and since no new cell divisions occur in this tissue once it is formed, the elongation rates mentioned above explain the increased size of the collenchyma ridge. Enlargement in the collenchyma ridge is due largely to cellulose thickenings laid down along adjacent cell walls. It appears that D_2O does not greatly inhibit the formation of cellulose; in fact, cell walls appear more rigid and distinct than the corresponding cell walls of control plant parts. However, it was noted that the cement-like intercellular carbohydrate material (calcium pectate) which imparts toughness and resiliency to the stem is produced in greatly reduced amounts. The deuteriated stem was very brittle and easily broken, whereas control plants could be handled without damage.

The number of volatile oil-containing glandular trichomes of the stem followed the pattern observed in the leaf. Since glandular trichomes are formed at the apical meristem and mature during the early differentiation of both leaf and stem, any factor such as D_2O which inhibits cell divisions at the meristem limits the formation of these epidermal cellular outgrowths. In the young D_2O stem there is a reduction in number per unit area of both one-celled and eight-celled glands when compared to the control stem. The effect is approximately the same in both types of glands. Once the stem has formed and developed, the spatial arrangement of the glands is a function of the degree of elongation of the stem. Since the D_2O stem did not elongate to the degree that its comparable control did, the glands were not as far apart, and the number of glands present in a comparable area was slightly greater, as would be expected. Again, the relative increase in both types of glands was about the same; the eight-celled gland count was not unusually high as observed in the leaf section.

Root.—Photomicrographs of root sections of control and plant grown in 70% D_2O nutrient are shown

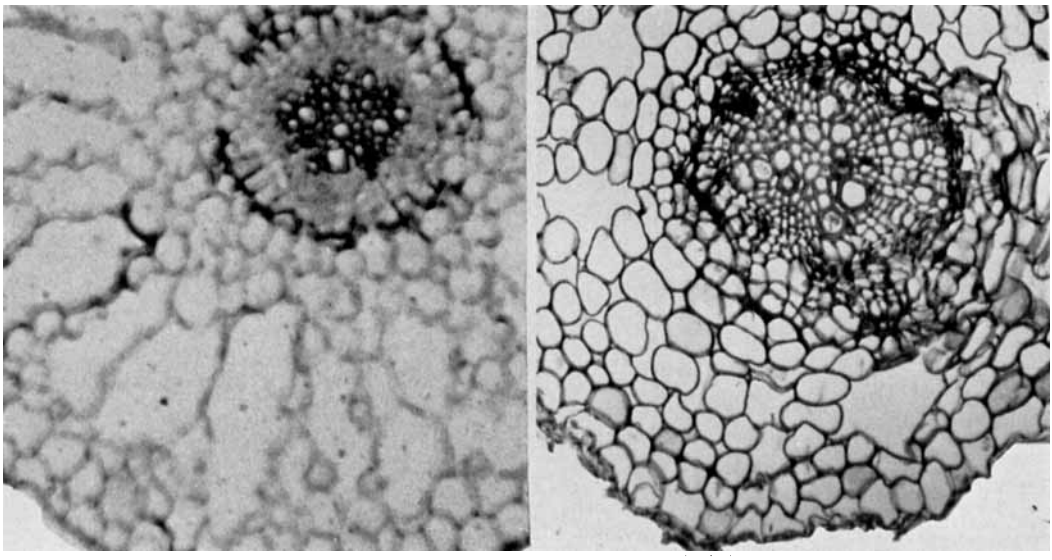


Fig. 5.—Transverse sections of mature roots taken 2 cm. below the junction of adventitious root and stem base of plants growing on (left) H_2O nutrient solution, (right) 70% D_2O nutrient solution. Magnification is approximately the same in both cases (× 100).

in Fig. 5. The histological measurements of the significant features are listed in Table III.

The root sections were cut from mature roots at a level 2 cm. below the point of origin from the stem base. Young root (from apex) was not studied because of problems in infiltrating and sectioning.

The diameter of the control root was significantly larger than that of the 70% D_2O root. However, the diameter of the stele in the two was almost identical. The cortex tissue in the control was over twice as thick as the deuteriated root. Cortical cells of the D_2O root had relatively thick cellulose walls that remained well organized during sectioning. The corresponding cells of the control had much thinner walls which ruptured easily during handling. The large intercellular air chambers between cortical cells appear to be a normal feature of peppermint roots, particularly when grown in liquid culture.

The number of primary xylem units in the stele was essentially the same; the wall thicknesses were not significantly different in the two cases. It is expected, therefore, that the endodermis, the single cellular layer marking the boundary of the stele, should also be the same in both cases.

There were a few cell layers of secondary xylem evident between primary xylem and phloem of the control root which had not developed in the D_2O grown root. This characteristic and the similar observation with the leaf and stem of the deuteriated plant indicate impairment in cell division, resulting in fewer cells in the cortex and in all the tissues arising from the cambium.

Table III.—Comparison of Histological Measurements in Root from Peppermint Grown in 70% D_2O Nutrient and in Aqueous Nutrient[a]

Feature	Mature Root	
	H_2O	70% D_2O
Root diameter	1087.5 ± 5.6[b]	716.5 ± 0.7
Stele diameter	336.2 ± 2.2	332.4 ± 2.6
Epidermal thickness	44.4 ± 4.1	38.8 ± 0.6
Cortex thickness	381.9 ± 3.7	187.8 ± 2.8
Endodermal thickness	24.5 ± 0.4	22.8 ± 0.5
No. of primary xylem units across stele	11.9 ± 0.1	13.1 ± 0.1
Phloem area		
Length	121.4 ± 2.8	156.4 ± 2.3
Width	48.2 ± 0.9	54.6 ± 1.4
No. of cells across cortex	9.6 ± 0.1	7.0 ± 0.0

[a] All measurements are in microns, and each represents the average of at least 10 samples taken from not less than three different sections. [b] Standard deviation.

SUMMARY

Deuterium oxide appears to exert its effect on the growth of peppermint, observed by histological examination, by inhibiting cell division presumably as a result of the disturbance of the rates of enzymatically controlled biosynthetic reactions. Parenchyma cells generally are enlarged with consequent changes in leaf and stem dimensions and other structural effects. Vascular tissue appears to be reduced in the deuteriated plants. The deuterium effects are more pronounced in growing tissues than in tissue exposed to deuterium after differentiation occurs.

Thus, deuterium is observed to exert a profound effect on both the macroscopic and microscopic morphology of the plant. The effects are complex and before generalizations can be profitably attempted, examination of other plant species is necessary. Such studies are now being conducted.

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Sustained-Release Principle in Human Subjects Utilizing Radioactive Techniques

By R. L. BOGNER and J. M. WALSH

Radioactive tracer studies were conducted in six human volunteers utilizing tritium-labeled phenylephrine to determine tritium blood levels obtaining at various time intervals after oral administration of the usual dosage form, the hydrochloride salt of the drug, and of a sustained-release preparation containing the drug as a protocolloid tannate complex. The results obtained from three subjects receiving each preparation showed that a sustained-release form provided a plateau-shaped blood level curve, whereas the hydrochloride salt rapidly yielded a high peak concentration which dropped comparatively sharply thereafter. The technique of incorporating a radioactive drug in different dosage forms and tracing the radioactivity in humans can assist the development of sustained-release drug preparations.

DURING THE PAST several years, an increased interest in long acting oral therapeutic agents has developed. The theoretical benefits derived from long acting medication are extension of optimal therapeutic effects and better dosage control and more uniform blood levels, with a consequent reduction in the incidence of undesirable side effects from intermittently high and low levels. Several types of oral sustained-release preparations have been devised in attempts to achieve these goals. However, the inadequacy of quantitative studies of drug absorption after orally administered sustained-release preparations have prompted criticism of the claims made for some of these products. Much of the supportive evidence have been projected from subjective observations in human therapy. Animal studies are of limited value in predicting gastrointestinal absorption in man, particularly over an extended number of hours.

The present report describes results obtained using the radioactive tracer technique to follow the appearance of a drug in plasma and urine of humans after oral administration of a sustained-release preparation based on the use of protocolloidal tannate complexes of therapeutic amines. In practice, an amine tannate, usually in combination with polygalacturonic acid, serves to provide the drug reservoir. Physical and chemical characteristics and *in vitro* studies of this type of therapeutic amine complex have been described (1).

The drug chosen for this study was phenylephrine made radioactive by random tritium labeling. Comparisons were made of plasma levels of tritium radioactivity following oral administration of tablets containing tritium-labeled phenylephrine as the hydrochloride and as the protocolloid tannate complex. The radioactive technique provides a sensitive and specific means of following absorption from the gastrointestinal tract into the systemic circulation and excretion *via* the kidneys.

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EXPERIMENTAL

Radioactive Phenylephrine Hydrochloride.—Phenylephrine hydrochloride was randomly labeled by the Wilzbach (2) gas exposure technique. A 5-Gm. quantity of solid, finely divided compound was exposed at 27° to 10 curies of tritium gas at a pressure of 0.39 Atm. for 2 weeks. Labile and adsorbed tritium atoms were exchanged by dissolving the initial tritiated product in water (5 Gm. 30 ml.) and removing the water with vacuum at room temperature. This exchange in a hydroxylic solvent was repeated, and the specific activity of the product was found to be 74.2 μc. per mg. Subsequently, an acidic methanol solution of the amine salt was chromatographed on Amberlite CG-45 type I resin, eluted with acidified ethanol and recrystallized from isopropyl alcohol. Repeated recrystallizations yielded a product with a constant specific activity of 11.4 μc. per mg., m.p. 143–144°. Paper chromatography and radioscanning demonstrated one radioactive peak as shown in Fig. 1.

Radioactive Phenylephrine Tannate.—A 0.3-Gm. quantity of phenylephrine-H^3 hydrochloride was dissolved in 2 ml. of distilled water, and a solution of 60 mg. sodium hydroxide in 2 ml. of distilled water was added to liberate phenylephrine-H^3 base which was kept from precipitating by warming the solution. A solution of 0.57 Gm. of tannic acid N.F. in 3 ml. of water was added to the warm solution of amine base; a brown gummy precipitate formed. This precipitate was ground in a mortar with ice, the mixture was filtered, and the finely dispersed solid material was washed repeatedly with cold water. The product was dried *in vacuo* at approximately 60° to yield a dry, free-flowing, light tan powder with a specific activity of 4.14 μc. per mg. and containing 29.8% phenylephrine-H^3 base.

Radioactive Tablets.—The tritium-labeled forms of phenylephrine hydrochloride and phenylephrine tannate were processed into radioactive tablet dosage forms by Irwin, Neisler and Co. on a single-punch experimental tablet machine. The radioactive

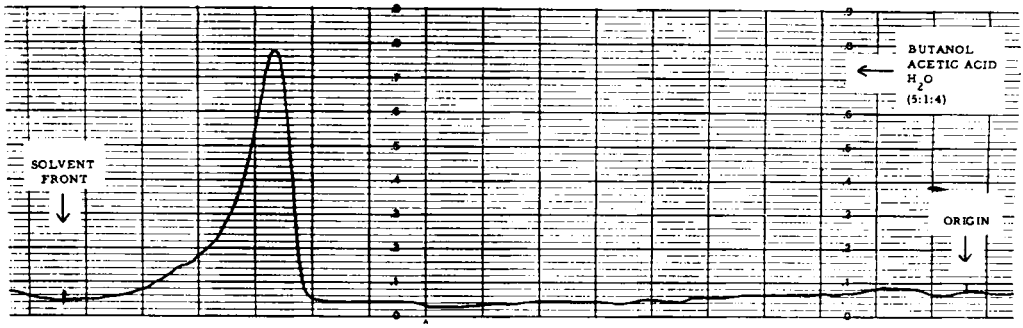


Fig. 1.—Radioscan of paper chromatogram of phenylephrine-H^3 hydrochloride.

TABLE I.—FEATURES OF SUBJECTS

Subject	Sex	Age	Radioactive Tablet
A	Female	39	Phenylephrine-H^3 tannate
B	Male	75	Phenylephrine-H^3 tannate
C	Male	54	Phenylephrine-H^3 tannate
D	Female	50	Phenylephrine-H^3 hydrochloride
E	Male	63	Phenylephrine-H^3 hydrochloride
F	Male	29	Phenylephrine-H^3 hydrochloride

tannate tablets were identical to commercial forms,[1] except that the stable nonradioactive content of the tannate of phenylephrine was replaced by 26.3 mg. (7.84 mg. phenylephrine-H^3 base) of the radioactive form of the drug representing 109 μc. The hydrochloride of radioactive phenylephrine was utilized in an analogous tablet for comparison in which 9 mg. of hydrochloride provided 7.39 mg. phenylephrine-H^3 base equivalent to 102.5 μc.

Preliminary Animal Studies.—Preliminary studies on plasma levels and urinary clearances of radioactive phenylephrine were carried out with two dogs after administration of the hydrochloride and tannate forms of the drug. The hydrochloride, as expected, was more rapidly both absorbed and eliminated during the test periods. Data from this preliminary screening served as bases for obtaining the required authorization to perform the human studies.

Administration to Human Volunteers.—The radioactive tablets were administered to six volunteers by Dr. Campbell Moses, Addison H. Gibson Laboratory, University of Pittsburgh. The features of the subjects are recorded in Table I.

All subjects had been admitted to the hospital for observations and diagnosis; subsequent studies revealed no abnormalities associated with disorders of absorptive or excretory functions.

Three subjects (*A*, *B*, and *C*) were each given a single tablet of phenylephrine-H^3 tannate and three other subjects (*D*, *E*, and *F*) were each given a single tablet of phenylephrine-H^3 hydrochloride. All doses were given on an empty stomach at 8 a.m. with 4 ounces of water following an overnight fast. Breakfast was served at 9 a.m., lunch at 1 p.m., and dinner at 5 p.m. Blood samples were obtained from each subject at 1, 2, 4, 6, 8, 12, 24, and 48 hours post-ingestion; plasma was immediately separated by centrifugation. Urine was collected as voided, and cumulative 24-hour collections were obtained.

[1] The product Rynatan contains 25 mg. phenylephrine tannate, 8 mg. chlorpheniramine tannate, 25 mg. pyrilamine tannate, and 32 mg. polygalacturonic acid plus inert excipients

TABLE II.—PLASMA LEVELS OF TRITIUM RADIOACTIVITY FOLLOWING ORAL ADMINISTRATION OF PHENYLEPHRINE-H^3 TANNATE AND PHENYLEPHRINE-H^3 HYDROCHLORIDE TABLETS

Subject	Time, hr.	Plasma Tritium, d.p.m./ml.	Equivalent Phenylephrine,[a] mcg./ml.
Tannate			
A	1	955	0.031
	2	2190	0.071
	4	2785	0.091
	6	1935	0.063
	8	1000	0.032
	12	880	0.029
B	1	990	0.032
	2	1960	0.064
	4	2800	0.091
	6	2660	0.086
	8	1950	0.063
	12	1440	0.047
C	1	785	0.025
	2	3550	0.115
	4	4160	0.135
	6	4860	0.158
	8	3380	0.110
	12	3400	0.111
Hydrochloride			
D	1	5300	0.172
	2	2860	0.074
	4	4105	0.133
	6	3750	0.122
	8	3450	0.112
	12	100	0.003
E	1	4500	0.146
	2	5290	0.171
	4	1500	0.049
	6	1450	0.047
	8	2200	0.071
	12	100	0.003
F	1	8570	0.278
	2	7470	0.242
	4	6350	0.206
	6	4670	0.152
	8	4100	0.133
	12	900	0.029

[a] Calculation based upon the assumption that the specific activity of the radioactive material in the plasma was identical to the specific activity of the administered material.

Analyses.—The total tritium radioactivity of each plasma sample was determined by a liquid scintillation counting technique. The procedure involved addition of 0.1 to 0.2 ml. plasma to 1 ml. of hydroxide of Hyamine 10-X[2] and subsequent addi-

[2] 1 *M* solution in methanol. Marketed by Rohm and Haas, Philadelphia, Pa.

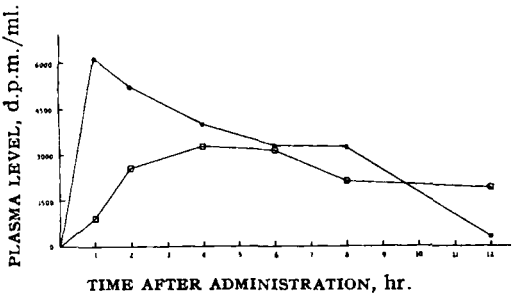


Fig. 2.—Average plasma levels of tritium radioactivity following oral administration of phenylephrine-H³ hydrochloride and tannate tablets. Key: ●, hydrochloride; □, tannate.

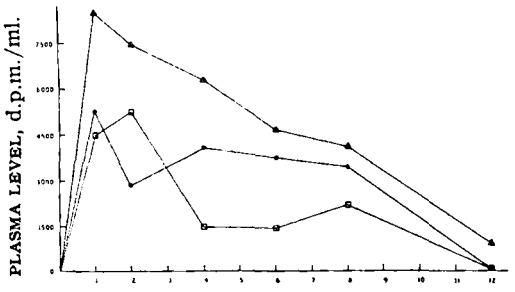


Fig. 3.—Plasma levels of tritium radioactivity following oral administration of phenylephrine-H³ hydrochloride tablets. Key: ●, subject *D*; □, subject *E*; Δ, subject *F*.

tion of 0.5 ml. ethanol and 15 ml. toluene-phosphor (0.6% PPO[3] and 0.02% POPOP[4]). The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer with counting chamber set at 3°, discriminator set at 10–100, and at voltage corresponding with the peak tritium counting rate. All sample counts were corrected for background and counting efficiency as determined from internal standards. Results were expressed as disintegrations per minute per milliliter (d.p.m./ml.).

Urine concentrations of tritium were also determined by liquid scintillation counting. Aliquots of 1 ml. of urine were placed in counting vials with 15 ml. dioxane-phosphor (0.7% PPO, 0.005% POPOP, and 8% naphthalene). All sample counts were again corrected for background and efficiency.

RESULTS

The plasma levels of tritium radioactivity in each of three humans following oral administration of phenylephrine-H³ hydrochloride tablets are listed in Table II. The tritium levels include the contributions from tritium-labeled phenylephrine as well as any tritium-containing metabolites. The tritium levels are also represented in terms of "equivalent phenylephrine concentrations" based upon the assumption, for purposes of calculation, that the specific activity of the radioactive material in the plasma was identical to the specific activity of the administered phenylephrine-H³.

Table II also shows corresponding plasma levels of tritium radioactivity and "equivalent phenylephrine concentrations" in humans following oral ingestion of phenylephrine-H³ tannate tablets.

The average plasma levels of the subjects receiving the hydrochloride and of those receiving the tannate are plotted in Fig. 2; the individual responses are shown in Figs. 3 and 4.

The 24-hour urine excretion of tritium radioactivity from each subject may be seen in Table III.

DISCUSSION

Examination of the average curves and the individual curves reveals relationships which are significant to the action of the sustained-release principle. Phenylephrine provided as the usual rapidly soluble salt form (hydrochloride) was quickly absorbed from the human gastrointestinal tract with resulting high blood levels attained by the first and second hours; appreciable levels were still present after 8 hours, but between the eighth and twelfth hours, the concentrations dropped off rapidly. The tannate complex provided a more gradual rise in tritium blood levels with a plateau rather than a sharp peak in the 2 to 8-hour range and with remarkably stable maintenance of blood level continued through the twelfth hour. Consistent individual blood levels were obtained in these humans with the sustained-release preparation, although the hydrochloride showed wide individual variations. Despite the use of only six humans, the measurement technique relates the variations in readings clearly to individual variations in absorption and elimination.

Figure 2 should be considered as a reflection of the relative absorption characteristics of drug from the two preparations and not as a suggestion of recommended absolute drug levels. The quantitative blood values obviously can be varied by variation in the absolute dosage. The shape of the two curves relative to one another is the point of primary significance.

The excretion data illustrated even more dra-

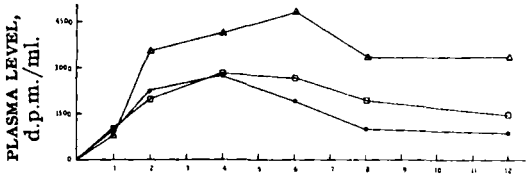


Fig. 4.—Plasma levels of tritium radioactivity following oral administration of phenylephrine-H³ tannate tablets. Key: ●, subject *A*; □, subject *B*; Δ, subject *C*.

TABLE III.—URINARY EXCRETION OF TRITIUM RADIOACTIVITY

Subject	Drug Form	Administered Radioactivity Excreted in 24 hr., %
A	Phenylephrine-H³ tannate	12.4
B	Phenylephrine-H³ tannate	15.9
C	Phenylephrine-H³ tannate	13.3
D	Phenylephrine-H³ hydrochloride	37.2
E	Phenylephrine-H³ hydrochloride	20.0
F	Phenylephrine-H³ hydrochloride	69.0

[3] 2,5-Diphenyloxazole.
[4] 1,4-Bis-2-(5-phenyloxazolyl-benzene).

matically than did the blood levels that absorption and excretion of phenylephrine from the hydrochloride show very wide individual variations, whereas the tannate complex sustained-release form showed an unusual degree of consistency of performance.

SUMMARY

Tritium-labeled phenylephrine hydrochloride and tannate were prepared and incorporated into ordinary and sustained-release type dosage forms, respectively.

Blood levels of tritium radioactivity in humans following oral ingestion of the ordinary hydrochloride dosage form followed a characteristic pattern of rapid rise and fall; blood levels following administration of a tannate complex dosage form rose more slowly but appeared to be more sustained.

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(2) Wilzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013(1957).

Electrochemical Oxidation of Chlorpromazine Hydrochloride

By F. HENRY MERKLE and CLARENCE A. DISCHER

The electrolytic oxidation of chlorpromazine hydrochloride has been studied. Controlled potential coulometry has shown the chlorpromazine undergoes a two-electron oxidation in dilute aqueous acid media. In 9 *N* sulfuric acid two successive one-electron oxidations, involving a stable semiquinone free radical intermediate, are identifiable. Polarographic studies with a rotating platinum electrode confirm the occurrence of two separate one-electron oxidation steps in 9 *N* sulfuric acid. The coulometrically determined *n* values are in agreement with these observations. Reaction mechanisms are proposed for the electrolytic oxidation of chlorpromazine under various conditions of acidity. Spectral absorbance curves are presented as supporting evidence for the indicated reaction mechanisms.

THE OXIDATION of chlorpromazine hydrochloride[1] by cerium (IV), iron (III), permanganate, and hydrogen peroxide as well as by photo-irradiation has been reported (1–3). It has also been shown that the intermediate species in the oxidation of chlorpromazine exists as a free radical (4). More recently, the existence of stable semiquinone free radicals of chlorpromazine and other *N*-substituted phenothiazine derivatives has been demonostrated (5). However, because of the inherent difficulties in the quantitative determination of the free radical species, their study has been restricted considerably.

This study was undertaken to establish an oxidation mechanism for chlorpromazine hydrochloride in aqueous media. Since chlorpromazine and its oxidation products are electrolytically active, controlled-potential coulometry offered a direct approach for this study. The oxidation at a platinum anode has proved to be an extremely useful method for the production of the intermediate free radical species. Moreover, this study indicates the possible use of controlled-potential coulometry as a quantitative analytical technique for chlorpromazine and its oxidized forms in the presence of one another.

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[1] Chlorpromazine hydrochloride and chlorpromazine 5-oxide were supplied by Smith Kline and French Laboratories, Philadelphia, Pa.

INSTRUMENTATION

An electronic controlled-potential coulometric titrator, model Q-2005 ORNL, was used to perform the electrolyses (6). Oxidations were performed in a cell designed to accept a cylindrical wire mesh rotating platinum electrode (1 in. diameter × 2 in. height) and approximately 100 ml. of solution. The reference (S.C.E.) and auxiliary (working cathode) electrodes were separated from the sample solution by agar plugs and fritted glass diaphragms. A triplet vacuum tube voltmeter, model 805, with a 7-in. scale served as the readout device.[2] Polarograms were obtained on the Sargent model XXI recording polarograph. An H-type cell was used with a rotating platinum microelectrode. A saturated calomel reference electrode was separated from the sample portion by an agar plug and fritted-glass diaphragm.

PROCEDURE AND RESULTS

Controlled Potential Data.—When chlorpromazine hydrochloride is electrolyzed at a potential of approximately +0.70 v. *versus* S.C.E. in 1 *N* sulfuric acid under an atmosphere of nitrogen, the solution gradually assumes a deep red. As the electrolysis is

[2] In the latter stages of this study readout voltages were measured with a Non-Linear Systems model 484 A digital voltmeter.

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The electrolytic oxidation of chlorpromazine hydrochloride has been studied. Controlled potential coulometry has shown the chlorpromazine undergoes a two-electron oxidation in dilute aqueous acid media. In 9 *N* sulfuric acid two successive one-electron oxidations, involving a stable semiquinone free radical intermediate, are identifiable. Polarographic studies with a rotating platinum electrode confirm the occurrence of two separate one-electron oxidation steps in 9 *N* sulfuric acid. The coulometrically determined *n* values are in agreement with these observations. Reaction mechanisms are proposed for the electrolytic oxidation of chlorpromazine under various conditions of acidity. Spectral absorbance curves are presented as supporting evidence for the indicated reaction mechanisms.

THE OXIDATION of chlorpromazine hydrochloride[1] by cerium (IV), iron (III), permanganate, and hydrogen peroxide as well as by photo-irradiation has been reported (1–3). It has also been shown that the intermediate species in the oxidation of chlorpromazine exists as a free radical (4). More recently, the existence of stable semiquinone free radicals of chlorpromazine and other *N*-substituted phenothiazine derivatives has been demonostrated (5). However, because of the inherent difficulties in the quantitative determination of the free radical species, their study has been restricted considerably.

This study was undertaken to establish an oxidation mechanism for chlorpromazine hydrochloride in aqueous media. Since chlorpromazine and its oxidation products are electrolytically active, controlled-potential coulometry offered a direct approach for this study. The oxidation at a platinum anode has proved to be an extremely useful method for the production of the intermediate free radical species. Moreover, this study indicates the possible use of controlled-potential coulometry as a quantitative analytical technique for chlorpromazine and its oxidized forms in the presence of one another.

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[1] Chlorpromazine hydrochloride and chlorpromazine 5-oxide were supplied by Smith Kline and French Laboratories, Philadelphia, Pa.

INSTRUMENTATION

An electronic controlled-potential coulometric titrator, model Q-2005 ORNL, was used to perform the electrolyses (6). Oxidations were performed in a cell designed to accept a cylindrical wire mesh rotating platinum electrode (1 in. diameter × 2 in. height) and approximately 100 ml. of solution. The reference (S.C.E.) and auxiliary (working cathode) electrodes were separated from the sample solution by agar plugs and fritted glass diaphragms. A triplet vacuum tube voltmeter, model 805, with a 7-in. scale served as the readout device.[2] Polarograms were obtained on the Sargent model XXI recording polarograph. An H-type cell was used with a rotating platinum microelectrode. A saturated calomel reference electrode was separated from the sample portion by an agar plug and fritted-glass diaphragm.

PROCEDURE AND RESULTS

Controlled Potential Data.—When chlorpromazine hydrochloride is electrolyzed at a potential of approximately +0.70 v. *versus* S.C.E. in 1 *N* sulfuric acid under an atmosphere of nitrogen, the solution gradually assumes a deep red. As the electrolysis is

[2] In the latter stages of this study readout voltages were measured with a Non-Linear Systems model 484 A digital voltmeter.

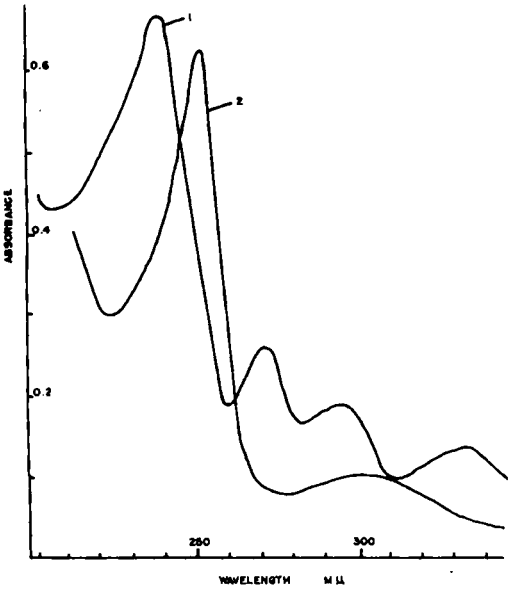


Fig. 1.—Ultraviolet spectral absorbance curves in 1 *N* sulfuric acid. Key: curve 1, chlorpromazine 5-oxide generated electrolytically from chlorpromazine; curve 2, chlorpromazine hydrochloride.

continued beyond this point, the solution becomes progressively lighter. When the oxidation is complete, as indicated by a negligible background current, the solution is again colorless. The final colorless oxidation product was shown to be chlorpromazine 5-oxide by comparison of its ultraviolet absorption spectrum with that of the pure material, Fig. 1. Equation 1 depicts a proposed oxidation of chlorpromazine to chlorpromazine 5-oxide *via* the colored free radical intermediate

$$\text{CLP} \rightarrow \text{CLP}^* \rightarrow \text{CLP-O} + 2e^- \quad \text{(Eq. 1)}$$

where CLP represents chlorpromazine, CLP-O represents chlorpromazine 5-oxide, and CLP* represents the colored free radical intermediate. The current-time integrals for a series of oxidations indicates an *n* value of 2.0, Table I.

When the electrolysis is interrupted at some point before completion and allowed to stand for 1000 to 2000 seconds under a nitrogen atmosphere, the red slowly fades. However, if the electrolysis is resumed, the red again intensifies. This process could be repeated many times during the course of a single electrolysis. The initial electrolysis current observed after standing is significantly larger than the current observed at the time when the electrolysis was interrupted.

These observations indicate that the unstable red free radical intermediate undergoes disproportionation, producing an electrolytically oxidizible species. Equation 2 is proposed for this disproportionation

$$\underset{\text{(dark red)}}{2\,\text{CLP}^*} \rightarrow \underset{\text{(colorless)}}{\text{CLP}} + \underset{\text{(colorless)}}{\text{CLP-O}} \quad \text{(Eq. 2)}$$

The changes observed in the ultraviolet spectrum of the electrolytically generated free radical support this reaction. Samples of chlorpromazine were oxidized electrolytically to maximum color (a dark red solution). The solution was then allowed to stand under nitrogen until the color faded. The ultraviolet spectrum of this solution was identical to that of an equimolar mixture of chlorpromazine and chlorpromazine 5-oxide, Fig. 2. Moreover, comparison of the ultraviolet spectra of the electrolytically generated free radical and that obtained using crystalline free radical, prepared as described in a previous report (7), indicate they are the same species.

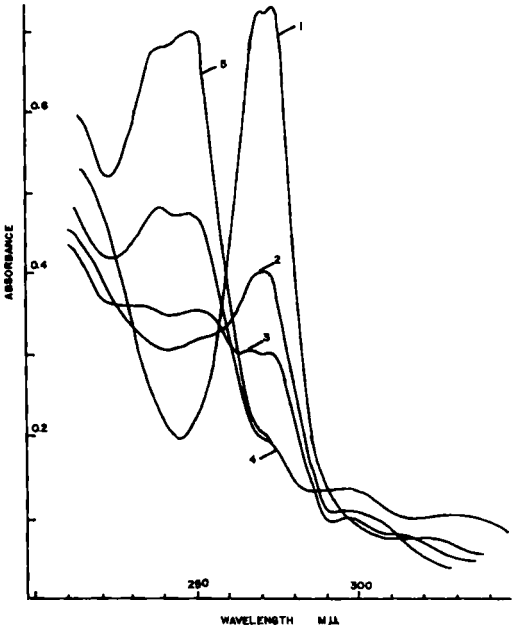


Fig. 2.—Ultraviolet spectra demonstrating the free radical disproportionation in 1 *N* sulfuric acid of the electrolytically generated product. Key: curve 1, spectrum immediately after generation; curve 2, spectrum after 15 minutes standing; curve 3, spectrum after 1 hour standing; curve 4, spectrum after 5 hours standing; curve 5, spectral curve of a synthetic equimolar mixture of chlorpromazine and chlorpromazine 5-oxide.

TABLE I.—*n* VALUES OBTAINED BY CONTROLLED-POTENTIAL ELECTROLYSES

Sample	Moles Taken, $\times 10^{-4}$	Supporting Electrolyte, H_2SO_4	Conditions	E *vs.* S.C.E.	*n* Values
CLP HCl[a]	1.407	1 *N*	Oxidation[c]	+0.7	1.96
CLP HCl[a]	1.407	9 *N*	Oxidation[c]	+0.5	0.98
CLP*	1.407	9 *N*	Oxidation[c]	+1.0	1.00
CLP HCl[a]	1.407	9 *N*	Oxidation[c]	+1.0	2.00
CLP*	1.407	9 *N*	Reduction[c]	+0.2	0.98
CLP-O HCl[b]	1.346	1 *N*	Reduction[d]	−1.0	1.92

[a] Based on a molecular weight of 355.33. [b] Based on a molecular weight of 371.33. [c] Rotating platinum gauze electrode. [d] Mercury pool electrode.

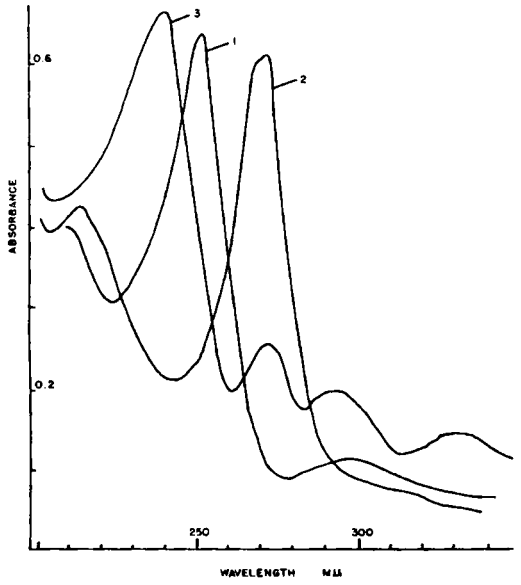


Fig. 3.—The ultraviolet spectral absorbance curves in 9 *N* sulfuric acid. Key: curve 1, chlorpromazine hydrochloride; curve 2, electrolytically produced free radical; curve 3, electrolytically produced chlorpromazine 5-oxide.

The complicating disproportionation of the free radical intermediate could be repressed by using 9 *N* sulfuric acid as the supporting electrolyte. A one-electron oxidation step could be isolated when electrolyzed at a potential of +0.50 v. *versus* S.C.E. (see Eq. 3). If after complete oxidation to the free

$$CLP \rightarrow CLP^* + e^- \qquad \text{(Eq. 3)}$$

radical, as indicated by negligible residual current, the potential of the working electrode was increased to approximately +1.0 v. *versus* S.C.E., the oxidation of the free radical species proceeds by a second one-electron step to the corresponding 5-oxide (see Eq. 4). If instead the potential of the working

$$CLP^* \rightarrow CLP\text{-}O + e^- \qquad \text{(Eq. 4)}$$

platinum electrode was lowered to approximately +0.2 v. *versus* S.C.E., the complete reduction of the free radical (CLP*) was possible (see Eq. 5).

$$CLP^* + e^- \rightarrow CLP \qquad \text{(Eq. 5)}$$

The coulometrically determined *n* values for the reactions, represented by Eqs. 3 and 4, show that each is a single-electron oxidation step, while the reaction represented by Eq. 5 indicates a one-electron reduction (Table 1). Ultraviolet spectral absorbance curves confirm the selective oxidation or reduction of the electrolytically produced free radical to chlorpromazine 5-oxide and chlorpromazine, respectively (Fig. 3).

In contrast to the two single oxidation steps occurring in 9 *N* sulfuric acid, the over-all reaction taking place in dilute aqueous acid media (1 *N* sulfuric acid) may now be represented

$$2\,CLP \xrightarrow{+0.7\text{ v.}} 2\,CLP^* + 2e^- \qquad \text{(Eq. 6)}$$

$$CLP \xleftarrow{\text{(spontaneous)}} \text{(from } 2\,CLP^*\text{)} \rightarrow CLP\text{-}O$$

The free radical, as produced by oxidation, undergoes spontaneous disproportionation to form chlorpromazine and chlorpromazine 5-oxide. This explains the *n* value of 2, observed when the electrolysis is conducted under these conditions.

Polarographic Data.—To demonstrate further the effect of the supporting electrolyte on the oxidation potentials of CLP and CLP*, polarographic studies were undertaken using a rotating platinum microelectrode. Polarograms obtained using 1 *N* sulfuric acid as the supporting electrolyte showed a single wave, with a $E_{1/2}$ of +0.6 v. *versus* S.C.E. When 9 *N* sulfuric acid was used, there appeared to be a separation of the single wave into two waves, with $E_{1/2}$ values of +0.37 and +0.95 v., respectively. The proposed reaction for the former is given by Eq. 3. That for the latter, somewhat less informative due to background current, is indicated by Eq. 4. The shift in half-wave potentials as a function of the supporting electrolyte is shown in Fig. 4.

Small additions of the crystalline free radical to solutions of chlorpromazine in 9 *N* sulfuric acid resulted in the appearance of a reduction wave with a $E_{1/2}$ of approximately +0.3 v. *versus* S.C.E. The second oxidation wave was more clearly defined as

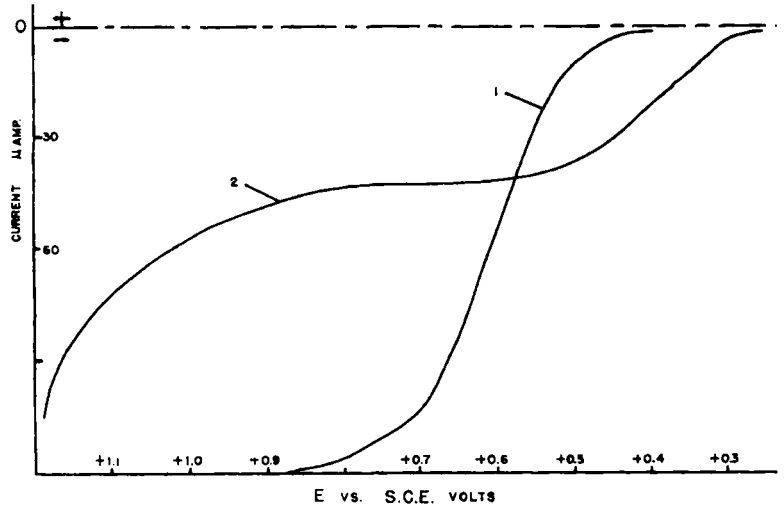


Fig. 4.—Polarograms obtained by the oxidation of chlorpromazine. Key: curve 1, 1 *N* sulfuric acid supporting electrolyte; curve 2, 9 *N* sulfuric acid supporting electrolyte.

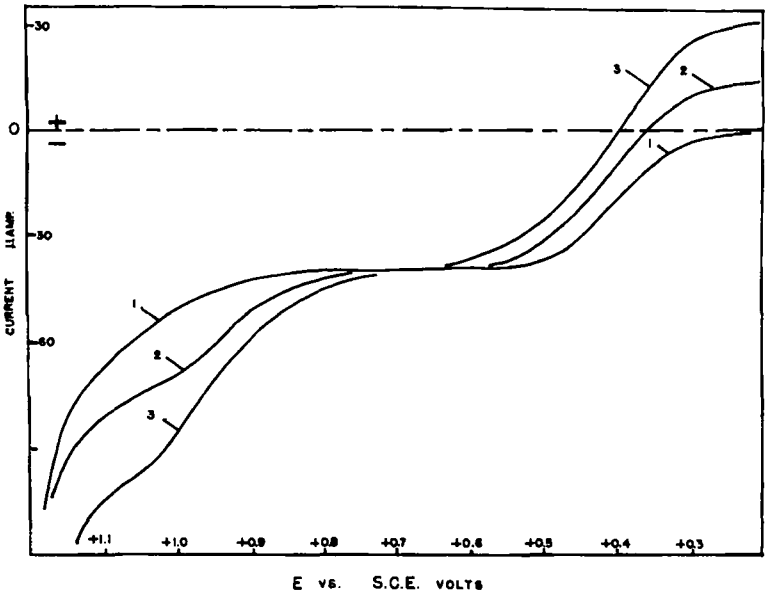


Fig. 5.—Polarograms demonstrating the effect of small additions of the solid free radical of chlorpromazine to the polarographic cell using 9 *N* sulfuric acid as the supporting electrolyte. Key: curve 1, no solid free radical added; curve 2, first addition of solid free radical; curve 3, second addition of solid free radical.

the concentration of added free radical was increased. This confirmed that the second wave was due to the oxidation of the free radical intermediate. The effect of addition of crystalline free radical is shown in Fig. 5.

SUMMARY

The coulometric oxidation of chlorpromazine in dilute aqueous acid solution was shown to proceed through a single-electron oxidation step to a colored free radical. This red free radical was shown to disproportionate rapidly to form chlorpromazine and chlorpromazine 5-oxide. This fact was verified by observing the changes in the ultraviolet absorption spectrum within a given period of time.

When chlorpromazine was electrooxidized in 9 *N* sulfuric acid, the free radical intermediate stabilized. Under these conditions it was possible to oxidize selectively chlorpromazine and chlorpromazine free radical, depending on the oxidation potentials selected. The reduction of chlorpromazine free radical was accomplished with equal facility.

Coulometrically determined *n* values are in complete agreement with the proposed oxidation-reduction reactions. Polarographic data demonstrated the effect of 9 *N* sulfuric acid in stabilizing the free radical intermediate, thereby enabling the selective oxidation or reduction of the species present.

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Synthesis of Some Tricyclic Analogs of Tetracycline

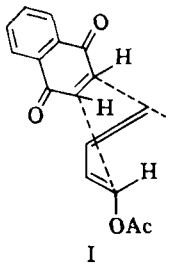
By HOWARD J. SCHAEFFER and GIRISH B. CHHEDA

Several tricyclic analogs of the tetracyclines were prepared through the Diels-Alder reaction. 1,4-Naphthoquinone was combined with 1-acetoxy-1,3-butadiene to give the adduct, 1-acetoxy-1,4,4a,9a-tetrahydroanthraquinone-9,10. Hydride reduction of the key adduct gave 1-acetoxy-1,4,4a,9,9a,10-hexahydro-9-hydroxy-10-ketoanthracene. This product, after several subsequent reactions, gave two isomeric 1,4,4a,-9,9a,10 - hexahydro - 9 - hydroxy - 9 - O - (2′ - tetrahydropyranyl) - 1,10 - dioxoanthracenes. The attempted Diels-Alder condensation of these isomers with 1-acetoxy-1,3-butadiene did not generate tetracyclic isomers. The synthesis and stereochemical assignment of these and other tricyclic compounds are described.

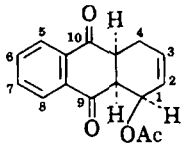
A NUMBER OF the attempted syntheses of linear tetracyclic compounds related to the naturally occurring antibiotics have employed multiple Claisen or Michael-type condensations for the construction of one or more rings of the desired tricyclic or tetracyclic compounds (1–4). It appeared that linear tetracyclic compounds could be synthesized from a properly activated intermediate by the Diels-Alder reaction. Therefore, using model compounds, we initiated a study of the synthesis of certain tricyclic and tetracyclic compounds in which the Diels-Alder reaction is employed.

The reaction of 1-acetoxy-1,3-butadiene (5) with 1,4-naphthoquinone was chosen as the first step in our study. The condensation of the diene and 1,4-naphthoquinone proceeded smoothly in refluxing benzene solution to give the adduct (II) in good yield (6, 7). The facile condensation of the diene with 1,4-naphthoquinone suggested that the diene must be predominantly *trans*, since it has been established that *cis*-1-substituted dienes are extremely sluggish in the Diels-Alder reaction and generally undergo polymerization (8–10).

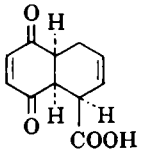
The spatial configuration of the adduct (II) has been assigned on the following grounds. On the basis of the well-known stereospecificity of the Diels-Alder reaction, we can assume that the newly formed ring in the adduct was *cis*-fused. The combination of the diene and quinone should proceed through the transition state (I) generally accepted for the Diels-Alder reaction, and thus would lead to an adduct with the stereochemistry as shown in II.[1] The steric orientation of the hydrogen at C_1 with respect to bridgehead hydrogens is capable of being derived from the classic "Alder-Stein Rules" (11). Few examples of similar steric situation can be cited. Woodward and his associates (12) have established that the adduct prepared from vinylacrylic acid and quinone has the stereochemistry shown in III.



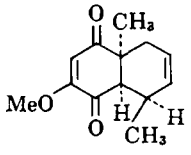
I



II

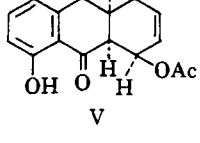


III



IV

Bloom (13) has proved that the stereochemistry of the adduct prepared from *p*-methoxytoluquinone and piperylene is correctly described by structure IV. Inhoffen and his associates (14) have made similar assumptions in assigning the stereochemistry of the adduct prepared from juglone[2] and 1-acetoxy-1,3-butadiene as shown in V.



V

From this knowledge we are most probably justified in assigning the stereochemical configurations of the three asymmetric centers in the adduct as shown in II.

Some preliminary studies with the key adduct (II) served to characterize its functions and gave valuable information about its reactivity in acidic and basic media. Treatment of the adduct with dilute solutions of methanolic ammonia or sodium

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Abstracted from a thesis submitted by G. B. Chheda to the Graduate School, State University of New York at Buffalo, in partial fulfillment of Doctor of Philosophy degree requirements.

[1] The chemical name for the adduct, according to the numbering shown in II, is 1-acetoxy-1,4,4a,9a-tetrahydroanthraquinone-9,10. This numbering of the tricyclic compounds is followed throughout the text.

[2] Juglone is 5-hydroxynaphthaquinone-1,4.

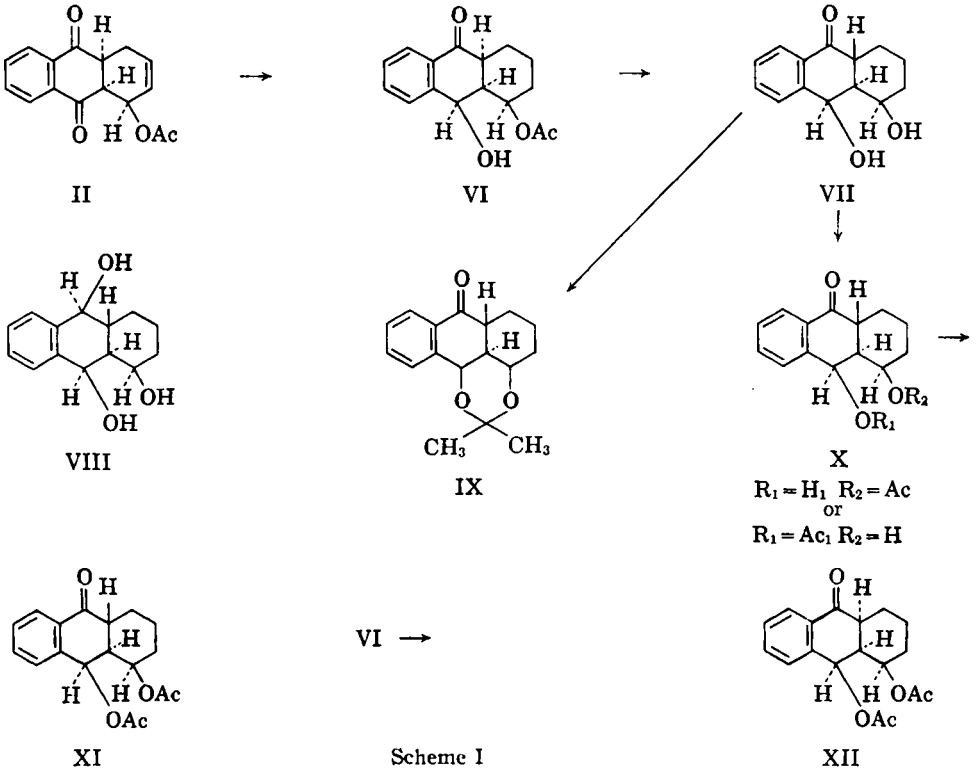
methoxide in methanol resulted in spontaneous elimination of acetic acid with concomitant oxidation of the alicyclic ring to give a quantitative yield of anthraquinone-9,10. These results establish that the acetate group of II cannot be removed until the activating effects of the keto groups have been removed. Therefore, a number of attempts were made to convert the ketone functions into their corresponding ketal by allowing II to react with ethyl orthoformate or with ethylene glycol catalyzed with *p*-toluenesulfonic acid or boron trifluoride; however, in all cases the only product which was isolated was anthraquinone-9,10.

Several attempts were made to convert the tricyclic adduct into a tetracyclic adduct by allowing II to react with an excess of 1-acetoxy-1,3-butadiene or 2,3-dimethyl-1,3-butadiene with and without a catalytic amount of aluminum chloride, but the only product which could be isolated was anthraquinone-9,10. Thus, it became apparent that a reduced form of adduct II would be more valuable for further synthetic studies.

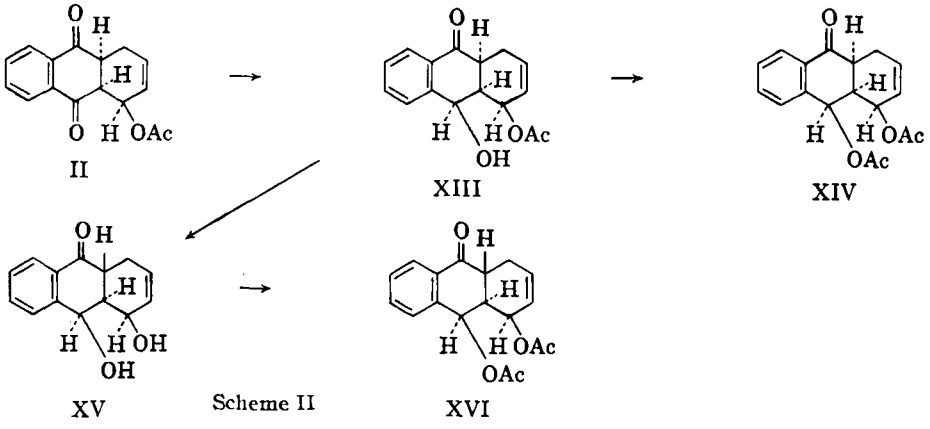
When II was hydrogenated using a palladium-on-charcoal catalyst, not more than 2 mol. equivalents of hydrogen could be introduced. A tetrahydro derivative (VI) of the adduct was obtained in a 45% yield, suggesting reduction of the double bond and of only one keto group. The infrared spectrum supported this data, in that it exhibited carbonyl absorption at 1690 cm.$^{-1}$ and an hydroxyl peak at 3450 cm.$^{-1}$; in addition, the ester carbonyl peak was lowered to 1715 cm.$^{-1}$ from 1740 cm.$^{-1}$ in the adduct (II). This lowering was probably due to the hydrogen bonding between the generated hydroxyl and the carbonyl group of the acetate, thus indicating that the reduction might have occurred at C_9. To establish that the reduced product had the structure VI, rather than the alternate possibility in which the hydrogenation had occurred at C_{10}, it was converted into a diol (VII) by treatment with a solution of methanolic ammonia. The limited solubility of the diol (VII) in boric acid solutions thwarted the study of changes in pH of boric acid solutions containing the diol (VII).

In addition to the indication from infrared spectra, certain preliminary chemical studies suggested that in the catalytic reduction of the adduct (II), the hydroxyl group was generated at C_9. To establish unequivocally that the hydroxyl group of acetoxy alcohol (VI) was located at C_9, we studied the preparation of an isopropylidene derivative in a model compound which definitely had hydroxyls at C_1 and C_9. The model compound was the triol (VIII) which was readily prepared in good yields by catalytic reduction of the diol (VII) with a rhodium-on-alumina catalyst. When the triol was stirred in an acetone solution containing anhydrous copper sulfate and a catalytic amount of sulfuric acid, an excellent yield of the desired isopropylidene derivative was obtained. Similarly, when the diol (VII) was stirred with acetone using a method identical to that for the model compound, a good yield of the isopropylidene derivative (IX) of the diol was obtained. The infrared spectra of the isopropylidene derivatives exhibited characteristic absorption at 1380 cm.$^{-1}$ for *gem*-dimethyl group. These results established that the structure VII was correct for the diol and that the acetoxy alcohol obtained by catalytic reduction of the adduct II had structure VI.

We found that during the alkaline methanolysis of acetoxy alcohol VI the *cis*-ring juncture was equili-



Scheme I



Scheme II

brated to *trans*. This was demonstrated as follows. The diol VII, when treated with one equivalent of acetic anhydride in pyridine gave a monoacetate (X) which melted at 145–146°. The infrared spectrum supported its gross structure since it exhibited peaks at 3490, 1735, and 1660 cm.$^{-1}$. The monoacetate (X) was thus clearly different from the acetoxy alcohol (VI) obtained from the adduct (II). The monoacetate (X) was reacetylated with excess acetic anhydride in pyridine; a diacetate (XI) was obtained, m.p. 172°. Similarly, when acetoxy alcohol (VI) was acetylated, a different diacetate (XII) was obtained, m.p. 158°. The infrared spectra of these two diacetates showed peaks for acetate and carbonyl groups but were not identical. The production of two different diacetates can be rationalized if it is assumed that during the removal of the acetate, the *cis*-ring juncture of VI underwent isomerization to *trans*. Parenthetically, it was found that the methanolysis of acetoxy alcohol (VI) by sodium methoxide gave the same isomerized diol (VII).

The introduction of asymmetry at C_9 in VI through catalytic reduction necessitates the assignment of configuration of this center relative to the configuration at the other asymmetric centers. Most probably the attack during catalytic reduction would occur on the convex face of the adduct (II), and thus the stereochemical orientation of the asymmetric centers in the acetoxy alcohol (VI) would be as depicted in VI.

Methanolysis of acetoxy alcohol (VI) by methanolic ammonia resulted in isomerization of position C_{4a}. Hence, the stereochemistry of the diol would be as assigned in formula VII. Because of this isomerization, the structure X for monoacetate is questionable with respect to the relative positions of acetate and hydroxy group. However, the stereochemistry of monoacetate would remain as shown in formula X. Stereochemically, the diacetates XI and XII differ at positions C_{4a}, XI being *trans*-fused and XII being *cis*-fused.

The stereochemical assignments of the triol (VIII) follow from that of the diol (VII), with the assumption that the attack during the reduction of C_{10} would occur on the α-face of the diol. Similarly, the steric orientations of the isopropylidene diol IX and the isopropylidene triol are capable of being derived from the parent compounds. The formation of six-membered isopropylidene derivatives from the diol and the triol besides proving the relative positions of hydroxyls offers support to the assignment of the *cis* configuration to the C_1 and C_9 hydroxyls.

After observing the results of the catalytic hydrogenation of the adduct (II), we became interested in investigating the chemical reduction of II. When the adduct was allowed to react with an excess of lithium aluminium hydride, two products were obtained: (*a*) an acetoxy alcohol (XIII) and (*b*) a product whose elemental analysis indicated that it was a diol.[3] The acetoxy alcohol (XIII) exhibited a peak at 1715 cm.$^{-1}$ for ester carbonyl and at 1690 cm.$^{-1}$ for a ketone. The position of acetate absorption was again lowered due to hydrogen bonding as it was in the hydroxy acetate (VI), suggesting that most probably the ketone which was in the vicinity of acetate has been reduced. To establish the position of the hydroxyl group in XIII, it was converted to the diol (XV) by treatment with methanolic ammonia. The diol (XV), when stirred in acetone containing copper sulfate and a catalytic amount of sulfuric acid, formed the isopropylidene derivative which exhibited infrared absorptions at 1380 cm.$^{-1}$ for the *gem*-dimethyl group, at 1690 cm.$^{-1}$ for carbonyl, and at 1660 cm.$^{-1}$ for a double bond. The formation of the isopropylidene derivative thus established the structure of the reduction product as that shown in XIII.

The preparation of the acetoxy alcohol (XIII) by lithium aluminum hydride reduction of II resulted in variable and frequently low yield of the desired product. Therefore, other methods of reduction of II were sought. After several variations, it was found that sodium borohydride reduction of II gave the acetoxy alcohol (XIII) in 65% yield.

For stereochemical studies, the alcohol (XIII) and the diol (XV) were allowed to react with an excess acetic anhydride in pyridine. The alcohol XIII gave a diacetate (XIV), m.p. 182°, and showed the expected peaks at 1735 cm.$^{-1}$ for ester carbonyl, at 1690 cm.$^{-1}$ for a ketone, and 1650 cm.$^{-1}$ for a double bond. However, the diacetate (XVI) obtained from the diol (XV) melted at 160–161° and exhibited the corresponding peaks at 1745, 1680, and 1660 cm.$^{-1}$ in its infrared spectrum. These results indicate that alcohol XIII underwent

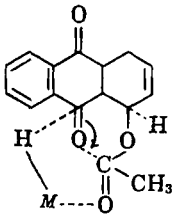
[3] Since we were interested mainly in the acetoxy alcohol (XIII), extensive structural work was not carried out on the diol. It was shown, however, that the diol was not merely deacetylated XIII (see text for structural studies on XIII).

an isomerization from a *cis*-ring juncture to a *trans* one during the treatment with methanolic ammonia in preparation of the diol (XV). That XIII had a *cis*-ring juncture was shown by the fact that catalytic hydrogenation of the double bond in XIII gave VI in excellent yields.

The assignment of the stereochemistry of the hydroxyl at C_9 in XIII is based on the following considerations. It has been shown by Beyler and Sarett (15) that reduction of *cis*-1,4-diketodecalin with lithium aluminum hydride gives only that diol in which the hydroxyl groups are *trans* to the bridgehead hydrogen atoms. Furthermore, it can be assumed from Cram's principle of asymmetric reduction (16) that the attacking group would enter from the least hindered side of the molecule. These data permitted us to assign the stereochemistry of the product obtained by the lithium aluminum hydride reduction of the adduct (II) as that shown in XIII. Inhoffen and his associates have also shown that the ring junctures remain *cis* after lithium aluminum hydride reduction of similar type of compounds (14).

The stereochemistry of the diol (XV) has been assigned as shown in XV from its progenitor XIII, which isomerizes at C_{4a} during ester hydrolysis. Thus, the diacetates XIV and XVI should be epimers at C_{4a}, diacetate XIV being *cis*-fused. The formation of an isopropylidene derivative from diol (XV) supported the *cis* orientation of C_1 and C_9 hydroxyls. In addition, XV on catalytic hydrogenation of the double bond was converted into VII, thereby supporting the assignment of the *trans*-ring juncture in XV.

The results of catalytic as well as chemical reduction of the adduct II indicate that the C_9 carbonyl is far more reactive than the C_{10} carbonyl, in spite of its being partially shielded by the C_1 acetoxy group. These observations prompt us to suggest that there may be participation by a neighboring acetoxy group in these reductions. For example, in the case of reductions with metal hydrides the adduct (II) may pass through the transition state



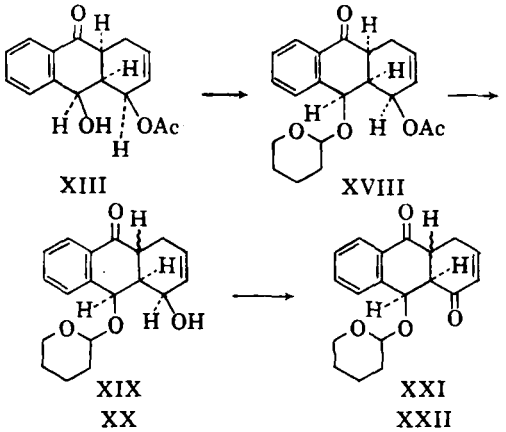
XVII

XVII wherein a six-membered ring may be formed. Inhoffen and his colleagues (14) have observed similar cases in the Grignard reactions of the quinone adducts with 1-acetoxy-1,3-butadiene.

For the attempted synthesis of tetracyclic compounds, the acetoxy alcohol (XIII) was a key intermediate, since it is substituted in a manner that ring C could be converted into a dienophile. As a preliminary study for preparing tetracyclic compounds, we attempted to condense 1-acetoxy-1,3-butadiene with the acetoxy alcohol (XIII); however, only starting material was recovered. The use of aluminum chloride to catalyze this condensation did not give better results. To carry out the reactions contemplated for the construction of a suitable intermediate which could serve as a good dienophile in Diels-Alder reaction, it became necessary to block the free hydroxyl group of acetoxy alcohol (XIII). When a slurry of acetoxy alcohol in anhydrous ether was stirred with 5 mol. equivalents of dihydropyran using *p*-toluenesulfonic acid as a catalyst, excellent yields of blocked material (XVIII) were obtained. Our choice of pyran as a blocking group was based on the fact that it would be stable if basic media, but easily removed in acid. The blocked ester (XVIII), as expected, did not show absorption for hydroxyl group in infrared spectrum, but exhibited other predicted absorptions for an ester, ketone, and ether. As expected, when XVIII was allowed to react with lithium aluminum hydride, the keto group at C_{10} and the acetoxy group at C_1 were reduced to alcohol functions.

When the pyranylated ester (XVIII) was hydrolyzed with 1 mol. equivalent of sodium hydroxide, two isomeric alcohols were obtained in almost equal yields (XIX and XX). The combined yield of the two isomers was about 40%. The residual syrup contained more of the low melting isomer (XX), as shown by infrared and oxidation studies, but it was difficult to separate by crystallization. The use of sodium methoxide and potassium hydroxide did not increase the yields of alcohols. Methanolic ammonia and *n*-butylamine did not remove the ester.

Isomerization studies were done in an attempt to determine the stereochemical differences of these two alcohols. When a methanol solution of the alcohol XIX was equilibrated in presence of 1 mol. equivalent of sodium hydroxide at reflux temperatures, 84% of the unchanged alcohol XIX was recovered. In a similar experiment on the isomer XX the recovery of unchanged alcohol was very low. These experiments, though inconclusive, indicate that the high melting isomer XIX may be *trans*-fused and the isomer XX may be *cis*-fused.



XIII XVIII

XIX XXI

XX XXII

It was necessary to oxidize the free hydroxyl to obtain a dienophile from these alcohols. When the high melting alcohol (XIX) was stirred with a chromium trioxide–pyridine complex in pyridine solution, a crystalline oxidation product (XXI) was obtained in moderate yield. The product melted at 113° and exhibited infrared absorption at 1680 $cm.^{-1}$ for carbonyl, 1620 $cm.^{-1}$ for a double bond, and 1135 $cm.^{-1}$ for ether. When the low melting alcohol (XX) was oxidized by a similar procedure, a different oxidation product (XXII) was obtained. This product has a melting point of 146–147°, and

it displayed infrared absorptions at 1690, 1675 cm.$^{-1}$ for carbonyl, and 1120 cm.$^{-1}$ for ether. The formation of two different oxidation products (XXI and XXII) from the two isomeric alcohols (XIX and XX) suggests that the alcohols as well as the oxidation products differ most probably in ring juncture.

Attempted Diels-Alder reaction of XXI with 1-acetoxy-1,3-butadiene under a wide variety of reaction conditions did not give the corresponding tetracyclic compound; a product which was formed in low yield in this reaction had an empirical formula of $C_{19}H_{20}O_4$ and appears to be a stereoisomer of the starting material (XXI). The attempted Diels-Alder reaction of XXII and 1-acetoxy-1,3-butadiene resulted in decomposition if high temperatures were employed, and the desired product could not be isolated. At low temperatures no reaction occurred. Thus, neither XXI nor XXII served as a useful dienophile in the Diels-Alder reaction.

EXPERIMENTAL[4]

1 - Acetoxy - 1,2,3,4,4a,9,9a,10 - octahydro - 9-hydroxy-10-ketoanthracene (VI).—A solution of 2.70 Gm. (10.0 mmoles) of II (6) in 200 ml. of ethanol was hydrogenated using 0.270 Gm. of a 5% palladium-on-charcoal catalyst in a Parr hydrogenator at an initial pressure of 38.8 p.s.i. Hydrogenation was continued until no more hydrogen was absorbed (19.2 mmoles). The catalyst was removed by filtration; the pale yellow filtrate was decolorized with charcoal and evaporated *in vacuo*. The syrupy residue was crystallized from a mixture of benzene-hexane and gave 1.26 Gm. (45.9%) of the desired product, m.p. 126°. After three recrystallizations of the crude material from a mixture of benzene-hexane, the analytically pure material was obtained, m.p. 130–131°. γ in cm.$^{-1}$ (KBr): 3450 (OH); 1715 (C=O acetate); 1690 (C=O). $\lambda_{max.}^{ethanol}$ 249.5 mμ, ($\epsilon \times 10^{-3}$) 7.06.

Anal.[5]—Calcd. for $C_{16}H_{18}O_4$: C, 70.04; H, 6.61. Found: C, 70.18; H, 6.64.

1,2,3,4,4a,9,9a,10 - Octahydro - 1,9 - dihydroxy-10-ketoanthracene (VII).—To a solution of 5.49 Gm. (20.0 mmoles) of VI in 50 ml. of methanol was added 100 ml. of 3.7% (w/v) ammonia in methanol. The mixture was allowed to stand at room temperature for 40 hours. The pink solution was decolorized with charcoal; the filtrate was evaporated under reduced pressure and gave 4.13 Gm. of crystalline solid. After crystallization from methanol, the crude product gave 2.12 Gm. of the desired product, m.p. 163°. Concentration of the mother liquor gave an additional 1.08 Gm. of the same material, m.p. 163°; yield, 3.20 Gm. (70.0%). After three recrystallizations of the crude material from methanol, the pure sample was obtained, m.p. 162–163°. γ in cm.$^{-1}$ (KBr): 3250 (OH); 1685 (C=O); 1600 (aromatic). $\lambda_{max.}^{ethanol}$ 247 mμ, ($\epsilon \times 10^{-3}$) 12.2.

Anal.—Calcd. for $C_{14}H_{16}O_3$: C, 72.29; H, 6.94. Found: C, 72.64; H, 7.06.

1,2,3,4,4a,9,9a,10 - Octahydro - 1,9,10 - trihydroxyanthracene (VIII).—A solution of 1.16 Gm. (5.00 mmoles) of VII in 200 ml. of ethanol was hydrogenated using 0.116 Gm. of rhodium-on-alumina catalyst in a Parr hydrogenator at an initial pressure of 50 p.s.i. The uptake of hydrogen stopped when 5.05 mmoles has been absorbed. The catalyst was removed by filtration, and the colorless filtrate was evaporated *in vacuo*. The white crystalline residue was recrystallized from a mixture of methanol-water and gave 0.782 Gm. of the desired product, m.p. 221°. Concentration of the mother liquor gave an additional 92 mg. of the desired product, m.p. 220°. The yield was 0.874 Gm. (74.6%).

Three recrystallizations of the crude product from methanol-water gave the analytical material, m.p. 220–221°. γ in cm.$^{-1}$ (KBr): 3200–3250 (OH); 2925 (CH).

Anal.—Calcd. for $C_{14}H_{18}O_3$: C, 71.79; H, 7.75; Found: C, 71.67; H, 7.82.

Isopropylidene Derivative of 1,2,3,4,4a,9,9a,10-Octahydro-1,9,10-trihydroxyanthracene.—To a solution of 0.117 Gm. (0.499 mmole) of VIII in 55 ml. of acetone were added 1 drop of sulfuric acid and 200 mg. of anhydrous copper sulfate. The reaction mixture was stirred for 72 hours at room temperature. After removing the copper sulfate by filtration, the filtrate was concentrated *in vacuo* at 20° to about half of the original volume. The concentrate was poured into 25 ml. of cold 2% sodium bicarbonate solution and refrigerated overnight. The white crystalline product was isolated by filtration, 0.070 Gm. (51.2%), m.p. 162–163°. The mother liquor was extracted with ether which, after drying over magnesium sulfate, then evaporation *in vacuo* yielded 0.028 Gm. of the crystalline material, m.p. 162–163°. The total yield was 0.098 Gm. (71.5%).

After three recrystallizations of the crude product from ethanol-water the analytically pure product was obtained, m.p. 162°. γ in cm.$^{-1}$ (KBr): 3500 (OH); 1385 (*gem*-dimethyl); 1110 (C—O—C).

Anal.—Calcd. for $C_{17}H_{22}O_3$: C, 74.42; H, 8.08. Found: C, 74.47; H, 8.14.

Isopropylidene Derivative of 1,2,3,4,4a,9,9a,10-Octahydro-1,9-dihydroxy-10-ketoanthracene (IX).—To a solution of 0.232 Gm. (1.00 mmole) of VII in 15 ml. of acetone were added 1 drop of sulfuric acid and 0.065 Gm. of anhydrous copper sulfate; the reaction mixture was stirred for 66 hours at room temperature. After removing the copper sulfate by filtration, the filtrate was poured into 15 ml. of cold 2% sodium bicarbonate solution and refrigerated for 1 hour. The white crystalline product was isolated by filtration: yield, 0.178 Gm. (64.9%), m.p. 155°. The mother liquor was extracted with ether which was then evaporated *in vacuo*. Recrystallization of the solid residue from ethanol-water yielded 0.024 Gm., m.p. 153°. The yield was 0.202 Gm. (73.7%) of the desired material.

Recrystallization of the crude material from ethanol-water gave the analytical sample, m.p. 156°. γ in cm.$^{-1}$ (KBr): 1685 (C=O); 1600 (aromatic); 1380 (*gem*-dimethyl); 1105 (C—O—C).

Anal.—Calcd. for $C_{17}H_{20}O_3$: C, 74.97; H, 7.40. Found: C, 75.11; H, 7.49.

iso - 1(9) - Acetoxy - 1,2,3,4,4a,9,9a,10 - octahydro-9(1) - hydroxy - 10 - ketoanthracene (X).—To a solution of 2.32 Gm. (10.0 mmoles) of VII in 25 ml. of pyridine was added 0.992 ml. (1.07 Gm., 1.05 mmoles) of acetic anhydride. The reaction mixture

[4] The ultraviolet spectra were determined on a Perkin-Elmer model 4000 A spectrophotometer; the infrared spectra were determined on a Perkin-Elmer 137 spectrophotometer. The melting points were determined on a Kofler Heizbank and are corrected.

[5] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

was allowed to stand at room temperature for 47 hours and then heated under reflux for 20 minutes. The reaction solution was poured into ice-water and was left in a refrigerator for 40 hours. The desired monoacetate crystallized and was isolated by filtration; yield, 1.43 Gm. (52.2%), m.p. 137–138°.

Two recrystallizations of the crude product from benzene-hexane raised the melting point to 145–146°. γ in cm.$^{-1}$ (KBr): 3490 (OH); 1735 (C=O ester); 1660 (C=O); 1600 (aromatic); 1225 (C—O—ester).

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.04; H, 6.61. Found: C, 70.68; H, 6.69.

iso - 1,9 - Diacetoxy - 1,2,3,4,4a,9,9a,10 - octahydro-10-ketoanthracene (XI) (Equilibrated).—To a solution of 0.274 Gm. (1.00 mmole) of X in 2 ml. of pyridine was added 0.3 ml. of acetic anhydride. The reaction mixture was allowed to stand at room temperature for 1 day, then poured into ice-water. After refrigeration for 2 hours, the white crystalline product was isolated by filtration; 0.294 Gm. (93.0%), m.p. 174°. Recrystallization of this material from benzene-hexane gave 0.220 Gm. (69.5%) of the desired product, m.p. 174°. Concentration of the mother liquor gave an additional 0.030 Gm. of the same material, m.p. 174°. The yield was 0.250 Gm. (79.2%).

Two recrystallizations of the crude product from ethanol gave the analytical material, m.p. 172°. γ in cm.$^{-1}$ (KBr): 1735 (C=O ester); 1680 (C=O); 1600 (phenyl); 1230 (C—O ester).

Anal.—Calcd. for $C_{18}H_{20}O_5$: C, 68.33; H, 6.37. Found: C, 68.04; H, 6.41.

1,9 - Diacetoxy - 1,2,3,4,4a,9,9a,10 - octahydro-10-ketoanthracene (XII) (Nonequilibrated).—To a solution of 0.274 Gm. (1.00 mmole) of VI in 2 ml. of pyridine was added 0.3 ml. of acetic anhydride. The reaction mixture was allowed to stand at room temperature for 1 day, then poured into ice-water. After refrigeration for 2 hours, the white crystalline product was isolated by filtration (0.291 Gm.), m.p. 148°. After recrystallization of the crude product, 0.150 Gm. (m.p. 159°) of the desired product was obtained. The filtrate, after concentration, gave 0.030 Gm. of the desired material, m.p. 155°. The yield was 0.180 Gm. (56.5%).

After two recrystallizations of the crude material from ethanol, the pure product was obtained, m.p. 158–159°. γ in cm.$^{-1}$ (KBr): 1740 (C=O ester); 1675 (C=O); 1600 (aromatic); 1225 (C—O—ester). $\lambda_{max.}^{ethanol}$ 247.5; ($\epsilon \times 10^{-3}$), 11.0.

Anal.—Calcd. for $C_{18}H_{20}O_5$: C, 68.33; H, 6.37. Found: C, 68.35; H, 6.49.

1 - Acetoxy - 1,4,4a,9,9a,10 - hexahydro - 9 - hydroxy-10-ketoanthracene (XIII).—To a stirred solution of 0.283 Gm. (7.50 mmoles) of sodium borohydride in 100 ml. of 95% ethanol was added with stirring over a period of 4 to 5 minutes a warm solution (at 40°) of 4.05 Gm. (15.0 mmoles) of II in 160 ml. of 95% ethanol. The reaction mixture was then stirred for 2 hours at room temperature. After cooling the reaction mixture in an ice bath, about 20 Gm. of ice and 60 ml. of water were added. After 5 minutes of additional stirring, 15 ml. of 5% hydrochloric acid was added; the whole reaction mixture was stirred for 15 minutes. The mixture was then cooled and filtered to remove the insoluble anthraquinone, 0.490 Gm. (15.7%). Concentration of the filtrate to a volume of about 80 ml. caused the crystallization of the desired product which was isolated by filtration; yield, 2.65 Gm. (65.0%) of XIII, m.p. 146–147°.

Two recrystallizations of the crude material from benzene-hexane gave the pure product, m.p. 149–150°. γ in cm.$^{-1}$ (KBr): 3490 (OH); 1715 (C=O, ester); 1690 (C=O); 1650 (C=C, sh); 1600 (aromatic); 1240 (C—O—ester). $\lambda_{max.}^{ethanol}$ 250, ($\epsilon \times 10^{-3}$) 9.09. TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{16}H_{14}O_4$: C, 70.57; H, 5.92. Found: C, 70.37; H, 6.12.

Lithium Aluminum Hydride Reduction of 1-Acetoxy-1,4,4a,9a-tetrahydroanthraquinone-9,10.—To a refluxing solution of 8.11 Gm. (30.0 mmoles) of II in 470 ml. of ether was added with stirring a slurry of 1.13 Gm. (30.0 mmoles) of lithium aluminum hydride in 550 ml. of ether over a period of 20 minutes. At the end of 2.5 hours of refluxing with stirring, the reaction mixture was cooled and 200 ml. of cold water was added, followed by the addition of 55 ml. of 5% hydrochloric acid. The mixture was stirred for a few minutes and then separated; the aqueous layer was extracted with ether (6 × 100 ml.). The organic phase, combined with ethereal extracts, was washed with 5% sodium bicarbonate solution (3 × 20 ml.), dried over magnesium sulfate, then concentrated *in vacuo* to about 40 ml. The precipitate of the crystalline diol, *iso*-1,4,4a,9,9a,10-hexahydro-1,9-dihydroxy-10-ketoanthracene, was isolated by filtration; yield 1.93 Gm. (27.9%), m.p. 163–164°.

The mother liquor gave, after concentration and cooling, 0.966 Gm. (11.8%) of XIII; m.p. 147–148°.

Two recrystallizations of the diol from methanol gave the pure product, m.p. 165–166°. γ cm.$^{-1}$ (KBr): 3470, 3325 (OH); 1665 (C=O); 1650 (C=C sh); 1600 (aromatic). TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{14}H_{14}O_3$: C, 73.02; H, 6.12. Found: C, 72.85; H, 6.19.

1,4,4a,9,9a,10 - Hexahydro - 1,9 - dihydroxy - 10-ketoanthracene (XV).—To a solution of 2.72 Gm. (10 mmoles) of XIII in 100 ml. of methanol was added 75 ml. of 3.7% (w/v) ammonia in methanol. After allowing the reaction mixture to stand at room temperature for 23 hours, the solution was filtered to remove 150 mg. of anthraquinone. The filtrate was evaporated *in vacuo;* the crude residue was dissolved in methanol, decolorized with charcoal, then allowed to crystallize from methanol. The desired product was isolated by filtration; yield, 1.43 Gm., m.p. 170°. The mother liquor after concentration gave 0.203 Gm. of the same diol, m.p. 168–169°; the total yield of the diol was 1.63 Gm. (70.8%).

After two recrystallizations of the crude product from methanol-water, the analytical sample was obtained, m.p. 171–172°. γ in cm.$^{-1}$ (KBr): 3390 (OH); 1685 (C=O), 1650 (C=C sh); 1600 (aromatic). $\lambda_{max.}^{ethanol}$ 250; ($\epsilon \times 10^{-3}$) 10.7. TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{14}H_{14}O_3$; C, 73.02; H, 6.13. Found: C, 72.96; H, 6.22.

Isopropylidene Derivative of 1,4,4a,9,9a,10-Hexahydro-1,9-dihydroxy-10-ketoanthracene.—To a solution of 231 mg. (1.00 mmole) of XV in 25 ml. of acetone was added 1 drop of concentrated sulfuric acid and 150 mg. of anhydrous copper sulfate. The reaction mixture was stirred for 66 hours at room

temperature. The copper sulfate was removed by filtration, the filtrate was poured into 20 ml. of cold 1% sodium bicarbonate solution. After cooling the solution, the crystalline white solid which separated was isolated by filtration; yield 111 mg., m.p. 154°. The filtrate was extracted with ether, which was then evaporated *in vacuo*. The residue, after two recrystallizations from ethanol-water yielded 26 mg. of the desired material, m.p. 151°. The total yield of the isopropylidene derivative was 137 mg. (51.0%).

Recrystallization of the crude material first from ethanol-water and then from acetone-water gave the analytical sample, m.p. 155–156°. γ in cm.$^{-1}$ (KBr): 1380, 1200, 773 (C-$(CH_3)_2$); 1690 (C=O); 1660 (C=C); 1600 (aromatic). $\lambda_{max.}^{ethanol}$ 247, ($\epsilon \times 10^{-3}$) 11.8.

Anal.—Calcd. for $C_{17}H_{18}O_3$; C, 75.53; H, 6.71. Found: C, 75.15; H, 7.01.

1,4,4a,9,9a,10 - Hexahydro - 1,9 - diacetoxy - 10-ketoanthracene (XIV).—To a solution of 504 mg. (1.85 mmoles) of XIII in 8 ml. of pyridine was added 2 ml. of acetic anhydride. The reaction mixture was allowed to stand at room temperature for 48 hours, then poured into 20 ml. of cold water. The mixture rapidly crystallized when placed in a cold room for 4 hours. The crystalline XIV was isolated by filtration; yield, 537 mg. (96.0%), m.p. 180°.

Recrystallization of the crude material from ethanol gave the analytical sample, m.p. 182°. γ in cm.$^{-1}$ (KBr): 1735 (C=O ester); 1690 (C=O); 1650 (C=C sh); 1260 (C—O— ester).

Anal.—Calcd. for $C_{18}H_{18}O_5$: C, 68.77; H, 5.77. Found: C, 68.93; H, 5.56.

iso - 1,4,4a,9,9a,10 - Hexahydro - 1,9 - diacetoxy-10-ketoanthracene (XVI).—To a solution of 224 mg. (0.974 mmole) of XV in 5 ml. of pyridine was added 1 ml. of acetic anhydride. The reaction mixture was allowed to stand at room temperature for 48 hours, then poured into 15 ml. of cold water. The solution, which crystallized immediately, was cooled in a refrigerator for a 4.5-hour period and filtered. A 241-mg. (79.0%) quantity of white needles of XVI, m.p. 160–161°, was obtained. Recrystallization of the crude product from ethanol did not change the melting point. γ in cm.$^{-1}$ (KBr): 1745 (C=O ester); 1680 (C=O); 1660 (C=C sh); 1225 (C—O— ester).

Anal.—Calcd. for $C_{18}H_{18}O_5$: C, 68.77; H, 5.77. Found: C, 68.86; H, 5.98.

1 - Acetoxy - 1,4,4a,9,9a,10 - hexahydro - 9 - hydroxy - 9 - O - (2′ - tetrahydropyranyl) - 10 - ketoanthracene (XVIII).—A mixture of 6.87 Gm. (0.025 mole) of XIII, 10.5 Gm. (0.125 mole) of dihydropyran, and 0.15 Gm. of *p*-toluenesulfonic acid in 25 ml. of anhydrous ether, was stirred for 5 hours at room temperature. At the end of 5 hours, the reaction was chilled in an ice-bath and the desired product was isolated by filtration, washed with ether, and dried *in vacuo* at room temperature; yield 4.83 Gm., m.p. 129°. The filtrate was washed with 5% sodium bicarbonate solution (2 × 5 ml.), then with water (2 × 5 ml.). The ethereal solution was then dried over magnesium sulfate and evaporated *in vacuo* to a thick syrup which crystallized after cooling. The crystalline product was isolated by filtration; 2.20 Gm., m.p. 120–122°. Recrystallization of this crude material (2.20 Gm.) gave 1.66 Gm. of desired product, m.p. 129–130° in three crops. Thus the total yield of XVIII was 6.49 Gm. (73.0%).

Three recrystallizations of the crude product from methanol-water gave the analytical sample, m.p. 130°. γ in cm.$^{-1}$ (KBr): 1750 (C=O ester); 1695 (C=O); 1655 (C=C sh); 1600 (aromatic); 1235 (C—O ester); 1075, 1030 (—C—O—C ether). TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{21}H_{24}O_5$: C, 70.76; H, 6.79. Found: C, 70.92; H, 6.97.

1,4,4a,9,9a,10 - Hexahydro - 1,9,10 - trihydroxy-9 - O - (2′ - tetrahydropyranyl)anthracene.—To a stirred suspension of 570 mg. (15.0 mmoles) of lithium aluminum hydride in 170 ml. of ether was added over a period of 2 minutes a solution of 3.56 Gm. (10.0 mmoles) of XVIII in 170 ml. of ether. After stirring for 8 hours at room temperature, the reaction mixture was chilled and 150 ml. of water was added. The mixture was stirred for 30 minutes, and 1% hydrochloric acid (100 ml.) was added; stirring was continued for an additional 15 minutes. The aqueous layer was separated from the ether phase and extracted with ether (3 × 100 ml.). The ether extracts were combined and washed with 2.5% sodium bicarbonate solution (2 × 40 ml.) and then with water (2 × 50 ml.). The ether was dried over magnesium sulfate and evaporated *in vacuo* to a syrupy residue (3.48 Gm.). The residue was triturated with ether which caused the crystals of a desired product to appear. Filtration of the mixture gave 1.03 Gm. (32.6%) of crystalline white material, m.p. 158–160°. For analysis a small sample was recrystallized first from benzene and then from methanol; m.p. 161–162°. γ in cm.$^{-1}$ (KBr): 3300 (broad) (OH); 1650 (C=C); 1010 (C—O—C ether).

Anal.—Calcd. for $C_{19}H_{24}O_4$: C, 72.12; H, 7.64. Found: C, 72.11; H, 7.41.

1,4,4a,9,9a,10 - Hexahydro - 1,9 - dihydroxy 9 - O - (2′ - tetrahydropyranyl) - 10 - ketoanthracene (XIX) and (XX).—To a solution of 10.7 Gm. (30.0 mmoles) of XVIII in 300 ml. of methanol was added a solution of 1.26 Gm. (31.5 mmoles) of sodium hydroxide in a mixture of 105 ml. of methanol and 45 ml. of water. The reaction solution was heated under reflux for 6 hours. The mixture was cooled and filtered to remove anthraquinone (60 mg.). The filtrate was diluted with water (400 ml.) and concentrated under reduced pressure to about 500 ml. which caused the crystallization of the yellow product, 5.51 Gm. melting over a wide range (122–128°). The filtrate was extracted with ether (5 × 200 ml.), and the combined extracts were washed with water (2 × 100 ml.). The ether was dried over magnesium sulfate and evaporated *in vacuo*. There was obtained 3.40 Gm. of a residual oil.

Recrystallization of 5.51 Gm. of the yellow product and crystallization of the syrup from methanol gave in two crops 1.60 Gm. (17.2%) of the high melting alcohol (XIX), m.p. 159–161°.

Several recrystallizations of the crude high melting alcohol (XIX) from methanol gave a pure product, m.p. 162–163°. γ in cm.$^{-1}$ (KBr): 3500 (OH); 1695 (C=O); 1660 (C=C sh); 1600 (aromatic); 1120, 1020 (C—O—C pyran). $\lambda_{max.}^{ethanol}$ 251, ($\epsilon \times 10^{-3}$) 10.7. TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{19}H_{22}O_4$: C, 72.58; H, 7.05. Found: C, 72.52; H, 7.07.

After separation of the above high melting isomer (XIX), the mother liquors, when allowed to crystallize further, gave 2.04 Gm. (21.5%) of another isomer (XX), m.p. 127–128°.

For analysis, a small sample of low melting alcohol (XX) was recrystallized five times from methanol. There was obtained a pure product, m.p. 128–129°. γ in cm.$^{-1}$ (KBr): 3500 (OH); 1680 (C=O); 1650 (C=C sh); 1040, 1060, 1120 (—C—O—C pyran); $\lambda_{max.}^{ethanol}$ 247, ($\epsilon \times 10^{-3}$) 11.0. TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{19}H_{22}O_4$: C, 72.58; H, 7.05. Found: C, 72.85; H, 7.30.

1,4,4a,9,9a,10 - Hexahydro - 9 - hydroxy - 9 - O-(2′ - tetrahydropyranyl) - 1, 10 - dioxoanthracene (XXI).—To a solution of 1.88 Gm. (6.00 mmoles) of XIX in 30 ml. of pyridine was added 61 ml. of solution of chromium trioxide-pyridine complex (17) in pyridine containing 1.19 Gm. (11.9 mmoles) of chromium trioxide (three times the required quantity). The dark brown reaction mixture was then stirred for 10 hours at room temperature. Ether (250 ml.) was added to the reaction mixture which was then stirred for 10 minutes. The precipitated red material was removed by filtration. The filtrate was washed with water (2 × 100 ml.), dried over magnesium sulfate, evaporated *in vacuo*, and gave 1.45 Gm. of residual syrup. The residue was dissolved in ethanol, treated with charcoal, and allowed to crystallize. There was obtained 1.01 Gm. (54.0%) of crystalline product, m.p. 108–110°. Two recrystallizations of the crude product from ethanol gave the pure product, m.p. 111–113° (oil bath). γ in cm.$^{-1}$ (KBr): 1680 (C=O); 1620 (C=C); 1600 (aromatic); 1250, 1135, 1035 (—C—O—C ether); $\lambda_{max.}^{ethanol}$ 235 (broad), ($\epsilon \times 10^{-3}$) 13.2. TLC (silica gel) ($CHCl_3$) one spot.

Anal.—Calcd. for $C_{19}H_{20}O_4$: C, 73.05; H, 6.45. Found: C, 73.27; H, 6.56.

A chloroform solution of 1,4,4a,9,9a,10-hexahydro-9-hydroxy-1,10-dioxoanthracene XXI was shaken with 5% hydrochloric acid and then evaporated *in vacuo* to a syrupy residue. Crystallization of the residue gave a 1,4,4a,9,9a-10-hexahydro-9-hydroxy-1,10-dioxoanthracene, m.p. 152–153°.

Recrystallization of this product from methanol gave the pure product, m.p. 155–156°. γ in cm.$^{-1}$ (KBr): 3525 (H); 1690 (CO=O); 1660 (C=O conjugated); 1600 (aromatic); 1620 (C=C sh).

Anal.—Calcd. for $C_{14}H_{12}O_3$: C, 73.67; H, 5.29. Found: C, 73.36; H, 5.44.

1,4,4a,9,9a,10 - Hexahydro - 9 - hydroxy - 9 - O-(2′ - tetrahydropyranyl) - 1, 10 - dioxoanthracene (XXII).—By an oxidation procedure similar to that used for XIX, 2.51 Gm. (8.00 mmoles) of XX gave 2.04 Gm. of a semisolid residue. Recrystallization of the residue from ethanol gave 1.11 Gm. (44.8%) of XXII, m.p. 144–145°. Two recrystallizations of the crude material from ethanol gave a sample for analysis, m.p. 146–147°. γ in cm.$^{-1}$ (KBr): 1690, 1675, (C=O). 1620 (C=C weak); 1600 (aromatic); 1300, 1120, 1030 (C—O—C ether); $\lambda_{max.}^{ethanol}$ 247, ($\epsilon \times 10^{-3}$) 11.2.

Anal.—Calcd. for $C_{19}H_{20}O_4$: C, 73.05; H, 6.45. Found: C, 73.18; H, 6.47.

Attempted Diels-Alder Reaction of 1,4,4a,9,9a,10-Hexahydro - 9 - hydroxy - 9 - O - (2′ - tetrahydropyranyl)-1,10-dioxoanthracene (XXI).—A mixture of 234 mg. (0.750 mmole) of XXI and 448 mg. (4.50 mmoles) of 1-acetoxy-1,3-butadiene in 20 ml. of benzene containing 10 mg. of hydroquinone was heated in a steel bomb at 130° for 66 hours. The cooled reaction mixture was evaporated *in vacuo*. The residue was triturated with cyclohexane, and a small amount of insoluble material was removed by filtration. Hexane was added to the filtrate which caused the separation of 12 mg. (3.5%) of a crystalline product, m.p. 137–140°. Two recrystallizations of the crude product from ether–petroleum ether gave the analytical sample which appears to be a stereoisomer of the starting material (XXI), m.p. 147–149°. γ in cm.$^{-1}$ (KBr): 1690 (C=O); 1650 (C=C sh); 1600 (aromatic).

Anal.—Calcd. for $C_{19}H_{20}O_4$: C, 73.05; H, 6.45. Found: C, 73.10; H, 6.61.

No further pure products could be obtained from the filtrate.

Conversion of 1-Acetoxy-1,4,4a,9,9a,10-hexahydro-9-hydroxy-10-ketoanthracene (XIII) into 1-Acetoxy - 1,2,3,4,4a,9,9a,10 - octahydro - 9 - hydroxy-10-ketoanthracene (VI).—A solution of 1.09 Gm. (4.00 mmoles) of XIII in 200 ml. of ethanol was hydrogenated using 108 mg. of 5% palladium-on-charcoal catalyst in a Paar hydrogenator at an initial pressure of 50 p.s.i. Hydrogenation was continued until no more hydrogen was absorbed. The catalyst was removed by filtration, and the filtrate was evaporated *in vacuo*. There was obtained 1.10 Gm. (101%) of crude crystalline residue. The crude material was recrystallized from methanol and gave 641 mg. (58.8%) of 1-acetoxy-1,2,3,4,4a,9,9a,10-octahydro-9-hydroxy-10-ketoanthracene (VI), m.p. 127–128°. An additional recrystallization of the product from ethanol gave the pure material, m.p. 128–129°, mixed m.p. 128–129° *with* an authentic sample of VI.

Conversion of 1,4,4a,9,9a,10-Hexahydro-1,9-dihydroxy-10-ketoanthracene (XV) into 1,2,3,4,4a,9,-9a,10 - Octahydro - 1,9 - dihydroxy - 10 - ketoanthracene (VII).—By a hydrogenation procedure similar to that described above, 161 mg. (0.693 mmoles) of XV was hydrogenated and gave 75 mg. (47%) of crude VII, m.p. 156–159°. Two recrystallizations of the crude product from methanol gave the pure sample, m.p. 162–163°, mixed m.p. 162–163°, with an authentic sample of VII.

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Determination of Moisture Content of Lactose by Neutron Thermalization

By GARNET E. PECK*, JOHN E. CHRISTIAN, and GILBERT S. BANKER

A procedure has been developed for the determination of moisture in an organic material by the thermalization of neutrons. The method developed utilized commercially available equipment designed for depth moisture determinations of soils. The moisture content of three series (replications) of water in lactose U.S.P. was determined at various concentration levels by the neutron method and by two official methods (gravimetric and Karl Fischer titration). The number of thermal neutrons produced at the various moisture concentrations prepared were plotted against the corresponding moisture content indicated by each of the two official moisture determination methods. Regression curves were prepared using least squares calculations. With *t* tests it was shown that the means of the slopes of the three series were estimates of the true slope of the regression curves, and 0.95 confidence limits were also set on the slopes. The applicability of this new method of determining the moisture content of pharmaceutical systems is discussed.

IN THE PREPARATION and evaluation of pharmaceutical products numerous methods of analysis are used to control the quality of the finished product. The control procedures for pharmaceutical products include in-process controls as well as the final release of the products for patient consumption. A determination which is critical in pharmaceutical processing of solids is the determination of moisture content. This determination usually requires the removal of a representative sample from a large mass of material with one of the official methods of the U.S.P. being used to determine the moisture content (1). A method has been developed for the sampling of large volumes of soil *in situ* (2–4), using a probe containing a radioactive source. The method determines both bound and unbound water. The purpose of this study was to evaluate this method with commercially available equipment for possible pharmaceutical applications in systems containing organic compounds. Since the proposed method is based on the thermalization of fast neutrons by the hydrogen atoms of water, it was necessary to investigate the ability of the nuclear system to measure the change in hydrogen atom content due to added water in the presence of large amounts of hydrogen atoms present in organic medicinals.

The moisture measurements by this nuclear method depend on the moderation of fast neutrons by the hydrogen atoms of water producing slow or thermal neutrons which are detectable by a boron trifluoride detector (2). The number of slow neutrons resulting from the moderation is directly proportional to the number of hydrogen atoms in the sample. Since the density of the material being studied would determine the amount of hydrogen atoms observed by the neutron source per unit volume, it is necessary to observe the relative density. The amount of absorption and backscattering of gamma rays by the outer electrons of the atoms in a given sample is a measure of the material's density (2). The amount of backscattering is inversely proportional to the density. In this study the gamma source was used only to observe any large changes in sample density.

EXPERIMENTAL

Since lactose is commonly used as a diluent and filler in many solid pharmaceutical preparations, it was chosen to represent organic substances commonly encountered in the manufacture of medicinals. Because of the sphere of influence of the neutron source used in this study, which was 12 in. in water, it was necessary to work with a 40 Kg. sample. Figure 1 illustrates the system used for the moisture determination procedure. The samples were evaluated in a standard container 35 cm. in diameter and 66 cm. deep. Supports were placed inside the drum to hold an access tube required to permit the insertion of the depth moisture and density probes. The supports also served to insure reproducible geometry of the system. The size of the container was within the limits suggested by previous work with sand, earth, and cement (5). The nuclear equipment used in this study was obtained from the Nuclear-Chicago Corp., Des Plaines, Ill. It consisted of their model P 19 depth moisture probe, model P 20 depth density probe, and model 2800 portable scaler.

The moisture probe is 15 in. in length and $1^1/_2$ in.

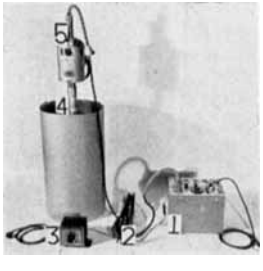


Fig. 1.—Moisture gauging equipment and sample container. Key: 1, scaler; 2, graduated cable; 3, attached external timer; 4, sample container and access tube; and 5, neutron probe inside paraffin shield.

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TABLE I.—CALIBRATION DATA FOR THE MOISTURE DETERMINATION OF LACTOSE, SERIES 3

Sample	Moisture Content—Method 1[a]	Moisture Content—Method 2[b]	Neutron[c] Count, c.p.m.	Gamma[c] Count, c.p.m.
1	5.0734	0.10	10,381.25	22,048.20
2	5.4479	0.48	10,616.25	22,310.15
3	6.0835	1.10	10,635.50	22,356.15
4	6.4655	1.70	10,761.25	22,256.55
5	6.7794	2.30	10,569.50	22,373.80
6	8.0475	3.10	11,026.75	22,660.90
7	8.5398	4.00	11,610.60	22,563.55
8	9.3001	4.90	11,987.05	22,443.35
9	10.4767	5.35	11,837.70	22,379.00
10	11.0067	5.70	12,503.80	22,423.70
11	11.4877	6.70	12,923.95	22,401.60
12	11.9584	6.90	12,928.65	22,418.60
13	12.1929	7.60	12,722.45	22,539.30
14	12.5308	8.70	13,318.05	22,297.95
15	12.7883	8.80	13,239.75	22,374.00

[a] Karl Fischer titration, in per cent. [b] Moisture balance method, in per cent. [c] Average of five 4-minute counts.

in diameter. The probe consists of the neutron source (radium-beryllium), detector, and preamplifier. The neutron output of the source was 6.78 $\times$ 10^4 neutrons per second. A boron trifluoride slow neutron detector is used in the probe. The probe is connected to the scaler with a cable which is graduated every 2 in. When not in use, the probe is stored in a paraffin shield. The density probe consisted of a gamma source (2.83 mc. of cesium-137), detector (three G. M. tubes), and a preamplifier. It was also connected to the scaler with a graduated cable. The portable scaler supplied has a built-in timing device; this timer was not accurate enough for this study. A Liebil-Flarshein, model 42 motor driven time switch was connected to the leads to the built-in timer. The scaler was operated in the A-C position.

Moisture Determination Procedure.—Certain preliminary observations were necessary before the final procedure was established. To insure that the system was operating properly, the moisture probe was inserted into a 20 gal. tank (16 in. in diameter and 18 in. deep) containing water. This water sample was used to study the effect of the height of the probe in the sample and to check the system before measuring lactose samples. In water the probe was operated at a height 4 in. from the bottom of the water container and 6 in. from the bottom of the sample container for the lactose. These heights gave the maximum readings for the respective samples. To check the reproducibility of the neutron count rate, the sample container was filled with lactose and counts were taken at the 6-in. level on 3 successive days. On each occasion the neutron probe was inserted to the appropriate level, counts taken, and the probe removed. Similar measurements were made in water. For the density measurements the position of the probe in the sample was at a height 4 in. from the bottom. All measurements were within the normal counting error of the respective systems.

Standard Curve Preparation.—The lactose used in this study was powdered by passage through a No. 60 mesh screen using the Fitzpatrick comminutor, model M,[1] with the hammers forward. The material was placed in the sample container and

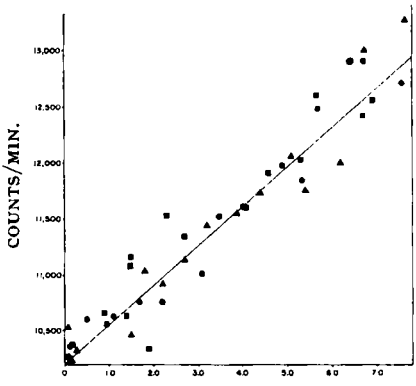


Fig. 2.—Standard curve for the moisture determination of lactose: neutron counts *vs.* moisture balance. Key: ■, Series 1; ▲, Series 2; and ●, Series 3.

nuclear measurements were taken. After removal of the lactose from the sample container, it was divided into two batches of 20 Kg. each. Distilled water was added to each batch and mixed using a Stokes 21 AA mixer[2] for 20 minutes. The batches were combined and passed through a suitable screen (No. 12 to No. 4 depending upon the amount of moisture added) using the Stokes oscillating granulator, model 43 A.[2] This was done to insure further complete distribution of the added water within the lactose. The moistened samples were stored in a single plastic bag for 24 hours before measurements were made with the probes. At low moisture contents this storage period was very important—to be certain that the added water was in equilibrium throughout the lactose sample. The following stepwise procedure was used for preparation and observation of the samples:

The lactose was added to the standard container by carefully scooping to avoid the entrapment of large voids within the container.

A 2-oz. composite sample was removed during the filling of the sample container for moisture determination by the Karl Fischer method and the moisture balance method.

The container was then shaken by hand to remove void spaces in the large sample volume.

The density probe was checked in its shield against the standard source (five 4-minute counts taken) after a 30 minute warm-up time and was then inserted into the sample *via* the access tube.

Five 4-minute counts were taken with the probe 4 in. from the bottom of the container.

The moisture probe was connected to the scaler, and the system warmed up for 30 minutes.

Five 4-minute counts were taken in the water tank with the probe inside an access tube and 4 in. from the bottom.

The probe was then placed in the sample container filled with the lactose, and five 4-minute counts were taken. After the density readings were taken, the density was adjusted by further shaking of the sample container to bring the density value close to a previous sample.

RESULTS AND ANALYSIS

To state that it is possible to determine the moisture content of an organic substance such as lactose,

[1] W. J. Fitzpatrick Co., Chicago, Ill.

[2] F. J. Stokes Corp., Philadelphia, Pa.

TABLE II.—EVALUATION OF MOISTURE CALIBRATION DATA FOR LACTOSE

Series	σ_x[a]	s_{yx}[b]	t test[c]	Slope[d]	Limits[e] on Slopes
		Karl Fischer Method			
1	2.0988	309.265	0.02618	397.361	±85.2297
2	2.1506	203.741	0.52519	409.175	±52.8375
3	2.5086	232.545	−0.57986	382.449	±51.6993
		Moisture Balance Method			
1	2.2669	348.818	−0.0145	360.693	±95.2322
2	2.3875	276.665	0.1278	365.156	±64.6296
3	2.8388	235.585	−0.0911	359.047	±46.2847

[a] Standard deviation of X (moisture content). [b] Standard error of estimate for the universe. [c] Critical region for t test: ±2.160 for $\alpha = 0.05$ and $n - 2$ degrees of freedom. [d] Slope in c.p.m. per 1.0% water content. [e] 0.95 confidence limits for slopes: $b \pm t_{0.025}\, s/\sigma_x\sqrt{N}$.

utilizing the principle of neutron thermalization, it must first be shown that a useful calibration curve can be prepared. Lactose contains sufficient hydrogen atoms ($C_{12}H_{22}O_{11}\cdot H_2O$) to produce a significant slow neutron count by itself when dry. In this study lactose was moistened with water and the change in slow neutron count was observed at the various moisture levels. Three series (replications) of the measurements were made. An example of the data collected in this study is given in Table I. The samples used to determine the moisture content by the Karl Fischer titration and the moisture balance were taken from the same composite samples of each series. Figure 2 is a plot of the slow neutron count for each sample *versus* the per cent moisture content obtained using the moisture balance. The equations for each series as determined by least squares calculations are

Series 1 $Y = 10{,}192.595 + 360.693\ X$
Series 2 $Y = 10{,}169.221 + 365.156\ X$
Series 3 $Y = 10{,}190.250 + 359.047\ X$

Because of the closeness of these values, it is impossible to show more than one regression curve for the data found in Fig. 2. This would indicate that the total reproducibility of this calibration method is satisfactory. A hypothesis was proposed to test the calculated slopes against the mean of these slopes. To test the hypothesis that each slope was either equal or unequal to the mean slope the following equations were used (6):

Standard deviation of X

$$\sigma_x = \frac{\sqrt{n\Sigma X^2 - (\Sigma X)^2}}{n} \qquad \text{(Eq. 1)}$$

Standard error estimate for the universe

$$s_{yx} = \sqrt{\frac{Y^2 - a\Sigma Y - b\Sigma XY}{n-2}} \qquad \text{(Eq. 2)}$$

Hypothesis test for the slope

$$t_{n-2} = \frac{b - b_{\text{mean}}}{\frac{s_{yx}}{\sigma_x\sqrt{n}}} \qquad \text{(Eq. 3)}$$

Table II is a summary of the results of the calculations. The t test used in this study was based on a 0.95 confidence level and $n - 2$ degrees of freedom. From these data it may be stated that the hypothesis cannot be rejected and that the slopes are equal, since all calculated t test values fall within the critical region. Therefore, when the moisture content was determined by the moisture balance method, each change of 1% moisture content was represented by an average change in slow neutron count of 361 c.p.m. as indicated by the calculated average slope.

Figure 3 represents another expression of the data from the three series of measurements, with the neutron counts being the same as the above, but the moisture content having been determined by Karl Fischer titration. The equations for the regression curves were also determined by the least squares method and are

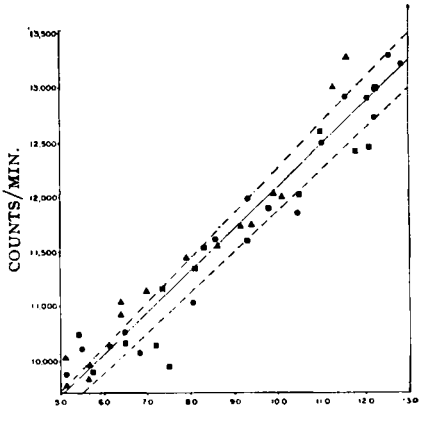
Series 1 $Y = 7{,}908.187 - 397.361\ X$
Series 2 $Y = 8{,}169.249 - 409.175\ X$
Series 3 $Y = 8{,}281.084 - 382.449\ X$

Table II illustrates the results from the same t test used for the moisture balance method. Again, for the three series of measurements the hypothesis that the slopes were equal to the mean slope could be accepted. Therefore, it may be stated that when the moisture content was determined by the Karl Fischer titrations, each change of 1% moisture content was represented by an average change in slow neutron count of 396 c.p.m. as indicated by the calculated average slope.

It is possible to set confidence limits on the calculated slopes using the following equation (6)

$$b'_{yx} = b_{yx} \pm \left(\begin{matrix} t,\ 2\text{ tails} = \\ n - 2\text{ d.f.}\end{matrix}\right)\frac{s_{yx}}{\sigma_x\sqrt{n}} \qquad \text{(Eq. 4)}$$

Table II shows the calculated 0.95 confidence limits with $n - 2$ of freedom. All the slopes fall within



% MOISTURE—KARL FISCHER TITRATION

Fig. 3.—Standard curve for the moisture determination of lactose: neutron counts *vs.* Karl Fischer titration. Key: ■, Series 1 - - - - - - -; ▲, Series 2 - - - - - - -; ●, Series 3 ———.

the limits, regardless of which slope is used to set these limits. This would indicate that any of the slopes of the regression curves are good estimates of the true slope, b'.

Table I includes the scattered gamma readings taken with the depth density probe. These measurements were made to observe and control, if necessary, the density within a given series of measurements. An attempt was made to correct for apparent differences in density within a series and from one series to the other; but when the corrections were applied, no improvements in the calibration curves resulted.

DISCUSSION

The neutron thermalization method of determining moisture in organic materials has two limitations that should be mentioned. First, the system used in this study requires a rather large sample. This is because the sphere of influence of the neutron probe is large, being 12 in. in water. Lactose, with its large number of hydrogen atoms per molecule, has a sphere of influence for the neutron source close to the value for pure water. If the sample size is inadequate, the neutron source will sample material outside the sample container. The sample size required for lactose was about 40 Kg.

The second principal limitation of this method is the complexity of achieving a satisfactory standard curve for the substance under study. A degree of scattering of the points of the calibration curves may be expected, requiring that a larger number of points be determined for the standard curves required for each compound or mixture. Some of the sources of error that may contribute to the scattering of the points are (*a*) a nonuniform incorporation of the added water, (*b*) variations in sample packing, (*c*) the normal slow neutron detection error which is about 1%, (*d*) the error in the determination of moisture content by the moisture balance or the Karl Fischer titration, and (*e*) any slight variation in geometry of the probe within the sample.

These errors may or may not be additive. However, with a sufficient number of samples at various moisture concentrations, a suitable calibration curve can be obtained which is reproducible.

The advantages of the neutron thermalization method for moisture determination are: (*a*) nondestructive testing of the test sample, (*b*) adaptability to continuous and automated programs, (*c*) elimination of the removal of small composite samples from large volumes of material with a concomitant reduction in sampling problems and sample error, and (*d*) the measurement of a system for an acceptable or unacceptable moisture level.

In certain pharmaceutical systems the neutron probe could be used simply to indicate an acceptable or unacceptable moisture concentration and would not require a complete calibration curve for various materials. The elimination of the need of removal of a composite sample from a quantity of material would be time saving as well as more accurate. The increase in accuracy of the moisture determination would be because the neutron probe analyzes a much larger sample because of its large sphere of influence. Most conventional methods analyze a small sample.

Two special areas of application are proposed. First, incoming bulk chemicals could be analyzed quickly and easily for moisture content by simply inserting an access tube, lowering the neutron probe, and measuring the slow neutron count rate. This would require only a simple calibration over a rather narrow range. Second, the use of the neutron probe for in-process control could enjoy wide application. For example, it would be possible to mount the probe inside the shaft of a large dough mixer or on one of the walls of the mixer. The neutron source could be then exposed at desired intervals to determine the extent of moistening taking place. This system could be calibrated for water or for other hydrogen containing moistening agents.

CONCLUSIONS

1. It was possible to prepare a calibration curve for the determination of moisture content of lactose by neutron thermalization using commercially available equipment.
2. The calibration curves for the moisture determination of lactose were prepared by plotting the slow neutron count *versus* the moisture determined by the moisture balance and by Karl Fischer titration. The best reproducibility was obtained using the neutron count *versus* the moisture balance for evaluation. However, acceptable reproducibility was obtained when the calibration curves were based on the Karl Fischer titrations.
3. A hypothesis test that the individual slopes (replications) were equal to the mean slope resulted in an acceptance of the hypothesis for the calibration curves based on the moisture balance method or the Karl Fischer titration.
4. In either case it was possible to set 0.95 confidence limits on the slopes.
5. For the series in which the moisture content was determined by the moisture balance a change of 1% moisture content was on the average indicated by a change in neutron count of 361 c.p.m.
6. For the series in which the moisture content was determined by the Karl Fischer method a change of 1% moisture content was on the average indicated by a change in neutron count of 396 c.p.m.

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Solubility of Benzoic Acid and Related Compounds in a Series of *n*-Alkanols

By F. A. RESTAINO* and A. N. MARTIN

The solubility of benzoic acid and esters of *p*-hydroxybenzoic acid were determined in a series of *n*-alkanols, and Hildebrand's solubility parameters were assigned to these compounds. The experimental results were compared with the theoretical solubility calculated from the Hildebrand equation for regular solutions. Fair agreement was found for methyl and ethyl *p*-hydroxybenzoic acid, but propyl and butyl *p*-hydroxybenzoic acid and benzoic acid agreed less favorably. Hildebrand's equation appears most applicable to solvents whose solubility parameters are quite similar to those of the solutes; it is strictly applicable to nonpolar rather than polar systems.

THERE IS scarcely anything more important for a practicing chemist than a knowledge of solubilities, but unfortunately he finds it more difficult to predict the solubility of a substance in a given solvent than to predict almost any other property. An attempt to remove this difficulty was made by Hildebrand and Scott (1) who developed equations restricted to nonpolar solvents. Negoro, *et al.* (2), have applied the Hildebrand treatment to pharmaceutical solutions. Chertkoff and Martin (3) showed that Hildebrand's equation for regular solutions is applicable to some polar systems where the polarity of solvent and solute are not significantly different from one another. This observation together with Burrell's (4, 5) series of articles entitled "solubility parameters" and Mortimer's (6, 7) adaptation of Hildebrand's idea of internal pressure encouraged further investigations of the Hildebrand-Scott equation in polar solvents.

Therefore, this investigation was undertaken to determine the solubility of benzoic acid and the esters of *p*-hydroxybenzoic acid in a series of *n*-alkanols at 25° and to compare these with the solubility predicted by Hildebrand's equation.

A regular solution, one in which heat is absorbed on mixing but no entropy change occurs, was assumed to apply for the solutions studied. A modified form of Hildebrand and Scott's (1) equation was used and applied to the systems studied. The equation is

$$\log x_2 = \frac{-\Delta H^f_m \quad (T_m - T)}{4.575\,(T_m T)} - \frac{V_2}{4.575\,T}\,(\delta_1 - \delta_2)^2\,\phi_1^2 \qquad \text{(Eq. 1)}$$

where x_2 is the mole fraction solubility of solute, ΔH^f_m is the heat of fusion of the solute at its melting point, T_m is the absolute melting temperature of the solute, T is the absolute temperature of the solution studied, δ_1 and δ_2 are the solubility parameters of solvent and solute, respectively, ϕ_1 is the volume fraction of solvent, and V_2 is the molar volume of the supercooled solute.

The solubility parameter is defined by Hildebrand and Scott (8) as the square root of the internal pressure of the substance. It is a measure of polarity so that the more polar a substance, the greater is its solubility parameter. The solubility parameters of the solvents are known and are listed in Table I. The parameters, the heats of fusion, and the molar volumes of the solutes must be determined before Hildebrand's equation can be applied.

EXPERIMENTAL

Reagents.—Normal propyl alcohol, Fisher certified reagent grade (b.p. 96.9 to 97.2°); *n*-amyl alcohol, Fisher certified reagent grade (b.p. 136.2 to 138.1°); and *n*-butyl alcohol, Fisher certified reagent grade (b.p. 116.6 to 117.8°) were used without further purification. Normal hexyl alcohol, Eastman Organic Chemicals (b.p. 156.5 to 157.5°) and *n*-octyl alcohol, Eastman Organic Chemicals (b.p. 194–195°) were redistilled.

A purified grade of Parasepts (esters of *p*-hydroxybenzoic acid), methyl, ethyl, propyl, and butyl obtained from Heyden Newport Chemical Corp. were recrystallized from chloroform until a sharp melting point was obtained. Benzoic acid, Fisher certified reagent, was also recrystallized from chloroform.

Procedure.—A slight excess of solid was added to approximately 40 ml. of a solvent contained in a screw-top bottle. A closure was made with aluminum foil, the top fixed tightly over the foil, and the

TABLE I.—SOLUBILITY PARAMETERS OF SOLVENTS AT 25° C.[a]

Solvent	Parameter
n-Propanol	11.9
n-Butanol	11.4
n-Pentanol	10.9
n-Hexanol	10.7
n-Octanol	10.3

[a] Taken from (9).

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whole sealed with several turns of electrical tape. The bottles were shaken in a constant temperature bath (Wilkens-Anderson Lo-Temp bath) at the desired temperature ±0.05° for 48 hours. The equilibrated solutions were then removed, filtered, and samples taken.

The analysis was done gravimetrically and spectrophotometrically on a Beckman model DU spectrophotometer at a predetermined wavelength.

The densities of the saturated solutions were determined with calibrated pycnometers.

RESULTS

An attempt was first made to assign Hildebrand's "solubility parameter" to the compounds studied. Since the solubility parameters (δ values) of the solvents were known, a plot of mole fraction solubility *versus* δ value of the solvents showed a maximum solubility which is assumed to correspond approximately to the δ parameter of the solute (see Figs. 1 and 2). The fact that the δ value of the solvent at maximum solubility yields the δ value of the solute has been discussed in an earlier paper (3). The parameters for the solutes ranged from 10.9 to 11.3 at 25°. The value of 11.2 determined for benzoic acid by this method is in agreement with the value, 11.3, reported by Chertkoff and Martin (3). The δ values are in doubt in most cases by about ±0.1. The δ value for butyl parasept is particularly difficult to establish because of the absence of a sharp maximum, as seen in Fig. 2. Other techniques must be used, one of which will be presented in a later report, for determining satisfactory δ values when the solubility data fails to produce a sharply defined maximum.

The heats of fusion for the esters of *p*-hydroxybenzoic acid were determined using Walden's method (10). He observed that $\Delta H_f = T_m K$, where ΔH_f is the heat of fusion, T_m is the melting point of the solid, and K is a constant. Walden found that K had a value of about 13.5 for many organic compounds. Mortimer (11), however, showed how K varied, depending upon the class of compounds. With this in mind, the heats of fusion for the esters of *p*-hydroxybenzoic acid were determined from a K obtained from the heat of fusion of benzoic acid (12). The constant K was determined as: ΔH_f of benzoic

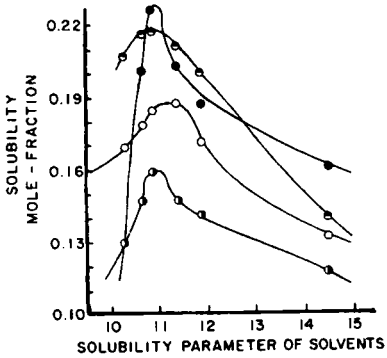


Fig. 1.—Determination of the solubility parameter of benzoic acid and esters of *p*-hydroxybenzoic acid at 25°C. Key: ◑, methyl *p*-hydroxybenzoate; ○, ethyl *p*-hydroxybenzoate; ◓, propyl *p*-hydroxybenzoate; ◒, butyl *p*-hydroxybenzoate; ●, benzoic acid.

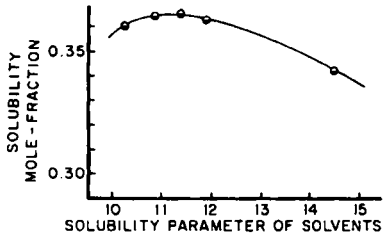


Fig. 2.—Determination of the solubility parameter of butyl *p*-hydroxybenzoate at 25°C.

acid = 4302 cal./mole, T_m of benzoic acid = 395.16, and K = 4302/395.16 = 10.89. If one assumes that the entropy of fusion and subsequently the crystal packing for each compound used were similar, then the heats of fusion can be calculated using the constant, K = 10.89. These were then labeled "apparent" heats of fusion and can be found in Table II.

TABLE II.—APPARENT HEATS OF FUSION

Ester	M.p., ° C.	$\Delta H_f'$
Methyl	126.5	4352
Ethyl	116.5	4243
Propyl	96.5	4026
Butyl	70.0	3737

To substantiate the use of K = 10.89, other compounds with similar structures were sought, *i.e.*, compounds with approximately the same substituent groups, corresponding in size, shape, and electronegativity to the hydroxyl group in the *para* position of benzoic acid. The only compound found for comparison was *p*-aminobenzoic acid. Using the K calculated, the heat of fusion for this compound was determined to be 5027 cal./mole. This value was in agreement with that reported in the literature (13).

Since the molar volume of the supercooled solid required in Hildebrand's equation is extremely difficult to obtain, it became necessary to use the "apparent molar volumes." These values were obtained (14) from knowledge of the densities and weights of solution, the density of solvent, and from the weight of solute. The apparent molar volume of benzoic acid thus determined was 101 ml./mole. This value was in agreement with the molar volume of the supercooled benzoic acid as reported in the literature (3). Accordingly, the apparent molar volumes of the parasepts should be in fair agreement with the molar volume. The apparent molar volumes of the compounds are reported in Table III.

TABLE III.—APPARENT MOLAR VOLUMES AT 25° C. (ml./mole)

Compd.	V_s[a]
Methyl *p*-hydroxybenzoate	121
Ethyl *p*-hydroxybenzoate	140
Propyl *p*-hydroxybenzoate	158
Butyl *p*-hydroxybenzoate	176
Benzoic acid	101

[a] The values reported are the average of several values obtained from the various solvents used.

Knowing the heats of fusion, the solubility parameter, and the molar volumes of the solids, one can now use Hildebrand's equation to calculate the predicted solubilities. Equation 1 was used for all calculations; the results appear in Table IV.

TABLE IV.—SOLUBILITY DATA AT 25° C.

Solvent	Density	Gm./Gm. s.s.	Mole Fraction Observed	Mole Fraction Calcd.
		Methyl *p*-Hydroxybenzoate		
n-Propanol	0.9221	0.2951	0.141	0.140
n-Butanol	0.8885	0.2622	0.147	0.152
n-Pentanol	0.8891	0.2458	0.159	0.155
n-Hexanol	0.8811	0.2052	0.147	0.153
n-Octanol	0.8672	0.1473	0.129	0.143
		Ethyl *p*-Hydroxybenzoate		
n-Propanol	0.9079	0.3626	0.171	0.175
n-Butanol	0.9071	0.3398	0.187	0.185
n-Pentanol	0.8961	0.2987	0.184	0.184
n-Hexanol	0.8888	0.2604	0.178	0.179
n-Octanol	0.8780	0.2065	0.170	0.162
		Propyl *p*-Hydroxybenzoate		
n-Propanol	0.9204	0.4363	0.205	0.244
n-Butanol	0.9146	0.3940	0.211	0.263
n-Pentanol	0.9059	0.3623	0.217	0.269
n-Hexanol	0.8992	0.3265	0.216	0.267
n-Octanol	0.8869	0.2655	0.207	0.255
		Butyl *p*-Hydroxybenzoate		
n-Propanol	0.9724	0.6466	0.361	0.423
n-Butanol	0.9592	0.6005	0.365	0.434
n-Pentanol	0.9527	0.5579	0.364	0.437
n-Hexanol	0.9467	0.5292	0.371	0.435
n-Octanol	0.9291	0.4565	0.360	0.418
		Benzoic Acid		
n-Propanol	0.8986	0.3182	0.187	0.159
n-Butanol	0.8963	0.2960	0.203	0.167
n-Pentanol	0.9007	0.2885	0.226	0.166
n-Hexanol	0.8786	0.2311	0.201	0.163
n-Octanol	0.8708	0.1219	0.129	0.150

The volume fraction of the solvent, ϕ_1, was generally assumed to be 1 as a first approximation. Then using the x_2 calculated, a new ϕ_1 was obtained. This process of iteration was used until x_2 became constant, as described by Chertkoff and Martin (3).

DISCUSSION

The results in Table IV show good agreement between the observed solubility and that calculated from Hildebrand's equation for the methyl and ethyl esters of *p*-hydroxybenzoic acid, but the agreement for the propyl and butyl esters of *p*-hydroxybenzoic acid and benzoic acid were not so good. It must be noted that the calculation involves the use of one point from the experimental curves (the maximum) for obtaining the δ value of the drug. Therefore, the theoretical or predicted solubilities are not completely independent of the experimental values, except in the case of benzoic acid.

The Hildebrand equation also may be used for predicting the solubility of a compound in mixtures of solvents where the δ values of the two solvents are not too different from one another and the δ value of the mixed solvent system is not too far removed from the value of the solute. This can be shown from the data obtained for the solubility of benzoic acid (δ = 11.2) in a solvent mixture of *n*-octanol (δ = 10.3) and *n*-propanol (δ = 11.9). The parameter of the solvent mixture is calculated to be 11.1. If we assume the molar volumes of the solvents to be additive, then the molar volume of the mixture will be 117 ml./mole. Substituting the necessary values in Eq. 1, the predicted mole fraction solubility for benzoic acid is 0.168. The observed mole fraction solubility was 0.164. Therefore, the per cent deviation from the predicted value is 2.4.

If we try to predict the solubility of the same compound in solvents with δ values on either side of that of the solute and with values of the individual solvents differing widely from each other, then we encounter greater differences between predicted and observed solubilities. For example, suppose we wish to predict the solubility of benzoic acid in a mixture of *n*-hexane (δ = 7.3) and ethanol (δ = 13.0[1]) whose value is 11.3. The predicted solubility for this system is 0.228, while the observed solubility is 0.170.[2] The per cent deviation from the predicted value now becomes 25.5.

The results reported here further substantiate the findings of Chertkoff and Martin (3) with respect to predicting the solubility of drugs in various solvents. The Hildebrand δ value seems to be a satisfactory parameter for estimating drug solubilities, and when the δ values of a large number of drugs and solvents are eventually determined and tabulated, presumably it will be a simple matter to choose a satisfactory solvent or solvent mixture for maximum solubility of a given drug. However, the methods and principles suggested here must be checked for other classes of drugs, and a table of values for these compounds must be calculated or determined experimentally. Experimental determination of the solubility in a series of solvents (the alcohols used here should prove satisfactory as hydrogen-bonded sol-

[1] Reported by Chertkoff and Martin (3); however, Burrell (9) reports the δ value of ethanol as 12.7.

[2] Data were taken from Chertkoff and Martin (3).

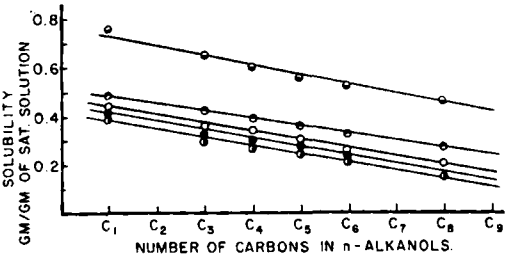


Fig. 3.—Solubility of benzoic acid and esters of *p*-hydroxybenzoic acid at 25°C. in a series of *n*-alkanols.

vents for a large number of drugs) and a plot of the data shown in Figs. 1 and 2 may provide values of satisfactory accuracy. It will be rare that the δ value of a drug can be calculated from the Hildebrand equation since energies of vaporization and molar volumes are not ordinarily available.

When mole fraction solubility was plotted against solvent δ values, a peak was observed in the curves. However, a linear relationship existed between the solubility (in grams of solute per gram of saturated solution) and the number of carbon atoms existing in the solvent. This seems to follow a relationship analogous to Traube's rule (15, 16) as observed from Fig. 3. Besides being linear, the lines for the various esters of *p*-hydroxybenzoic acid appear to be nearly parallel to one another, which leads one to believe that the forces involved in dissolution of the various esters in a series of *n*-alkanols are more or less constant from one solute to the next.

CONCLUSION

The Hildebrand equation appears most applicable to hydrogen-bonding solvents whose solubility parameters are quite similar to those of the solutes; in such cases, it will give a fair estimate of the drug solubility even though the system is a polar one. Optimum solubility may be obtained by using a mixture of solvents having a composite δ value equal to that of the solute, but when using a mixed solvent system, the observed solubility will, in general, be somewhat less than that predicted by the Hildebrand equation.

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Factors Influencing the Properties of Films Used for Tablet Coating I

Effects of Plasticizers on the Water Vapor Transmission of Cellulose Acetate Phthalate Films

By LEON LACHMAN and ARGE DRUBULIS

The water vapor transmission of thin plasticized cellulose acetate phthalate films was studied at 90, 76, and 52 per cent relative humidities. The effect of the plasticizers, triacetin, diacetin, diethyl phthalate, dimethyl phthalate, Citroflex-2, Citroflex-A2, and dibutyl tartrate on this property was determined. In general, a decrease followed by an increase in water vapor transmission was observed with increasing plasticizer concentration. The concentration at which the water vapor transmission is at a minimum appears to be the same for all the plasticizers investigated.

THE USE OF polymeric substances, such as cellulose acetate phthalate, for coating tablets and particles has been widely accepted in recent years. The reports that have appeared in the pharmaceutical literature during this time have dealt primarily with the development of polymeric substances as film formers (1–6), the formulation of film forming agents for use in tablet and particle coating (7–12), the development of test procedures for film coated tablets (13–15), and the improvement of processes for the application of these agents onto pharmaceutical dosage forms (16–20).

Although work in the above areas has been

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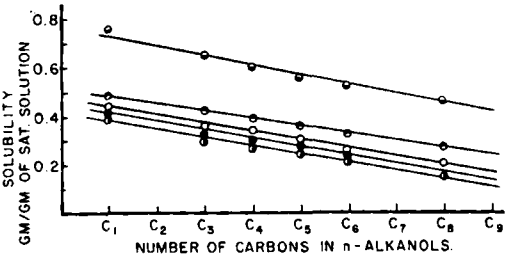


Fig. 3.—Solubility of benzoic acid and esters of *p*-hydroxybenzoic acid at 25°C. in a series of *n*-alkanols.

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TABLE I.—APPARENT WATER VAPOR TRANSMISSION (*WVT*) VALUES (Gm. 24 HR.$^{-1}M^{-2}$) AT 90, 76, AND 52% RELATIVE HUMIDITIES FOR PLASTICIZED CELLULOSE ACETATE FILMS[a]

Plasticizer	R.H.,[b] %	Plasticizer, % 1.0	2.0	3.0	4.5	6.0	9.0
Triacetin	90	62.4	58.6	52.7	97.9	105.6	261.0
	76	44.7	35.5	44.2	67.2	98.9	175.6
	52	45.3	47.0	25.9	44.2	60.5	86.4
Diacetin	90	125.8	77.7	61.9	129.6	162.1	254.1
	76	74.3	50.9	56.6	65.3	94.8	138.2
	52	54.7	42.6	41.3	43.2	45.1	179.5
Diethyl phthalate	90	83.5	94.0	86.4	83.5	118.0	261.0
	76	52.4	51.8	38.8	67.2	95.9	166.9
	52	40.3	25.9	30.7	21.1	79.6	162.1
Citroflex-2	90	75.8	72.9	62.3	87.4	126.8	217.7
	76	48.0	63.3	39.3	62.4	114.1	199.5
	52	36.5	31.3	34.6	50.8	75.8	151.6
Citroflex-A2	90	89.3	77.7	61.4	83.5	126.5	195.9
	76	74.9	66.2	53.7	51.2	121.4	149.8
	52	44.3	36.5	34.0	45.2	88.9	133.2
Dimethyl phthalate	90	94.0	86.4	69.1	75.8	94.0	193.9
	76	44.2	40.3	43.2	60.4	98.8	157.4
	52	42.3	38.4	33.6	54.7	75.8	140.1
Dibutyl tartrate	90	103.7	70.0	64.3	87.4	108.4	162.1
	76	58.6	56.6	54.3	78.7	103.6	165.0
	52	53.7	44.2	52.6	73.9	76.8	111.2

[a] *WVT* values for unplasticized films: 90% R.H. = 142.0; 76% R.H. = 85.7; 52% R.H. = 61.9. [b] Relative humidity.

rather extensive, little has been reported on the evaluation of the critical physical-chemical parameters of these substances and how these parameters may be influenced by the formulations used. Consequently, it has been the practice to develop formulations empirically without any understanding of what these parameters are or how they may be influenced by the other components in the film forming systems. Furthermore, even less is known concerning the effects that changes in these parameters have on the finished coated pharmaceutical product.

Water vapor transmission represents one physical property of considerable significance for film coatings. There is no doubt that the permeation of water vapor through a coating must be considered in the over-all evaluation of the stability of the active drug in a medicinal product. Components added to film forming agents as a part of a formulation may easily affect this property and ultimately alter the stability of the preparation.

The present investigation was designed to study the influence of plasticizers on the water vapor transmission of free cellulose acetate phthalate films. Data have been obtained at three relative humidity conditions for several plasticizers, including phthalates, citrates, glycerol acetates, and a tartrate.

EXPERIMENTAL

Preparation of Cellulose Acetate Phthalate Solutions.—Solutions containing 10% cellulose acetate phthalate and varying amounts of the plasticizers, triacetin, diacetin, diethyl phthalate, dimethyl phthalate, Citroflex-2,[1] Citroflex-A2,[1] and dibutyl tartrate were prepared on a per cent weight basis in a solvent system containing an equal quantity of acetone and anhydrous ethanol. The solutions were prepared by dissolving the cellulose acetate phthalate in a mixture of the acetone and the plasticizer and adding the alcohol when solution was complete. The finished solutions were passed through several layers of gauze.

Preparation of Cast Films.—The solutions were cast as sheets of 25 mil wet thickness onto highly polished glass plates using a Gardner film casting knife[2] equipped with a micrometer blade adjustment. The sheets were dried at ambient temperature and stripped from the plates at 18 hours. The resulting films were approximately 2 mils thick.

Determination of Water Vapor Transmission (WVT).—The water vapor transmission (*WVT*) was determined by a modification of ASTM method E-96-53T, "Measuring Water Vapor Transmission of Materials in Sheet Form." Ten grams of Drierite, as the desiccant, were placed into a Gardner-Park permeability cup.[2] A disk of the film under investigation was cut from each sheet and clamped tightly between two polyethylene gaskets under a flat retaining ring. The retaining ring was held in place by a ring cover screwed down onto the cup, resulting in a gas-tight seal. The exposed area of the sample was 25 cm.^{2} The charged cup was weighed and placed into a desiccator containing a saturated solution of either zinc sulfate heptahydrate, sodium acetate trihydrate, or sodium hydrogen sulfate monohydrate to provide the relative humidity conditions of 90, 76, and 52%, respectively. Weighings of the sample cup were made at definite intervals throughout the day.

A preliminary study was undertaken to determine the effect of the quantity of desiccant used, the position of the cup, and the nature of the film surface exposed. Samples were run in which (*a*)

[1] Marketed by Charles Pfizer & Co., Inc.
[2] Gardner Laboratory, Inc.

both 10 and 20 Gm. of Drierite were used, (*b*) the cups were placed in the desiccator in inverted and upright positions, and (*c*) both surfaces of the film were exposed; *i.e.*, the upper "orange-peeled" and the smooth underside. No appreciable difference in the water vapor transmission values was observed for these conditions. In view of these results, either surface of the film was exposed, 10 Gm. of desiccant was used, and the cups were maintained in an upright position throughout the study.

To determine whether water vapor was sorbed by the film during the water vapor transmission determination, several samples of film disks were weighed before and after the determination. No increase in weight was observed for any of the samples tested. Thus, it was assumed that the weight increase obtained for the sample cup during a determination was due to the water vapor which permeated the film.

RESULTS AND DISCUSSION

The water vapor transmission values for cellulose acetate phthalate films plasticized with the varying concentrations of the plasticizers studied are presented in Table I. The values were obtained from the slopes of the plots of weight increase of the sample cup *versus* time, using

$$WVT = \frac{[g]\ [24]}{[t]\ [a]}$$

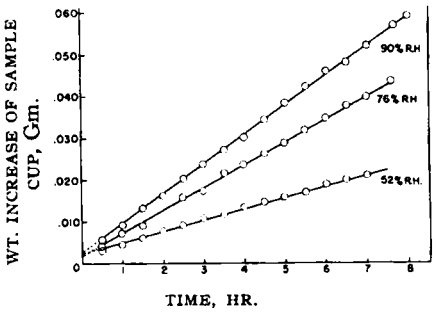


Fig. 1.—Water vapor permeation as a function of time for the diethyl phthalate plasticized films. Cellulose acetate phthalate: 1% diethyl phthalate.

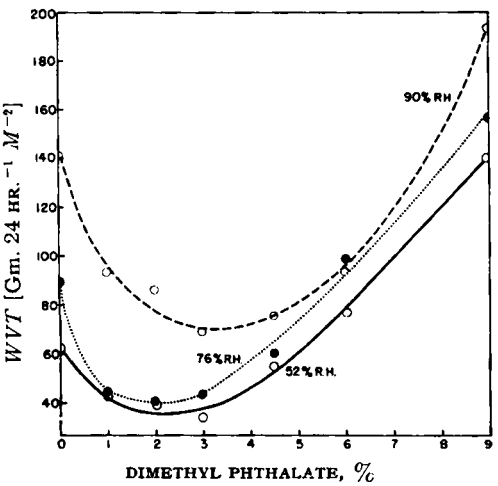


Fig. 2.—Effect of plasticizer concentration on *WVT* for the dimethyl phthalate plasticized films.

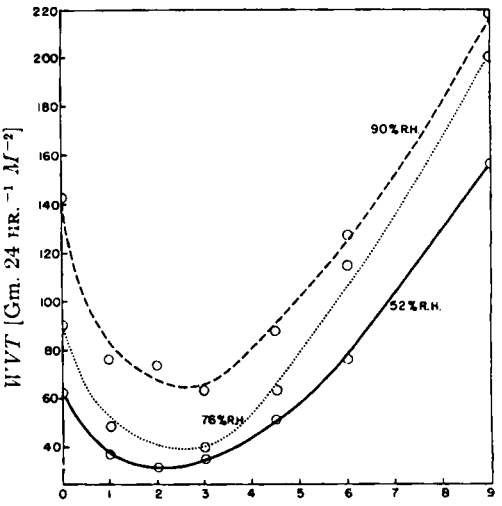


Fig. 3.—Effect of plasticizer concentration on *WVT* for the Citroflex-2 plasticized films.

where *WVT* = "apparent" water vapor transmission in grams 24 hours$^{-1}M^{-2}$, g = weight gain in grams, t = time in hours during which the gain was observed, and a = exposed area of the specimen in square meters (M.^{2}).

Typical plots from which these values were calculated for films plasticized with 1% diethyl phthalate are shown in Fig. 1.

The *WVT* values obtained in this study should by no means be considered as absolute, but rather as relative for the experimental conditions. This limitation is necessary because the relative humidity conditions obtained with saturated salt solutions contained in desiccators change as the samples are removed and actually are not at the theoretical values for the entire sample time.

As a check, samples were tested under conditions in which the salt solutions were continuously stirred to allow the humidities within the desiccators to reach equilibrium quickly once the samples were replaced after weighing. The length of time between weighings was also increased severalfold. Under these conditions, the samples were at relative humidity conditions closer to the theoretical values for a longer period of time. The results obtained for these samples were of a different magnitude, but of the same relative order as the data presented above. In view of these results, it would appear that the findings of this investigation, in general, are indicative of what occurs in these plasticized films.

Table I shows that the permeation of water vapor for all the systems studied is similarly dependent on the concentration of the plasticizer in the film. Figures 2 and 3, respectively, are plots of *WVT* *versus* per cent plasticizer for the dimethyl phthalate and the Citroflex-2 plasticized systems. The dependency appears to be one that shows a decrease in permeation for increasing plasticizer concentration up to about 3% and an increase in permeation with increasing plasticizer concentration beyond this point.

In general, this dependency, exhibiting a minimum *WVT* value, is similar to that obtained by Kirk-

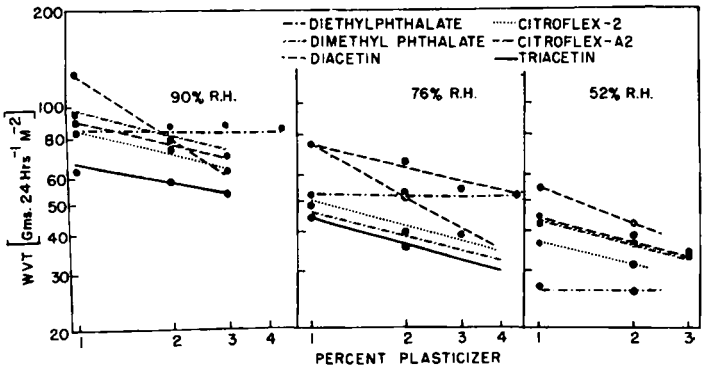


Fig. 4.—Effect of plasticizer concentration on *WVT* for lower concentrations.

patrick (21) for cellulose acetate films plasticized with certain sulfonamides and phthalates. It may possibly be explained if the plasticizer is considered as an integral part of the film distributed within the interstices of the polymer. If the permeation of water vapor through the film is primarily by passage through the interstices, then it would be expected that as these spaces become filled with plasticizer the rate of water vapor permeation would decrease.

The rate at which the water vapor permeation decreases for plasticizer concentrations between zero and 3% can readily be seen when the logarithm of *WVT* is plotted against the logarithm of per cent plasticizer, as shown in Fig. 4. It is evident from the slopes of these plots that the change in permeation is greatest for diacetin, while essentially no change in permeation occurs with increasing concentrations of plasticizer for diethyl phthalate. For the other plasticizers studied, the change in permeation with plasticizer concentration is approximately the same, as indicated by the parallel lines in Fig. 4.

It would seem that the change of *WVT* with respect to plasticizer concentration at these lower concentrations is specifically dependent on some physical property of the plasticizers, and that this property is approximately the same for dimethyl phthalate, triacetin, Citroflex-2, Citroflex-A2, and dibutyl tartrate, but different for diacetin and diethyl phthalate. Of the properties for these plasticizers listed in Table II, the water solubility correlates with the data presented.

Thus, diacetin with an infinite water solubility exhibits the greatest influence on the rate at which *WVT* changes with respect to plasticizer concentration. The water insoluble diethyl phthalate, on the other hand, apparently does not exhibit any influence on the rate at which *WVT* changes with plasticizer

TABLE II.—PHYSICAL PROPERTIES

Plasticizer	Mol. wt.	B.p., °C.	Soly. in H_2O, Gm./100 ml.
Triacetin	218.20	259	7.17
Diacetin	176.17	176	∞
Diethyl phthalate	222.2	296.1	insol.
Citroflex-2	276	127	6.5
Citroflex-A2	318	132	0.72
Dimethyl phthalate	194.18	282	0.5
Dibutyl tartrate	262.30	203	0.49

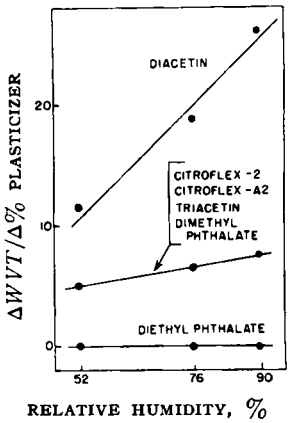


Fig. 5.—Relative humidity dependency of the rate of change of *WVT* with respect to plasticizer concentration for lower concentrations.

concentration. This is indicated by the resulting lines of zero slope. The effects of triacetin, dimethyl phthalate, Citroflex-2, and Citroflex-A2 appear to be intermediate. The tenfold difference in water solubility between Citroflex-A2 and triacetin, for example, apparently is not sufficient to influence the permeation; thus the rate at which *WVT* changes is the same for these plasticizers.

The relationships presented above appear to hold for these systems at the three relative humidity conditions studied. Furthermore, a plot of the change of *WVT* with respect to per cent plasticizer (Δ *WVT*/Δ% plasticizer) *versus* per cent relative humidity shown in Fig. 5 indicates that in all probability the influence of the plasticizer on the water vapor permeation or transmission is linearly dependent on the relative humidity.

As indicated above, the *WVT* values for all systems studied go through a minimum as the concentration of plasticizer is increased from zero to 9%. Apparently, any plasticizer added beyond the concentration present at the point of minimum *WVT* can no longer be held as an integral part of the film and thus may be "squeezed out" and may separate as a thin layer on the surface of the film. It is at this point that an increase in water vapor permeation with increasing plasticizer concentration is observed. It appears that, although the absolute permeation may be dependent on the specific thin layer of plasticizer present on the surface of the film, the over-all change in *WVT* with respect to

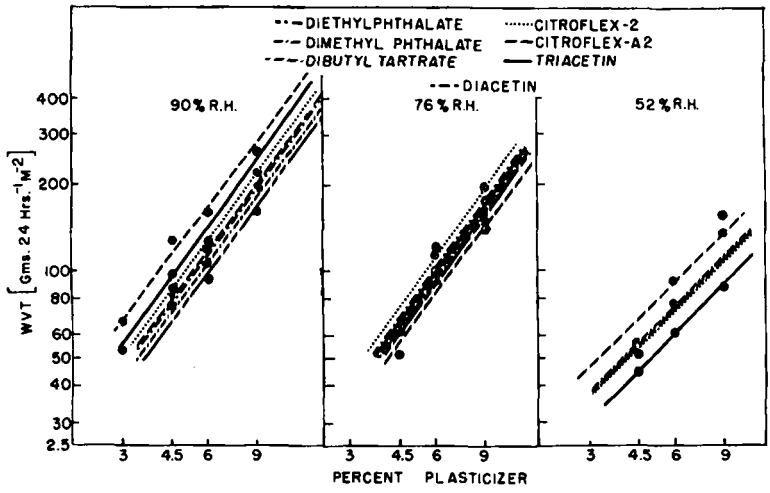


Fig. 6.—Effect of plasticizer concentration on WVT for higher concentrations.

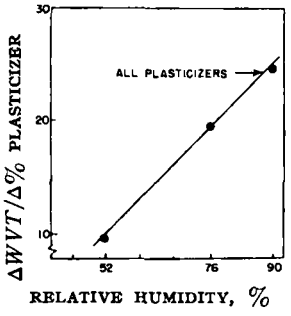


Fig. 7.—Relative humidity dependency of the rate of change of WVT with respect to plasticizer concentration for higher concentrations.

plasticizer concentration is not dependent on the specific plasticizer used. An explanation that can be offered is that once the plasticizer has filled the interstices of the polymer, further addition of plasticizer has only a dilution effect on the polymer. As the polymer is diluted, the permeation of water vapor would be expected to increase. Furthermore, since the plasticizers studied are of reasonably similar molecular mobility, they would tend to affect the permeation in a similar manner. This can be seen from the logarithmic plots in Fig. 6, where the slopes of the graphs of WVT *versus* per cent plasticizer for higher concentrations of plasticizer are the same for all the plasticizers studied.

As in the case of the lower plasticizer concentrations, when the change in WVT with respect to per cent plasticizer is plotted against the per cent relative humidity, a linear relationship results. The dependency can be seen in Fig. 7. Again, this shows that the influence of the plasticizer on the water vapor transmission is dependent on the relative humidity.

SUMMARY

The effects of several plasticizers on the water vapor transmission of cellulose acetate phthalate films have been presented. The data tend to indicate that for all the plasticizers studied (*a*) a decrease in water vapor permeation occurs with increasing plasticizer concentration up to about 3%, followed by an increase in permeation with further increase in plasticizer concentration, (*b*) the rate at which WVT changes with respect to plasticizer concentration is dependent on the specific plasticizer used for the lower concentrations, but not for the higher concentrations, and (*c*) that this rate is linearly dependent on relative humidity for both low and high plasticizer concentrations.

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Complexation of Organic Substances in Aqueous Solution by Hydroxyaromatic Acids and Their Salts II

Influence of Halogen, Nitro, and Other Substituents and Correlation of Binding Tendencies Toward Prednisolone, Theophylline, and Phenacetin

By TAKERU HIGUCHI and FRANCIS D. PISANO*

Complexing tendencies of (*a*) variously substituted halo, methyl, nitro, and amino benzoates, (*b*) several phthalates, (*c*) dihydroxyphthalates, (*d*) dihydroxy and halohydroxy-2-naphthoates, and (*e*) pamoate have been evaluated by the solubility technique. The rather generalized binding effects observed earlier were again observed. Of all the salts studied, sodium pamoate exhibited the strongest tendency with a stability constant of 160 L./mole for prednisolone.

THE GENERAL APPROACH, techniques, and objectives of these studies have already been reported (1). In this paper the authors present additional data on hitherto uninvestigated systems which were selected to elicit further information on factors which control the intensity of these type interactions. The present studies were concerned specifically with the effects of alkyl, halo, nitro, carboxyl, and amino substituents and of enlargement of the size of the aromatic anionic group.

EXPERIMENTAL

Reagents

The following reagents were recrystallized from water, ethanol, methanol, or mixtures of these: isophthalic acid, m.p. 338–343°; terphthalic acid, m.p. >340°; 2,3-naphthalenedicarboxylic acid,[1] m.p. 238–239°; 3,4-dimethylbenzoic acid,[1] m.p. 165–167°; 3,4-dichlorobenzoic acid,[2] m.p. 205–206°; 5-iodosalicylic acid,[1] m.p. 194–197°; 4-aminosalicylic acid,[2] m.p. 147–148°; 2-chlorobenzoic acid,[3] m.p. 139–140°; 4-chlorobenzoic acid,[3] m.p. 235–236°; 4-bromobenzoic acid,[3] m.p. 254–256°; 4-iodobenzoic acid,[3] m.p. 271–273°; 4-nitrobenzoic acid,[3] m.p. 232–237°; *p*-toluic acid,[3] m.p. 178–180°; 5,5-methylenedisalicylic acid,[3] m.p. 215–220° with decomposition; 3,6-dihydroxy, 2-naphthoic acid,[4] m.p. 256–260°; theophylline U.S.P., m.p. 270–272°; phenacetin U.S.P., m.p. 134–136°; prednisolone alcohol,[5] m.p. 240–242°; sodium pamoate,[6] m.p. >330°.

Dowex 1-X4 anion exchange resin,[7] 50–100 mesh as the chloride form, was washed with water and methanol prior to use.

The following were used without further purification: potassium acid phthalate, sodium bicarbonate, potassium bicarbonate, bromine, iodine, hydrochloric acid, methanol, chloroform, *n*-propanol, all analytical reagent grade, and ethanol U.S.P.

2,3-Dihydroxyterphthalic Acid.—This was prepared from catechol using the Marasse modification of the Kolbe-Schmidt reaction described by Cameron, *et al.* (2). The reaction was carried out in a model MBT 250 high pressure magne dash autoclave.[8] The isolated product, recrystallized from aqueous methanol, melted at 315–317° with decomposition. Neutralization equivalent: found, 108.4; calcd. for the monohydrate, 108.1.

4,6-Dihydroxyisophthalic Acid.—This was prepared from resorcinol using the above procedure. The product, recrystallized from aqueous ethanol, melted at 332–333°. Neutralization equivalent: calcd.: 99.1; found: 99.8.

2,5-Dihydroxyterphthalic Acid.—This was prepared using the Kolbe-Schmidt synthesis on the dipotassium salt of gentisic acid (2, 3). The reaction was run for 6 hours at 160° and 1500 p.s.i. CO_2 pressure. The isolation was carried out as above and the product recrystallized from aqueous methanol. The crystals darkened but did not melt at 330°. Neutralization equivalent: calcd., 99.1; found, 99.2.

3,6-Dihydroxy-2-naphthoic Acid.—This was prepared from the potassium salt of 2,7-naphthalenediol using the Kolbe-Schmidt synthesis (2, 3). The reaction was run for 4 hours at 130° under CO_2 pressure of 1100 p.s.i. The product, recrystallized from aqueous ethanol, melted at 256–258°. Neutralization equivalent: calcd., 204.2; found, 207.0;

4-Iodo-3-hydroxy-2-naphthoic acid. This was prepared by dropwise introduction of an iodine-potassium solution into a stoichiometric amount of 3-hydroxy-2-naphthoic acid dissolved in sodium bicarbonate solution. The product was precipitated with dilute phosphoric acid and recrystallized from

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[1] Aldrich Chemical Co., Inc.
[2] Matheson Coleman and Bell, Division of the Matheson Co., Inc.
[3] Eastman Organic Chemicals.
[4] Pfister Chemical Works.
[5] Courtesy of The Upjohn Co., Kalamazoo, Mich.
[6] Courtesy of Parke, Davis and Co., Detroit, Mich.
[7] J. T. Baker Chemical Co.
[8] Autoclave Engineer, Inc., Erie, Pa.

aqueous methanol. The crystals decomposed with the liberation of iodine vapor at 210°. Neutralization equivalent: calcd., 314.1; found, 317.5.

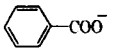
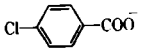
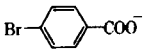
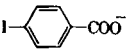
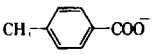
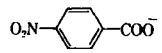
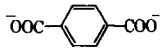
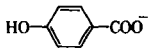
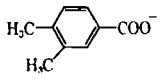
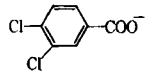
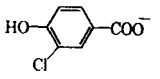
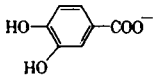
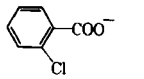
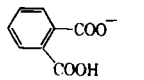
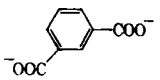
4-Bromo-1-hydroxy-2-naphthoic Acid.—This was prepared by the dropwise addition of a methanolic bromine solution to a stoichiometric quantity of 1-hydroxy-2-naphthoic acid in methanol. The product which was precipitated by the addition of cold water and recrystallized from dilute ethanol melted at 250–251° with decomposition. Neutralization equivalent: calcd., 267.1; found, 268.1.

PROCEDURE

Solubility Studies.—Except for potassium acid phthalate, sodium pamoate, and sodium 3,5-diiodosalicylate, the salt forms of the acids were prepared *in situ*. Stock solutions of the salts were prepared by dissolving and bringing to volume an accurately weighed quantity of acid and enough sodium bicarbonate so that the acid was completely converted to the salt, and the resulting solution was 0.1 *M* with respect to the bicarbonate. Increments of the stock solution were bureted or pipeted into 15-ml. screw cap vials containing a constant excess of the substrate, usually three to 10 times the solubility in water. The solution was brought to a constant final volume (generally 10 ml.) with the required amount of 0.1 *M* sodium bicarbonate solution.

Equilibration was effected by rotation of the vials in a water bath thermostated at 30.0 ± 0.1°. The time necessary for equilibration was determined to be less than 24 hours when phenacetin and theophylline were the substrates and less than 72 hours for prednisolone. After equilibration, an

TABLE I.—APPARENT STABILITY CONSTANTS[a] (L./MOLE) FOR THE INTERACTION OF SOME SUBSTITUTED BENZOATE SALTS

Compd.	Theophylline	Phenacetin
1  Benzoate	5.3[b]	1.0[b]
2  4-Chlorobenzoate	14	2.1
3  4-Bromobenzoate	16	2.7
4  4-Iodobenzoate	23	3.4
5  4-Methylbenzoate	7.7	1.3
6  4-Nitrobenzoate	9.0	3.3
7  Terphthalate	3.9	0.42
8  4-Hydroxybenzoate	12[b]	1.2
9  3,4-Dimethylbenzoate	16	2.6
10  3,4-Dichlorobenzoate	52	4.3
11  4-Hydroxy-3-chlorobenzoate	34	2.7
12  3,4-Dihydroxybenzoate	14[b]	1.5[b]
13  2-Chlorobenzoate	1.6	0.85
14  Acid phthalate	12	1.4
15  Isophthalate	1.9	0.27

[a] Calculated from the mass action equation assuming a 1:1 stoichiometry. [b] Data from Higuchi and Drubulis (1) included for comparison.

aliquot of the supernatent liquid was withdrawn and analyzed for total substrate.

Analysis.—Theophylline was separated from the complexing agent by one of two extraction procedures, depending on the partition coefficient of the acid form of the complexing agent. In most cases the theophylline was partitioned into an organic phase of 10% by volume of propanol in chloroform from an aqueous 0.5 *M* sodium bicarbonate solution. In those cases in which the acid form of the complexing agent was particularly water insoluble, the separation was effected by extraction with 1.0 *M* hydrochloric acid from an organic phase of 10% by volume diethylether in chloroform.

Prednisolone was isolated with an ion exchange column of Dowex 1-X4 resin. The aliquots were placed on the column and eluted slowly with methanol. Retention of the complexing agent was verified by prior elution of a solution containing only complexing agent.

Phenacetin was similarly isolated, except that 50% methanol in water was used as the eluent. In some cases an extraction procedure was employed in which the phenacetin was partitioned into chloroform from an aqueous 0.5 *M* bicarbonate solution.

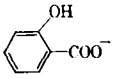
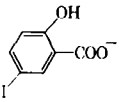
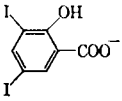
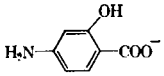
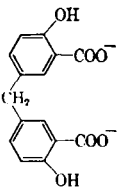
The solutions containing the substrate were appropriately diluted and the absorbance read on the Cary model 11MS spectrophotometer at the wavelength of maximum absorbance. Beer's law determinations of the substrate in each of the corresponding solvents were previously made, and the concentrations of the substrates were calculated from these.

RESULTS AND DISCUSSION

Results of these studies together with some of the pertinent data from the earlier study are shown in Tables I–IV expressed generally as apparent stability constants at the saturation concentration of the three solubilized compounds. For details of calculation and treatment reference should be made to the previous work (1).

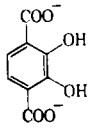
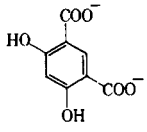
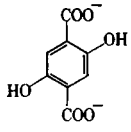
It is apparent from the tables that in every instance the intensities of interaction are in the order theophylline > prednisolone > phenacetin. The interactions with the xanthine were quite strong, often so strong that more than one molecule

Table II.—Apparent Stability Constants[a] (L./Mole) for the Interactions of Some Substituted Salicylates

Compd.	Theophylline	Phenacetin	Prednisolone
16  Salicylate	20[b]	1.9[b]	7.3[b]
17  5-Iodosalicylate	1:1.0[c]	5.8	27
18  3,5-Diiodosalicylate	1:1.3[c]	12	58
19  4-Aminosalicylate	20	1.9=	...
20  5,5′-Methylenedisalicylate	1:1.0[c]	12	62

[a] Calculated from the mass action equation assuming a 1:1 stoichiometry. [b] Data of Higuchi and Drubulis (1) included for comparison. [c] Stoichiometric ratios (salt:theophylline).

TABLE III.—APPARENT STABILITY CONSTANTS[a] (L./MOLE) FOR THE INTERACTION OF SOME DIHYDROXYPHTHALATES

Compd.	Theophylline	Phenacetin	Prednisolone
21  2,3-Dihydroxyterphthalate	107	3.9	18
22  4,6-Dihydroxyisophthalate	110	5.3	22
23  2,5-Dihydroxyterphthalate	166	5.3	...

[a] Calculated from the mass action equation assuming a 1:1 stoichiometry.

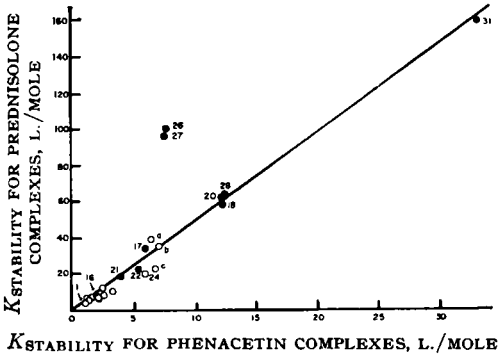
of theophylline was brought into solution for each molecule of salt added. In these instances the molar ratios are given since stability constants calculated on a 1 to 1 basis are meaningless.

The trend observed by Higuchi and Drubulis (1) which suggested that these interactions are extremely generalized seems to be confirmed by the present study. The nature of this relationship is readily evident in the plot, shown in Fig. 1, of the stability constants of prednisolone against the stability constants of phenacetin for each of the several complexing agents. The data of Higuchi and Drubulis have been included for comparison purposes. Figure 1 shows that the majority of the compounds fall essentially on a straight line going through the origin. Thus, the ratio of stability constants for prednisolone and phenacetin is essentially for the weakest complexing pairs the same as for the strongest complexing combinations. Such a correlation is interesting, particularly since prednisolone and phenacetin are structurally quite dissimilar, and the complexing agents involved represent a variety of substituted benzoates, naphthoates, phthalates, and substances such as the methylene derivatives, sodium pamoate and 5,5′-methylenedisalicylate—the group as a whole consisting of a spectrum of differently oriented substituents.

With such systems, if the behavior depended significantly on localized interactions, one may well expect a fair degree of "lock and key" effect, in which case certain orientations of the functional groups would particularly tend to favor formation with one of the drugs rather than the other. Since it is unlikely that prednisolone and phenacetin have similarly oriented sites of interaction, the absence of a lock and key effect must mean that the interaction with these substrates is largely of a nonspecific nature.

As indicated by the plot, the weakest binder studied with these two substrates was benzoate, the various substituents all causing an increase in the stability constants. The strongest of the complexing agents was sodium pamoate. Between those two lie the various compounds in an order which leads to several new observations.

It can be seen, for example, from the relative position of 2,5-dihydroxybenzoate and 2-hydroxy-5-iodobenzoate that iodo substitution has a greater effect on the stability of the complex than does the hydroxyl substitution. The large stabilizing effect of the iodo group is seen by the position of 3,5-diiodosalicylate. The same trends are observed with the naphthalene derivatives, the unsubstituted



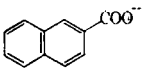
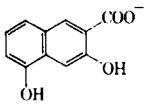
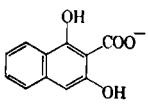
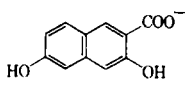
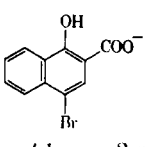
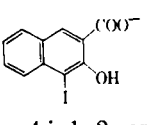
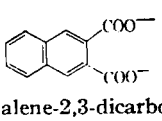
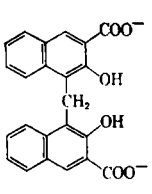
K STABILITY FOR PHENACETIN COMPLEXES, L./MOLE

Fig. 1.—A plot of the stability constants for prednisolone and for phenacetin with each of the indicated complexing agents. The unfilled circles represent data of Higuchi and Drubulis (1). The numbers refer to the compounds numbered in Tables I–IV. The unidentified circles represent the mono- and dihydroxybenzoates. The letters *a*, *b*, and *c* represent 3-hydroxy-2-naphthoate, 1-hydroxy-2-naphthoate, and 2-hydroxy-1-naphthoate, respectively.

naphthoate being the poorest complexing agent, and the hydroxy and halo substituted naphthoates complexing to a much greater extent. The naphthalene derivatives, however, showed more complexing ability than the corresponding benzene derivative.

Rationalizations of such effects can be devised either on the basis of a donor-acceptor theory, which maintains that intermolecular interaction involves the π orbitals of the aromatic nucleus with the electrons of the donor, or on the basis of the hydrophobic bonding mechanism. The strong

TABLE IV.—APPARENT STABILITY CONSTANTS[a] (L./MOLE) FOR THE INTERACTION OF SOME 2-NAPHTHOATE DERIVATIVES

Compd.	Theophylline	Phenacetin	Prednisolone
24  2-Naphthoate	1:1.2[b,c]	5.8[b]	20[b]
25  3,5-Dihydroxy-2-naphthoate	1:1.7[c]	11	...
26  1,3-Dihydroxy-2-naphthoate	...	7.6	100
27  3,6-Dihydroxy-2-naphthoate	...	7.5	97
28  1-Hydroxy-4-bromo-2-naphthoate	1:1.7[c]	12	64
29  3-Hydroxy-4-iodo-2-naphthoate	1:1.7[c]	17	...
30  Naphthalene-2,3-dicarboxylate	37	1.1	...
31  Pamoate	1:2.2[c]	33	160

[a] Calculated from the mass action equation assuming a 1:1 stoichiometry. [b] Data from Higuchi and Drubulis (1) included for comparison. [c] Stoichiometric ratios (salt:theophylline).

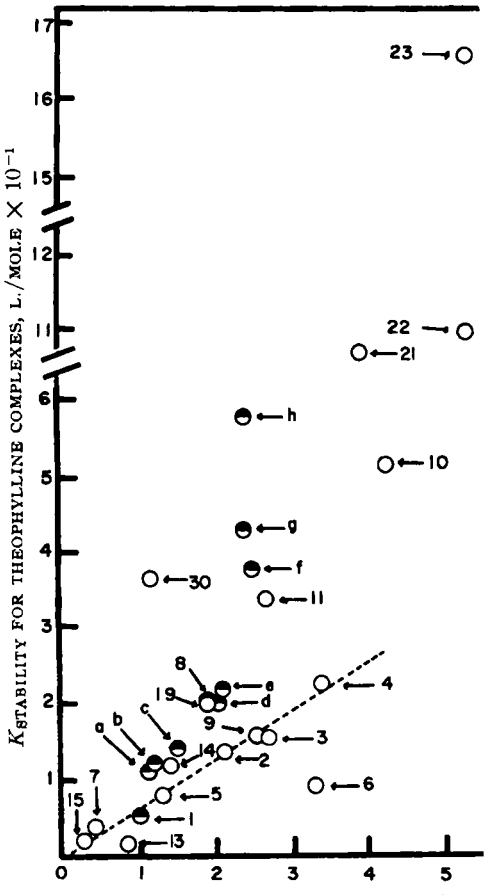


Fig. 2.—Plot showing the stability constants for complex formation of theophylline against the stability constants for complex formation of phenacetin with each of the indicated complexing agents. The numbers refer to the compounds numbered in Tables I–IV. The half closed circles refer to data of Higuchi and Drubulis (1). Key: *a*, 3-hydroxybenzoate; *b*, 4-hydroxybenzoate; *c*, 3,4-dihydroxybenzoate; *d*, 3,5-dihydroxybenzoate; *e*, 3,4,5-trihydroxybenzoate; *f*, 1-naphthoate; *g*, 2,5-dihydroxybenzoate; *h*, 2,4-dihydroxybenzoate.

binding with the halogenated compounds, for example, may be accounted for by the halogen atoms' electron withdrawing ability which would tend to make the aromatic nucleus more electrophilic and thereby more favorable for interaction with the substrate electrons. The enhanced binding over that of the unhalogenated derivatives, however, may be due, on the other hand, to the increase in the size and hydrophobic character of the complexing agents associated with halogen substitution, an effect which would tend to increase the amount of hydrophobic bonding.

The increased tendency toward complex formation observed with the expansion from a benzene ring to a naphthalene system would be expected on the basis of either hypothesis. Donor-acceptor complexing would tend to be facilitated by the increase in the size of the conjugated system, and the increase in the hydrophobic surface would certainly tend to increase the "squeezing out" effect.

The puzzling but substantial enhancement provided by the hydroxyl substitutions noticed earlier is confirmed in the present study. Since phenolic hydroxyl groups interact with water molecules to a considerable degree, they would not necessarily be expected to enhance hydrophobic bonding. Hydroxyl functions, moreover, are not generally considered as strong electron withdrawing groups, but from a consideration of their *ortho-para* directing nature are sometimes thought of as pushing electrons into the ring; any significant increase in the electrophilicity of the ring, therefore, seems unlikely. Higuchi and Drubulis (1) have discounted hydrogen bonding with the phenolic hydrogens as a major contributing factor in the formation of these complexes. This leaves the reason for the stronger complexing behavior of *meta* and *para* hydroxy compounds somewhat unresolved.

The strong binding observed with the *ortho* substituted hydroxy compounds and the dihydroxyphthalates may possibly be explained on the basis of increased area of the π cloud above and below the plane of the aromatic molecule. If the *ortho* hydroxy hydrogen forms a hydrogen bond with the carboxylate group, the rotation about the bond between C_1 and the carboxylate carbon would be arrested and the π electrons of the carboxylate function may resonate with the π electrons of the aromatic ring, and in effect increase the area of the π cloud. It can also be rationalized that in addition to the increase in the resonance effect, the planar configuration of the *ortho* hydrocarboxylates can increase the interaction by affording less steric hindrance to hydrophobic bonding.

The strong complex forming tendencies of the bis methylene derivatives, sodium pamoate and disodium 5,5′-methylenedisalicylate, may have been expected in view of the binding tendencies of sodium 3-hydroxy-2-naphthoate (K = 39 L./mole with prednisolone and 6.3 L./mole with phenacetin) and sodium salicylate. The fact that the stability constants for the methylene derivatives were not two but four to eight times that of the latter compounds on molar basis indicates that the effects which the additional hydroxy naphthoate and salicylate groups have on the compounds apparently are not necessarily those in which the extent of interaction are statistically additive. The significant displacement of the dihydroxy naphthoates from the line in Fig. 1 may mean a specific interaction with prednisolone or a specific hindrance to complex formation with phenacetin.

The stability constants for complex formation of theophylline and phenacetin with the various complexing agents listed in Tables I–IV are shown plotted against each other in Fig. 2. Those interactions where the phase solubility diagrams showed slopes of approximately one or greater for the theophylline system were excluded, since it was neither possible nor rational to calculate the stability constants based on a 1:1 stoichiometry in these instances. Again the data of Higuchi and Drubulis (1) are included for comparison.

It is obvious from the plot that the clearly linear relationship found between the formation constants of phenacetin and prednisolone does not exist in this case. The increasingly higher constants for theophylline with the stronger binders may be due at least in part to the tendency of the

xanthine to form higher complexes. The apparent 1:1 stability constants calculated from the phase solubility diagrams would then tend to be an exaggerated measure of the complexing tendencies. The linear relationship, perhaps for this reason, appears to hold better for the weaker complexing agents. It may be that for these compounds, the same mechanism operates in the complex formation with both phenacetin and theophylline, but with the stronger binders a second superimposed mechanism has a pronounced stabilizing effect on the theophylline complexes.

In view of the relative behavior of phenacetin interactions with respect to the steroid system, the pronounced scatter observed in Fig. 2 suggests that theophylline is subject to a much more specific type binding than the other two. It seems more logical to attribute the observed deviations to theophylline rather than to the phenacetin systems in view of the latter's notable correlation with the steroid system. In every case, however, the xanthine is substantially more strongly bound than phenacetin, a behavior which may be partly attributable to the planarity conferred on theophylline by its fused ring structure and partly perhaps to its ability to share electrons with bound molecules.

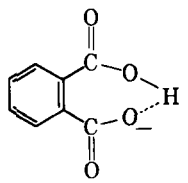
It appears from Fig. 2 that theophylline tends to have a greater relative affinity for the hydroxyl and carboxylate ion substituted compounds than for the halo and alkyl substituted compounds compared to phenacetin. The dashed line on the plot, drawn through the points representing the purely halogenated and alkylated carboxylate salts, appears to suggest a linear correlation within this class. These complexing agents may be expected to participate to a lesser degree than the phenolic hydroxy and carboxylate ion substituted compounds in specific interactions with the xanthine. It may be that electron sharing may be a more important factor in complexation with theophylline than it is with phenacetin.

Although much of the discussion on the complexation with prednisolone and phenacetin (Fig. 1) can apply to the data of theophylline and phenacetin in Fig. 2, some of the complexing agents not included in the former plot may warrant additional discussion. The rather poor complexing ability of isophthalate and terphthalate and 2,3-naphthalenedicarboxylate compared to plain benzoate and naphthoate again may be explained on the basis of the donor-acceptor theory or by hydrophobic bonding. The carboxylate ions of the phthalates may tend to push electrons into the ring and therefore decrease the electrophilicity of the aromatic nucleus. The introduction of the charged carboxylate ion might possibly also be detrimental to hydrophobic bonding in view of the possible additional hydration of the molecule.

The threefold increase in the stability constant for the binding of *p*-nitrobenzoate over unsubstituted benzoate with phenacetin may perhaps be better explained on the basis of the nitro group's electron withdrawing properties rather than on increased hydrophobic bonding, since it may be expected that the nitro group does not necessarily impart increased hydrophobic character to the carboxylate salt. The comparatively slight increase in the complex formation constant with theophylline (5.3 L./mole to 9.0 L./mole) in view of the marked increase in the phenacetin constant is somewhat surprising. Since it is uncertain whether this behavior represents unexpectedly weak complexing with theophylline or exceptionally strong complexing behavior with phenacetin, further explanations could only be speculative.

The similarity of the binding constants of *p*-aminosalicylate and salicylate places the role of the amino group in doubt. A comparison of the water solubilities of aniline and benzene suggests that the amino function possibly reduces the hydrophobic character of the compound and thereby lessens this type interaction. In addition, the amino group is generally referred to as electron donating, and on this basis a reduction in the electrophilicity of the aromatic nucleus may be expected to reduce any donor-acceptor interaction. It seems, however, that the amino group in the *para* position has apparently little effect on the binding behavior of salicylate with either theophylline or phenacetin.

Some evidence in Table I suggests the importance of the planarity of the complexing agent. For example, *ortho* substitution of chlorine hinders complex formation rather markedly, even though *para* substitution of chlorine significantly enhances it. It has been shown (4) that in the crystal structure of *o*-chlorobenzoic acid, the plane of the carboxyl group is 13.7° out of phase with the plane of the ring. In solution, restraining van der Waals forces operative in the crystal are disrupted, and it may be expected that in the ionized form the plane of the carboxylate ion and the benzene ring are out of phase to an even greater degree. When the *ortho* substituted function is a COOH group, however, the binding is increased substantially over that of benzoate. It may be that hydrogen bonding between the acidic hydrogen and the *ortho* carboxylate oxygen maintains the structure planar represented by



This greater planarity for reasons mentioned previously may render complex formation more favorable.

It has been reported by Eckert (5) that caffeine and procaine hydrochloride form a complex which exhibits a characteristic charge-transfer absorption band. In the present study with the same system, the existence of a rather sharp absorption band at 340 mμ was verified. A procedure similar to Eckert's was used with the theophylline 2,4-dihydroxybenzoate system, and absorption was observed to occur between 350 and 330 mμ, the existence of specific charge-transfer absorption being impossible to determine below 350 mμ due to the great absorption by the individual components. The possibility of charge-transfer absorption bands was also investigated with theophylline and 4-bromobenzoate. The spectrum of a mixture of the two components using as blanks two consecutively aligned cells with the corresponding concentrations of the individual components showed a rather broad absorption band with a maximum at approximately

295 mμ. The same experiment repeated with sodium 2-chlorobenzoate produced a similar band at 295 mμ. The above procedure repeated with the hydrochloride salt of 2-dimethylaminomethyl-4-methylphenol instead of the benzoates revealed a peak at approximately 307 mμ very similar in its sharpness to that obtained with the procaine hydrochloride-caffeine system.

These results indicate that theophylline appears to participate in charge-transfer complexation. The observed spectra with both the benzoates and the protonated Mannich base suggest theophylline's ability to share electrons with both positively and negatively charged aromatic compounds. It is evident, however, that further investigation of a more quantitative nature would be required to determine whether these charge-transfer interactions account for the total interaction by theophylline in aqueous solutions.

CONCLUSIONS

Solubility studies on the interactions of theophylline, prednisolone, and phenacetin with substituted benzoates, phthalates, and naphthoates produced the following observations.

Complex formation between aromatic carboxylate salts and the above drugs occurred in every instance, but with varying extents.

Introduction of substituents on the aromatic ring appears to have a marked effect on the binding tendencies.

There is a surprising absence of any significant "lock and key" specificity, particularly among the complexes of the aromatic acid salts with prednisolone and phenacetin.

The results suggest that both hydrophobic bonding and donor-acceptor interactions involving the nucleus of the aromatic carboxylates contribute to formation of the complexes.

The binding with theophylline appears to be at least partially due to specific charge-transfer interactions.

The extent of complex formation of the aromatic carboxylate salts with theophylline, phenacetin, and prednisolone is increased by (*a*) substitution of halo groups *meta* and *para* to the carboxylate, the enhancement increasing in the order chloro, bromo, and iodo; (*b*) methyl, nitro, and hydroxyl groups substituted *meta* and *para* to the carboxylate; (*c*) placement of carboxylic acid and hydroxy groups *ortho* to the carboxylate ion; (*d*) further substitution of the above substituents on the aromatic carboxylates; (*e*) expansion of a benzenoid system to a naphthalene structure and by generally increasing the planar surface of the substituted acid salts.

The extent of interaction of these systems is decreased by halogen substitution on the ring *ortho* to the carboxylate group and by substitution of carboxylate ion.

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Emulsifying Effects of Some Nonionic Surfactants on a Nonaqueous Immiscible System

By R. V. PETERSEN, R. D. HAMILL, and J. D. McMAHON

Representative members of seven classes of nonionic surfactants and combinations of these agents were tested by hand methods of trituration for their ability to induce emulsification of glycerin and olive oil. No relation between HLB values and emulsifying capacity, method of mixing, or emulsion type was apparent. On the other hand, the chemical nature of the surfactant appeared to have an effect on the method of mixing and emulsion type. Only stearate ester surfactants induced emulsification when the surfactant was first added to olive oil. In addition, only stearate ester surfactants induced glycerin-in-oil emulsification.

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Various nonionic surfactants have been used in formulations which have been administered orally to humans (2). Ester type products are hydrolyzed in the digestive tract in a manner similar to edible fats and oils. The fatty acid portions are available for nutrition like those from natural fats, while the polyol moieties are eliminated (3). The glyceryl monostearate products (GMS 165,[1] glyceryl monostearate, self-

[1] Marketed as Arlacel 165 by Atlas Chemical Industries, Inc., Wilmington, Del.

295 mμ. The same experiment repeated with sodium 2-chlorobenzoate produced a similar band at 295 mμ. The above procedure repeated with the hydrochloride salt of 2-dimethylaminomethyl-4-methylphenol instead of the benzoates revealed a peak at approximately 307 mμ very similar in its sharpness to that obtained with the procaine hydrochloride-caffeine system.

These results indicate that theophylline appears to participate in charge-transfer complexation. The observed spectra with both the benzoates and the protonated Mannich base suggest theophylline's ability to share electrons with both positively and negatively charged aromatic compounds. It is evident, however, that further investigation of a more quantitative nature would be required to determine whether these charge-transfer interactions account for the total interaction by theophylline in aqueous solutions.

CONCLUSIONS

Solubility studies on the interactions of theophylline, prednisolone, and phenacetin with substituted benzoates, phthalates, and naphthoates produced the following observations.

Complex formation between aromatic carboxylate salts and the above drugs occurred in every instance, but with varying extents.

Introduction of substituents on the aromatic ring appears to have a marked effect on the binding tendencies.

There is a surprising absence of any significant "lock and key" specificity, particularly among the complexes of the aromatic acid salts with prednisolone and phenacetin.

The results suggest that both hydrophobic bonding and donor-acceptor interactions involving the nucleus of the aromatic carboxylates contribute to formation of the complexes.

The binding with theophylline appears to be at least partially due to specific charge-transfer interactions.

The extent of complex formation of the aromatic carboxylate salts with theophylline, phenacetin, and prednisolone is increased by (*a*) substitution of halo groups *meta* and *para* to the carboxylate, the enhancement increasing in the order chloro, bromo, and iodo; (*b*) methyl, nitro, and hydroxyl groups substituted *meta* and *para* to the carboxylate; (*c*) placement of carboxylic acid and hydroxy groups *ortho* to the carboxylate ion; (*d*) further substitution of the above substituents on the aromatic carboxylates; (*e*) expansion of a benzenoid system to a naphthalene structure and by generally increasing the planar surface of the substituted acid salts.

The extent of interaction of these systems is decreased by halogen substitution on the ring *ortho* to the carboxylate group and by substitution of carboxylate ion.

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[1] Marketed as Arlacel 165 by Atlas Chemical Industries, Inc., Wilmington, Del.

emulsifying, and GMS 169,[2] glyceryl monostearate, nonself-emulsifying) are types of partial ester emulsifiers long accepted for use as ingestible ingredients (3). Various sucrose derivatives, particularly the esters, have been found to be quite nontoxic; hence, their use as emulsifying agents in pharmaceuticals has been suggested (4–7).

Nonaqueous emulsions, such as those described in this report, may be of pharmaceutical or cosmetic value since they are composed primarily of edible, nontoxic ingredients and can be formulated to exhibit a wide range of physical properties. Some possible uses might be as topical application bases for dermatologicals, particularly for hydrolyzable drugs, as emollient bases for cosmetic preparations, or as nutrient preparations.

In 1949, Griffin (8) devised a method of classification of nonionic surfactants based on the ratio of hydrophilic to lipophilic (polar to nonpolar) properties. Subsequently, other investigators have proposed various ways of expressing polar-nonpolar relations of surfactants (9, 10). The values used throughout this report are based on the Griffin proposals.

Although the emulsifying effects of nonionic surfactants on water-oil systems and the validity of the HLB scale as a guide to the estimation of the effects of these agents on aqueous systems have been extensively studied, very little is known regarding the emulsifying effects of nonionic surfactants on nonaqueous systems or regarding the utility of established HLB values as predictive tools for their effects in such systems. For these reasons, this study was undertaken. The results obtained serve as a basis for this report.

EXPERIMENTAL

The methods employed throughout this study were the same as reported in a previous paper which described the emulsifying effects of *ionic* surfactants on glycerin and olive oil (1). Briefly, in *Method I* the surfactant was added to the requisite weight of glycerin. An equal weight of olive oil was added in small portions, with vigorous trituration after each addition. The quantities of reagents and procedure in *Method II* were the same as in *Method I*, except for the order of mixing. In *Method II*, the surfactant was first added to the olive oil. The glycerin was then added in small portions, with rapid trituration after each addition. Heat was applied when necessary to effect solution or dispersion of the surfactant in the glycerin or olive oil, but all reagents were returned to room temperature before emulsification was attempted. Commercial grades of surfactants were used without further purification. The glycerin and olive oil were U.S.P. grade.

To study the influence of the hydrophile-lipophile-balance (HLB) value on emulsifying capacity, order of mixing, and emulsion type, blends of Arlacels and polysorbates were prepared. Solid surfactants required heating in a water bath to effect blending. The proportion of each surfactant in a blend was calculated by alligation methods or by reference to the Atlas HLB computing graph.[3] These methods depend on a linear relation of the HLB value of a mixture with that of the pure components.

The criteria used to evaluate the products were based on three observations: emulsifying capacity, miscibility of emulsions with added olive oil or glycerin, and microscopic examination. Emulsifying capacity was evaluated on the basis of the appearance of the preparation after standing for 7 days. If any degree of separation into layers occurred within this period of time, the preparation was considered unstable. If no separation was detectable, the product was considered an emulsion. Figure 1 illustrates six representative preparations as they appear after standing 7 days. These preparations served as standards for evaluation of all other preparations. Bottle *1* contains 2% polyoxyethylene lauryl ether (POL 35)[4] in equal weights of glycerin and olive oil and is representative of preparations classified as *separations*. Bottle *2* contains 2% GMS 165. This, and similar preparations, are classified as *partial separations*. In most instances, preparations classified as partial separations appeared emulsified when first made, but exhibited some degree of separation on standing for 1 week. Bottles *3* to *6* contain a clear emulsion, an opaque emulsion, a creamy, white emulsion, and a semisolid, white emulsion, respectively. No. *3* was induced by 2% POL 30[5] by *Method I;* No. *4* by 5% GMS 165 by *Method I;* No. *5* by 4% of a combination of sorbitan monostearate (S-60)[6] and polyoxyethylene sorbitan monostearate (PS-60)[7] at HLB 6 by *Method I;* and No. *6* by 4% S-60 by *Method II*. These four and similar preparations are classified as *emulsions*.

Miscibility of emulsions with added olive oil or glycerin was used for determining emulsion type. This observation was based on the usual assumption that emulsions mix readily with additional liquid corresponding to the external phase, but not the internal phase.

Microscopic examination of emulsions prepared from olive oil which contained 1.0% dissolved D&C Red No. 18 dye was employed to confirm emulsification and to verify emulsion type.

RESULTS

Several of the nonionic surfactants tested and blends of these agents were effective in producing emulsions of glycerin and olive oil. A summary of the results is given in Table I.

[2] Marketed as Arlacel 169 by Atlas Chemical Industries, Inc., Wilmington, Del.

[3] Atlas Chemical Industries, Inc., Wilmington, Del.

[4] Marketed as Brij 35 by Atlas Chemical Industries, Inc., Wilmington, Del.

[5] Marketed as Brij 30 by Atlas Chemical Industries, Inc., Wilmington, Del.

[6] Marketed as Arlacel 60 by Atlas Chemical Industries, Inc., Wilmington, Del.

[7] Marketed as Tween 60 by Atlas Chemical Industries, Inc., Wilmington, Del.

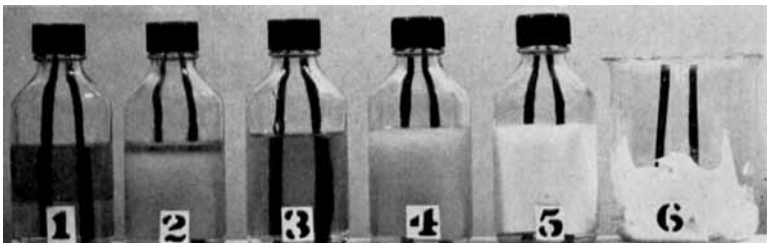


Fig. 1.—Physical appearance of representative preparations. Key: *1*, a separation; *2*, a partial separation; *3*, a clear emulsion containing 2% POL 30; *4*, an opaque emulsion containing 5% GMS 165; *5*, a creamy, white emulsion containing 4% of a combination S-60 and PS-60 at HLB 6; *6*, a semisolid, white emulsion containing 4% S-60.

TABLE I.—EMULSIFYING EFFECTS OF SOME NONIONIC SURFACTANTS AND COMBINATIONS ON EQUAL WEIGHT OF GLYCERIN AND OLIVE OIL OBSERVED 7 DAYS FOLLOWING PREPARATION

Surfactant or Blend	HLB[a]	%	Method I[b]	Method II[b]	Appearance of Emulsion	Type of Emulsion
S-20	8.6	4	Emulsion	Separation	Clear	o/g
PS-20	16.7	4	Emulsion	Separation	Clear	o/g
S-20 + PS-20 (83:17)[c]	10.0	4	Emulsion	Separation	Clear	o/g
S-20 + PS-20 (58:42)	12.0	4	Emulsion	Separation	Clear	o/g
S-20 + PS-20 (33:67)	14.0	4	Emulsion	Separation	Clear	o/g
S-20 + PS-20 (9:91)	16.0	4	Emulsion	Separation	Clear	o/g
S-40	6.7	4	Emulsion	Separation	Opaque	o/g
PS-40	15.6	4	Emulsion	Separation	Clear	o/g
S-40 + PS-40 (85:15)	8.0	4	Partial sep.	Separation	...	...
S-40 + PS-40 (63:37)	10.0	4	Partial sep.	Separation	...	...
S-40 + PS-40 (40:60)	12.0	4	Partial sep.	Separation	...	...
S-40 + PS-40 (18:82)	14.0	4	Partial sep.	Separation	...	...
S-60	4.7	4	Emulsion	Emulsion	Semisolid, white	o/g
PS-60	14.9	4	Emulsion	Separation	Clear	o/g
S-60 + PS-60 (87:13)	6.0	4	Emulsion	Emulsion	Creamy, white	o/g
S-60 + PS-60 (68:32)	8.0	4	Emulsion	Emulsion	Creamy, white	o/g
S-60 + PS-60 (48:52)	10.0	4	Emulsion	Partial sep.	Opaque	o/g
S-60 + PS-60 (29:71)	12.0	4	Emulsion	Partial sep.	Opaque	o/g
S-60 + PS-60 (9:91)	14.0	4	Emulsion	Separation	Clear	o/g
S-80	4.3	4	Emulsion	Separation	Clear	o/g
PS-80	15.0	4	Emulsion	Separation	Clear	o/g
S-80 + PS-80 (84:16)	6.0	4	Emulsion	Separation	Clear	o/g
S-80 + PS-80 (66:34)	8.0	4	Emulsion	Separation	Clear	o/g
S-80 + PS-80 (47:53)	10.0	4	Emulsion	Separation	Clear	o/g
S-80 + PS-80 (28:72)	12.0	4	Emulsion	Separation	Clear	o/g
S-80 + PS-80 (9:91)	14.0	4	Emulsion	Separation	Clear	o/g
S-83	3.7	4	Emulsion	Separation	Clear	o/g
S-85	1.8	4	Emulsion	Separation	Clear	o/g
GMS 165	11.0	2	Partial sep.	Partial sep.	...	...
GMS 165	11.0	5	Emulsion	Emulsion	Opaque	g/o
GMS 169	4.0	2	Partial sep.	Partial sep.	...	...
GMS 169	4.0	5	Emulsion	Emulsion	Opaque	g/o
POL 30	9.5	2	Emulsion	Separation	Clear	o/g
POL 30	9.5	5	Emulsion	Separation	Clear	o/g
POL 35	16.9	2	Separation	Separation	...	...
POL 35	16.9	5	Separation	Separation	...	...
POS 45	11.1	2	Separation	Separation	...	...
POS 45	11.1	5	Separation	Separation	...	...
POS 52	16.9	2	Partial sep.	Separation	...	...
POS 52	16.9	5	Partial sep.	Partial sep.	...	...
POS 52	16.9	10	Partial sep.	Partial sep.	...	...
DPM 600	...	2	Emulsion	Separation	Creamy, white	o/g
DPM 600	...	5	Emulsion	Separation	Creamy, white	o/g
OHS 80	...	2	Emulsion	Separation	Opaque	o/g
OHS 80	...	4	Emulsion	Separation	Opaque	o/g
PPO L-64[d]	...	4	Separation	Separation	...	...
PPO L-64	...	8	Separation	Separation	...	...
PPO F-68[d]	...	4	Separation	Separation	...	...
PPO F-68	...	8	Separation	Separation	...	...

[a] HLB values given in Atlas Catalog of "Products for Cosmetic Formulations," Atlas Powder Co., Wilmington, Del., 1957 [b] See text. [c] Figures in parentheses indicate the ratio of Arlacel:polysorbate in a blend. [d] Polyoxyethylene derivatives o polypropylene oxides. Marketed as Pluronic L-64 and Pluronic F-68 by the Wyandotte Chemical Corp., Wyandotte, Mich.

It should be emphasized that the results reported in the table were derived only from those preparations which contained equal weights of olive oil and glycerin. Indeed, on several occasions emulsification was apparent at lower concentrations of oil, but not the 50% level. For example, in the experiments using a blend of polyoxyethylene sorbitan monopalmitate (PS-40)[8] and sorbitan monopalmitate (S-40)[9] at HLB 10, an emulsion resulted when 43% oil was used, whereas partial separation occurred in the preparations containing 50% oil.

In a few instances, preparations listed as emulsions in Table I showed a partial separation after standing for 2 or 3 months. Preparations made from POL 30, sorbitan monolaurate (S-20)[10]–polyoxyethylene sorbitan monolaurate (PS-20)[11] combinations, S-40–PS-40 combinations, sorbitan monooleate (S-80)[12]–polyoxyethylene sorbitan monooleate (PS-80)[13] combinations, or from sorbitan trioleate (S-85)[14] showed a layer of oil on top with a gradual lightening of color on the bottom after 90 days. Products thus separated could not be restored to their original emulsified state by simple shaking. Emulsions made from S-60, PS-60, combinations of S-60 and PS-60, sorbitan sesquioleate (S-83),[15] GMS 165, GMS 169, or sucrose dipalmitate (DPM 600)[16] showed little or no tendency to separate and were considered the most stable products.

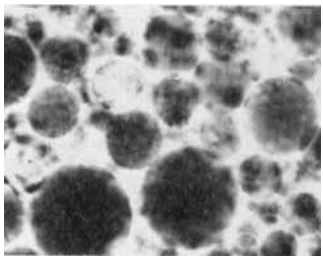


Fig. 2.—Photomicrograph of an emulsion containing 4% of a combination of S-60 and PS-60 (HLB 6) in equal weights of glycerin and olive oil, stained with D&C Red No. 18 dye. The emulsion was deaerated under reduced pressure prior to being photographed (520× magnification).

As shown in Table I, *Method I* was effective in all systems which contained Arlacels, polysorbates, combinations of Arlacels and polysorbates (except combinations of S-40 and PS-40), POL 30, DPM 600, or octakis(2-hydroxypropyl)sucrose OHS 80[17]; whereas *Method II* was effective only in those preparations which contained GMS 165, GMS 169, S-60, or combinations of the latter with PS-60. Table I shows also that all of the emulsions were of the oil-in-glycerin (o/g) type, except for those prepared from GMS 165 or GMS 169. Figure 2 is a photomicrograph of an o/g emulsion containing 4% of a combination of S-60 and PS-60 at HLB 6. Figure 3 is a photomicrograph of a glycerin-in-oil (g/o) emulsion containing 5% GMS 165. Mixed emulsification can be detected in the latter figure. Some degree of mixed emulsification was observed also in many other preparations.

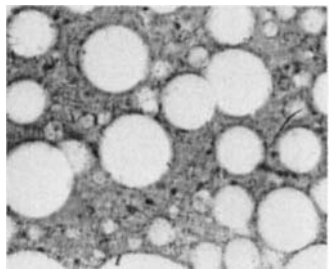


Fig. 3.—Photomicrograph of an emulsion containing 5% GMS 165 in equal weights of glycerin and olive oil, stained with D&C Red No. 18 dye (260× magnification).

All of the preparations which contained GMS 165, GMS 169, or high proportions of S-60 were opaque, creamy, or semisolid preparations. The incorporation of large amounts of air was characteristic of preparations containing relatively high proportions of S-60, but not of the other opaque emulsions. Trituration of glycerin and S-60, in the absence of olive oil, produced foams which retained the entrapped air for several days. Deaeration under reduced pressure of small portions of S-60-containing emulsions resulted in almost complete clarification. In all other instances where opaque emulsions formed, the opacity was not due to entrapped air, and clarification could not be achieved by application of reduced pressures. Incomplete solubility of the surfactant may have contributed to their appearance in such cases.

Preparations made from S-60 or its blends, by *Method II*, required heavy trituration for long periods of time (20–40 minutes) after addition of all glycerin before emulsification was effected.

DISCUSSION

The results of these studies indicate that neither the capacity to produce an emulsion of glycerin and olive oil, the comparative efficacy of *Methods I* and *II*, nor the type of emulsion formed can be related to the HLB value of the surfactant used.

Evidence for the lack of correlation between the capacity to produce an emulsion and the HLB value of the surfactant is shown by the fact that emulsification resulted from the use of agents with HLB values from 1.8 (S-85) to 16.7 (PS-20), and by the fact that separations and partial separations occurred also within this range. To cite a specific example—at HLB 10, combinations of S-20 and PS-20, S-60 and PS-60, or S-80 and PS-80 produced emulsions, whereas a combination of S-40 and PS-40 at the same HLB value failed to produce an emulsion. Immediately after manipulation it appeared that emulsification of the latter was effected, but partial separation occurred within 7 days.

Emulsions were obtained by *Method I* from surfactants having HLB values from 1.8 (S-85) to 16.7 (PS-20) and by *Method II* from agents having HLB values from 4.0 (GMS 165) to 11.0 (GMS 169). In one series of experiments it appeared originally that emulsification had been induced by an agent with an HLB value of 16.9 (polyoxyethylene

[8] Marketed as Tween 40 by Atlas Chemical Industries, Inc., Wilmington, Del.
[9] Marketed as Arlacel 40 by Atlas Chemical Industries, Inc., Wilmington, Del.
[10] Marketed as Arlacel 20 by Atlas Chemical Industries, Inc., Wilmington, Del.
[11] Marketed as Tween 20 by Atlas Chemical Industries, Inc., Wilmington, Del.
[12] Marketed as Arlacel 80 by Atlas Chemical Industries, Inc., Wilmington, Del.
[13] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.
[14] Marketed as Arlacel 85 by Atlas Chemical Industries, Inc., Wilmington, Del.
[15] Marketed as Arlacel 83 by Atlas Chemical Industries, Inc., Wilmington, Del.
[16] Marketed as Sucrodet D-600 by the Millmaster Chemical Corp., New York, N. Y.
[17] Marketed as Hyprose SP-80 by the Dow Chemical Co., Midland, Mich.

stearate, POS 52[18]) by *Method II;* however, these preparations failed to remain stable for 7 days and are thus shown in Table I as partial separations. These data indicate that no correlations can be drawn between HLB values and the relative efficacy of *Methods I* and *II*.

Based on the fact that glycerin is considered to be a polar substance, it was expected that surfactants having high HLB values would favor o/g emulsification and those having low HLB values would favor g/o emulsification. This, however, did not result. Oil-in-glycerin emulsions were produced from agents ranging in HLB values from 1.8 (S-85) to 16.7 (PS-20), and glycerin-in-oil emulsions resulted from the use of agents having HLB values of 4.0 (GMS 165) and 11.0 (GMS 169). The fact that the HLB value of a surfactant could not be correlated with emulsion type in oil-glycerin systems is in direct conflict with the fact that there is a close correlation between HLB values and emulsion type in oil-water systems.

The chemical nature of the surfactant appears to influence the efficacy of the methods employed and the resulting emulsion type. Thus, the stearate ester surfactants, such as GMS 165 (glyceryl monostearate, self-emulsifying), GMS 169 (glyceryl monostearate, nonself-emulsifying), S-60 (stearate ester of sorbitol anhydride), and combinations of the latter with PS-60 (stearate ester of polyoxyethylene sorbitol anhydride), all produced emulsions by both methods and, except for S-60 and its combinations with PS-60, resulted in emulsions of the glycerin-in-oil type. It should be emphasized that these are the only emulsions produced by *Method II* and are the only g/o emulsions produced by either *Method I* or *Method II*. To test this interpretation, another stearate surfactant was used in an attempt to emulsify this system. Preparations containing POS 52 were made by both *Methods I* and *II*. These preparations, as shown in Table I, exhibited partial separation within 7 days. They did, however, remain stable for at least 24 hours. Microscopic observation of the dye-containing emulsions made from POS 52 revealed that they were of the g/o type. This was further substantiated by the fact that they mixed readily with added olive oil, but not with glycerin.

[18] Marketed as Myrj 52 by Atlas Chemical Industries, Inc., Wilmington, Del.

The above observations regarding stearates are in accord with previous work reported from our laboratories (1) which demonstrated that the only *ionic* surfactants tested which produced g/o emulsions were sodium stearate and stearyltrimethylammonium chloride. Thus, it might be concluded that the chemical nature, more than the HLB value, influenced the method of mixing and the type of emulsion produced.

SUMMARY

Several nonionic agents, including S-20, S-40, S-60, S-80, S-83, S-85, GMS 165, GMS 169, PS-20, PS-40, PS-60, and PS-80, POL 30 and 35, POS 45 and 52, DPM 600, OHS 80, PPO L-64 and F-68 were tested for their capacity to emulsify the nonaqueous system, glycerin and olive oil. Blends of Arlacels and polysorbates at various HLB values also were prepared and tested. Stable emulsions resulted from the use of several of these agents and combinations. The HLB value of a given surfactant, or blends of two surfactants, could not be correlated either with its ability to induce emulsification, its effect on emulsion type, or its influence on method of mixing the emulsion components. It was shown that the chemical nature of the surfactant may be somewhat determinant in the type of emulsion formed and on the order of mixing of the components. In all cases where g/o emulsions formed and/or emulsification was successful by first incorporating the surfactant into the oil (*Method II*), the surfactant was a derivative of stearic acid.

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Dissolution Rate Studies on Methylprednisolone Polymorphs

By GERHARD LEVY and JOSEPHINE A. PROCKNAL

Dissolution rates (DR) of methylprednisolone polymorphs I and II were determined by the rotating disk method as a function of rate of rotation (RR). The results show that the DR–RR relationship differs from that normally expected in the case of diffusion-controlled dissolution from rotating disks. The experimental data confirm the observations by Hamlin, *et al.* (5), that differences in DR between methylprednisolone polymorphs diminish at higher agitation intensities and indicate that this is due to an intrinsic property of the drug and is not caused by particular apparatus or methodology. From DR–RR data on methylprednisolone I, it was possible to determine the approximate agitation intensities, in terms of rotating disk r.p.m., obtained with five commonly used dissolution methods. These values are shown to be in reasonable agreement with those calculated independently from aspirin and salicylic acid data.

STUDIES OF intrinsic dissolution rates can be useful in evaluating the prospective absorption rate and physiologic availability of drugs which, when administered orally in solid form, are absorption-rate limited by the dissolution process (1, 2). Such studies are particularly pertinent to steroids because of the poor water-solubility of most of these and the difficulties encountered in determining their physiologic availability, due to the small doses customarily employed.

Recently (3), one of the authors (G. L.) has presented an equation (and experimental verification) evolved from an equation developed by Cooper and Kingery (4), which relates the effect of rate of rotation (RR) to the diffusion-controlled rate of dissolution (DR) of substances from the face of a rotating disk, *viz.*,

$$DR = K(RR)^{0.5} \qquad \text{(Eq. 1)}$$

Equation 1 assumes that (*a*) the surface area of the disk remains constant during dissolution, (*b*) the concentration of the dissolving substance in the bulk medium is very much less at all times than its solubility, and (*c*) the effective weighted average viscosity of the boundary layer and the effective weighted average diffusion coefficient of the dissolving species in the boundary layer are independent of rate of rotation (and thus independent of effective boundary layer thickness).

On the basis of Eq. 1 and our experimental data, we have shown (3) that the *ratio* of the dissolution rates of compounds is independent of rotation rate, provided that these compounds behave according to Eq. 1 or to a more general relationship

$$DR = K(RR)^{a} \qquad \text{(Eq. 2)}$$

where the value of the exponent a is the same for all the respective compounds.

The above relationships are useful since they permit one to compare the *in vitro* dissolution rate of a new drug with that of an older drug. In this way one may, in certain instances, deduce the relative *in vivo* dissolution rate of the new drug from absorption data obtained previously with the older drug. This is particularly so if pK's are similar or if the solubility of both drugs is independent of pH, but requires that dosage form properties (including particle size) be essentially the same in each case.

Hamlin, *et al.* (5), have reported recently results of experiments which show that differences in dissolution rates of methylprednisolone polymorphs I and II, observed at low agitation intensities, disappeared at higher agitation intensities. These results could be due to loss of sensitivity encountered with some of the apparatus and methodology used or it could be due to an intrinsic property of the polymorphs. The latter must be reflected by one of the following: (*a*) one or both polymorphic forms do not behave according to Eq. 1, (*b*) one or both forms do not behave according to Eq. 2, or (*c*) both forms behave according to Eq. 2 but differ in the value of exponent a. These possibilities have been investigated by the authors, and the results of the study are the subject of this report.

It was possible to make further use of the experimental data since they permitted an estimation of agitation intensities obtained with various apparatus and methods used in different laboratories. Independent estimations of these values were also made (using dissolution rate data obtained with other drugs), in order to check our methodology and treatment of data.

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EXPERIMENTAL

Materials.—Methylprednisolone polymorphs I and II were used in the forms in which they were obtained. Physicochemical descriptions of these two materials have been published previously by W. I. Higuchi, *et al.* (6). Acetylsalicylic acid and salicylic acid were of U.S.P. grade.

Dissolution Rate Determinations.—Pure methylprednisolone was compressed into flat-faced pellets of 1.27 cm. diameter at a pressure of 3570 Kg./cm.[2] with a modified Carver hydraulic press. Dissolution rate measurements were made in distilled water (methylprednisolone) and in 0.1 *N* hydrochloric acid (aspirin and salicylic acid) at 37 ± 0.1° by the rotating disk and beaker methods. Details of these methods and the respective apparatus have been reported in previous publications by Levy and Sahli (7) and by Levy and Hayes (8), respectively. Some of the rotating disk experiments (particularly the low-speed runs) were carried out with a newly developed precision apparatus (9). The duration of most runs was 2–4 hours, with sampling at 30 minute intervals. Data shown in Fig. 1 represent averages of three to five individual experiments. The beaker method, employed previously only for conventional tablets, was modified for intrinsic dissolution rate determinations by mounting the drug pellet with paraffin wax on a microscope cover glass (such that one face of the pellet remained exposed) and attaching the cover glass to the center of the bottom of the beaker with a water-insoluble adhesive. Samples of dissolution media were analyzed spectrophotometrically at 248 mμ (methylprednisolone) or 302 mμ (salicylic acid) after appropriate dilution with water (in the case of methylprednisolone), 0.1 *N* hydrochloric acid (in the case of salicylic acid), or alkaline hydrolysis followed by dilution with 0.1 *N* hydrochloric acid (in the case of aspirin).

RESULTS AND DISCUSSION

Figure 1 shows the dissolution rates of methylprednisolone forms I and II, determined by the rotating disk method, as a function of rate of disk rotation. Polymorph I follows the relationship expressed by Eq. 2, but the slope value is 0.37 rather than 0.5 as might be expected (3). Polymorph II apparently does not obey either Eqs. 1 or 2, since

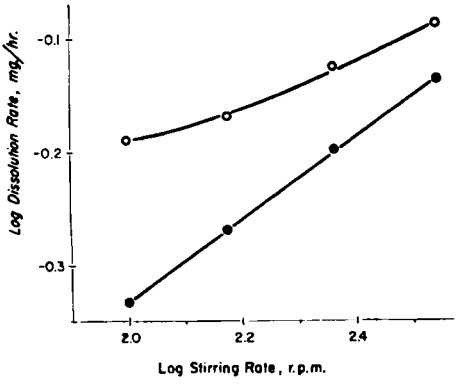


Fig. 1.—Dissolution rates of methylprednisolone polymorph I (●) and polymorph II (○) from rotating disks (1.27 cm.[2]) as a function of rate of disk rotation.

the log-log plot yielded a slight curve. The latter statement is made with reservations, since the reproducibility of dissolution runs with polymorph II was not as good as that with polymorph I.[1]

Figure 1 shows that differences in dissolution rate between forms I and II tend to disappear at higher agitation intensities.[2] This confirms the observations reported by Hamlin, *et al.* (5). In addition, since all measurements were made with the same apparatus, and since the utility of the method to yield proper data (0.5 slope values where applicable) has already been demonstrated (3), it may be concluded that the decrease in dissolution rate differences at higher agitation intensities is probably an intrinsic characteristic of the drug and not due to the apparatus or methodology used by Hamlin, *et al.* (5). The reasons for this unusual behavior are not readily apparent and are the subject of further investigations.

One important implication of the experimental results is that *in vitro* dissolution rate data obtained with compounds behaving like the methylprednisolone polymorphs may be misleading biopharmaceutically if the agitation intensity of the test procedure is either too high *or* too low. Thus, one would expect that very low agitation intensity procedures may yield good correlation with *in vivo* data obtained with pellet implants, but that correlation with data obtained from gastrointestinal absorption experiments may be better with *in vitro* data obtained at somewhat higher agitation intensities. It is certainly appropriate to characterize the dissolution-rate dependence of drugs on agitation intensity as part of their biopharmaceutical evaluation. Among the factors which are already known to account for unusual dissolution rate-agitation rate relationships are surface film formation (10, 11), interfacial reaction-controlled dissolution (5), surface effervescence (12), and surface-pitting (3).

The linearity of the log DR–log RR plot for methylprednisolone I and the good reproducibility of the data suggested to us the possibility of using our results as a "standard curve" to determine the relative agitation intensity encountered with other published dissolution procedures. The desirability of obtaining such information to permit comparison of dissolution data reported by different workers using different methodology has been emphasized strongly (13). Using the regression equation $y = 0.368x - 1.068$ which represents the straight line fitted to the methylprednisolone I data by the method of least squares [where x is log RR (in r.p.m.) and y is log DR (in mg./1.27 cm.[2]/hr.)], agitation intensities for the hanging pellet, Wruble 6 r.p.m., Wruble 12 r.p.m., and Souder and Ellenbogen 40 r.p.m. procedures were calculated from the data of Hamlin, *et al.* (5). Agitation intensity for the beaker procedure was calculated from data collected in our own laboratory. The agitation intensities are expressed in terms of rotating disk equivalent (RDE) r.p.m. This permits comparison of intensities derived from data obtained with different compounds without having to know their respective solubilities and diffusion coefficients.

[1] Similar differences in reproducibility are apparent from the data of Hamlin, *et al.* (5).

[2] It is possible that polymorph II may actually dissolve less rapidly than polymorph I at some sufficiently high agitation rate. Technical limitations prevented determinations of dissolution rates at such high rates of disk rotation.

TABLE I.—AGITATION INTENSITY EQUIVALENTS (IN TERMS OF ROTATING DISK R.P.M.) OF VARIOUS DISSOLUTION METHODS

Method	Dissolution Rate (mg./cm.²/hr.)	RDE (r.p.m.)[b]
I. Based on Methylprednisolone Polymorph I[a]		
Hanging pellet	0.091[c]	2.3
Wruble apparatus, 6 r.p.m.	0.203[c]	20
Wruble apparatus, 12 r.p.m.	0.276[c]	46
Beaker	0.266	42
Souder and Ellenbogen apparatus, 40 r.p.m.	0.630[c]	435
II. Based on Salicylic Acid[d]		
Beaker	14.6	50
III. Based on Acetylsalicylic Acid[e]		
Wruble apparatus, 6 r.p.m.	12.8[f]	14
Beaker	18.9[g]	49

[a] Calculated from equation $Y = 0.368x - 1.068$, where x is log r.p.m. and y is log dissolution rate in mg./1.27 cm.²/hr. in water at 37°. [b] RDE = rotating disk equivalent. [c] Data from Hamlin, *et al.* (5). [d] Calculated from equation $y = 0.495x + 0.320$, where x is log r.p.m. and y is log dissolution rate in mg./cm.²/hr. in 0.1 *N* HCl at 37°. [e] Calculated from equation $y = 0.515x + 0.406$, where x is log r.p.m. and y is log dissolution rate in mg. salicylic acid equivalent/cm.²/hr. in 0.1 *N* HCl at 37°. [f] Data from Hamlin (14). [g] In terms of salicylic acid.

Table I shows the calculated RDE values for five published dissolution procedures. In view of a number of other imponderables related to the use of data collected in more than one laboratory, no attempt has been made to treat the results statistically. The RDE values listed in Table I should therefore be looked upon as reasonable approximations only.

The use of the regression equation to derive RDE values which fell beyond the range of experimental data from which the equation was calculated was based on the assumption that the linearity of the log DR *versus* log RR plot is maintained over a much wider range, particularly at lower rates. While extension of our rotating disk studies to very low rotation rates was impracticable due to the extremely low dissolution rate of the steroid, it was thought necessary to carry out at least one DR determination at a very low RR. Accordingly, the dissolution rate of methylprednisolone I was determined at 4 r.p.m. in a 6-hour run with 1-hourly sampling of medium. This yielded a dissolution rate of 0.12 mg./hour, compared with a theoretical value of 0.14 mg./hour calculated from the regression equation. Considering the extent of extrapolation and the experimental difficulties, this can be considered a very satisfactory agreement and justification for use of the regression equation over the indicated range.

An independent estimation of some RDE values was possible with data obtained from two other drugs, namely aspirin and salicylic acid. Using regression equations obtained in a previous study (3), an RDE value of 49 and 50 r.p.m., respectively, was obtained for the beaker method, which may be compared with a value of 42 r.p.m. calculated from the methylprednisolone I data. The agreement is quite good and also affords further support for extrapolation of the methylprednisolone data and the reasonable correctness of the slope value. RDE values for the Wruble 6 r.p.m. procedure were 20 and 14 r.p.m. from methylprednisolone and aspirin data, respectively. Considering the marked sensitivity of DR to changes in RR at the lower RR range, one may conclude that these RDE values are also in reasonably good agreement.

The RDE values obtained for the rotating bottle and tube methods may appear surprisingly high when compared with the rate of wheel rotation, but these high agitation intensities probably are due to the added agitation (and turbulence) caused by the to-and-fro movement of the void space in the containers. The RDE estimations for the Wruble and Souder and Ellenbogen methods apply only to instances where the solid drug is immobile (held rigidly in the container) and not where drug solids move freely in the dissolution medium. The latter case will usually involve much greater agitation (5) which varies with particle size and specific gravity of the drug, among others (1), and is probably not readily reproducible. It may seem unusual that an RDE value is provided for the hanging pellet procedure, since it involves an immobile object immersed in unstirred medium. However, solvent does flow over the pellet surface due to the difference in density of the drug-saturated boundary layer and the bulk medium. The rate of this flow is a function of the viscosity of the medium and the difference in density between the boundary layer and bulk medium (15). This difference is a function of the solubility of the drug and its effect in changing the density of the solvent. Thus, an RDE value for the hanging pellet is justified, but it is not a constant. It will differ with each drug, depending on the viscosity and density conferred by the drug on the boundary layer. Nelson has pointed out previously that dissolution from a hanging pellet and from stirred media represent different types of processes, with only the latter usually following Noyes-Whitney kinetics (15).

The experimental results described in this report clearly point up a new difficulty which may be encountered in dissolution rate studies pursued for biopharmaceutical purposes, and it appears prudent to obtain dissolution rate-agitation intensity profiles rather than to determine dissolution rates of substances at only one agitation intensity. Further studies in this and other laboratories may show whether the unexpected DR–RR relationship observed with methylprednisolone polymorphs is characteristic of steroids and certain other classes of compounds in general or whether it is a rare and unusual phenomenon.

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Preservatives for Poliomyelitis (Salk) Vaccine II

Formaldehyde and Esters of *p*-Hydroxybenzoic Acid

By J. M. TRACY, D. G. GLASS, M. J. NICHOLSON, and HILLIARD PIVNICK

During the manufacture of poliomyelitis vaccine, 92 p.p.m. of formaldehyde was added to inactivate the virus. After 2 years at 4°, 70–80 p.p.m. was still present. If not neutralized by bisulfite, this concentration of formaldehyde was a potent antibacterial agent, but had little antimycotic activity. The addition of toxoids and pertussis vaccine to poliomyelitis vaccine to form DPT polio caused no immediate loss of formaldehyde but after about 10 months at 4° and 25° there were losses of about 50 and 80 per cent, respectively. The loss of 50 per cent of the formaldehyde decreased the preservative activity. The methyl and propyl parabens in a ratio of 10:1 and a final concentration of 0.165 per cent were only moderately antibacterial, but were effective antifungal agents. The addition of parabens to vaccine containing nonneutralized formaldehyde gave a mixture of preservatives which was inhibitory against high concentrations of both bacterial and fungal contaminants. Parabens also prevented loss of formaldehyde from DPT polio vaccine.

A PREVIOUS STUDY (1) showed that benzethonium chloride possessed limited usefulness as a preservative for inactivated poliomyelitis vaccine (Salk). However, the antibiotics and formaldehyde added to the vaccine during the manufacturing process possessed considerable antibacterial activity. The antibiotics are added to the tissue culture used for proliferation of the poliomyelitis virus to prevent the growth of bacterial contaminants; in the finished vaccine 135 p.p.m. of streptomycin and 10 p.p.m. of neomycin were found. Formalin is added to a concentration of 1:4000 (92 p.p.m. of formaldehyde) to inactivate the virus. The residual formaldehyde (70–80 p.p.m.) may be neutralized with bisulfite or it may be left without neutralization. If not neutralized, the formaldehyde serves as an antibacterial agent.

Neither the antibiotics nor the formaldehyde, at the concentration used in poliomyelitis vaccine, possess marked antimycotic activity. However, the esters of *p*-hydroxybenzoic acid (parabens) have excellent antimycotic activity (2, 3) and do not destroy poliomyelitis antigen (4). Because of the possibility of complementary activity of antibiotics, formaldehyde, and parabens against both bacteria and fungi, we studied mixtures of these materials in poliomyelitis vaccine. This report presents the results of the study.

EXPERIMENTAL

Poliomyelitis vaccine with and without neutralization of formaldehyde by bisulfite, the combination of pertussis vaccine, toxoids, and poliomyelitis vaccine (DPT polio vaccine), HB597 medium, and the microbial cultures have been described previously (1).

Antibiotics, added to the tissue cultures used for growing the poliomyelitis virus, were present in the finished vaccine (1). To obtain comparable concentrations of antibiotics in HB597 medium, we added 140 p.p.m. of streptomycin and 2 p.p.m. of neomycin.

Cultures used for challenge were four strains of *Pseudomonas aeruginosa*, four unidentified *Pseudomonas* species, a *Proteus* species, *Shigella flexneri*, *Salmonella paratyphi C*, two strains of *Staphylococcus aureus*, *Saccharomyces ellipsoideus*, *Debaromyces kloeckeri*, *S. rouxii*, *Rhodotorula glutinis*, *Rhizopus oryzae*, *Fusarium lini*, and *Circinella spinosa*. Preparation of cultures, numbers of cells used in the challenge, and criteria for growth in the challenged preparations have been described (1).

Formaldehyde was measured by the method of Nash (5) and a modification of this method (6). The former measures total formaldehyde, *i.e.*, free formaldehyde plus that which is combined with bisulfite, while the latter method measures only free formaldehyde.

Esters of *p*-hydroxybenzoic acid (parabens) were added as a 29% w/v ethanolic solution to obtain a mixture containing 1.5 mg. of the methyl ester and 0.15 mg. of the propyl ester per milliliter. Higher concentrations crystallized when the vaccine was stored at 4°. Parabens were assayed by the method of Jones, *et al.* (7).

RESULTS

The antimicrobial activity of formaldehyde and antibiotics was tested in HB597 medium. This medium is a component of the tissue culture used for growing poliomyelitis virus and comprises most of the volume of the finished poliomyelitis vaccine.

Table I shows that HB597 medium supported growth of all bacteria, even when the challenge dose was diluted to 10^{-6} (approximately 100 viable cells per challenge), but the medium was less suitable for growth of small inocula of yeasts and molds. The addition of 46 p.p.m. of formaldehyde to HB597 medium inhibited the growth of bacteria, but did not affect yeasts and molds. Increasing the form-

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TABLE I.—PRESERVATIVE EFFECT OF FORMALDEHYDE AND ANTIBIOTICS IN HB597 MEDIUM

Formaldehyde Added, p.p.m.	Dilution[a] of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged — Antibiotics Absent: Bacteria	Antibiotics Absent: Yeasts and Molds	Antibiotics Present: Bacteria	Antibiotics Present: Yeasts and Molds
0	None	13/13	7/7	11/13	7/7
	10^{-3}	13/13	6/7	6/13	6/7
	10^{-6}	13/13	2/7	5/13	4/7
46	None	7/13	7/7	3/13	7/7
	10^{-3}	1/13	6/7	1/13	6/7
	10^{-6}	0/13	4/7	0/13	2/7
92	None	2/13	6/7	1/13	6/7
	10^{-3}	1/13	3/7	0/13	2/7
	10^{-6}	0/13	2/7	0/13	1/7

[a] Undiluted challenge cultures furnished challenge doses of approximately 1×10^8 bacterial cells or 1×10^7 yeast or mold cells.

aldehyde to 92 p.p.m. gave an increased antibacterial activity and possibly some antimycotic activity. The addition of antibiotics to HB597 medium furnished moderate antibacterial activity but no antimycotic activity. Mixtures of antibiotics and formaldehyde possessed greater antibacterial activity than either preservative by itself, but the antimycotic activity of the mixture appears to be due only to the formaldehyde.

Table II shows that the residual formaldehyde in poliomyelitis vaccine contributed considerable antibacterial activity and that neutralization of formaldehyde by bisulfite destroyed this antibacterial activity. The loss of antibacterial activity resulting from neutralization of the formaldehyde confirmed the work of Taylor and Moloney (6), who showed that the Nash method of analysis for formaldehyde neutralized by bisulfite was not a true analysis of the free formaldehyde, whereas their modification of the assay measured free formaldehyde.

Table III shows that formaldehyde in poliomyelitis vaccine was stable for 2 years and that the addition of pertussis vaccine and toxoids to form DPT polio vaccine caused no immediate loss of formaldehyde. As will be shown later, however, prolonged storage of DPT polio vaccine may result in loss of formaldehyde.

The failure of formaldehyde to inhibit yeasts and molds showed that some antimycotic compound should be added. Table IV shows that parabens possessed a marked antifungal activity in HB597 medium but only moderate antibacterial activity. The mixture of parabens with formaldehyde, how-

TABLE II.—EFFECT OF NEUTRALIZING FORMALDEHYDE IN POLIOMYELITIS VACCINE[a] BY ADDITION OF BISULFITE

Vaccine Tested	Formaldehyde, p.p.m. Nash	Formaldehyde, p.p.m. Modified Nash	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
Poliomyelitis vaccine (Lot 155)	82	73	None	2/13	6/7
			10^{-3}	0/13	2/7
			10^{-6}	0/13	1/7
Poliomyelitis vaccine (Lot 155) with bisulfite	63	8	None	11/13	7/7
			10^{-3}	3/13	5/7
			10^{-6}	0.13	6/7

[a] Antibiotics present.

TABLE III.—STABILITY OF THE ANTIBACTERIAL ACTIVITY OF FORMALDEHYDE IN POLIOMYELITIS VACCINE

Prepn. Tested	Storage Period at 4° C.	Formaldehyde, p.p.m. Nash	Formaldehyde, p.p.m. Modified Nash	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
HB597[a]	0	4	4	None	13/13	7/7
				10^{-3}	13/13	6/7
				10^{-6}	13/13	5/7
Poliomyelitis[b] vaccine	0	82	73	None	2/13	6/7
				10^{-3}	0/13	2/7
				10^{-6}	0/13	1/7
Poliomyelitis[b] vaccine	1 yr.	69	62	None	2/13	6/7
				10^{-3}	0/13	4/7
				10^{-6}	0/13	1/7
Poliomyelitis[b] vaccine	2 yr.	76	70	None	1/13	6/7
				10^{-3}	0/13	4/7
				10^{-6}	0/13	0/7
DPT[b] polio vaccine	7 days	76	76	None	3/13	6/7
				10^{-3}	0/13	5/7
				10^{-6}	0/13	3/7

[a] Formaldehyde and antibiotics absent. [b] Formaldehyde not neutralized; antibiotics present.

TABLE IV.—ANTIMICROBIAL EFFECT OF PARABENS MIXED WITH FORMALDEHYDE IN HB597 MEDIUM

Parabens, %	Formaldehyde, p.p.m. Nash	Formaldehyde, p.p.m. Modified Nash	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
0	4	8	None	13/13	7/7
			10^{-3}	13/13	5/7
			10^{-6}	13/13	5/7
0	52	53	None	10/13	7/7
			10^{-3}	4/13	4/7
			10^{-6}	0/13	3/7
0.165	4	8	None	7/13	3/7
			10^{-3}	5/13[a]	0/7
			10^{-6}	3/13[b]	0/7
0.165	50	54	None	0/13	1/7
			10^{-3}	0/13	0/7
			10^{-6}	0/13	0/7

[a] 4/5 cultures which grew were *Ps. aeruginosa*; 1/5 was *S. aureus*. [b] 2/3 cultures which grew were *Ps. aeruginosa*; 1/3 was *S. aureus*.

ever, inhibited growth of all bacteria and six of seven yeasts and molds when the undiluted inoculum was used for challenge.

Table V confirms that formaldehyde with the antibiotics is an excellent antibacterial mixture, even in DPT polio, but that the addition of parabens is necessary for antifungal activity.

The stability of preservatives in DPT polio vaccine was studied. Table VI shows that there was no immediate loss of parabens when poliomyelitis vaccine was mixed with other antigens to form DPT polio but within 7 months there was a decrease from 0.165 to 0.14%. After 11 months' storage at 4°, there was a further decrease to 0.12% (Table VII). This loss of parabens did not result in a significant decrease in antimicrobial activity but did cause a drop in pH, shown by the phenol red indicator present in the vaccine.

Table VII shows that the stability of formaldehyde in DPT polio vaccine during storage for 9 to 11 months depended on both temperature and the presence of parabens. In the absence of parabens, about 50 and 85% of the formaldehyde was lost when temperatures of storage were 4° and 25°, respectively. This resulted in a marked loss of antibacterial activity. In the presence of parabens, formaldehyde was stabilized and retained its antibacterial activity.

Studies similar to those reported in Table VII were done by adding 25 p.p.m. of benzethonium chloride (BEC) to DPT polio vaccine. The BEC was reduced to 3–4 p.p.m. by the addition of pertussis vaccine (1), and this residuum had no effect on the stability of the formaldehyde.

TABLE V.—ANTIMICROBIAL ACTIVITY OF PARABENS IN DPT POLIO VACCINE

Prepn. Tested	Dilution of Challenge Culture	No. of Tubes with Growth / No. of Tubes Challenged: Bacteria	Yeasts and Molds
HB597[a]	None	13/13	7/7
	10^{-3}	13/13	6/7
	10^{-6}	13/13	4/7
DPT[b] polio vaccine	None	1/13	6/7
	10^{-3}	0/13	6/7
	10^{-6}	0/13	3/7
DPT polio vaccine with 0.165% parabens	None	1/13	3/7
	10^{-3}	0/13	0/7
	10^{-6}	0/13	0/7

[a] Formaldehyde and antibiotics absent. [b] Formaldehyde not neutralized; antibiotics present.

DISCUSSION

The most important requirements for preservatives in vaccines are activity against diverse microorganisms, compatibility with the vaccine, stability, and lack of toxicity.

Antimicrobial activity is necessary to prevent the growth of microorganisms which may be introduced when samples are withdrawn from multiple-dose containers (1, 8–10). The antimicrobial agent should prevent the growth of large numbers of a wide variety of bacteria, yeasts, and molds. Preferably, it should destroy them quickly, although failure to act quickly should not invalidate an otherwise useful compound.

Compatibility of the preservative with the vaccine may be considered from several standpoints. The preservative must not lower the potency of the vaccine below permissible levels; it should not form a precipitate; and it should not be inactivated by the vaccine. The compatibility must be maintained for periods of time and under conditions of storage and transportation which would not adversely affect the antigen if the preservative were not added.

Adequate stability of the preservative during the shelf life of the vaccine is essential. However, if breakdown occurs, it should not result in a marked decrease of the antimicrobial activity, visible changes, or destruction of the antigen. In addition, the preservative should not precipitate in the cold or be absorbed by the rubber stopper.

These numerous and exacting requirements make it unlikely that a universal preservative will be found which will be suitable for all vaccines. In-

TABLE VI.—PARABEN CONTENT OF POLIOMYELITIS AND DPT POLIO VACCINES

Poliomyelitis Vaccine: Lot No.	Paraben content, %	DPT Polio Vaccine: Lot No.[a]	Paraben Content, %
1	0.15	1A	0.16
2	0.16	2A	0.16
3	0.17	3A	0.16
4	0.17	4A	0.17
5	0.17	5A	0.17
6	0.17	6A	0.14

[a] Lots 1A–5A were analyzed within 2 weeks, and Lot 6A was analyzed 7 months after preparation.

TABLE VII.—RECOVERY OF PRESERVATIVES IN DPT POLIO VACCINE AFTER PROLONGED STORAGE

Vaccine No.	Preservatives Added	Preservatives Recovered—Formaldehyde, p.p.m.	Preservatives Recovered—Parabens, %	Tubes with Growth / Tubes Challenged B[c]	Tubes with Growth / Tubes Challenged Y and M[d]
		11 Mo. at 4° C.			
486	Form[a]	25		9/13	4/6
467	Form	45			
468	Form	40			
484	Form + *para*[b]	71	0.13	0/13	1/6
488	Form + *para*	71	0.12	1/13	0/6
		9[f] Mo. at 25° C.			
486	Form	11		11/13	6/6
484	Form + *para*	67	0.10	1/13	1/6
488	Form + *para*	64	0.10	1/13	0/6
HB597	None[e]			13/13	6/6

[a] 92 p.p.m. formaldehyde. [b] 92 p.p.m. formaldehyde and 0.165% parabens. [c] Bacteria—10^{-3} dilution of inoculum used for challenge. [d] Yeasts and molds—10^{-1} dilution of inoculum used for challenge. [e] Control on ability of inoculum to initiate growth in HB597 medium. [f] Stored 1–2 months at 4° C. and then 9 ± 1 month at 25° C.

deed, it is difficult to find a single preservative which fulfills all requirements for a single vaccine.

Falk and Applington (11) recognized the deficiencies of a single preservative for vaccines and showed that mixtures of thimerosal with phenol were superior to either compound alone. We also have shown that mixtures of preservatives are useful in poliomyelitis vaccine. The mixtures we have used include the two antibiotics, formaldehyde and methyl and propyl esters of *p*-hydroxybenzoic acid.

The antibiotics added to the tissue culture to prevent bacterial contamination during manufacture have considerable stability (1, 12). The mixture of streptomycin and neomycin has the advantages of a wide spectrum of antibacterial activity, low toxicity, and apparent compatibility with the antigen. The antibiotics fail, however, to control heavy contamination with most bacteria or even light contamination with fungi and some strains of *Ps. aeruginosa*.

Formaldehyde, which may also be present in poliomyelitis vaccine (13–16) has excellent antibacterial activity against most bacteria and is especially important because of its activity toward *Ps. aeruginosa* (17). This species is one of the most difficult organisms to inhibit (18–20), and even a few cells of some strains can initiate growth in the presence of both the antibiotics and *p*-hydroxybenzoate esters at the concentrations used in this investigation. The excellent stability of formaldehyde in poliomyelitis vaccine, its compatibility with other antigens, its ready solubility, and its lack of toxicity at levels which are adequate for antibacterial activity make it a useful preservative. Formaldehyde has been used for the preservation of solutions of penicillin and streptomycin (21) and suspensions of procaine penicillin (22). Schmidt-Lorenz has recently reviewed the use of formaldehyde as a preservative (23).

The concentration of formaldehyde in poliomyelitis vaccine may vary considerably. Although formalin is added to a concentration of 1:4000 (92 p.p.m.) to inactivate the virus, the residual formaldehyde may be neutralized by bisulfite (16) or it may be left without neutralization. If left without neutralization, we have found 60–80 p.p.m. Other workers have found more (13, 14). It is permissible to inactivate the virus for vaccine without using formaldehyde (16), and some manufacturers use β-propiolactone for inactivation.

The antimicrobial activity of the parabens has been described in numerous publications; a comprehensive review is available (3). The main advantages of the parabens are their demonstrated ability to inhibit fungi, their lack of harm to antigens and, in DPT polio vaccine, their stabilizing effect on formaldehyde. Their disadvantages are their low solubility at temperatures at which vaccines are usually stored (4–10°), their moderate antibacterial activity, especially against *Ps. aeruginosa* (18, 19), and a lowering of pH when vaccines containing parabens are stored.

The stability of formaldehyde in poliomyelitis vaccine and its instability in DPT polio vaccine during prolonged storage is unexplained. Also unexplained is the ability of parabens to stabilize the formaldehyde in DPT polio vaccine. Any hypothesis concerning these phenomena should take into account the following observations: (*a*) formaldehyde is stable in DPT polio vaccine for at least 7 days after the poliomyelitis vaccine is mixed with the toxoids and pertussis; (*b*) pertussis vaccine alone or mixed with toxoids is far more stable than pertussis vaccine mixed with polio vaccine (24); and (*c*) the addition of Versene to DPT poliomyelitis vaccine decreases degradation of pertussis (25). It is possible that the instability of formaldehyde and pertussis vaccine in DPT polio vaccine are related.

One hypothesis concerning the instability of formaldehyde and pertussis vaccine is that poliomyelitis vaccine contains proteolytic enzymes which slowly break down pertussis cells. This breakdown produces sites which react with formaldehyde. Unfortunately, this and several other hypotheses do not accommodate all of the known observations and additional work must be done.

The value of a preservative in a vaccine can only be established when adequate tests have been conducted concerning not only its antimicrobial activity but also its compatibility with the antigens which it preserves. Such tests have been conducted by various departments in these laboratories. To date, there has been no contraindication concerning the use of the antibiotics, formaldehyde, and parabens in the concentrations mentioned.

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By G. J. PAPARIELLO, H. LETTERMAN, and R. E. HUETTEMANN

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THE DEVELOPMENT of rapid analysis of unit dose formulations is a problem which has only recently come into the forefront. In 1961, Head (1) investigated the possible use of solid state fluorescence for the analysis of individual intact tablets and demonstrated that fluorescence was not totally acceptable for this purpose. The author did, however, suggest that X-ray diffraction techniques might possibly be more useful.

Taking advantage of this suggestion, an investigation into quantitative X-ray diffraction was initiated in this laboratory with the view that it might be possible to develop this technique to assay individual intact tablets both rapidly and automatically. It was felt that this investigation could uncover the answers to the following questions: What adaptations and precautions are necessary to obtain acceptable reproducibility in the analysis of an intact tablet by X-ray diffraction techniques? What concentration of a drug within the normal tablet matrix can be detected by these techniques? What precision can be obtained and how rapid can an analysis be run on individual intact tablets?

The theory of quantitative X-ray diffraction analysis is authoritatively considered by Klug and Alexander (2), while a brief but lucid account has recently been given by Shell (3). Consequently, a treatment of the underlying theory will not be considered here. However, it must be noted that the attainment of high precision in intensity measurements of diffracted X-rays demands careful attention to a number of factors, even when one is dealing with a carefully packed powdered sample. These factors—the particle size of the crystallite, preferred orientation, etc.—were considered in this work but could not be dwelled upon because of the nature of the sample. It is understood that an attempt at doing quantitative X-ray diffraction work without careful precautionary sample preparation is unusual and may be viewed with distress by a purist.

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mide[1] tablets N.F. by this technique and the factors influencing this method.

EXPERIMENTAL

X-Ray Equipment.—A General Electric XRD-5 diffraction spectrogoniometer was employed in this work. X-ray radiation was provided by a copper target high intensity X-ray tube operated at 50 KVP and 15, 20, or 31 ma. A 3° beam slit, along with a medium resolution soller slit and a 0.1° detector slit made up the slit system used for most of this work. A 4° beam axis to target angle and a time constant of 1.0 seconds were employed. A gas proportional counter (General Electric No. 6 tube) was used as the detection device, its output being graphically displayed on a Leeds and Northrup Speedomax recorder. The scanning speed of the goniometer used throughout this study was either 2 or 0.2° per minute.

Sample Holders.—Powdered samples were packed in the normal shallow Bakelite trays $1^1/_4 \times 1$ in. in size; intact tablet samples were mounted in a specially designed sample holder fabricated out of Bakelite. This holder is illustrated in Fig. 1.

Qualitative Pattern Examination.—Powdered samples were finely powdered, passed through a

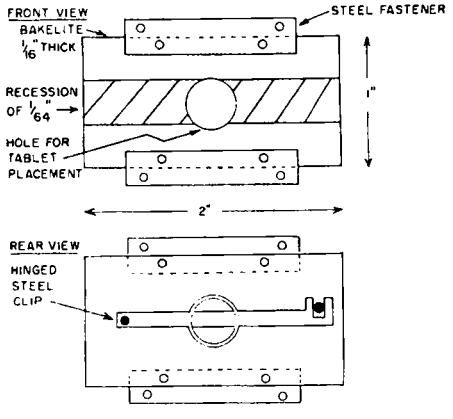


Fig. 1.—Intact tablet sample holder.

TABLE I.—TABLET FORMULATIONS INVESTIGATED

Tablet[a]	Active Component Wt. (%) in Total Formulation Wt.
Methylphenidate hydrochloride, 10 mg.	7
Hydrochlorothiazide U.S.P., 50 mg.	17
Tolazoline hydrochloride U.S.P., 25 mg.	17
Tripelennamine hydrochloride U.S.P., 50 mg.	25
Glutethimide N.F., 500 mg.	77

[a] Marketed as Ritalin, Esidrix, Priscoline, Pyribenzamine, and Doriden, respectively, by Ciba Pharmaceutical Co., Summit, N. J.

standard No. 320 sieve, and carefully packed in a shallow Bakelite tray. These samples were scanned from 5 to 30° 2θ. Intact tablets were merely placed in the specially designed intact tablet sample holder and scanned from 5 to 30° 2θ.

Quantitative Analysis of Glutethimide Tablets.—A tablet is placed in the intact tablet sample holder with its bisection facing forward. The tablet is oriented in the holder so that its bisection is perpendicular to the path of the X-ray beam. The sample holder is fastened in place on the diffractometer. A scan is made from 16 to 18° 2θ using a linear count range of 2000 counts per second and the instrumental conditions as previously indicated. This procedure is repeated for every sample tablet and the standard tablet.

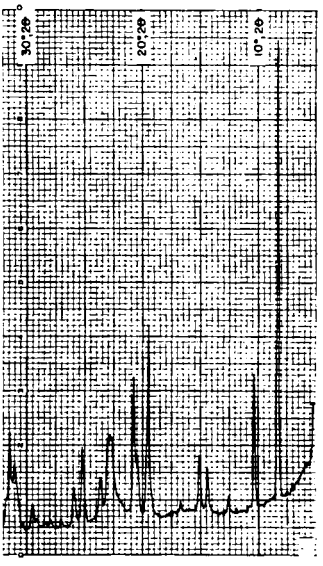


Fig. 2.—X-ray diffraction pattern of methylphenidate hydrochloride powder.

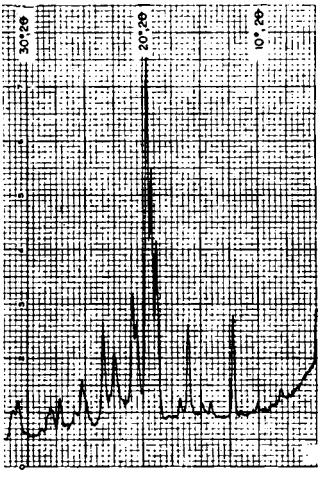


Fig. 3.—X-ray diffraction pattern of powdered methylphenidate hydrochloride tablet formulation.

For each tablet measure the height of the peak from its base is at approximately 17° 2θ. The equation (height of sample peak/height of standard peak)·100 = per cent glutethimide in tablet compares the peak intensity of the sample to that of the standard to obtain the analytical result.

RESULTS AND DISCUSSION

X-Ray Diffraction Examination of Representative Tablet Formulations.—The purpose of this part of the study was to determine what tablet formulations might possibly be analyzed by X-ray diffrac-

[1] Marketed as Doriden by Ciba Pharmaceutical Co., Summit, N. J.

tion techniques. From the outset it was understood that absorption and matrix interference preclude the use of this method for very small dosage tablet formulations. Nevertheless, there are many other tablet formulations where the percentage of drug weight in total formulation weight is at a level where analysis might be possible. Tablet formulations whose percentage of drug weight in total formulation weight was from 7 to 77% were considered. The commercial tablet formulations investigated are listed in Table I.

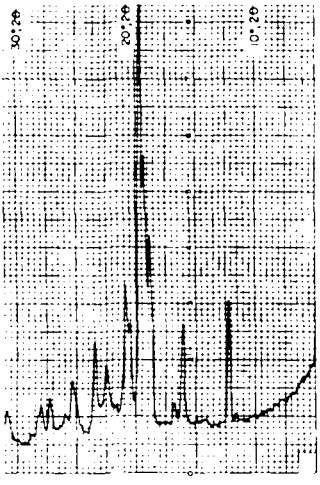


Fig. 4.—X-ray diffraction pattern of powdered methylphenidate hydrochloride placebo tablet formulation.

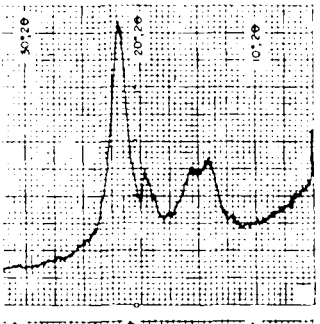


Fig. 5.—X-ray diffraction pattern of an intact methylphenidate hydrochloride tablet.

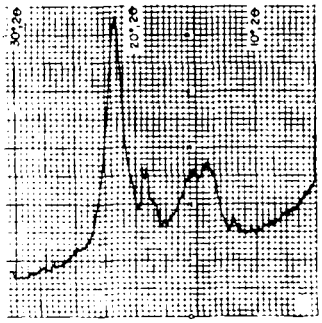


Fig. 6.—X-ray diffraction pattern of an intact methylphenidate hydrochloride placebo tablet.

The manner in which these various formulations were examined is quite simple. First a diffraction pattern of the powdered drug itself is obtained, and the most intense diffraction peaks noted. Then a diffraction pattern of the powdered tablet formulation containing the drug in question is taken. This pattern is studied, and one seeks out those diffraction peaks which were previously noted to be large on the pattern of the pure drug. A diffraction pattern of the powdered placebo formulation will aid in this examination, since the other components in a formulation have diffraction patterns of their own and can obscure or interfere with that of the drug itself. The final step is obtaining and examining diffraction patterns of the intact tablet and the intact placebo tablet.

Those patterns obtained for the methylphenidate hydrochloride investigation are shown in Figs. 2 through 6 as an illustration of the type of results obtained. By examining Figs. 2, 3, and 4, one will notice that a good many of the strong diffraction peaks seen in the methylphenidate hydrochloride powder pattern are obscured or absorbed in the pattern of the powdered formulation. However, if one were interested in performing the analysis on the powder rather than the intact tablet, it would be entirely possible since there are still measurable peaks at 8.1°, 14.3°, and 14.9° 2θ in the powdered formulation diffraction pattern. Figures 5 and 6 clearly demonstrate that the number and intensity of the diffraction peaks obtained from the dense intact tablet sample definitely diminishes. It can be seen that there are no diffraction peaks present which might reflect the amount of methylphenidate hydrochloride present in the intact tablet. All the intact tablet patterns of the compounds studied except for glutethimide were like those of methylphenidate hydrochloride and were determined to be unsuitable for use in an analytical method.

Diffraction patterns for powdered samples of glutethimide itself, glutethimide tablet formulation, and glutethimide placebo formulation indicated that most of the diffraction peaks were due to the glutethimide itself (see Figs. 7, 8, and 9). Seventy-seven

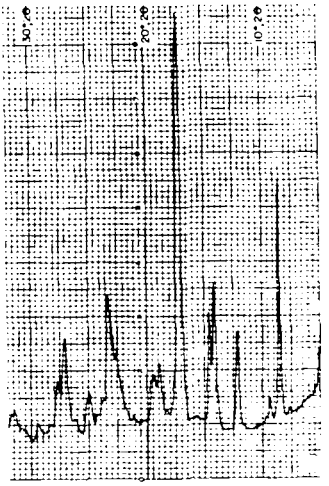


Fig. 7.—X-ray diffraction pattern of glutethimide powder.

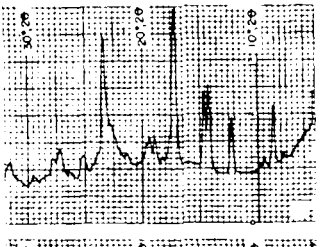


Fig. 8.—X-ray diffraction pattern of powdered glutethimide tablet formulation.

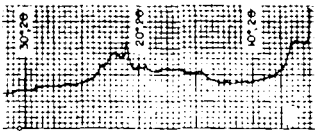


Fig. 9.—X-ray diffraction pattern of powdered glutethimide placebo tablet.

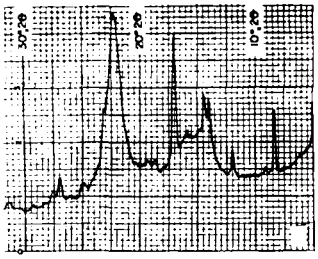


Fig. 10.—X-ray diffraction pattern of an intact glutethimide tablet.

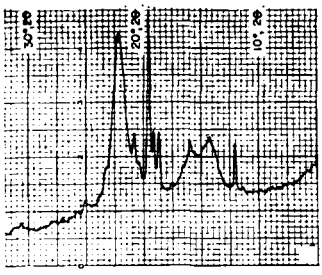


Fig. 11.—X-ray diffraction pattern of an intact glutethimide placebo tablet.

per cent of the tablet formulation is glutethimide, making it the predominant crystalline component in the formulation. Thus these diffraction results were expected. From an examination of Figs. 10 and 11 it can be seen that, although decreased, the glutethimide peaks in the intact tablet pattern are still distinguishable. The most intense diffraction peak, that at 17° 2θ, was chosen as the peak to use in an attempt to analyze glutethimide intact tablets quantitatively.

Quantitative Analysis of Glutethimide Tablets.—Having found a diffraction peak which was intense enough to warrant investigation in the case of glutethimide, it was then necessary to show that this peak did indeed reveal the quantity of glutethimide present in an intact tablet. Proof of this nature was obtained by preparing tablets of known composition and analyzing them by the diffraction technique. That is, several small batches of tablet formulation were prepared, and tablets were manually compressed using a Stokes model E tablet press.[1] Measurements were made on these tablets with ultraviolet spectrophotometry and X-ray diffraction techniques. A plot of the results of these analyses indicate that the intensity of the peak at 17° 2θ for the intact tablet is dependent on the quantity of glutethimide present in the tablet. Figure 12 shows that over a rather large concentration range this dependence is linear.

It was originally intended to use the tablets prepared by the above indicated method as standards for the analysis of the glutethimide production tablets. When this was attempted, however, it was noted that the diffraction intensities for the production tablets were less than that for the standard tablets. This observation was investigated and was shown to be a pressure effect. The pressure used in compressing the production tablets is, of course, much greater than that used in preparing the standard tablets. This pressure effect might be due to preferred orientation or a reduction in the size of the crystallites. It has been reported that the presence of a large proportion of crystals less than 0.2 μ in size in a sample will result in sufficient line broadening to produce an abnormally low peak height (4).

The existence of this pressure effect made it impossible to use the specially prepared standard tablets. It was necessary to use a production tablet as a standard tablet. Such a tablet was obtained by measuring the peak intensity at 17° 2θ for a group of production tablets. Each time a set of two tablets having identical peak height intensities was found—one member of the set was assayed by ultraviolet spectrophotometry, while the other was saved intact to be used as a standard.

[1] The authors are grateful to Dr. Arge Drubulis, who prepared these special tablets.

Table II compares the ultraviolet spectrophotometric and the diffraction analyses of 10 glutethimide tablets. The results for the diffraction analysis are given in peak height units (millimeters) and can be converted to milligrams per tablet by use of a standard production tablet. However, this conversion is not necessary if one is checking for dosage uniformity since limits in millimeters can be specified. The analysis of the intact tablets by diffraction took 25 minutes, whereas the ultraviolet spectrophotometric analysis of the powder of these tablets took over 2 hours. The reproducibility obtainable by the spectrophotometric method is greater than that obtained by the diffraction technique, but the diffraction technique's reproducibility is certainly acceptable if one were performing dosage uniformity analysis.

The monogram and scoring of a tablet have an effect on the absorption and scattering of the X-ray radiation. Consequently, one must always present the same surface similarly positioned in the X-ray beam if any sort of reproducibility is hoped for. Even when this precaution is taken, the reproducibility of measurements when the tablet is taken from the sample holder and then replaced in a similar manner is only ±2.5%. These results, however, are not greatly different from those obtained by Klug for the quantitative X-ray diffraction determination of powdered samples (5).

Shifting of the analytical peak from 16.93° to 17.18° 2θ has been noted. This shifting is the reason for obtaining a recorded scan and measuring the peak height rather than obtaining a count for a preset time at exactly 17.00° 2θ. Such peak shifting is not unusual when one is using large scanning speeds and large time constant values.

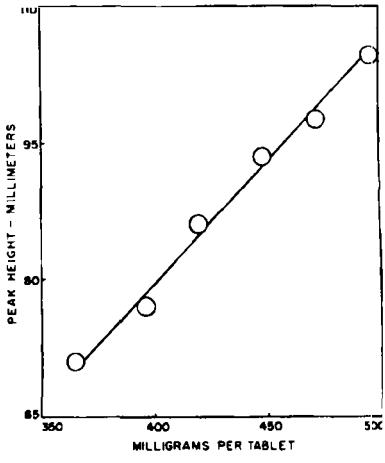


Fig. 12.—Relationship between the milligrams of glutethimide present in the intact tablet and the measured peak height intensity at 17° 2θ.

TABLE II.—COMPARISON OF DIFFRACTION AND ULTRAVIOLET SPECTROPHOTOMETRIC ANALYSES OF INDIVIDUAL GLUTETHIMIDE TABLETS

Tablet No.	Peak Ht., mm.	Deviation from Mean, mm.	Deviation from Mean, %	U.V. Analysis mg./Tablet	Deviation from Mean, mg.	Deviation from Mean, %
1	128	1	0.5	498	3	0.6
2	130	1	0.5	491	4	0.6
3	131	2	1.8	489	6	1.1
4	133	4	2.7	511	16	3.3
5	132	3	1.9	497	2	0.6
6	136	7	4.8	496	1	0.2
7	120	9	6.6	484	11	2.1
8	133	4	2.8	493	2	0.2
9	119	10	7.1	491	4	0.7
10	129	0	0.3	495	0	0.1
Av.	129	4	2.9	495	5	1.0

SUMMARY

This investigation has demonstrated that the percentage of drug weight in the total formulation weight must be large before one can consider the use of X-ray diffraction intact tablet analysis. From the data collected, it appears as if the drug must be at least 50% of the total formulation weight before analysis by this diffraction procedure could be contemplated. Of course, this judgement will depend on the crystalline character of the drug and the absorption effects of the tablet matrix.

It has also been shown that intact glutethimide tablets can be assayed by this X-ray diffraction technique with a reproducibility of ±3%.

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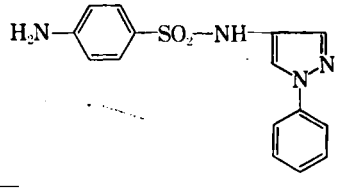
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Drug Standards

Qualitative and Quantitative Tests for Sulfaphenazole

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

N' - (1 - PHENYLPYRAZOLYL - 5)SULFANILAMIDE; $C_{15}H_{14}N_4O_2S$; mol. wt. 314.37. The structural formula of sulfaphenazole may be represented



Received December 31, 1963, from the Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, D. C. 20037.
Accepted for publication January 31, 1964.
Physicians Products Co., Inc., Petersburg, Va., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

Physical Properties.—Sulfaphenazole occurs as a white to cream-colored, fine crystalline powder, m.p. 178–182°, U.S.P. XVI Class I. It is freely soluble in acetone, sparingly soluble in alcohol, and practically insoluble in water. Sulfaphenazole dissolves in dilute mineral acids and in solutions of alkali hydroxides.

Identity Tests.—To about 100 mg. of sulfaphenazole, add 5 ml. of diluted hydrochloric acid, and boil gently for about 5 minutes. Cool in an ice bath, add 4 ml. of a solution of sodium nitrite (1 in 100), dilute to 10 ml. with water, and place the mixture in an ice bath for 10 minutes. To 5 ml. of the cooled mixture, add a solution of 50 mg. of betanaphthol in 2 ml. of sodium hydroxide solution (1 in 10): an orange-red precipitate is formed.

TABLE II.—COMPARISON OF DIFFRACTION AND ULTRAVIOLET SPECTROPHOTOMETRIC ANALYSES OF INDIVIDUAL GLUTETHIMIDE TABLETS

Tablet No.	Peak Ht., mm.	Deviation from Mean, mm.	Deviation from Mean, %	U.V. Analysis mg./Tablet	Deviation from Mean, mg.	Deviation from Mean, %
1	128	1	0.5	498	3	0.6
2	130	1	0.5	491	4	0.6
3	131	2	1.8	489	6	1.1
4	133	4	2.7	511	16	3.3
5	132	3	1.9	497	2	0.6
6	136	7	4.8	496	1	0.2
7	120	9	6.6	484	11	2.1
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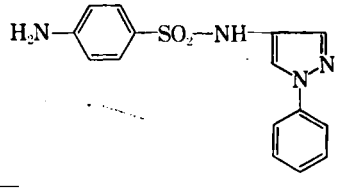
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Identity Tests.—To about 100 mg. of sulfaphenazole, add 5 ml. of diluted hydrochloric acid, and boil gently for about 5 minutes. Cool in an ice bath, add 4 ml. of a solution of sodium nitrite (1 in 100), dilute to 10 ml. with water, and place the mixture in an ice bath for 10 minutes. To 5 ml. of the cooled mixture, add a solution of 50 mg. of betanaphthol in 2 ml. of sodium hydroxide solution (1 in 10): an orange-red precipitate is formed.

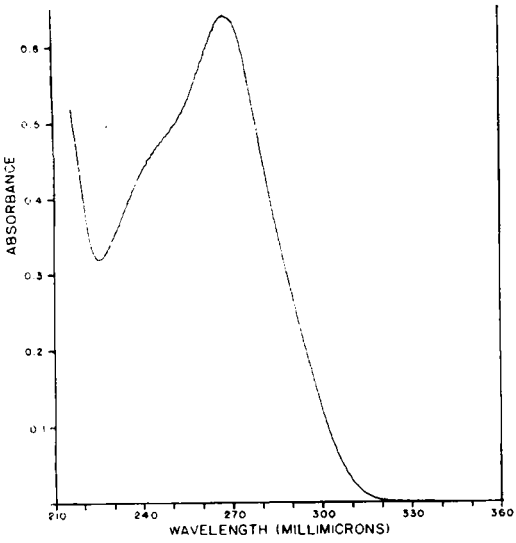


Fig. 1.—Ultraviolet absorption spectrum of sulfaphenazole in alcohol (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

To about 20 mg. of sulfaphenazole suspended in 5 ml. of water add, dropwise, sodium hydroxide T.S. until dissolved, then add 2 or 3 drops of cupric sulfate T.S.: a characteristic light bluish-green precipitate forms which remains unchanged on standing.

A 1:100,000 solution of sulfaphenazole in alcohol exhibits an ultraviolet absorbance maximum at about 268 mμ [absorptivity (1%, 1 cm.) about 640] and a minimum at about 225 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of sulfaphenazole in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests.—Dry about 1 Gm. of sulfaphenazole, accurately weighed, at 105° for 4 hours: it loses not more than 0.5% of its weight.

Char about 1 Gm. of sulfaphenazole, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.2%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of sulfaphenazole in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U. S. P. XVI heavy metals test, method I: the heavy metals limit for sulfaphenazole is 20 p.p.m.

Digest 2 Gm. of sulfaphenazole with 100 ml. of carbon dioxide-free water at about 70° for 5 minutes, cool at once to about 20°, and filter. To 25 ml. of the filtrate, add 2 drops of phenolphthalein T.S. and determine the acidity by titration with 0.1 *N* sodium hydroxide: not more than 0.25 ml. is required for neutralization. Retain the remainder of the filtrate for the chloride and sulfate limit tests.

Determine the chloride content of sulfaphenazole by the U.S.P. XVI chloride limit test, using a 25-ml. portion of the filtrate prepared in the test for acidity: the sample shows no more chloride than corresponds to 0.1 ml. of 0.02 *N* hydrochloric acid (140 p.p.m.).

Determine the sulfate content of sulfaphenazole by the U.S.P. XVI sulfate limit test, using a 25-ml. portion of the filtrate prepared in the test for acidity: the sample shows no more sulfate than corresponds to 0.2 ml. of 0.02 *N* sulfuric acid (400 p.p.m.).

Assay.—Weigh accurately about 500 mg. of sulfaphenazole, previously dried at 105° for 4 hours, and transfer to a 250-ml. beaker. Add 20 ml. of hydrochloric acid and 50 ml. of water, stir until dissolved, cool to room temperature, add about 25 Gm. of crushed ice, and slowly titrate with 0.1 *M* sodium nitrite, stirring vigorously, until a glass rod dipped into the titrated solution produces an immediate blue ring when touched to starch iodide paper. When the titration is complete, the end point is reproducible after the mixture has been allowed to stand for 1 minute. Each milliliter of 0.1 *M* sodium nitrite is equivalent to 31.44 mg. of $C_{15}H_{14}N_4O_2S$. The amount of sulfaphenazole found is not less than 99.0% and not more than 101.0% of the weight of the sample taken.

DOSAGE FORMS OF SULFAPHENAZOLE

Sulfaphenazole Tablets

Identity Test.—Finely powder a number of sulfaphenazole tablets. To a portion of the powder equivalent to about 100 mg. of sulfaphenazole, add 5 ml. of diluted hydrochloric acid, boil gently for about 5 minutes, and filter. Cool the filtrate in an ice bath, add 4 ml. of a solution of sodium nitrite (1 in 100), dilute to 10 ml. with water, and place the mixture in an ice bath for 10 minutes. To 5 ml. of the cooled mixture, add a solution of 50 mg. of betanaphthol in 2 ml. of sodium hydroxide solution (1 in 10): an orange-red precipitate is formed.

Assay.—Weigh and finely powder not less than 20 sulfaphenazole tablets. Weigh accurately a portion of the powder, equivalent to about 500 mg. of sulfaphenazole, and transfer to a 250-ml. beaker. Proceed as directed in the *Assay* in the monograph for sulfaphenazole beginning with "Add 20 ml. of hydrochloric acid. . . ." Each milliliter of 0.1 *M* sodium nitrite is equivalent to 31.44 mg. of $C_{15}H_{14}N_4O_2S$. The amount of sulfaphenazole found is not less than 95.0% and not more than 105.0% of the labeled amount.

Sulfaphenazole Suspension

Identity Tests.—To a volume of sulfaphenazole suspension equivalent to about 100 mg. of sulfaphenazole, add 5 ml. of diluted hydrochloric acid and proceed as directed in the *Identity Test* in the monograph for sulfaphenazole tablets beginning with ". . . boil gently for about five minutes. . . ."

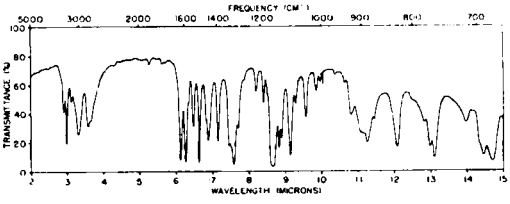


Fig. 2.—Infrared spectrum of sulfaphenazole in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

Transfer a volume of sulfaphenazole suspension equivalent to about 500 mg. of sulfaphenazole to a glass-stoppered centrifuge tube, add 15 ml. of water, and shake vigorously. Centrifuge, decant and discard the supernatant liquid, and wash the residue with two additional 15-ml. portions of water, centrifuging and decanting each time. Transfer the residue of sulfaphenazole to a piece of filter paper or porous plate and allow to air dry. Prepare a 0.5% dispersion of the dry residue in potassium bromide and compress. The infrared spectrum in the range of 2 to 15 μ exhibits absorbance maxima at the same wavelengths as a similar preparation of sulfaphenazole reference standard.

Assay.—Determine the specific gravity of sulfaphenazole suspension. Weigh accurately an amount of suspension equivalent to about 500 mg. of sulfaphenazole and transfer to a 250-ml. beaker. Proceed as directed in the *Assay* in the monograph for sulfaphenazole beginning with "Add 20 ml. of hydrochloric acid. . . ." Each milliliter of 0.1 *M* sodium nitrite is equivalent to 31.44 mg. of $C_{15}H_{14}N_4O_2S$. By means of the specific gravity, calculate the amount of sulfaphenazole present in the volume of the suspension taken. The amount of sulfaphenazole found is not less than 93.0% and not more than 107.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminologies for solubility, melting range, reagents, etc., have been used wherever feasible.

Sulfaphenazole,[1] synthesized by Schmidt and Druey (1), is a rapidly absorbed, long-acting sulfa drug. The tests and standards provided in these monographs are closely patterned after those present in U.S.P. XVI and N.F. XI for similar sulfonamides and their dosage forms.

Identity Tests.—The chemical tests provided give results which are characteristic of many and do not serve to distinguish sulfaphenazole from other sulfa drugs. Such distinction can be made by comparing the ultraviolet and infrared spectra with those which have been published for a number of the official sulfonamides (2). A short review of publications dealing with identification methods for sulfonamides was prepared by Woods and Schneller (3).

Quantitative Methods.—The functional groups common to most sulfonamides are the primary aromatic amine and the acidic hydrogen of the sulfonamide group. Upon these are based most of the analytical methods which have been used for the determination of these compounds. Because of the presence of the amine group, sulfonamides have been determined volumetrically by diazotization, bromination, iodination, and by nonaqueous titration with acetous perchloric acid. The acidic hydrogen has been titrated argentimetrically and acidimetrically with alkali metal alkoxides in nonaqueous media. Among the many colorimetric methods which have been used for the sulfonamides, most prominent is the diazotiazation and coupling method of Bratton and Marshall. These and other methods were extensively discussed by Woods and Schneller (4).

The diazotization reaction made use of in the nitrite titration assay procedure which is common to many of the official sulfonamide monographs is irreversible and complex, the kinetics being influenced by such factors as the basicity of the amine group, the acidity of the medium, and the presence of a catalyst such as bromide ion. The methods of end point detection include the use of (*a*) external indicators such as starch iodide paste or paper, acriflavine, and 3,7-thiaxanthenediamine-5,5-dioxide (Eastman 8776), (*b*) internal indicators such as orange IV, metanil yellow, and diphenylbenzidine disulfonic acid, (*c*) photometric titration, and (*d*) various electrometric methods. The latter include amperometric, conventional potentiometric (at zero current), potentiometric at constant current, and dead-stop titration methods. The various methods may have advantages in increased accuracy, precision, sensitivity, speed,[2] or applicability to unusual situations, but the classical starch iodide method usually suffices for most pharmaceutical control purposes. Analysis of bulk sulfaphenazole gave an average value of 100.2 ± 0.1%, while analysis of commercial tablets (500 mg.) and suspension (100 mg. per milliliter) gave respective average values of 100.1 ± 0.1% and 103.7 ± 0.1% of the labeled amounts.

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(4) *Ibid.*, pp. 137–179.

[1] Marketed as Sulfabid by Physicians Products Co., Inc., Petersburg, Va.

[2] Especially true of potentiometry at constant current with automatic recording equipment.

Spray Congealing: Particle Size Relationships Using a Centrifugal Wheel Atomizer

By MORTON W. SCOTT*, MANFORD J. ROBINSON, JOHN F. PAULS, and RUSSELL J. LANTZ

Stearic acid–ethylcellulose mixtures were spray congealed using a specially instrumented Niro laboratory model spray dryer. Thirty-five runs were conducted under various operating conditions. The variables studied included atomizer wheel speed (11,900 to 43,100 r.p.m.), feed rate (32 to 280 Gm. per minute), and feed viscosity (0.098 to 1.995 poise). The volume-surface mean diameter, D_{vs}, for each congealed product was obtained from Fisher subsieve sizer measurements. The influence of each variable on the mean particle size is correlated by simple exponential equation which relates D_{vs} to feed viscosity, peripheral wheel velocity, and to feed rate per unit wetted wheel periphery. Predicted particle sizes closely agree with those determined experimentally.

THE USE OF spray congealing techniques for modifying the physical, chemical, and/or physiological behavior of pharmaceutical materials has gained increasing attention within recent years. Robinson and co-workers, in their development of sustained-release sulfonamides (1, 2), extended the conventional spray congealing or "prilling" unit operation (3) to pharmaceutical formulations. The general technique was applied by others in the preparation of tasteless forms of several water-soluble vitamins (4) and iron salts (5) and in formulating stabilized, sugar-based flavoring agents (6). The use of spray congealing in conjunction with fluidization procedures for the manufacture of free flowing particles containing vitamin A and other liquid materials has been reported (7). Spray congealing methods also have been applied to citric acid, sodium bicarbonate, and other pharmaceutical materials in order to modify their moisture sensitivity, rate of solubility, and compatibility (8).

These spray congealed products generally are prepared by suspending the active ingredient in a melt of a suitable "coating" or matrix material, then atomizing the hot liquid mixture into a cool chamber. Conventional spray dryers operating with a cool inlet air often are used for this purpose. Feed rates and temperature conditions are adjusted to insure rapid congealing of the atomized liquid droplets. The powder collected as product consists of individual, more or less, spherical particles, each of which contains bits of active ingredient suspended in a matrix of the coating agent (8).

Waxes, fatty acids and alcohols, plastics, sugars, and other materials which are solids at room temperature and melt without decomposition can be used as matrix agents. The behavior of the final congealed product depends in part on the properties (solubility, hydrophobicity, permeability, etc.) of the specific matrix selected. The characteristics of the congealed material also are governed by the particle size of the product. This factor is particularly important in controlling the bulk density, grittiness, and the distribution and coverage of the active ingredient in the matrix.

The engineering literature includes a number of reports in which relationships between the particle size characteristics of atomized materials and the conditions of atomization have been studied (9–13). The majority of work has concerned nozzle-type atomizers (14–17). Centrifugal wheel-type atomizers of the type commonly used in pharmaceutical operations have been studied, but usually in connection with spray



Fig. 1.—Centrifugal wheel atomizer. Wheel has radius = 2.50 cm., 24 vanes, vane height = 5.82 mm., width of vane opening = 3.51 mm.

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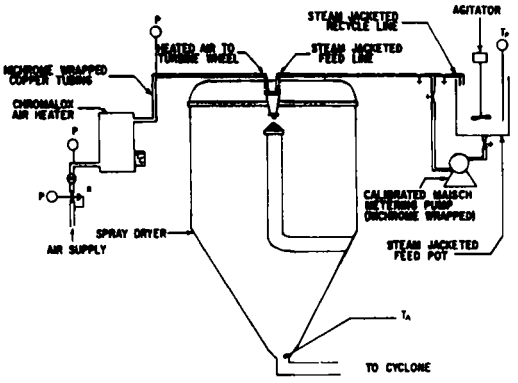


Fig. 2.—Schematic diagram of equipment used for spray congealing. Instruments and controls include: T_p, pot thermometer; P, pressure gauge; R, reducer value; N, needle valve; T_C, thermostatic control; T_A, outlet air thermocouple; ⋈ jacketed gate valve.

drying applications (18–21). Information of direct value in the spray congealing of pharmaceutical systems has not appeared in the literature previously.

The object of the present study was to develop the relationships between the average particle size of a spray congealed product and several of the more important process variables. A centrifugal wheel-type atomizer was used, and the variables studied included liquid feed rate, feed viscosity, and atomizer wheel speed. The experimental system was designed so that the results would have particular application to spray congealing procedures of interest in pharmacy.

EXPERIMENTAL

Materials.—Molten stearic acid (single-pressed) thickened with various proportions of dissolved ethylcellulose[1] was used as the test system. Melt viscosities for each mixture were determined using a Epprecht viscometer.[2] Over the range of ethylcellulose concentrations (0 to 4.0% w/w) and temperature (68.0 to 72.0°) studied, all systems showed Newtonian flow characteristics. Measurements of surface tension at 68 to 72° were obtained with a Du Nouy tensiometer using a hot water jacketed Petri dish as a sample holder.

Equipment.—A Niro laboratory spray dryer[3] operating with inlet air at ambient temperature (25–27°) was used for the spray congealing studies. The Niro unit employs an air-driven, rotating, vaned wheel for atomization of the liquid feed. A single wheel of the type shown in Fig. 1 was used for all runs

A calibrated Maisch metering pump,[4] electrically heated by nichrome wire windings, was used to deliver feed material to the atomizer. Flow rates were calculated by measuring the liquid volume pumped per unit time through the steam-traced feed line leading to the atomizer. This measure of flow rate was repeated at the start and end of each run. The rates obtained by these measurements agreed with each other within ±1.0%. Cyclone losses were estimated from material balance calculations. The over-all equipment setup used in these studies is illustrated in Fig. 2.

Continuous readings of atomizer wheel speeds during each run were obtained using a Minitron proximity impulse generator[5] mounted opposite a steel extension of the wheel shaft. A small hole was drilled through this extension to break the magnetic field of the generator, thus creating two impulses per wheel revolution. The signals from the generator were amplified and fed to a Hewlett-Packard tachometer indicator[6] which gave direct dial readings of wheel speed in revolutions per minute.

Wheel speeds were controlled by regulating the turbine air pressure through a pressure reducer and needle valve assembly which is standard equipment on the Niro unit. Wheel speed was maintained substantially constant (±200 r.p.m.), during each run.

Feed temperature at the wheel was obtained from a thermocouple which had been introduced down the atomizer feed tube and terminated at the liquid entrance to the wheel. In all cases, this temperature measurement was within ±0.5° of the feed temperature measured at the feed pot.

Figure 3 presents a schematic diagram of the

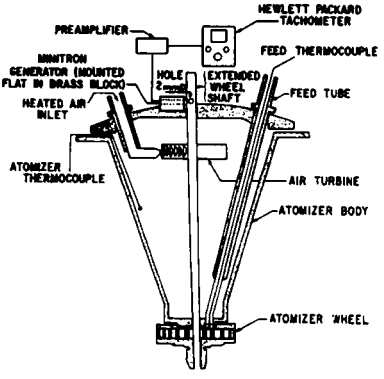


Fig. 3.—Schematic diagram of atomizer and instrumentation.

atomizer head and its accompanying instrumentation.

Operating Procedure.—At the start of each run the atomizer head was brought to approximately 75° by using heated air at 80° to drive the air turbine. This preheating prevented the melt from congealing in the atomizer feed lines or on the wheel during the run. Molten feed then was circulated through the pump and feed lines until a constant flow rate and a constant melt temperature in the range of 68–72° was obtained. The atomizer wheel was brought to approximately the desired speed, and feed to the wheel started. Slight corrections in the turbine air pressure, which were required to compensate for the increased load on the wheel, were made immediately. The small amount of product (5–10 Gm.) collected in the cyclone during this start-up period was dis-

[1] Ethocel, 50 cps., Dow Chemical Co., Midland, Mich.
[2] American Optical Co., Pittsburgh, Pa.
[3] Nichols Engineering and Research Corp., New York, N. Y.
[4] Mechanical Product Corp., Chicago, Ill.
[5] Minatron Corp., Belle Mead, N. J.
[6] Model 500 C, Hewlett-Packard, Inc., Palo Alto, Calif

carded. Approximately 2000 Gm. of product was prepared in each test. The entire equipment assembly was dismantled and cleaned between runs.

For these studies, feed rates were varied from 32 to 280 Gm. per minute. Wheel speeds ranged from 11,900 to 43,100 r.p.m. The viscosities examined were from 0.098 to 1.995 poise. Viscosity values were obtained from predetermined relationships between viscosity and temperature for each stearic acid–ethylcellulose mixture and from the measured temperature of the feed at the wheel.

The order in which the operating variables were changed from run to run followed a predetermined randomized design so that experimental bias would be minimized. Four sets of duplicate experiments were conducted to establish the reproducibility of the results.

The experimental conditions used in each run are listed in Table I.

Particle Size Determinations.—Samples taken for particle size analysis were obtained as coned and quartered aliquots of the total product collected in each run. To confirm the reliability of this sampling procedure, analyses were occasionally performed on duplicate samples obtained by repeating the cone and quartering procedure. In all cases examined, the agreement of the results was within ±2%.

The particle size was determined with the Fisher subsieve sizer. Density values required for these determinations were obtained for each composition by the (liquid) pycnometer method.

TABLE I.—EXPERIMENTAL CONDITIONS USED IN SPRAY CONGEALING RUNS

Run	System (Wt. % Ethylcellulose Combined with Stearic Acid)	Feed Rate, F (Gm./Min.)	Feed Temp., °C.	Wheel Speed, W_s (r.p.m.)
1	0.0	47	67.5	20300
2	0.0	170	68.0	20500
3	0.0	168	68.0	31900
4	0.0	32	68.5	41300
5	0.0	280	70.5	17600
6	0.0	32	69.0	43100
7	0.0	33	69.0	22300
8	0.0	33	71.0	30400
9	0.0	110	68.5	38200
10	0.0	110	69.0	25300
11	0.0	170	69.5	33200
12	0.0	110	69.5	16800
13	0.0	112	68.5	28900
14	0.0	272	69.5	18500
15	0.0	46	68.5	11900
16	0.0	111	69.0	35600
17	0.0	46	72.0	29600
18	0.0	46	71.5	20300
19	0.0	272	72.0	21600
20	0.0	168	70.0	23600
21	0.0	168	70.0	29400
22	0.0	46	71.0	39000
23	0.0	32	70.5	18400
24	0.0	110	70.0	25200
25	0.0	168	70.0	31800
26	0.0	46	70.0	29600
27	2.5	110	72.0	34300
28	2.5	46	71.5	38500
29	2.5	110	70.5	20300
30	2.5	168	71.5	30700
31	2.5	272	72.0	27900
32	1.5	32	72.0	24500
33	1.5	46	70.5	30500
34	4.0	32	70.5	37100
35	4.0	110	69.5	24000

TABLE II.—VALUES FOR THE EXPERIMENTAL OPERATING VARIABLES AND AVERAGE PARTICLE SIZES

Run	Feed Rate Per Unit Wetted Wheel Periphery,[a] F_p (Gm./Sec./cm.)	Peripheral Wheel Velocity,[b] V_p (cm./Sec.)	Feed Viscosity, μ (poise)	Mean Surface-Volume Particle Size of Product,[c] D_{vs} (μ)
1	0.0174	5300	0.108	25.4
2	0.0630	5400	0.107	31.6
3	0.0622	8300	0.107	25.7
4	0.0118	10800	0.106	17.4
5	0.104	4600	0.102	38.2
6	0.0118	11300	0.105	18.2
7	0.0122	5800	0.105	26.5
8	0.0122	8000	0.100	19.3
9	0.0408	10000	0.106	22.3
10	0.0408	6600	0.105	26.4
11	0.0630	8700	0.104	25.8
12	0.0408	4400	0.104	32.2
13	0.0415	7600	0.106	25.1
14	0.101	4800	0.106	39.7
15	0.0170	3100	0.106	35.6
16	0.0412	9300	0.105	23.5
17	0.0170	7700	0.098	22.1
18	0.0170	5300	0.099	26.5
19	0.101	5700	0.098	39.7
20	0.0622	6200	0.102	31.5
21	0.0622	7700	0.102	26.9
22	0.0170	10200	0.100	18.1
23	0.0118	4800	0.102	28.4
24	0.0408	6600	0.102	27.3
25	0.0622	8300	0.102	25.8
26	0.0170	7700	0.102	23.1
27	0.0408	9000	0.613	23.4
28	0.0170	10100	0.619	18.1
29	0.0408	5300	0.671	30.9
30	0.0622	8000	0.624	25.7
31	0.101	7300	0.613	29.0
32	0.0118	6400	0.328	22.0
33	0.0170	8000	0.341	18.8
34	0.0118	9700	1.845	17.9
35	0.0408	6300	1.940	28.4

[a] Calculated from feed rate, F, as listed in Table I by

$$F_p = \frac{F\ (\text{Gm./min.})}{60(\text{sec./min.}) \times P\ (\text{cm.})}$$

where P is the total periphery of vane openings of the wheel, P = 18.66 (mm./vane opening) × 24 (vane openings) = 45 cm. [b] Calculated from wheel speed, W_s, listed in Table I by

$$V_p = \frac{2\pi R(\text{cm./rev.}) \times W_s\ (\text{r.p.m.})}{60\ (\text{sec./min.})}$$

where R = wheel radius = 2.5 cm. [c] Fisher subsieve sizer values.

Particle size distributions were obtained also using the Sharples Micromerograph.

RESULTS

To establish a correlation between the operating variables (feed rate, F, feed viscosity, μ, and wheel speed, W_s) and the average particle size, a simple exponential equation was assumed to apply.[7] Equation 1 served as the starting point

$$D_{vs} = K(F)^a(W)^b(\mu)^c \qquad \text{(Eq. 1)}$$

where D_{vs} is the mean volume-surface diameter of the product,[8] F is the feed rate, μ is the feed viscos-

[7] It was assumed thereby that effects produced by the interaction of the variables would not be significant. This assumption appears to be justified by the results.

[8] Average particle sizes obtained by Fisher subsieve sizer analysis are D_{vs} values.

ity, W_s is the wheel speed, a, b, c are dimensionless constants, and K is a constant (proportionality factor) with dimensions necessary to make the equation dimensionally correct.

It was considered desirable to include appropriate terms in the final correlation to account for the differences in atomizer wheel size which may be employed with units of different production capacities and which have been studied by other workers (18, 20). Equation 1 confined itself to data obtained with a single wheel size. To remove this limitation, various methods of expressing feed rate, F, and the wheel speed, W_s, which were independent of wheel size, were tested in Eq. 1. Equation 1 was best modified by expressing wheel speed in terms of peripheral wheel velocity and feed rate in terms of feed rate per unit wetted wheel periphery. Equation 2 shows this form of the relationship

$$D_{vs} = K(F_p)^a(V_p)^b(\mu)^c \qquad \text{(Eq. 2)}$$

where F_p is the feed rate per unit wetted wheel[1] periphery, grams per second per centimeter, V_p is the peripheral wheel velocity, centimeters per second, and K, a, b, c, and μ are as defined in Eq. 1.

The values of F_p, V_p, and feed viscosity for each experimental run are listed in Table II. The average particle size of the product obtained in each run is given in Table II also.

Equation 2 was set in logarithmic form and the constants K, a, b, and c calculated from the experimental data by the method of least squares. The best fit to the experimental data was obtained by Eq. 3

$$D_{vs} = 5240F_p^{0.171}V_p^{-0.539}\mu^{-0.017} \qquad \text{(Eq. 3)}$$

where F_p is the feed rate per unit wetted wheel periphery, grams per second per centimeter, V_p is the peripheral wheel velocity, centimeters per second, and μ is the feed viscosity, poise.

D_{vs} values were calculated from Eq. 3 using the values of F_p, V_p, and μ established in each run (Table II). These calculated average particle sizes are compared with those determined experimentally in Fig. 4.

DISCUSSION

The instrumentation developed for this study allowed close control of the spray congealing process. As a result, essentially constant operating conditions

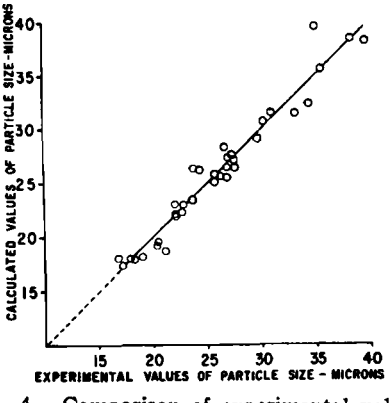


Fig. 4.—Comparison of experimental values of average particle size (D_{vs}) with values predicted by Eq. 3.

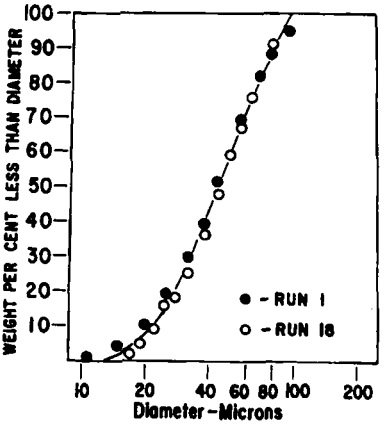


Fig. 5.—Micromerograph data for runs *1* and *18* illustrating reproducibility of results.

could be maintained during the course of each run. It was also possible to set each variable at its predetermined level quickly at the start of each run and thereby minimize product contamination during the start-up period. The Minitron generator and Hewlett-Packard tachometer system for measuring wheel speed was particularly useful, since preliminary trials showed that neither friction tachometers nor stroboscopic techniques could be applied for measuring this variable.

The particle size characteristics of the spray congealed products appear to be reproducible. This is indicated, for example, by comparison of the average particle size values obtained in duplicate runs *3* and *25* (25.7 and 25.8 μ, respectively), duplicate runs *17* and *26* (22.1 and 23.1 μ, respectively), and duplicate runs *4* and *6* (17.4 and 18.2 μ, respectively). Particle size distributions obtained with the Sharples Micromerograph are shown in Fig. 5 for duplicate runs *1* and *18* and further demonstrate the reproducibility of results.

Cyclone losses in the present experiments were estimated to be less than 0.3%. The particles passing the cyclone were most probably those with the smallest diameters. While the presence of these particles would modify average particle size values if calculated on the basis of particle count,[9] their influence on Fisher D_{vs} averages would not be significant.[10]

In developing final relationships between the particle size and the operating variables, several techniques of measuring and expressing the average particle size were tested. Correlations were generated using average particle size values obtained by Micromerograph analysis and expressed both in terms of D_{vs} and D_{mm} (mass median diameter). These correlations, however, did not fit the experimental data so closely as the correlation obtained using Fisher D_{vs} values (Eq. 3).

The final correlation derived in this study is given by Eq. 3 and shows that particle size increases with

[9] As obtained from microscopic analysis for example.

[10] This was shown by calculation of D_{vs} values for an extreme hypothetical system containing 99.7% (by weight) of particles with diameter equal to 10 μ plus 0.3% of particles with diameter equal to 1 μ. This D_{vs} value differed by only 2.5% from the value calculated assuming no small particles were present in the system. The conclusion also was confirmed experimentally. This was done by blending 2.0% by weight of particles having a measured Fisher D_{vs} of 17.4 μ with 98% of particles having a D_{vs} of 39.7 μ. The Fisher value obtained with the mixture was 39.6 μ.

decreasing wheel speed (peripheral velocity), increasing feed rate, and decreasing feed viscosity. The present results indicate that particle size varies inversely as the 0.54 power of the peripheral velocity. This value is in reasonable agreement with Wallman and Blyth's value of 0.6 (21) and Meyer's value of 0.69 (20). However, Friedman, *et al.*, found that particle size varied inversely as the 0.9 power of the peripheral velocity (18).

The present correlation also shows that particle size varies directly as the 0.17 power of feed rate. This result closely agrees with the results of Wallman and Blyth (0.11), Friedman, *et al.* (0.2), and Meyer (0.21). Thus, feed rate appears to exert less influence on particle size than wheel speed. This points to the relative importance of controlling wheel speed to obtain spray congealed products with reproducible size characteristics.

The correlation expressed in Eq. 3 shows that feed viscosity has little if any influence on particle size. The negative value of the exponent, however, is surprising in view of the reports of others which indicate that particle size tends to increase with increasing viscosity (13, 18).

The close agreement of the particle sizes calculated by Eq. 3 and the experimentally determined values are evident in Fig. 5. Table III summarizes data on the deviation of calculated and experimental values. The average deviation for all runs was 1.0 μ. The deviation was less than 1.8 μ in 31 of the 35 runs.

TABLE III.—COMPARISON OF EXPERIMENTALLY DETERMINED AVERAGE PARTICLE SIZE WITH PREDICTED VALUES

Run	Exptl. Value of Particle Size[a] (μ)	Predicted Value of Particle Size[b] (μ)	Deviation (μ)
1	25.4	26.9	1.5
2	31.6	33.2	1.6
3	25.7	26.3	0.6
4	17.4	17.2	0.2
5	38.2	39.4	1.2
6	18.2	16.8	1.4
7	26.5	24.1	2.4
8	19.3	20.3	1.0
9	22.3	22.1	0.2
10	26.4	27.7	1.3
11	25.8	25.7	0.1
12	32.2	34.4	2.2
13	25.1	25.7	0.6
14	39.7	38.3	1.4
15	35.6	35.8	0.2
16	23.5	23.0	0.5
17	22.1	22.0	0.1
18	26.5	26.9	0.4
19	39.7	35.0	4.7
20	31.5	30.8	0.7
21	26.9	27.4	0.5
22	18.1	18.9	0.8
23	28.4	26.6	1.8
24	27.3	27.7	0.4
25	25.8	26.3	0.5
26	23.1	22.0	1.1
27	23.4	22.7	0.7
28	18.1	18.4	0.3
29	30.9	30.1	0.8
30	25.7	26.0	0.3
31	29.0	29.6	0.6
32	22.0	22.3	0.3
33	18.8	21.1	2.3
34	17.9	18.0	0.1
35	28.4	27.0	1.4
		Av. Dev. =	$\pm 1.0\ \mu$

[a] Fisher subsieve sizer values. [b] Values predicted by Eq. 3.

These results also suggest that second and higher order interactions of the variables are not involved in the atomization.

Changes in surface tension of the liquid feed have been reported to have a small but significant effect on particle size (18). In the present study, surface tension was relatively constant at 27.8 dynes per cm. and did not vary by more than $\pm 1.0\%$ for any of the test systems. The correlation, therefore, could not be extended to include this variable. The influence of surface tension is contained, however, in the statement of the constant K in Eq. 3. This constant would be expected to take higher numerical values with increasing surface tension.

The stearic acid–ethylcellulose system selected for this study is representative of other wax mixtures which may be used as matrix agents in spray congealing operations. The system exhibited Newtonian flow characteristics over the complete range of test conditions. This simplified the estimation of feed viscosity at the point of atomization, *i.e.*, at the wheel periphery. It should be noted, however, that concentrated suspensions of active ingredients in molten wax often are of interest in spray congealing processes. These systems may show non-Newtonian behavior. For such cases, the viscosity at the wheel will vary with sheer rate, and therefore with wheel speed. Viscosity is not in effect an independent variable and must be estimated independently for each wheel speed used.

CONCLUSIONS

The results of this study show that the average particle size (D_w) of spray congealed stearic acid varies directly with the 0.17 power of the feed rate, inversely with the 0.54 power of the peripheral wheel velocity, and inversely with the 0.02 power of feed viscosity. Except for the latter variable, the correlations developed here are in general agreement with those previously reported for spray drying operations. The lack of agreement regarding the influence of feed viscosity is of little practical concern since changes in viscosity over the limits studied in this report (and in the reports of others) exerted negligible effects on particle size.

Average particle sizes calculated by the equations derived in this report showed excellent agreement with the experimental values. The average deviation of the predicted values from the experimental values was $\pm 1.0\ \mu$. The results of duplicate runs indicate that products with reproducible particle size characteristics are obtainable.

In the absence of other information, the correlation developed here should be useful in arriving at first estimates of the wheel speed and feed rate required to obtain a given desired particle size. When specifications for the product have been established in laboratory runs, the correlating equation (Eq. 3) may help in approximating scale-up factors for the pilot and production level operations.

The instrumentation developed in the study was reliable, simple to construct, and provided close control of the congealing process.

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Change of Ethylene Oxide Concentration During Gaseous Sterilization Process

By D. SATAS

The change of ethylene oxide concentration during the operation of laboratory and production sterilizers is reported. The rate of sorption is high at the beginning and decreases toward the end of a sterilization cycle.

ETHYLENE OXIDE (ETO) gas and its mixtures with carbon dioxide and fluorocarbons are widely used in the chemical sterilization of various pharmaceutical and hospital products. The principal use is for the sterilization of products which cannot be sterilized by steam without damage to their appearance or functionality. With an increasing utilization of heat-sensitive plastic materials, the importance of ETO as an efficient and economical sterilizing agent is growing.

This paper considers the change of ETO concentration during sterilization in laboratory and production size sterilizers, and discusses some of the experiences with a 10% ETO 90% CO_2 mixture at the Chicago Division of the Kendall Co.

Methods of ETO Analysis.—Chemical and infrared gas analyses were employed to assay the ETO concentration in the gaseous phase.

The reaction between epoxide compounds and hydrochloric acid to form the corresponding chlorohydrin was utilized to determine the ETO concentration (1). The reagent was prepared by dissolving 810 Gm. of $CaCl_2 \cdot H_2O$ and 95 ml. of concentrated HCl in 1400 ml. of water. Although several gas sampling techniques provided adequate accuracy, it was most convenient to bubble the gas slowly through gas washing bottles equipped with fritted glass cylinders and filled halfway with 150 ml. of reagent. Two wash bottles in series were used. At bubbling rates of 150–200 ml./minute and ETO concentrations of 10% or below, practically all ETO was absorbed in the first bottle. The gas flow rate was measured by a rotameter in series with the washing bottles. The amount of ETO absorbed was determined by titration of the excess acid with NaOH.

Infrared gas analysis is especially suitable for quick continuous determination of ETO and has been successfully used also by other workers (2). A known mixture of ETO and N_2 was used for calibration of the instrument. Such mixtures should be prepared carefully to assure their homogeneity. If any ETO is condensed in the calibration gas container, the discharge concentration will vary and might result in erroneous calibration of the instrument. For this reason, other more difficultly condensible gas mixtures (*n*-butane in N_2) can be used more conveniently for checking the analyzer once it has been calibrated with ETO.

ETO Changes in the Laboratory Sterilizer.—The change of ETO concentration in an 80-L. laboratory sterilizer manufactured by American Sterilizer Co.

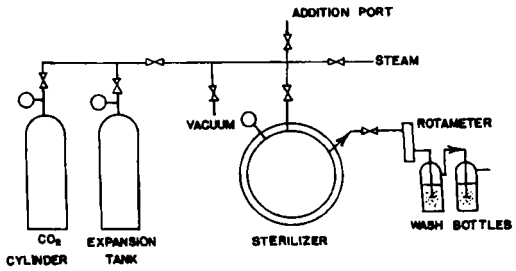


Fig. 1.—Schematic diagram of the equipment used for the laboratory sterilizer tests.

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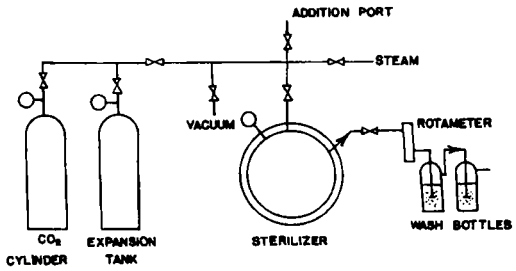


Fig. 1.—Schematic diagram of the equipment used for the laboratory sterilizer tests.

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was investigated under various conditions. A mixture of 10% ETO and 90% CO_2 was used for these experiments. Figure 1 shows the setup used for these tests. Liquid ETO is introduced through the addition port into evacuated sterilizer, and the proper amount of CO_2 is allowed to enter the sterilizer from the expansion tank. The rotameter and wash bottles are connected to the sterilizer for the assay of ETO concentration in the manner described above.

Figure 2 shows the change of ETO content in the sterilizer without a load at two different temperatures and two different water surface areas available for gas absorption. Water was introduced by placing pans of 10 to 70 sq. in. surface area into the sterilizer. The quantity of water was 1 L. in both cases.

The linearity of the curves suggests that the rate of ETO sorption is independent of ETO concentration in the gaseous phase and that the diffusion of ETO in water might be the rate-controlling step. The difference between curves A and B, however, cannot be attributed to the effect of temperature on ETO diffusivity in water. Increase of temperature might cause eddy diffusion in water and possibly some condensation of water on the cooler sterilizer surfaces, rendering the analysis of data difficult.

The sorption of ETO was sharply increased when a load of 8 lb. of cotton was placed in the sterilizer (Fig. 3). If the load is wetted with 100 ml. water, the ETO removal from the gas is further accelerated,

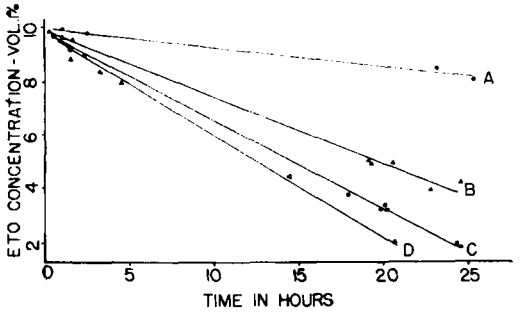


Fig. 2.—ETO removal in an empty laboratory sterilizer at different temperatures and available water surfaces for gas absorption. Key: (A) 80°F., 10 sq. in.; (B) 140°F., 10 sq. in.; (C) 80°F., 70 sq. in.; (D) 140°F., 70 sq. in. Total pressure 30 p.s.i.g.

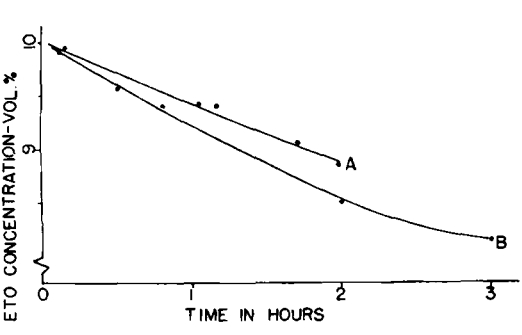


Fig. 3.—ETO sorption by cotton (8 lb.) in a laboratory sterilizer at 140°F., and 30 p.s.i.g. total pressure. Key: (A) dry load; (B) wetted with 100 ml. of water.

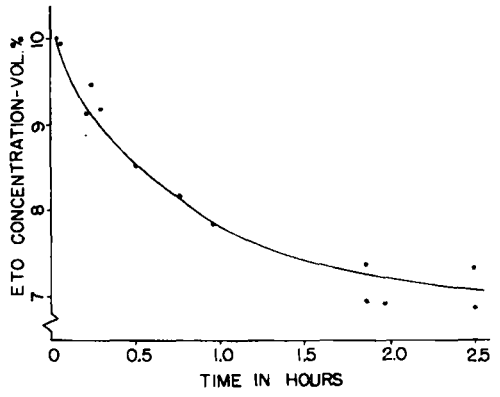


Fig. 4.—Change of ETO concentration in a laboratory sterilizer during a simulated sterilization cycle.

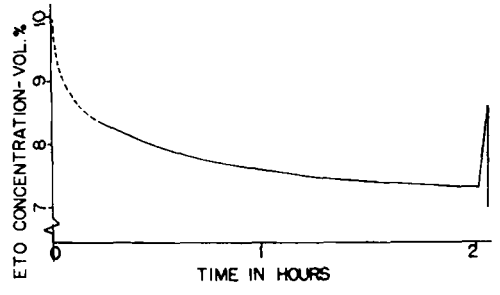


Fig. 5.—Typical ETO concentration change in a production sterilizer.

as evident from the differences between curves A and B.

The change of ETO concentration during a simulated sterilization cycle of a product containing cellulosic and plastic materials is shown in Fig. 4. Steam was introduced to preheat and humidify the load. Formation of fine condensate droplets increased the rate of ETO sorption and the amount of the gas absorbed by the load.

ETO Change in the Production Sterilizer.—Sterilizing gas can be introduced into the sterilizer either directly from a compressed gas cylinder or through auxiliary equipment designed to heat the gas. The ETO concentration change was followed in a 200-cu. ft. sterilizer by continuous infrared analysis as shown in a typical curve (Fig. 5). In this case, the gas was allowed to warm up in a jacketed expansion tank before introduction into the sterilizer. The sorption of ETO by the load of cellulosic and plastic material was faster than in the labora-

TABLE I.—CHANGE OF ETO CONCENTRATION IN STERILIZATION OF VARIOUS PRODUCTS

Product	Av. ETO Concn.—Vol. %: First Reading at the Start of Cycle	Av. ETO Concn.—Vol. %: At the End of Cycle	Difference Between Nominal 10% Concentration and Last Reading
Porous, fine cotton	7.6	6.1	3.9
Mixture of 1 and 3	7.9	6.3	3.7
Cotton gauge goods	8.1	7.0	3.0
Plastic and cellulosic materials	8.3	7.2	2.8
Heavy cotton goods	9.4	8.8	1.2

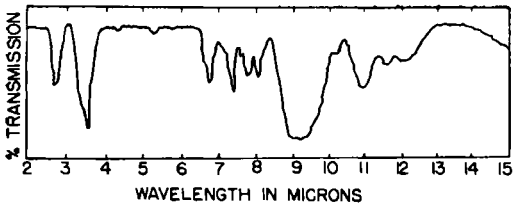


Fig. 6.—Infrared absorption of polyethylene glycol.

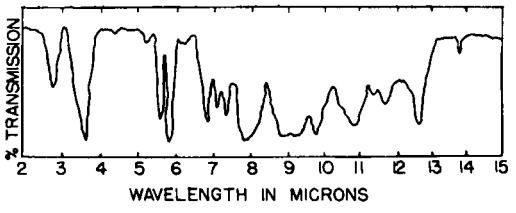


Fig. 7.—Infrared absorption of ethylene oxide polymerization products.

tory sterilizer. The first reading was invariably below the initial nominal 10% ETO concentration. The sudden increase in concentration at the ends of the cycles occurs during discharge, when the pressure drops from 30 p.s.i.g. to atmospheric, and some ETO is released by the load. Such a release was also observed by El Khishen in the fumigation of grain (4).

The ETO consumption varied with different products as summarized in Table I. ETO consumption appears to be related to the surface area of the product rather than the weight. The products which exhibited the highest rate of sorption initially, as shown by the decrease of ETO concentration from the initial 10% to the value given by the first reading (second column Table I), also consumed more ETO during the sterilization.

Polymerization Products of ETO.—ETO polymerization products, an oily viscous fluid of dark brown, are found in the expansion tank where the ETO–CO_2 mixture is stored at about 140° F. The residue was analyzed by the infrared method, and the spectra are similar to that of polyethylene glycol (Figs. 6 and 7). The additional bands at 5.5, 5.7, and 14 μ could be attributed to ethylene carbonates. The results agree with other reports (5, 6).

Summary and Discussion.—The rate of ETO sorption is rapid at the start of a sterilization cycle and decreases after 30 minutes to 1 hour. An increase of moisture content increases the rate of sorption as well as the amount of ETO removed from the gas by the product. The absorbed gas does not remain in the load, but a part is released when the pressure in the sterilizer decreases.

If the sterilization takes place *via* diffusion of ETO in the aqueous phase to bacteria as suggested by Mayr (3), then the rate of ETO absorption, most of which is probably dissolved by water in the load, and the amount of ETO removed from the gaseous phase, become a measure of the process efficiency. The removal is facilitated by forced diffusion and increased moisture content. Too much moisture, however, might cause a nonuniform distribution of ETO in the aqueous solution throughout the load, since a portion of ETO might be removed by the condensate on the outside of packages before the gas actually reaches the sterilization site. The importance of relative humidity in the vicinity of the spores and the danger of stripping the gas of ETO before the spores are reached has also been emphasized by Opfell and co-workers (7). An excessive amount of moisture would also decrease the ETO concentration in the aqueous phase, and thicker water film would hinder the diffusion of the sterilizing agent.

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(3) Mayr, G., *ibid.*, p. 96.
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(5) Baize, T. H., *Ind. Eng. Chem.*, 53, 903(1961).
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Notes

Synthesis of 3-Alkylsydnone-4-carboxylic Acids

By LEMONT B. KIER*, DEVINDRA DHAWAN, and MELVIN J. FREGLY

The synthesis of the first of the alkylsydnone carboxylic acids is reported. A brief review of the effects of 3-isopropylsydnone-4-carboxylic acid on urinary output and blood pressure is included.

DURING THE COURSE of our studies on structure-activity relationships, the authors have made a number of 3-alkylsydnones (1, 2) and 3,4-dialkylsydnones (3) in an effort to determine the effect of ring substituents on physical parameters and biological action. A significant influence on partition coefficient and convulsive activity by the 3-alkyl group of the monosubstituted sydnones (2) was noted. The influence of two alkyl groups on the sydnone ring, however, did not follow any predictable or readily apparent pattern in the compounds studied.

In an effort to learn more about the influence of ring substitution, we have begun a program of studying the effect on biological activity produced

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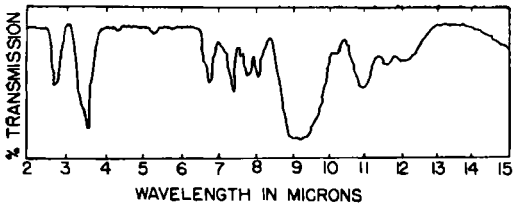


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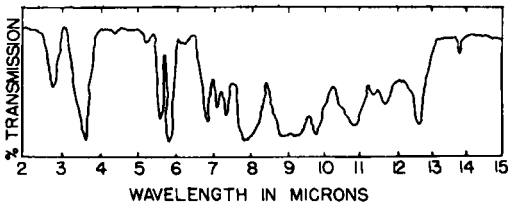


Fig. 7.—Infrared absorption of ethylene oxide polymerization products.

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In an effort to learn more about the influence of ring substitution, we have begun a program of studying the effect on biological activity produced

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by introducing ring substituents other than short alkyl groups. This communication deals with the preparation and preliminary pharmacological study of two 3-alkylsydnone-4-carboxylic acids. A more detailed pharmacological evaluation and comparison with other sydnones is being published elsewhere (4).

Early attempts to prepare sydnone-4-carboxylic acids (I) included the oxidation of 3-phenyl-4-methylsydnone (5), the reaction of potassium cyanide with 3-phenyl-4-bromosydnone (6), and the reaction of carbon dioxide with the Grignard compound derived from 3-phenyl-4-bromosydnone (7). Kato and Ohta (8) successfully prepared the first sydnone-4-carboxylic acid—namely, 3-phenylsydnone-4 carboxylic acid. In their procedure they obtained the lithium compound by treating 3-phenyl-4-bromosydnone with *n*-butyl lithium. Subsequent treatment with carbon dioxide gave the acid.

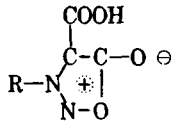
We have modified this procedure slightly and have obtained the first reported 3-alkylsydnone-4-carboxylic acids—namely, the 3-isopropyl and the 3-*sec*-butyl acids. The two 4-bromosydnones were prepared by direct bromination of an acetic acid solution of the 3-alkylsydnones. The two bromosydnones were crystalline and stable compounds. Commercial *n*-butyl lithium in hexane was used to form the lithium compounds which were then carbonated *in situ* with dry ice. Both sydnone acids were crystalline and easily purified.

3-Isopropylsydnone-4-carboxylic acid as the sodium salt was studied along with other sydnones for their effects. A more detailed treatment of these findings will be published later (4). The 3-isopropylsydnone-4-carboxylic acid had only a moderate effect in raising urinary output and excretion rates of sodium, potassium, and chloride. The effect was less than for the alkyl and dialkylsydnones studied. There was no effect on the blood pressure of hypertensive rats compared with a moderate activity for the other sydnones tested. The acid was much less toxic than the other sydnones and produced none of the convulsions previously reported (1).

EXPERIMENTAL[1]

3-Isopropyl-4-bromosydnone.—A solution was prepared containing 5 Gm. of 3-isopropylsydnone (2) and 5 Gm. of fused sodium acetate in 40 ml. of glacial acetic acid. A 5-Gm. quantity of bromine was added to this with stirring and cooling. After 10 minutes, the solution was diluted with 400 ml. of water and extracted with ether. The ether solution

[1] All melting points are corrected. Analyses were performed by Galbraith Laboratories, Knoxville, Tenn.



(I)

was washed with sodium bicarbonate solution until neutral, then dried. The residue from the ether was crystallized from ethanol, m.p. 80 to 81.5°, yield 70%.

Anal.—Calcd. for $C_5H_7BrN_2O_3$: C, 28.98; H, 3.38; Br, 38.64; N, 13.52. Found: C, 29.00; H, 3.65; Br, 38.60; N, 13.48.

3-Isopropylsydnone-4-carboxylic Acid.—A solution of 10 ml. of *n*-butyl lithium (15% in hexane) was added dropwise to a suspension of 2 Gm. of 3-isopropyl-4-bromosydnone in 10 ml. of dry ether at −50° with stirring. After 30 minutes, the reaction mixture was poured over 5 Gm. of powdered dry ice. After all of the dry ice had sublimed, the solution was shaken with water. The aqueous phase was washed with ether and then acidified. The precipitate was filtered and crystallized from ethanol, m.p. 143–144°, yield 60%.

Anal.—Calcd. for $C_6H_8N_2O_4$: C, 41.86; H, 4.68; N, 16.28. Found: C, 42.01; H, 4.81; N, 16.25.

3-sec-Butyl-4-bromosydnone.—This compound was prepared as previously described. The material was crystallized from ethanol, m.p. 61 to 62.5°, yield 80%.

Anal.—Calcd. for $C_6H_9BrN_2O_2$: C, 32.58; H, 4.07; Br, 36.19; N, 12.67. Found: C, 32.74; H, 4.16; Br, 35.98; N, 12.80.

3-sec-Butyl-4-carboxylic Acid.—This compound was prepared as previously described. The material was crystallized from ethanol, m.p. 88–89°, yield 57%.

Anal.—Calcd. for $C_7H_{10}N_2O_4$: C, 45.16; H, 5.67; N, 15.05. Found: C, 45.33; H, 6.04; N, 15.32.

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Precision Apparatus for Dissolution Rate Determinations

By GERHARD LEVY and WILLIAM TANSKI, JR.

An apparatus particularly useful for intrinsic dissolution rate determinations by the rotating disk method is described. It affords precision speed control, wide range of rotation rates, good shaft concentricity, and maintains constant speed over extended periods of time. The apparatus is particularly useful for dissolution rate studies at very low rates of rotation.

THE USEFULNESS of the rotating disk method for intrinsic dissolution rate determinations based either on periodic assays of the medium (1) or on determination of weight loss of the disk (2) has been demonstrated and discussed in a number of recent publications (1–7). It is frequently necessary to determine the dissolution rate of a given drug at different rates of agitation in order to characterize fully its dissolution behavior and to elucidate the mechanism governing the rate of dissolution (6, 8). This makes it desirable to have available an apparatus which affords rates of rotation encompassing as much as two orders of magnitude. The stirrer shaft must be free of vibration and other extraneous (nonconcentric) motion, particularly at higher speeds, and the apparatus must maintain a given rate of rotation accurately for extended periods of time, despite motor heating. The latter requirement is particularly important at the lower speeds, in view of the marked effect of rate of rotation on dissolution rate at lower rotation rates, and the longer duration of experiments performed at low rotation rates. These requirements are not satisfied by the usual commercial stirrers which we have examined. The electronically controlled stirrer which we have used in previous studies (9) permits precision speed control, but is not functional at very low speeds and does not offer a vernier device for rapid regulation of speed. The apparatus to be described in this communication was designed to fulfill the stated needs.

A model T-8 Sigmamotor pump[1] consisting of a 1/8 hp., 110 v., 60 cycle, 1725 r.p.m. single phase explosion-proof motor connected to a model 14 Revco Zero Max speed changer (with vernier control), which in turn is connected to a Sigmamotor pump head, provided the basic equipment. The pump head was removed, and two vertical support rods were attached to the base of the unit in its stead. A vertical sliding bar was attached to the support rods. Clamps were provided to secure the vertical bar at any desired height. A heavy duty flexible shaft (the speedometer cable from a truck) was coupled to the output shaft of the speed changer. The flexible shaft terminates at the cross-bar, where it was coupled to a short (9 cm.), 0.75 cm. diameter, rigid stainless steel shaft. Vertical motion of the latter was prevented by a sleeve at the cross-bar, while nonconcentric motion was minimized by keeping the steel shaft short. A disk holder (1) can be attached to the end of the steel shaft with another accurately machined sleeve. The disk holder can be removed easily by loosening two setting screws in the sleeve. Immersion of the disk holder into and removal from the dissolution medium are accomplished by lowering and raising, respectively, of the cross-bar. The apparatus is shown in Fig. 1.

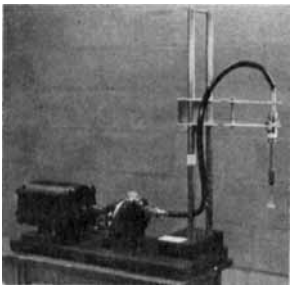


Fig. 1.—Precision apparatus for dissolution rate determinations. (See text for detailed description.)

The vernier speed control is calibrated readily by means of a suitable tachometer (at higher speeds) or by stop-watch (at lower speeds). The equipment has been operated continuously for up to 48 hours without difficulty. Dissolution rate experiments at speeds as low as 4 r.p.m. and of 6 hours duration have been carried out without measurable variation in rate of rotation, evidenced by periodic direct speed determinations and by the constancy of the dissolution rate data. The range of rotation rates of the apparatus is approximately 3 to 400 r.p.m.

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[1] Manufactured by Sigmamotor, Inc., Middleport, N. Y.

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Activation of Pancreatic Lipase by Pharmaceutical Bile Products

By IRVING MELCER and E. A. LAZO-WASEM*

The relative effectiveness of pharmaceutical bile products to activate pancreatic lipase was compared using an assay technique which attempts to reflect *in vivo* conditions of human intestinal digestion.

POTENTIATION OF pancreatic lipase by bile has been known for seven decades (1). However, the mechanism of action remains obscure (2). Contributing factors to this lack of knowledge have been the unavailability until recently (3) of preparations of high purity pancreatic lipase and the diverse, nonspecific methodology used for lipolysis measurements. The same factors have militated against clear-cut ranking of the relative effectiveness of commercial bile acids and their conjugated salts as activators of pancreatic lipase.

EXPERIMENTAL

Materials.—The lipase preparations and bile derivatives used were commercial products manufactured in our laboratory. Dehydrated, defatted preparations of swine pancreas of equal lipase content were used throughout the study. The bile products tested included desiccated ox and hog biles; ox bile extract N.F., which constitutes the alcohol soluble portion of bile; sodium cholate, dehydrocholate, desoxycholate, and hyodesoxycholate, all of purity approaching 100%; and sodium taurocholate, 70% pure, consisting primarily of taurine conjugates of cholic and, to a small extent, of desoxycholic acids.

Methods.—Two assay methods were utilized. The first was the procedure of Tietz, *et al.* (4), which employs an olive oil substrate pre-emulsified with gum arabic (*Method A*). This technique was used to test the effect of prior substrate emulsification on lipolytic activity. The remainder of the investigation—the comparison of lipolytic activation by pharmaceutical bile products—was carried out using *Method B*. The latter is an adaptation (5) of the procedure of Sammons, *et al.* (6). In the method, attempting to reflect *in vivo* conditions, the common dietary lipid olive oil is the substrate. Emulsification is brought about by the addition of physiological concentrations of bile constituents, intestinal motility is reproduced by agitation, and the reaction is carried out at 37° and pH 7.8. In both *Methods A* and *B*, fatty acids liberated during the digestion period are titrated and the extent of lipolysis expressed as the amount of alkali consumed.

The first series of experiments was designed to show the role of substrate emulsification in activation of lipase by bile using *Method A*. The point was studied further employing *Method B*. Polysorbate emulsifiers[1] were aded to the incubation medium and the effect of further addition of ox bile extract determined. In the second series of experiments, carried out with *Method B*, the relative lipase activating effect of bile derivatives was determined. A weighed amount of lipase was homogenized in a Waring Blendor, model 702A, with a suitable amount of water; aliquots representing 0.5 mg. of enzyme were incubated with the substrate containing the bile preparations dissolved in the 5 ml. of buffer required by the procedure (5). In this manner the total volume of the reaction mixture was kept constant. After a 30-minute digestion period, the reaction was stopped by the addition of 5 drops of concentrated HCl, the liberated fatty acids were extracted into benzene, and titrated with alcoholic NaOH with phenolphthalein as indicator (5).

Results.—Addition of ox bile extract increased the enzymatic release of fatty acids from a pre-emulsified oil substrate (Table I). The same effect was demonstrated when utilizing a nonemulsified olive oil substrate, since, while the addition of polysorbate emulsifiers resulted in an increase in the rate of lipolysis, added ox bile extract again produced a further increase (Table II). The level of polysorbates added corresponded to a very narrow region in which potentiation of lipase activity could be demonstrated, presumably by substrate emulsification. In agreement with Minard (7), larger amounts of added emulsifiers had an inhibitory effect.

Table III summarizes the relative effectiveness of bile derivatives as lipase activators. In the first series two chemically distinct biles, desiccated hog and ox biles, proved to be similarly effective when tested at a concentration of 0.5%; the partially purified ox bile extract was slightly more potent.

TABLE I.—EFFECT OF BILE ON THE LIPOLYSIS OF A STABLE OLIVE OIL EMULSION BY PANCREATIC LIPASE

Lipase (mg./ Digestion Flask)	Ox Bile Extract N.F. (mg./ Digestion Flask)	Lipolytic Activity[a] (ml. 0.05 *N* NaOH/ Digestion Flask)
0.123	None	1.5
0.308	None	3.4
0.616	None	5.4
0.616	25	12.5

[a] *Method A*, employing lipase lot 109302.

TABLE II.—COMPARATIVE EFFECTIVENESS OF BILE AND POLYSORBATE EMULSIFIERS IN THE ACTIVATION OF PANCREATIC LIPASE

% Concn. of Additive in 5 ml. of Buffer Added to Digestion Mixture			Lipolytic Activity[a] (ml. 0.05 *N* NaOH/mg. Enzyme)
Emulsifier		Ox Bile Extract N.F.	
None		None	0.8
None		0.5	2.9
Polysorbate 80	0.01%	None	1.1
Polysorbate 80	0.05%	None	1.5
Polysorbate 80	0.20%	None	0.7
Polysorbate 80	3.20%	None	0.2
Polysorbate 80	6.40%	None	0.1
Polysorbate 80	0.05%	0.5	2.6
Polysorbate 65	0.05%	None	1.3
Polysorbate 65	0.05%	0.5	2.8

[a] *Method B*, employing lipase lot 109104.

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Previous report: Melcer, I., and Lazo-Wasem, E. A., *Federation Proc.*, **20**, 222(1961).

[1] Marketed as Tween 65 and Tween 80 by the Atlas Powder Co., Wilmington, Del.

* Present address: Strong Cobb Arner Inc., Cleveland, Ohio.

TABLE III.—COMPARATIVE ACTIVATION OF PANCREATIC LIPASE BY BILE DERIVATIVES

% Concn. of Bile Derivative in 5 ml. of Buffer Added to Digestion Mixture		Lipolytic Activity[a] (ml. 0.05 N NaOH/mg. Enzyme)
None	...	1.0
Hog bile powder	0.5	2.2
Ox bile powder	0.5	2.5
Ox bile extract N.F.	0.2	2.4
Ox bile extract N.F.	0.5	2.9
Ox bile extract N.F.	1.0	3.3
Ox bile extract N.F.	4.0	4.5
Ox bile extract N.F.	8.0	5.4
Ox bile extract N.F.	16.0	6.0
Sodium cholate	0.1	2.9
Sodium cholate	0.25	3.3
Sodium cholate	0.5	3.7
Sodium cholate	2.0	4.2
Sodium cholate	4.0	3.8
Sodium desoxycholate	0.1	3.5
Sodium desoxycholate	0.25	3.8
Sodium desoxycholate	0.5	5.0
Sodium desoxycholate	1.0	5.5
Sodium desoxycholate	2.0	6.2
Sodium desoxycholate	5.0	5.9
Sodium cholate + Sodium desoxycholate	0.25 + 0.1	3.4
Sodium hyodesoxycholate	0.1	3.5
Sodium taurocholate	0.25	1.7
Sodium taurocholate	0.5	2.9
Sodium dehydrocholate	0.25	0.8

[a] *Method B*, employing lipase lots 109104, 112753, and 115037.

Sodium cholate at a level of 0.25% and sodium desoxycholate at a level of 0.1% showed equivalent effects. Sodium cholate, the sodium salt of cholic acid (3,7,12-trihydroxycholanic acid), represents about 50% of dry ox bile and sodium desoxycholate, the sodium salt of desoxycholic acid (3,12-dihydroxycholanic acid), accounts for 20%. The activating effect of 0.5% ox bile extract, therefore, can be accounted for by either of its constituent bile acids, no additive effects of the latter being apparent. This was confirmed by a separate experiment in which the two pure bile salts were added simultaneously in the same concentrations present in 0.5% ox bile extract. The sodium salt of dehydrocholic acid, a chemically modified bile acid prepared by oxidation of cholic acid and lacking the characteristic hydroxyl groups of native bile acids, was without effect.

On an absolute weight basis, desoxycholate was the most potent activator—as much as a sixfold increase in activity was obtained. Within certain limits, the effect was correlated with added bile acid with a linear logarithmic relationship prevailing over a twentyfold range in concentration (Fig. 1). Similar results were obtained when lipolytic potentiation by ox bile extract, which contains desoxycholate, was tested (Table III).

In a further series of tests, the predominant and characteristic bile acid of hog bile, hyodesoxycholic acid (3,6-dihydroxycholanic acid), was compared to its isomeric counterpart of ox bile, desoxycholic acid. Both acids produced significant enzymatic activation of approximately the same magnitude at equal concentrations in the reaction mixture.

Since bile acids occur only as the glycine or taurine conjugates in native bile (2), the effect of one such compound was tested in a third series. Sodium taurocholate was chosen because this bile salt has become something of a standard for study in this field (2, 8–10). This bile salt did not achieve the degree of activation produced by the unconjugated bile acids in our study. Interpretation of the results is complicated because the material employed, representative of commercially available preparations, was only 70% pure.

DISCUSSION

Sobotka (1) has reviewed the early literature concerning the effect of bile acids in pancreatic lipolysis, including the pioneer work of Japanese investigators which established that different bile acids may differ in their ability to activate lipase. Independently, workers have claimed to achieve as much as a fourteenfold increase in the extent of the reaction by the use of bile, while others have observed no such activation under their test conditions (11). Confusion has probably arisen from the lack of a sufficiently pure pancreatic lipase which leads to investigations with diverse and heterogeneous pancreas preparations and from the complex methodology encompassing titrimetric, manometric, nephelometric, and colorimetric procedures with substrates which have varied from simple aliphatic esters to long-chain mixed triglycerides (12). Thus, some authors have attributed the accelerating effect of bile acids to mere substrate emulsification (13); others held that bile facilitates adsorption of the enzyme onto emulsified fat droplets (reviewed in *Reference 2*); Borgstrom has concluded that the level of taurocholate employed influences the pH optimum of the lipolytic reaction and hence its rate (10). More recently, Desnuelle attributed activation by taurocholate to a conversion of lipolysis by the latter from a non-temperature dependent reaction to a faster temperature dependent one (14).

This last observation was confirmed by the work of De Moerloose (15). This author employed "pancreatic lipase preparations" not further characterized and a titrimetric procedure based on the use of a pre-emulsified olive oil substrate. Using the sodium salts of three bile acids, a descending order of activation was observed for desoxycholic, cholic, and taurocholic acids. The activation was attributed to a facilitation of contact between enzyme and substrate independent of the surface-active or solubilizing properties of the bile salts employed.

Our observations indicate that the physical state of the substrate plays a role in the over-all reaction, shown by the potentiating effect of added polysorbate emulsifiers. However, the increased and

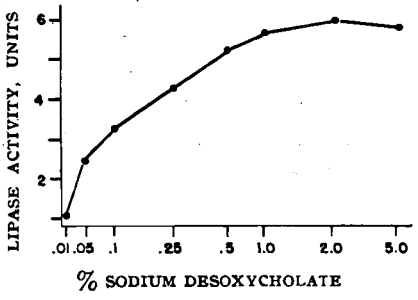


Fig. 1.—Effect of sodium desoxycholate on activation of pancreatic lipase (*Method B*).

additive effect of bile shows that this cannot be the entire explanation. Similar increases in activity produced by added taurocholate were reported by Goldman, *et al.* (8), who employed emulsified fats of extremely small droplet size as substrate—and more recently, by Constantin and co-workers (9), utilizing an emulsified olive oil substrate and a pancreatic lipase claimed to be homogeneous.

In the present study both ox and hog bile proved to be excellent activators of lipolysis, in spite of their distinct chemical make-up. Ox bile consists primarily of cholic and desoxycholic acids, with conjugation divided about equally between glycine and taurine; hog bile contains chiefly hyodesoxycholic and hyocholic acids, with exclusive glycine conjugation (1, 2). The dihydroxy acids, desoxycholic and hyodesoxycholic, had the greatest potentiating activity, with the trihydroxy acid, cholic, somewhat less. Dehydrocholic acid, lacking hydroxyl groups, did not show an effect *in vitro*, although *in vivo* it would be expected to exert a pronounced activating effect mediated by bile acids made available through its potent choleretic action (1). The foregoing structural specificity hints at an actual involvement of bile acids in lipolysis by some yet undescribed mechanism.

SUMMARY

Crude and purified bile derivatives showed activating properties on the course of pancreatic lipolysis *in vitro* when employing assay methods utilizing both emulsified and nonemulsified olive oil substrates. Of the salts of purified bile acids tested, desoxycholate was the most potent—a sixfold increase in lipolysis was achieved. Considerable activation was also obtained with hyodesoxycholate, cholate, and taurocholate and with ox and hog biles. In contrast, dehydrocholate did not have *in vitro* effect.

ADDENDUM

Recently an article appeared [Fritz, P. J., and Melius, P., *Can. J. Biochem. Physiol.*, **41**, 719(1963)], which advanced the theory that bile salts activate lipolysis by splitting the enzyme-diglyceride complex, thus increasing the action of lipase on the triglyceride ester. The activating effect was thought *not* to be due to simple emulsification. Glyco and taurocholate were superior to desoxycholate in contrast to the present findings and those of others (1, 15).

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Ceric Sulfate as Permanganate Replacement in Pharmacopeial Assays

By M. M. TUCKERMAN, N. DOCTOFSKY, R. PRICE, R. RAKE, S. RALPH, S. SHENK, and B. A. WISHNEV

Standard 0.1 *N* potassium permanganate was replaced by standard 0.1 *N* ceric sulfate and orthophenanthrolene T. S. indicator in the official assay of ferrous sulfate U.S.P., hydrogen peroxide U.S.P., and sodium perborate N.F. Statistical analysis of the results using the *t* test shows no sufficient reason to doubt that the two methods yield the same mean value at the 10 per cent significance level.

ONE OF THE tests of a rational philosophy is that it contain no unnecessary axioms. One of the tests of a rational system of quantitative analysis might well be that it contain no unnecessary reagents. The "United States Pharmacopeia" and "National Formulary" do not meet this criterion.

This paper focuses attention on the use of strong oxidizing agents as standard solutions in titrimetry. Experience has shown the utility of three agents: ceric ion, dichromate, and permanganate. Of these, dichromate has slowly lost its status, being used now only as a precipitating agent in the determination of quinacrine. With the change in assay of most of the calcium preparations from oxidation of the precipitated oxalate with standard permanganate to a complexometric titration, the need for a standard permanganate solution has decreased. Materials requiring standard permanganate solution may be divided into five classes: (*a*) ferrous sulfate U.S.P., exsiccated ferrous sulfate U.S.P., hydrogen peroxide solution U.S.P., and sodium perborate N.F.; these are titrated directly. (*b*) Dibasic calcium phosphate U.S.P. and cherry juice U.S.P.; these require titration of precipitated calcium oxalate. (*c*) Lead monoxide N.F., lead subacetate solution N.F., and diluted lead subacetate solution N.F.; these require titration of oxalic acid left in the filtrate after precipitation of lead oxalate. (*d*) Sodium nitrite U.S.P. which requires a residual titration involving both standard permanganate and standard oxalic acid.

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TABLE I.—COMPARISON OF RESULTS FOR FERROUS SULFATE U.S.P.

Analyst	Permanganate Trial A	Permanganate Trial B	Permanganate Trial C	Ceric Sulfate Trial A	Ceric Sulfate Trial B	Ceric Sulfate Trial C
1	100.5	100.5	100.3	99.0	98.3	98.9
2	100.6	100.2	100.3	99.1	99.3	99.0
3	99.8	99.8	99.8	102.8	102.8	102.8
4	100.1	100.4	100.3	99.9	99.8	100.0
5	100.2	99.9	100.4	99.1	99.8	99.4
6	102.6	101.6	101.5	102.9	102.7	102.5
Av. ($\overline{X}$)			100.49			100.46
S.D. (σ)			1.87			4.46
sp				1.327		
t				0.1356		
For $\alpha = 0.10$				$t > \lvert 1.69 \rvert$		

TABLE II.—COMPARISON OF RESULTS FOR HYDROGEN PEROXIDE SOLUTION U.S.P.

Analyst	Permanganate Trial A	Permanganate Trial B	Permanganate Trial C	Ceric Sulfate Trial A	Ceric Sulfate Trial B	Ceric Sulfate Trial C
1	2.32	2.30	2.34	2.50	2.51	2.51
2	2.18	2.22	2.34	2.18	2.23	2.36
3	2.94	2.93	2.96	2.95	2.96	2.93
4	2.40	2.31	2.30	2.48	2.36	2.34
5	2.93	2.95	2.95	2.90	2.93	2.88
Av. ($\overline{X}$)			2.551			2.604
S.D. (σ)			0.548			0.470
sp				0.3104		
t				0.3543		
For $\alpha = 0.10$				$t > \lvert 1.70 \rvert$		

TABLE III.—COMPARISON OF RESULTS FOR SODIUM PERBORATE N.F.

Analyst	Permanganate Trial A	Permanganate Trial B	Permanganate Trial C	Ceric Sulfate Trial A	Ceric Sulfate Trial B	Ceric Sulfate Trial C
1	9.60	9.55	9.47	9.51	9.60	9.61
2	9.60	9.51	9.48	9.48	9.36	9.60
3	9.56	9.55	9.47	9.81	9.76	9.79
4	9.83	9.80	9.72	9.69	9.72	9.78
5	9.65	9.58	9.37	9.68	9.64	9.56
6	9.39	9.44	9.31	10.33	10.30	10.29
Av. ($\overline{X}$)			9.549			9.751
S.D. (σ)			0.230			0.461
sp				2.215		
t				0.2736		
For $\alpha = 0.10$				$t > \lvert 1.69 \rvert$		

(*e*) Titanium dioxide N.F., potassium permanganate U.S.P., and potassium permanganate tablets N.F.

The purpose of this paper is to compare the results of titrations using permanganate and ceric sulfate for substances in the first group. Other methods of assay will be proposed at a later time for the other groups.

METHOD

The assays reported in the tables were performed by senior pharmacy students who had taken 1 year of quantitative analysis, during which time they had experience with both permanganate and ceric ion. All assays were carried out by the official methods of the "United States Pharmacopeia" (1) or "National Formulary" (2) and again carried out substituting standard 0.1 *N* ceric sulfate solution (1) and orthophenanthrolene T.S. (1) for the permanganate. No other changes were made in the procedure. The samples used were a single solid sample of ferrous sulfate U.S.P., a single solid sample of sodium perborate N.F., and a previously unopened bottle of commercially prepared hydrogen peroxide solution U.S.P. containing 0.05% acetanilid as a preservative.

RESULTS AND DISCUSSION

The results obtained for ferrous sulfate U.S.P. are listed in Table I, those for hydrogen peroxide solution U.S.P. in Table II, and those for sodium perborate N.F. in Table III. The results, viewed as a whole, show that although there is generally good agreement between the three values obtained by any one analyst, there is considerable variation between analysts. This variation is shown by the values of the standard deviations. Although these may seem excessively large to one who is accustomed to comparing only the results of his own three repetitive analyses, they are actually in agreement with other studies of laboratory and analyst variability (3).

The results for hydrogen peroxide solution U.S.P. are a cause for concern because of the large standard deviation. The cause for the deviation has not been investigated, but it may, in part, be caused by the small (2-ml.) sample size, since the accuracy of measurement of a sample containing oxygen bubbles is questionable, and decomposition of the sample catalyzed by traces of heavy metals adsorbed on the walls of the glass equipment used, is possible.

This study again emphasizes the danger in placing confidence in a single assay, even when done by a trained person using a well established technique. It also shows the danger of placing confidence in replicate assays performed in parallel by the same analyst, even when the standard deviation for the replicates appears acceptable.

CONCLUSIONS

As shown by the statistical data included in the tables, there is no reason to think that the replacement of permanganate with ceric sulfate changes the mean value of the assay results. Although smaller values for the standard deviations might have been obtained by rejecting some of the results, all data collected have been reported.

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Vasopressin Tachyphylaxis

By P. N. PATIL, A. TYE, and J. W. NELSON

Pretreatment with D-*levo*- ephedrine is known to augment the pressor effect of vasopressin in dogs. It also delayed the development of vasopressin tachyphylaxis in this study. Methoxamine did not produce the same effects. Reserpine augmented the pressor response and arrested the tachyphylaxis. Increasing doses of vasopressin then produced increases in pressor response, an effect which suggests a possible bioassay for vasopressin. No cross tachyphylaxis to pressor effects were observed between vasopressin and angiotensin.

GEILING AND CAMPBELL (1) and Jones and Schlapp (2) have noted the decrease in pressor response to successive doses of posterior pituitary extract. Hogben, *et al.* (3), studied the development of tolerance to posterior pituitary lobe extract in the spinal cat and concluded that it is a function of dose and time interval between doses. By spacing repeated doses at appropriate time intervals, they were able to obtain pressor responses of the same character and magnitude as the one produced by the first injection. Woodbury and Wilks (4) found that in ouabain-treated animals tachyphylaxis to vasopressin developed less readily than in control animals; they suggested that vasopressin tachyphylaxis is a "pseudo-tachyphylaxis." Gardier and Abreu (5) showed that tolerance to vasopressin can be prevented by bilateral carotid sinus denervation and midcervical vagotomy. We investigated the effects of pretreatment with D-*levo*-ephedrine, methoxamine, and reserpine in this study. The sympathomimetic amine, D-*levo*-ephedrine, is a good antagonist of the coronary constriction produced by vasopressin (6); methoxamine is a sympathomimetic amine that produces peripheral effects without cardiotonic effects (7, 8); reserpine blocks carotid and vagal reflexes (9, 10). Cross tachyphylaxis between angiotensin and vasopressin and the effects of renin were also studied.

EXPERIMENTAL

Twenty-five mongrel dogs of each sex, weighing from 7 to 11 Kg., were anesthetized with 35 mg./Kg. of pentobarbital i.p. Both vago-sympathetic nerves were severed. With the usual hemodynamic setup, blood pressure was recorded from the right carotid artery on a kymograph. The trachea was always cannulated. All drugs were dissolved in physiological saline and injected into the femoral vein *via* an indwelling catheter.

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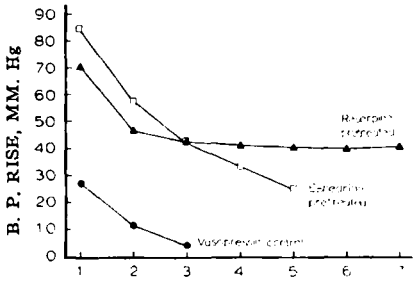


Fig. 1.—The effects of reserpine and D-*levo*-ephedrine pretreatment on vasopressin tachyphylaxis. Each point represents the average blood pressure rise of four to five dogs. The standard errors of the mean varied from 9 to 20% of the average values.

The reserpinized dogs were prepared by administering 0.5 mg./Kg. reserpine i.p. each day for 2 days; the blood pressure was measured as above on the third day. Anesthesia was produced with 15 to 20 mg./Kg. of pentobarbital i.p. in these dogs. Additional pentobarbital was given when necessary to maintain surgical anesthesia.

The following drugs were used: D-*levo*-ephedrine HCl, methoxamine HCl,[1] vasopressin,[2] reserpine phosphate,[3] angiotensin,[4] and hog renin.[5]

RESULTS

In control dogs, vasopressin 0.3 u./Kg. at 30-minute intervals resulted in a progressive reduction of the pressor effect. Tachyphylaxis was practically

[1] Marketed as Vasoxyl by Burroughs Wellcome and Co., Inc., Tuckahoe, N. Y.
[2] Marketed as Pitressin by Parke, Davis and Co., Detroit, Mich.
[3] Marketed as Serpasil by Ciba Pharmaceutical Products, Inc., Summit, N. J.
[4] Marketed as Hypertensin-Ciba by Ciba Pharmaceutical Products, Inc., Summit, N. J. Courtesy of Dr. A. J. Plummer.
[5] Courtesy of Dr. O. M. Helmer, Eli Lilly and Co., Indianapolis, Ind.

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GEILING AND CAMPBELL (1) and Jones and Schlapp (2) have noted the decrease in pressor response to successive doses of posterior pituitary extract. Hogben, *et al.* (3), studied the development of tolerance to posterior pituitary lobe extract in the spinal cat and concluded that it is a function of dose and time interval between doses. By spacing repeated doses at appropriate time intervals, they were able to obtain pressor responses of the same character and magnitude as the one produced by the first injection. Woodbury and Wilks (4) found that in ouabain-treated animals tachyphylaxis to vasopressin developed less readily than in control animals; they suggested that vasopressin tachyphylaxis is a "pseudo-tachyphylaxis." Gardier and Abreu (5) showed that tolerance to vasopressin can be prevented by bilateral carotid sinus denervation and midcervical vagotomy. We investigated the effects of pretreatment with D-*levo*-ephedrine, methoxamine, and reserpine in this study. The sympathomimetic amine, D-*levo*-ephedrine, is a good antagonist of the coronary constriction produced by vasopressin (6); methoxamine is a sympathomimetic amine that produces peripheral effects without cardiotonic effects (7, 8); reserpine blocks carotid and vagal reflexes (9, 10). Cross tachyphylaxis between angiotensin and vasopressin and the effects of renin were also studied.

EXPERIMENTAL

Twenty-five mongrel dogs of each sex, weighing from 7 to 11 Kg., were anesthetized with 35 mg./Kg. of pentobarbital i.p. Both vago-sympathetic nerves were severed. With the usual hemodynamic setup, blood pressure was recorded from the right carotid artery on a kymograph. The trachea was always cannulated. All drugs were dissolved in physiological saline and injected into the femoral vein *via* an indwelling catheter.

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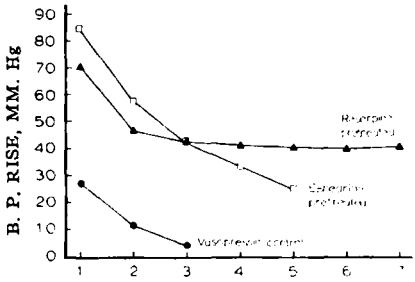


Fig. 1.—The effects of reserpine and D-*levo*-ephedrine pretreatment on vasopressin tachyphylaxis. Each point represents the average blood pressure rise of four to five dogs. The standard errors of the mean varied from 9 to 20% of the average values.

The reserpinized dogs were prepared by administering 0.5 mg./Kg. reserpine i.p. each day for 2 days; the blood pressure was measured as above on the third day. Anesthesia was produced with 15 to 20 mg./Kg. of pentobarbital i.p. in these dogs. Additional pentobarbital was given when necessary to maintain surgical anesthesia.

The following drugs were used: D-*levo*-ephedrine HCl, methoxamine HCl,[1] vasopressin,[2] reserpine phosphate,[3] angiotensin,[4] and hog renin.[5]

RESULTS

In control dogs, vasopressin 0.3 u./Kg. at 30-minute intervals resulted in a progressive reduction of the pressor effect. Tachyphylaxis was practically

[1] Marketed as Vasoxyl by Burroughs Wellcome and Co., Inc., Tuckahoe, N. Y.
[2] Marketed as Pitressin by Parke, Davis and Co., Detroit, Mich.
[3] Marketed as Serpasil by Ciba Pharmaceutical Products, Inc., Summit, N. J.
[4] Marketed as Hypertensin-Ciba by Ciba Pharmaceutical Products, Inc., Summit, N. J. Courtesy of Dr. A. J. Plummer.
[5] Courtesy of Dr. O. M. Helmer, Eli Lilly and Co., Indianapolis, Ind.

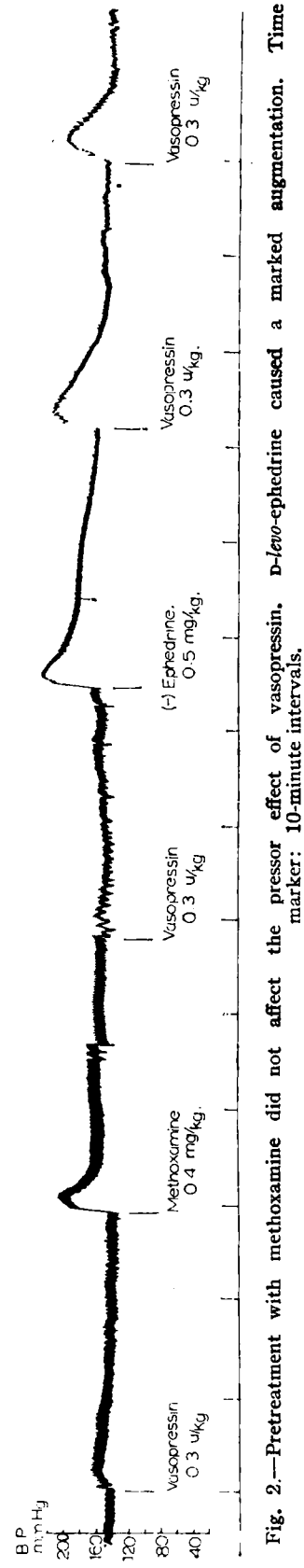


Fig. 2.—Pretreatment with methoxamine did not affect the pressor effect of vasopressin. D-*levo*-ephedrine caused a marked augmentation. Time marker: 10-minute intervals.

complete after two to four such injections (Fig. 1). D-*levo*-ephedrine hydrochloride given in doses of 0.2, 0.5, 1.0, 3.0, and 10.0 mg./Kg., 15 to 30 minutes before the injection of vasopressin, caused a marked augmentation of the pressor response to the latter drug. Doses above 0.5 mg./Kg. of D-*levo*-ephedrine HCl did not cause an increase in this augmentation. Vasopressin repeated at 30-minute intervals in ephedrine pretreated animals still resulted in tachyphylaxis, but the development was slower. A second injection of D-*levo*-ephedrine HCl augmented once more the pressor response to vasopressin. The pressor response to the second dose of D-*levo*-ephedrine HCl was lower than the response to the first dose, indicating that ephedrine tachyphylaxis was unaffected. Figure 1 shows the effect of pretreatment with 0.5 mg./Kg. of D-*levo*-ephedrine HCl on the pressor effects of repeated doses of vasopressin.

Pretreatment with methoxamine in doses of 0.1 to 0.4 mg./Kg., unlike D-*levo*-ephedrine, failed to augment vasopressin's pressor effect (Fig. 2). Pretreatment with reserpine, 0.5 mg./Kg. per day for 2 days, resulted in an augmented pressor effect from vasopressin. A second dose of vasopressin resulted in a somewhat reduced pressor response, but tachyphylaxis was then arrested, and succeeding doses continued to produce the same pressor response. At this stage, increasing doses of vasopressin resulted in increasing pressor response. The results of one such experiment are shown in Fig. 3.

Since cross tachyphylaxis between long acting sympathomimetic amines is well established, we investigated the possibility of cross tachyphylaxis between the octapeptides, vasopressin and angiotensin. Angiotensin itself, 1 mcg./Kg. every 20 minutes for five doses, did not produce tachyphylaxis; intervening doses of vasopressin had no effect on the pressor response to succeeding doses of angiotensin.

Renin is known to produce tachyphylaxis and to reduce or block the pressor response to angiotensin (11, 12). In our experiments, animals given renin 3 G. u./Kg. every 30 minutes for two or three doses exhibited complete tachyphylaxis. Subsequent doses of angiotensin suffered a reduction in pressor effect, but vasopressin effects were essentially unaffected.

DISCUSSION

True tachyphylaxis may be defined as a diminution in response to repeated doses of a drug due to increasing receptor saturation. The suggestion of Woodbury and Wilks (4) that vasopressin produces a pseudo-tachyphylaxis was based on their finding that ouabain offsets the tachyphylactic effect by increasing the force of cardiac contraction. Our results are compatible with this view, for ephedrine, which is known to oppose the coronary vasoconstrictor effects of vasopressin (7), slowed down the development of tachyphylaxis with more regularity than ouabain; while the sympathomimetic amine methoxamine, which has no cardiotonic effects, did not affect the tachyphylaxis. Ephedrine not only slowed down the development of tachyphylaxis but also enhanced the pressor response to vasopressin. This action probably involves not only cardiotonic effects, but also peripheral effects and abolishment of reflexes (13).

Reserpine arrested the development of vasopressin tachyphylaxis, probably by blocking re-

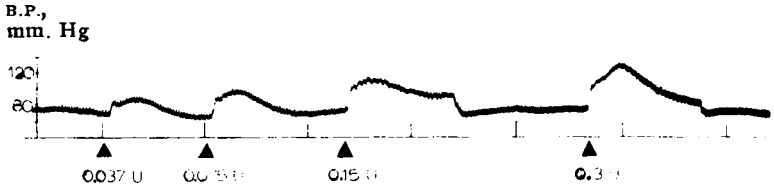


Fig. 3.—Effects of increasing doses of vasopressin after arrest of tachyphylaxis in the reserpinized dog. All doses were in units per kilogram. Time marker: 10-minute intervals.

flexes. Once tachyphylaxis was arrested, dose-response effects were observable. This observation suggests that the use of reserpinized dogs for the bioassay of vasopressin should be investigated.

Our results with angiotensin agreed with those of Page, *et al.* (14). In the pure synthetic form, it was not tachyphylactic and did not affect vasopressin tachyphylaxis. Although both angiotensin and vasopressin are octapeptides, the differences between their structural formulas are sufficient to make it not surprising that there was no cross tachyphylaxis. For the same reason it was expected that, though renin blocked the pressor response to angiotensin, it would not affect the pressor response to vasopressin.

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Synthesis of Potential Antineoplastic Agents I

By WILLIAM D. ROLL

Four new derivatives of *o,o'*-diphenamide have been prepared to evaluate their anticarcinogenic activity: *o,o'*-bis(*N*-2-mesyloxyethyl)diphenamide, *o,o'*-bis[*N*-2-(3-chloropropionamido)ethyl]diphenamide, *o,o'*-bis[*N*-2-(3-bromopropionamido)-ethyl]diphenamide, and *o,o'*-bis(*N*-2-mercaptoethyl)diphenamide.

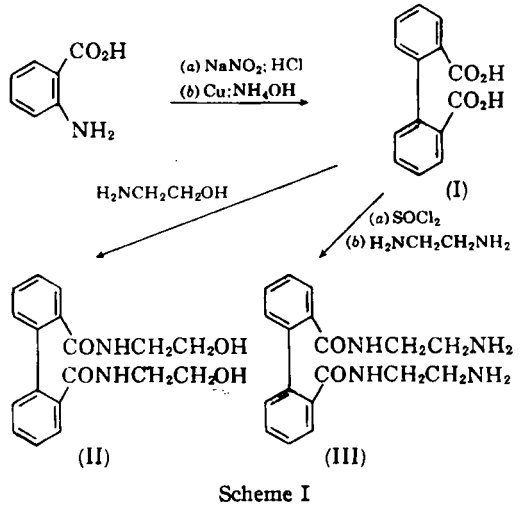
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The synthetic procedure used for the preparation of these analogs of *o,o'*-diphenamide may be briefly outlined as follows. *o,o'*-Diphenic acid (I), synthesized by the procedure described by Atkinson and Lawler (3), was converted to *o,o'*-bis(*N*-2-hydroxyethyl)diphenamide (II), by a procedure similar to that described by Wenker (4). Treatment of II with methanesulfonyl chloride yielded *o,o'*-bis(*N*-2-mesyloxyethyl)diphenamide (IV).

Treatment of I with thionyl chloride gave *o,o'*-diphenoyl chloride (VII) (5, 6). The acyl halide reacted with ethylene diamine at low temperatures to give the amide *o,o'*-bis(*N*-2-aminoethyl)diphenamide (III). Acylation of the amino analog (III) with 3-chloropropionyl chloride or ethyl 3-chloropropionate and 3-bromopropionyl chloride or methyl 3-bromopropionate gave *o,o'*-bis[*N*-2-(3-chloropropionamido) ethyl]diphenamide and *o,o'*-bis[*N*-2-(3-bromopropionamido)ethyl]diphenamide, compounds V and VI, respectively.

To introduce the mercaptoethyl side chain, diphenic acid (I) was converted to the acyl halide (VII) initially. It was allowed to react with



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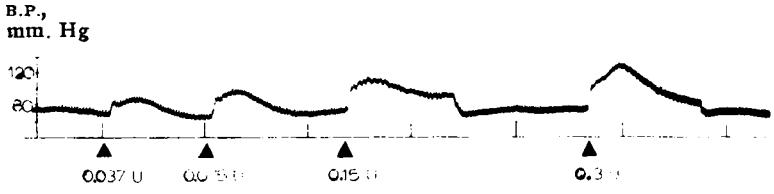


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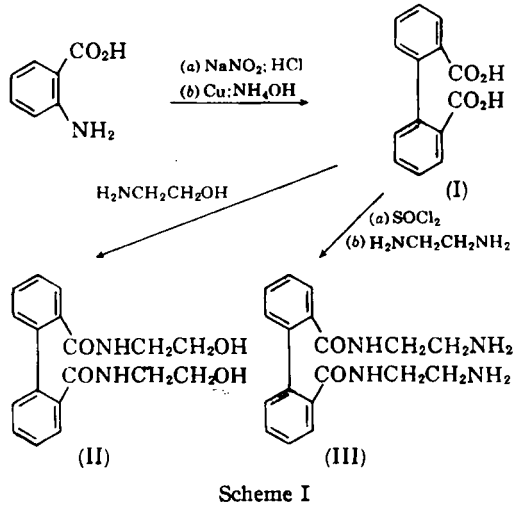
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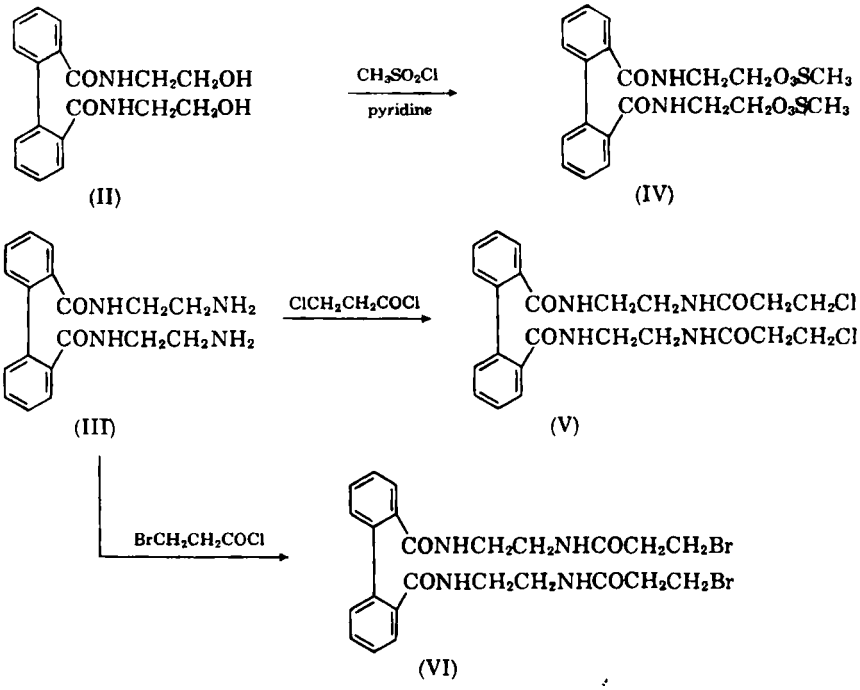
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Scheme II

sodium hydrosulfide to give *o,o'*-thiodiphenic acid (VIII) by a procedure similar to that described in the literature for the synthesis of thiobenzoic acid (7–9). The thio acid gave *o,o'*-bis(*N*-2-mercaptoethyl)diphenamide (IX) on treatment with ethylenimine (10) by a procedure analogous to that described by Kuhn and Quadbeck (11).

EXPERIMENTAL

The sequence of synthetic reactions is shown by Schemes I, II, and III.

o,o'-Diphenic Acid (I).—The procedure used for the synthesis of diphenic acid was that described in "Organic Syntheses" (3).

o,o'-Bis(N-2-hydroxyethyl)diphenamide (II).—A mixture of 4.8 Gm. (0.02 mole) of I and 2.4 Gm. (0.04 mole) of 2-aminoethanol was heated in an open flask until the loss in weight corresponded to 2 moles of water. The crude material was crystallized from absolute methanol to give 4.4 Gm. (68%) of product melting at 130–131°. The compound did not form a salt with HCl, and the infrared spectrum showed a broad band at 3.1 μ. This would indicate that the amide was formed rather than the ester.

Anal.—Calcd. for $C_{18}H_{20}N_2O_4$: C, 65.84; H, 6.14; N, 8.53. Found: C, 66.08; H, 6.10; N, 8.48.

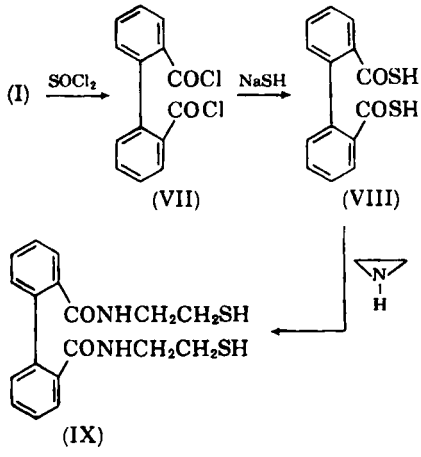
o,o'-Bis(N-2-mesyloxyethyl)diphenamide (IV).—A solution of 1.3 Gm. (0.004 mole) of II in 2.5 ml. of anhydrous pyridine was cooled to 0°. A 1.4-Gm. (0.012 mole) quantity of methanesulfonyl chloride, previously cooled to 0°, was added to this in a dropwise manner. The mixture was allowed to stand for 1 hour, then poured with rapid stirring into ice water. The product was removed on a cold Buchner funnel and washed several times with cold water. The product was crystallized from absolute methanol to give 0.8 Gm. (42%) of product melting at 192–193°.

Anal.—Calcd. for $C_{20}H_{24}N_2O_8S_2$: C, 49.57; H, 4.99; N, 5.78; S, 13.23. Found: C, 49.65; H, 5.10; N, 5.70; S, 13.34.

o,o'-Bis(N-2-aminoethyl)diphenamide (III).—To a cold (0°) 2.4-Gm. (0.04 mole) solution of ethylenediamine and 5% aqueous sodium hydroxide was added a chloroformic solution containing 4.8 Gm. (0.02 mole) of *o,o'*-diphenoyl chloride (VII). The mixture was shaken vigorously for 5 minutes, and the crude amine was removed by filtration. The crude material was crystallized twice from dry chloroform to give 4.0 Gm. (61%) of product melting at 220–221°.

Anal.—Calcd. for $C_{18}H_{22}N_4O_2$: C, 66.23; H, 6.79; N, 17.17. Found: C, 65.98; H, 6.83; N, 17.30.

o,o'-Bis[N-2-(3-chloropropionamido)ethyl]diphenamide (V).—A solution of 2.0 Gm. (0.016 mole) of 3-chloropropionyl chloride in dry chloroform was



Scheme III

added dropwise to a 2.6-Gm. (0.008 mole) solution of the amine (III) in chloroform at room temperature. When the addition of the acyl halide was complete, the reaction mixture was refluxed until the evolution of hydrogen chloride had ceased. The reaction mixture was concentrated *in vacuo* and the crude amide removed by filtration. The crude product was placed in a Soxhlet apparatus and extracted with dry chloroform for several hours. The product melted at 225–226°, yield 3.2 Gm. (79%).

Anal.—Calcd. for $C_{24}H_{28}Cl_2N_4O_4$: C, 56.81; H, 5.56; Cl, 13.98; N, 11.02. Found: C, 57.01; H, 5.48; Cl, 14.01; N, 11.22.

o,o′-Bis[N-2-(3-bromopropionamido)ethyl]diphenamide (VI).—The same procedure described above for the preparation of the chloropropionamide analog was used. To a 2.6-Gm. (0.008 mole) solution of the amine (III) in dry chloroform was added 2.5 Gm. (0.016 mole) of 3-bromopropionyl chloride (in dry chloroform). A slightly longer reflux period was necessary for completion of the reaction than with compound V. The product weighed 3.4 Gm. (57%) and melted at 244–245°.

Anal.—Calcd. for $C_{24}H_{28}Br_2N_4O_4$: C, 48.34; H, 4.73; Br, 26.80; N, 9.40. Found: C, 48.50; H, 4.65; Br, 26.74; N, 9.44.

o,o′-Diphenoyl Chloride (VII).—The procedure used for the synthesis of diphenoyl chloride and the results obtained have been previously described (5, 6).

o,o′-Thiodiphenic Acid (VIII).—A 2.8-Gm. (0.01 mole) quantity of diphenoyl chloride (VII) was added to a solution of sodium sulfhydrate, prepared from 3.7 Gm. (0.04 mole) of NaSH in 25 ml. of distilled water. The mixture was stirred and heated on a steam bath for 3 hours. It was cooled to 0°, surrounded by an ice bath, and slowly acidified with a cold solution of 3 *N* hydrochloric acid. The product was removed by filtration, washed with cold water, and dried overnight in a desiccator. The yield of cream-colored crystals was 1.4 Gm. (50%), m.p. 118–119°.

Anal.—Calcd. for $C_{14}H_{10}O_2S_2$: S, 23.37. Found: S, 23.40.

o,o′-Bis(N-2-mercaptoethyl)diphenamide (IX).—Ethylenimine, 0.4 Gm. (0.009 mole), was added slowly to a solution of 1.1 Gm. (0.004 mole) of thiodiphenic acid (VIII) in 20 ml. of absolute ethanol with constant stirring at 0°. The reaction mixture, under a nitrogen atmosphere, was allowed to come to room temperature, and the solvent was removed *in vacuo*. The residue was crystallized from ethanol–ether to give 1.2 Gm. (82%) of yellow crystals, m.p. 129–130°. The infrared spectrum showed a well defined band at 2600 $cm.^{-1}$(−sh).

Anal.—Calcd. for $C_{18}H_{20}N_2O_2S_2$: C, 59.97; H, 5.60; N, 7.77; S, 17.79. Found: C, 59.98; H, 5.65; N, 7.85; S, 17.82.

DISCUSSION

The *N*-chloropropionamido and *N*-bromopropionamido derivatives of diphenamide, compounds V and VI, respectively, were also prepared by treating a cold methanolic solution of *o,o′*-bis(*N*-2-aminoethyl)diphenamide with ethyl 3-chloropropionate and methyl 3-bromopropionate. However, an extended reaction period at 0° was required, and the yields of the haloamides were considerably lower than when the acyl chlorides were employed.

Preliminary pharmacological studies indicate that compounds V and VI are nontoxic in doses up to 90 mg./Kg. in mice.

In the synthesis of *o,o′*-thiodiphenic acid (VIII), two minor products were also isolated. *o,o′*-Monothiodiphenic acid had a melting point of 125–126°.

Anal.—Calcd. for $C_{14}H_{10}O_3S$: C, 65.10; H, 3.90; S, 12.42. Found: C, 64.96; H, 3.79; S, 12.50.

o,o′-Diphenoyl sulfide had a melting point of 91–92°.

Anal.—Calcd. for $C_{14}H_8O_2S$: C, 69.98; H, 3.36; S, 13.34. Found: C, 69.84; H, 3.40; S, 13.42.

SUMMARY

Four new derivatives of *o,o′*-diphenamide have been synthesized to evaluate their anticarcinogenic activity: *o,o′*-bis[*N*-2-(3-chloropropionamido)ethyl]-diphenamide, *o,o′*-bis[*N*-2-(3-bromopropionamido)-ethyl]diphenamide, *o,o′*-bis(*N*-2-mesyloxyethyl)diphenamide, and *o,o′*-bis(*N*-2-mercaptoethyl)diphenamide. Two intermediate products, *o,o′*-bis(*N*-2-aminoethyl)diphenamide and *o,o′*-bis(*N*-2-hydroxyethyl)diphenamide, and two by-products, *o,o′*-monothiodiphenic acid and *o,o′*-diphenoyl sulfide, were also reported.

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Stereospecific Hydrogenations Using Palladium-on-Silica Gel Catalysts

By ROBERT E. PADGETT, JR., and ROBERT L. BEAMER

Studies have been conducted on the stereospecific nature of catalysts prepared by depositing palladium upon silica gels which have been precipitated in the presence of optically active alkaloids. So far, only the gels formed in the presence of the cinchona alkaloids have proved to be active as carriers for asymmetric catalysts. The substrate for this work was α-methylcinnamic acid affording a slight excess of (+)-dihydro-α-methylcinnamic acid on reduction. Details of the preparation of the carriers and catalysts and studies made on their stereoselective properties are given.

In an earlier paper Beamer, *et al.* (1), presented evidence supporting the existence of substrate-specific and stereospecific centers in palladium-on-carbon catalysts. These data were in accord with the three-point contact theory proposed by Ogston (2) to explain stereospecificity in enzymes. This work also indicated the possible design of an asymmetric synthesis resembling those catalyzed by enzymes but using a nonbiologic catalyst such as palladium. That such a synthetic reaction is not impossible is evidenced by the work of Akabori with palladized silk fibroin (3) and Smith using specially treated palladized charcoal (4).

The present work is concerned with further studies into the stereospecific hydrogenations catalyzed by certain palladized silica gels (5). The gels used in these experiments were prepared by precipitation from a sodium silicate solution using hydrochloric acid in the presence of the various alkaloids of the cinchona family. Although other alkaloids have been employed in these experiments (strychnine, brucine, ephedrine, and monobenzoylephedrine), so far only the gels prepared with the cinchona alkaloids have proved to be active as carriers for asymmetric catalysts. The substrate for this work was α-methylcinnamic acid affording a slight excess of (+)-dihydro-α-methylcinnamic acid on reduction. Oximes such as acetophenone oxime, α-oximinopropiophenone, and 3-pentyl-*p*-methoxyphenylketoxime either could not be reduced using these catalysts or upon reduction produced optically inactive products.

Prior to our work, Dickey (6) and Haldeman and Emmett (7) had demonstrated adsorption specificity and Beckett and Anderson (8) had shown stereoselective adsorption on similarly prepared gels.

EXPERIMENTAL

Reagents.—α-Methylcinnamic acid (K and K Laboratories), quinine sulfate N.F., quinidine sulfate U.S.P., cinchonine sulfate U.S.P. IX, cinchonidine sulfate U.S.P. X, sodium silicate solution (technical, 40–42° Be) were used.

Procedure.—The preparation of our gels was essentially that used by Dickey (6). The desired quantity of alkaloidal salt (sulfate) was dissolved in 130 ml. of 5.7 *N* hydrochloric acid, and this solution was added to 42 Gm. of sodium silicate solution (technical grade) which had been diluted to 200 ml. with distilled water. After standing overnight, the entire solution formed a jelly which was dried under a current of air in a hood. The dried gel was triturated, and the material which passed through a No. 40 sieve was washed with methyl alcohol in a Soxhlet extractor for 48 hours, after which time all the alkaloid had been removed. The absence of any alkaloid could be demonstrated by the lack of rotation of plane polarized light and the lack of precipitation by alkaloidal reagents in samples of the wash liquor. Furthermore, small quantities of the gel were not charred when placed in a Bunsen flame.

The catalysts were prepared by the method of Hartung (9) using 100 mg. of palladous chloride per gram of gel and sodium acetate as a buffer. Hydrogenations were performed in absolute ethanol on a low-pressure Parr hydrogenation apparatus at about 60 p.s.i.g. using 1.0 Gm. of catalyst. Heating was accomplished using an infrared heat lamp.

These catalysts all possessed a low order of activity, requiring about 32 hours for the reduction of 0.05 mole of α-methylcinnamic acid. After 24 hours, an additional 1.0 Gm. of the catalyst was added and the hydrogenation continued for another 8 hours. No kinetic studies were attempted because of the abnormally long reduction period and the lack of an adequate temperature control.

Following hydrogenation, the catalyst was removed by filtration, and the alcohol evaporated at room temperature leaving an oily residue or crystals depending on the extent of reduction. The product was distilled *in vacuo* collecting the fraction boiling from 125–130°/0.5 mm. Hg (uncorrected).[1] The optical rotation was taken in benzene using a 1.0 dm. tube and a Kern Aarau polarimeter. Literature values for the boiling point and the specific rotation of dihydro-α-methylcinnamic acid were 160°/13 mm. Hg and 27.06° (benzene), respectively (10).

RESULTS

Silica gels prepared in the presence of quinine, quinidine, cinchonidine, and cinchonine produced stereospecific catalysts when used as carriers for palladium as shown in Table I. Within experimental error, palladized gels made in the presence of quinine and cinchonidine were twice as effective in producing an optically active product as those prepared using quinidine and cinchonine. Also, no enhancement or lowering of quinine and cinchonidine induced stereoselectivity was observed when equal mixtures by weight of quinine and cinchonidine, cinchonidine and cinchonine, quinine and cinchonine, or quinine and quinidine were used to prepare the gels.

The stereoselectivity of the gels does not appear to decrease significantly with age. So far, our evidence indicates a decrease in catalytic activity by the palladized 6-month-old gel. Further data must be obtained before definite statements can be made

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[1] The product was also identified by its neutralization equivalent.

TABLE I.—RESULTS OF THE HYDROGENATION EXPERIMENTS USING α-METHYLCINNAMIC ACID

Carriers	α-Methyl Cinnamic Acid Concn., %[a]	$[\alpha]_D^{25}$	Optical Yield, %[b]	Over-all Yield, %
QN 0.5 SG[c]	59.9	+0.87	3.21	81.1
QDN 0.5 SG[c]	44.1	+0.45	1.66	68.0
CN 0.5 SG[c]	63.2	+0.47	1.74	77.1
CDN 0.5 SG[c]	64.8	+0.88	3.25	87.9
CDN 0.25-CN 0.25 SG[d]	52.8	+0.86	3.18	70.9
QN 0.25-CN 0.25 SG[d]	57.6	+0.87	3.21	78.1
CDN 0.25-QN 0.25 SG[d]	55.5	+0.86	3.18	67.7
QDN 0.25-QN 0.25 SG[d]	65.5	+0.84	3.10	79.9
Plain SG	49.5	0.00	0.00	44.3
Aged QN SG (6 mo.)	39.5	+0.85	3.14	48.2

[a] Weight in volume in benzene. [b] Per cent optical yield is calculated by

$$\% \text{ optical yield} = \frac{[\alpha]_D^{25} \text{ (exptl.)} \times 100}{[\alpha]_D^{25} \text{ (lit.)}}$$

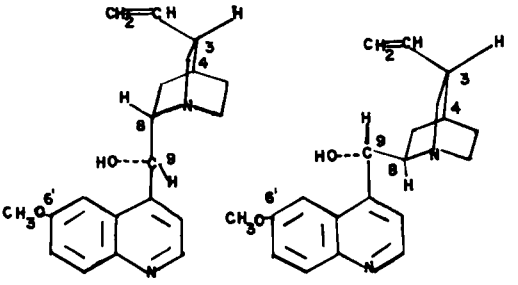
[c] Quinine silica gel which was prepared using 0.5 Gm. of quinine sulfate in the gel preparation. QDN = quinidine sulfate; CN = cinchonine sulfate; CDN = cinchonidine sulfate. [d] Silica gels prepared using mixtures of the individual alkaloids as indicated.

regarding the effects of age on the gel. Experiments are in progress to demonstrate the effects of alkaloid concentration on the stereoselectivity of the gels and will be reported at a later date.

It should be re-emphasized that no alkaloid was present during the hydrogenations of α-methylcinnamic acid, having been removed by the washing procedure. Evidently, the alkaloids have induced a change in the silica gel itself, making it stereoselective. Also, increased catalytic activity was noted in the palladized cinchona-treated gels over the palladized plain gel.

Only dextrorotatory product was obtained no matter which alkaloid was employed in the preparation of the gel. The difference noted was in the respective optical yields of the product. This might be explained by reference to the steric structures of the cinchona alkaloids as determined by Woodward (11) and given in Fig. 1. Note that quinine and quinidine are not antimers but diastereoisomers. They possess opposing configurations about carbon atoms eight and nine, but the configurations about carbons three and four are the same in the two alkaloids. Quinine and quinidine differ from cinchonidine and cinchonine by possessing a methoxy group at position 6′. Configurationally, cinchonidine corresponds with quinine and cinchonine with quinidine.

The experimental data indicate that the asymmetric inductive effects from quinine or cinchonidine might result from a potentiation of the asymmetric induction stemming from carbon atoms three and four by the asymmetric induction from carbons eight and nine. On the other hand, asymmetric induction from carbons eight and nine possibly opposes that from carbons three and four in the asymmetric inductive effects stemming from quinidine and cinchonine.



Quinine Quinidine

Fig. 1.—Steric structures of cinchona alkaloids.

SUMMARY

Stereoselective reduction of α-methylcinnamic acid was accomplished using palladium-on-silica gel catalysts. The silica gels prepared in the presence of cinchona alkaloids all produced dextrorotatory product. When used as carrier for palladium, the gels prepared in the presence of quinine and cinchonidine produced approximately twice the optical yields of (+)-dihydro-α-methylcinnamic acid as those prepared in the presence of quinidine or cinchonine.

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Phosphorus-Nitrogen Compounds I. Carboxy and Carbethoxy-Substituted Aryl Derivatives

By LINDLEY A. CATES* and TONY E. JONES

Since certain aromatic compounds in which carboxy and carbethoxy groups are situated *para* to an amino group possess medicinal value, a number of similar organophosphorus derivatives were prepared. The compounds that were synthesized include three phosphorodiamidic acids, two phosphoramidates, a phosphorodiamidic chloride, and a phosphenimidic amide.

PHOSPHORODIAMIDIC ACIDS are generally prepared by the hydrolysis of the corresponding phosphorodiamidic chlorides which can be derived from the reaction of either 2 or 4 moles of a primary or secondary amine with 1 mole of phosphorus oxychloride in an inert solvent[1] (1, 2). A modification of this procedure involves the interaction of a phosphorus oxychloride-pyridine complex with the amine in chloroform solution. This method was employed by Audrieth and Toy (3) for the preparation of *N*-substituted derivatives of phosphoryl triamides using a ratio of $POCl_3$:amine:C_6H_5N = 1:6:3. This scheme can also be used to prepare phosphorodiamidic chlorides in good yield. However, the poor solubility of some amines in chloroform, and of the $POCl_3$–C_6H_5N complex in solvents other than chloroform (*i.e.*, dioxane), restricts the applications of this method. Primary amines (2 moles) react smoothly with phosphoryl halides (1 mole) in inert solvents[1] to form the corresponding phosphoramidates in a manner analogous to the preparation of phosphorodiamidic acids (4).

Michaelis (2) prepared an ethyl phosphoramidate by reacting the chloro derivative with sodium ethoxide. The action of a nonaqueous alkaline reagent on a phosphorodiamidic chloride, however, results in dehydrohalogenation of the compound with the formation of a phosphenimidic amide (2, 5). Such a product results from the reaction of *N,N'* - di(*p* - carbethoxyphenyl)phosphorodiamidic acid with sodium ethoxide. Treatment of a carbethoxy-substituted phosphorodiamidic acid with an aqueous alkaline reagent under controlled conditions yields a hemiester.

It has been shown that some compounds containing a phosphorus-nitrogen bond have high solubilities in various solvents (3). This property is attributed to the pronounced hydrogen bonding tendencies displayed by the phosphoryl group. Since the solubilities of such compounds are remarkable even in substances of large molecular weight, some difficulty is encountered in their isolation and purification.

Several organophosphorus compounds containing carboxyl groups are reported as having high melting points, and in some cases no definite melting points are indicated (6, 7). These latter compounds apparently decompose over a range without having true melting points.

p-Aminobenzoic acid (PABA) and its esters have been extensively investigated for various therapeutic applications. The acid is considered to be an essential nutrilite for a number of microorganisms whose biological activity is dependent upon incorporation into the pteroylglutamic acid molecule. PABA has also been shown to be valuable in treating rheumatic fever and human rickettsial infections (8). It is also interesting that large amounts of PABA are reported to cause a striking lowering of the leucocyte counts in some cases of myelogenous leukemia (9). Alkyl esters of *p*-aminobenzoic acid are well known for their local anesthetic properties. Substitution of the amine nitrogen of *p*-aminobenzoates in certain cases (*i.e.*, tetracaine) has resulted in increased anesthetic potency; whereas similar alterations of simple esters often produce a loss of activity.

To ascertain the effect of phosphorus substitution on the physiological activity of PABA and its ethyl ester, certain organophosphorus compounds have been synthesized. Compounds containing the N—P link are quite stable at biological pH (10); however, a potentially free amino group is present in these substances since hydrolysis of this bond may be accomplished through the enzymatic influence of phosphamidases. This enzymatic hydrolysis constitutes the rationale for the selective cytotoxic activity of some alkylating antineoplastic agents (11).

EXPERIMENTAL[2]

N,N' - di(*p* - Carboxyphenyl)phosphorodiamidic Acid (I).—A mixture of 60 mmoles of *p*-aminobenzoic acid in 200 ml. of reagent dioxane and 15 mmoles of phosphorus oxychloride were stirred under reflux for 1 hour. The formed precipitate was separated from the cooled reaction mixture and stirred with dilute hydrochloric acid. The suspension was filtered, the residue washed with water, and recrystallized from boiling water to yield the white crystalline product.

N,N' - di(*p* - Carbethoxyphenyl)phosphorodiamidic Acid (II).—A solution of 50 mmoles of ethyl *p*-aminobenzoate in 150 moles of dry benzene was slowly added to 100 ml. of dry benzene containing 25 mmoles of phosphorus oxychloride. No reaction was apparent at 5°, but a precipitate began to form upon heating. The reaction mixture was refluxed for 4 hours and allowed to stand overnight. The reaction mixture was then taken to dryness with an air stream and steam bath. Recrystallization of the residue from hot, dilute ethanol yielded the white crystalline product.

N,N' - di(*p* - Carbethoxyphenyl)phosphorodiamidic Chloride (III).—A mixture of 100 mmoles of

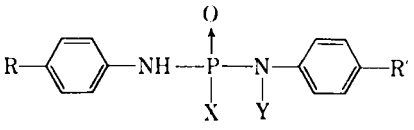
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[1] We found reagent dioxane to be a good solvent when *p*-aminobenzoic acid constitutes the amine in this reaction.

[2] See Tables I and II.

TABLE I.—PHOSPHORODIAMIDIC ACIDS, CHLORIDE, AND PHOSPHENIMIDIC AMIDE



No.	R	R′	X	Y	M.p., ° C.[a]	Analysis % C Calcd.	C Found	H Calcd.	H Found
I	COOH	COOH	OH	H	200 dec.	50.0	49.4	3.9	4.2
II	COOEt	COOEt	OH	H	200–202 dec.	55.1	55.0	5.4	5.2
III	COOEt	COOEt	Cl	H	172–173	56.5	57.0	5.3	5.8
IV	COOH	COOEt	OH	H	154–157	52.8	52.5	4.7	4.8
V	COOEt	COOEt	..	..	188–189	57.7	57.7	5.1	6.0

[a] All melting points are uncorrected.

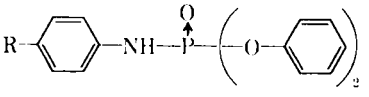
ethyl *p*-aminobenzoate, 17 mmoles of phosphorus oxychloride, and 50 mmoles of pyridine were reacted in reagent chloroform according to the method of Audrieth and Toy (3). The pink, waxy residue was washed with dilute hydrochloric acid and water until the washings gave a negative chloride test with silver nitrate T.S. Recrystallization of the residue from ethanol following Norit A treatment yielded the white crystalline product.

N - (*p* - Carboxyphenyl) - N′ - (*p* - carbethoxyphenyl)phosphorodiamidic Acid (IV).—A suspension of 1.3 mmoles of III in 50 ml. of 10% sodium hydroxide was stirred and heated at 70° for 15 minutes. The reaction mixture was filtered and the colorless filtrate acidified with concentrated hydrochloric acid to form, upon cooling, the white crystalline product.

N,N′ - di(*p* - Carbethoxyphenyl)phosphenimidic Amide (V).—A mixture of 2.7 mmoles of III in 25 moles of absolute ethanol and 20 mmoles of sodium metal previously reacted with 20 ml. of absolute ethanol was heated and stirred for 45 minutes. The formed suspension was added to 300 ml. of cold water, and the resulting solution was rendered neutral with concentrated hydrochloric acid. Cooling of this solution produced a white precipitate that was collected and suspended in 10% sodium carbonate solution. The suspension was heated and stirred for 15 minutes, filtered, and the residue washed with water until the washings were neutral. Recrystallization of the residue from dilute ethanol yielded the white crystalline product.

Diphenyl *p*-Carbethoxyphenylphosphoramidate (VI).—When 100 mmoles of ethyl *p*-aminobenzoate and 50 mmoles of diphenylphosphoryl chloride in 250 ml. of dry benzene were heated, a precipitate formed which disappeared at reflux temperature. The reaction mixture was allowed to reflux for 1 hour, and the clear solution evaporated to near dryness with a warm air stream. The residue was stirred with an excess of dilute hydrochloric acid to yield a pink waxy solid which was recrystallized from ethanol-dilute hydrochloric acid to yield the white product in the form of needles.

TABLE II.—PHOSPHORAMIDATES



No.	R	M.p., ° C.[a]	Analysis % P Calcd.	P Found
VI	COOEt	151–152	7.8	7.9
VII	COOH	194–195	8.4	8.2

[a] All melting points are uncorrected.

Diphenyl *p*-Carboxyphenylphosphoramidate (VII).—A mixture of 100 mmoles of *p*-aminobenzoic acid and 50 mmoles of diphenylphosphoryl chloride in 250 ml. of reagent dioxane was refluxed for 1 hour. The reaction mixture was cooled and the formed brown precipitate was collected. This material was boiled with water and filtered. The pink crystals that developed in the cooled filtrate were stirred with excess glacial acetic acid. The residue was washed with water and recrystallized from boiling water to yield the product in the form of white needles.

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REVIEWS

Tablet Making. Second Edition. By ARTHUR LITTLE and K. A. MITCHELL. The Northern Publishing Co., Ltd., 37 Victoria St.; Liverpool 1, England, 1963. 115 pp. 14 × 22 cm. Price 25/- net.

In terms of both unit and dollar volume tablets represent the most important of all dosage forms. Nevertheless, efforts to remove the art and provide a solid scientific base for the various processes making up tablet technology have met with considerable resistance. The second edition of this book, one of the few dealing with the subject, contributes little to the critical approach needed in this field.

Emphasis is placed upon the practical or vocational aspects of tablet making with brief discussions of mixing, drying, compressing, and coating. The chapter dealing with compressing equipment and mechanical trouble shooting should be useful to beginners. The authors statement that "the operation of the DryCota should present no serious problems to any tablet maker who remembers the principles of his trade (sic!) and considers logically the method of operation of the machine" is open to question.

Aside from a reference to a published bibliography on tableting, the only specific reference in the entire book is to a paper on film coating and there is no further discussion of this important development. Also missing is a discussion of the factors controlling intertablet weight and potency variation.

Particularly unfortunate is the verbal and photographic exclusivity afforded a single manufacturer of tableting and associated equipment. Under such circumstances the book approaches the appearance and concept of promotional literature. The definitive textbook on tablet technology remains to be written.

Reviewed by Jack Cooper
Ciba Pharmaceutical Company
Summit, New Jersey

Pediatric Therapy. Edited by HARRY C. SHIRKEY, B.S. (Pharm.), M.D. The C. V. Mosby Co., 3207 Washington Blvd., St. Louis 3, Missouri, 1964. 1144 pp. Price $16.50.

This book, although intended primarily for physicians specializing in pediatrics, will be of interest to pharmacists because of its over-all emphasis on therapy rather than diagnosis, and its sections on drug treatment and on poisoning and specific antidotes.

Part I, Drug Treatment, consists of twelve chapters on such subjects as "The Placebo—Its Clinical Significance," "Reactions to Drugs," "Principles in Choice of Drugs," and "Physical and Chemical Considerations in Choice of Drugs for Pediatric Use." There are eighteen additional parts concluding with a 48-page table of pediatric dosages.

The Editor, himself a distinguished practitioner and teacher, is to be commended for compiling chapters from 72 contributors into such a comprehensive, readable, and well-illustrated text.

Reviewed by George P. Provost
American Society of Hospital Pharmacists
Washington, D.C.

Accepted Dental Remedies 1964. American Dental Association, 222 E. Superior St., Chicago 11, Ill., 1963. xiv + 220 pp. 15 × 22.5 cm. Price $3. Paperbound.

The twenty-ninth edition of the widely recognized handbook of dental therapeutics is presented. Attention is given to recent reports of liver necrosis in a few individuals administered halothane for general anesthesia. The discoloration of teeth that may follow administration of some of the tetracyclines in late pregnancy, infancy, and early childhood is noted and precautions in providing closed chest cardiac massage are noted. The section on mouthwashes has been completely rewritten. Monographs are presented for drugs having recognized value in dentistry, drugs of uncertain status more recently proposed for dental uses, and some drugs once employed extensively but generally regarded as obsolete. Only brands which the ADA Council on Dental Therapeutics has accepted as having demonstrated value in dentistry are listed.

NOTICES

Kirk-Othmer Encyclopedia of Chemical Technology. 2nd Ed. Aluminum Compounds to Azo Dyes. Vol. 2. Executive Editor ANTHONY STANDEN. Interscience Publishers, Inc., 605 Third Ave., New York 16, N. Y., 1963. xv + 910 pp. 19 × 27 cm. Price: $45 single copy, $35 subscription.

Psychopharmacology Handbook, Vol. II, Animal Research in Psychopharmacology, Central Nervous System Effects. Edited and compiled by the Psychopharmacology Service Center, Research Grants and Fellowships Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland. Available from Superintendent of Documents, U. S. Govt. Printing Office, Washington, D. C. 20402. xvi + 529 pp. 20 × 26 cm. Price $3. Paperbound.

Handbook of Cosmetic Science. An Introduction to Principles and Applications. Edited by H. W. HIBBOT. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y., 1963. xi + 556 pp. 15.5 × 23 cm.

Chemical Constitution and Biological Activity. 3rd Ed. By W. A. SEXTON. D. Van Nostrand Co., Inc., 120 Alexander St., Princeton, N. J., 1963. xxviii + 517 pp. 16 × 25 cm. Price $19.75.

Experimental Pharmacogenetics. By HANS MEIER. Academic Press Inc., 111 Fifth Ave., New York 3, N. Y., 1963. xi + 213 pp. 15.5 × 23.5 cm. Price $7.50.

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NOTICES

Kirk-Othmer Encyclopedia of Chemical Technology. 2nd Ed. Aluminum Compounds to Azo Dyes. Vol. 2. Executive Editor ANTHONY STANDEN. Interscience Publishers, Inc., 605 Third Ave., New York 16, N. Y., 1963. xv + 910 pp. 19 × 27 cm. Price: $45 single copy, $35 subscription.

Psychopharmacology Handbook, Vol. II, Animal Research in Psychopharmacology, Central Nervous System Effects. Edited and compiled by the Psychopharmacology Service Center, Research Grants and Fellowships Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland. Available from Superintendent of Documents, U. S. Govt. Printing Office, Washington, D. C. 20402. xvi + 529 pp. 20 × 26 cm. Price $3. Paperbound.

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REVIEWS

Tablet Making. Second Edition. By ARTHUR LITTLE and K. A. MITCHELL. The Northern Publishing Co., Ltd., 37 Victoria St.; Liverpool 1, England, 1963. 115 pp. 14 × 22 cm. Price 25/- net.

In terms of both unit and dollar volume tablets represent the most important of all dosage forms. Nevertheless, efforts to remove the art and provide a solid scientific base for the various processes making up tablet technology have met with considerable resistance. The second edition of this book, one of the few dealing with the subject, contributes little to the critical approach needed in this field.

Emphasis is placed upon the practical or vocational aspects of tablet making with brief discussions of mixing, drying, compressing, and coating. The chapter dealing with compressing equipment and mechanical trouble shooting should be useful to beginners. The authors statement that "the operation of the DryCota should present no serious problems to any tablet maker who remembers the principles of his trade (sic!) and considers logically the method of operation of the machine" is open to question.

Aside from a reference to a published bibliography on tableting, the only specific reference in the entire book is to a paper on film coating and there is no further discussion of this important development. Also missing is a discussion of the factors controlling intertablet weight and potency variation.

Particularly unfortunate is the verbal and photographic exclusivity afforded a single manufacturer of tableting and associated equipment. Under such circumstances the book approaches the appearance and concept of promotional literature. The definitive textbook on tablet technology remains to be written.

Reviewed by Jack Cooper
Ciba Pharmaceutical Company
Summit, New Jersey

Pediatric Therapy. Edited by HARRY C. SHIRKEY, B.S. (Pharm.), M.D. The C. V. Mosby Co., 3207 Washington Blvd., St. Louis 3, Missouri, 1964. 1144 pp. Price $16.50.

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Part I, Drug Treatment, consists of twelve chapters on such subjects as "The Placebo—Its Clinical Significance," "Reactions to Drugs," "Principles in Choice of Drugs," and "Physical and Chemical Considerations in Choice of Drugs for Pediatric Use." There are eighteen additional parts concluding with a 48-page table of pediatric dosages.

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Chemistry in the Service of Medicine. Edited by F. N. L. POYNTER. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1963. vii + 207 pp. 14 × 22 cm. Price $5.

Henderson's Dictionary of Biological Terms. 8th Ed. By JOHN H. KENNETH. D. Van Nostrand Co., Inc., 120 Alexander St., Princeton, N. J., 1963. xv + 640 pp. 15 × 23 cm. Price $12.50.

Precis de Therapeutique et de Pharmacologie. Supplement (1963) a la Neuvieme Edition. By RENE HAZARD. Masson et Cie, Editeurs, Libraries de L'Academie de Medecine, 120, Boulevard Saint-Germain, Paris, France, 1963. 158 pp. 13.5 × 20 cm. Paperbound. Price 10 F.

Index Psychopharmacorum. By WALTER POLDINGER and PAUL SCHMIDLIN. Verlag Hans Huber Bern und Stuttgart, Marktgasse 9, Berne, Switzerland, 1963. 69 pp. 15 × 23 cm. Price DM 14.80.

Grundriss der galenischen Pharmazie. By ERIK SANDELL. Govi-Verlag, Pharmazeutischer Verlag, 6 Frankfurt am Main 7, Germany, 1962. 392 pp. 15.5 × 23 cm. Price DM 52.50.

Grundlagen der Arzneimittelforschung un der synthetischen Arzneimittel. By JAKOB BUCHI. Birkhauser Verlag, Basel 10, Switzerland, 1963. 744 pp. 17 × 25 cm. Price Fr. 96.

Progress in Drug Research. Vol. 5. Edited by ERNST JUCKER. Birkhauser Verlag, Basel 10, Switzerland, 1963. 654 pp. 17 × 25 cm.

Travaux des Laboratoires de Matiere Medicale et de Pharmacie Galenique de la Faculte de Pharmacie de Paris. By M. M. JANOT and R. PARIS. Vigot Freres, Editeurs, 23 Place de L'Ecole-de-Medecine, Paris, France, 1963. 14.5 × 23 cm. Paperbound.

Cosmetic Science 1962. Edited by A. W. MIDDLETON. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y., 1963. xv + 270 pp. 15.5 x 23 cm. Price $12.50. (Contains papers presented at the Second Congress of the International Federation of Societies of Cosmetic Chemists.)

Elsevier's Medical Dictionary. Compiled by A. SLIOSBERG, M.D. American Elsevier Publishing Company, Inc., 52 Vanderbilt Ave., New York 17, N. Y., 1964. 1588 pp. 15 × 23 cm. Price $45. (A compilation of medical terms and their foreign language equivalents in French, Italian, Spanish, and German.)

First International Pharmacological Meeting. Bradykinin and Vasodilating Polypeptides. Vol. 9. Part 1, Edited by M. R. E. SILVA, and U. S. VON EULER. Part 2, *Pharmacology of the Lung.* Edited by D. M. AVIADO. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y., 1963. viii + 193 pp. 15.5 x 23 cm. Price $15.

Perception and the Conditioned Reflex. By YE. N. SOKOLOV. Pergamon Press, Ltd., Headington Hill Hall, Oxford, England, 1963. ix + 309 pp. 15 x 23 cm.

Kremers and Urdang's History of Pharmacy. 3rd Ed. Revised by GLENN SONNEDECKER. J. B. Lippincott Company, East Washington Square, Philadelphia 5, Pa., 1963. xii + 464 pp. 18 x 26 cm. Price $9.50.

Methoden Der Organischen Chemie (Houben-Weyl). Vierte, Vollig Neu Gestaltete Auflage. Band VI/2 Sauerstoff-Verbindungen 1, Teil 2. Georg Thieme Verlag, Herdweg 63, 7000 Stuttgart 1, Germany, 1963. U. S. Agent: Intercontinental Medical Book Corp., New York 16, N. Y. xlviii + 952 pp. 17 × 26 cm. Price DM 220. —(Moleskin) DM 198.—(Vorbestellpreis).

Pharmazeutisches Jahrbuch 1958. Edited by WOLFGANG EBERHARDT and BERNHARD JACKOWSKI. Govi - Verlag G.m.b.H., Pharmazeutischer Verlag, Frankfurt am Main, Germany, 1963. xii + 549 pp. 17 × 24 cm.

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The Rat Brain. By JOACHIM F. R. KONIG and RENATE A. KLIPPEL. The Williams and Wilkins Company, Baltimore 2, Md., 1963. 162 pp. 21.5 × 28.5 cm. Price $15.

Readings in Pharmacology. Edited by B. HOLMSTEDT and G. LILJESTRAND. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y., 1963. x + 395 pp. 15.5 × 23.5 cm. Price $7.50.

The Sebaceous Glands (Advances in Biology of Skin, Vol. 4). Edited by W. MONTAGNA, R. A. ELLIS, and A. F. SILVER. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y., 1963. xii + 260 pp. 15 × 23 cm. Price $12.50.

Pharmacology of the Carotid Body Chemoreceptors. By S. V. ANICHKOV and M. L. BELEN'KII. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y., 1963. xiv + 225 pp. 14 × 22 cm. Price $8.50.

Lehrbuch der Allgemeinen Pharmakognosie. By E. STEINEGGER and R. HANSEL. Springer-Verlag, 1 Berlin 31 (Wilmersdorf), Heidelberger Platz 3/(Berlin-West), Germany. 595 pp. 17 × 25 cm. Price DM 69.

Contribution a L'Etude de L'Analyse, du Metabolisme et de la Biogenese des Alcaloides et aes Amines Pyrocatechiques du Genet a Balai. By FRANCOIS JAMINET. Editions Arscia S. A., 60, rue de l'Etuve, Bruxelles I, Belgium, 1963. 260 pp. 16 × 24 cm. Paperbound.

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JANUARY TO DECEMBER, 1964

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Review Article

Pharmaceutical Sciences—1963. Part I

A Literature Review

By WAYNE McKEEHAN and LEWIS C. LAPPAS

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[Contents for Part II will accompany the concluding part of this Review Article next month.]

THIS SURVEY of the literature pertaining to pharmaceutical sciences is the first sequel to the 1962 review published in THIS JOURNAL (1, 2). In maintaining continuity, more than 100 periodicals and *Chemical Abstracts'* Pharmaceuticals and Pharmacodynamics sections published during 1963 were searched for appropriate material. Those areas which are reviewed annually in other publications were omitted from this review. Although not all-inclusive, this comprehensive report is directed toward presenting a compilation of the current developments in several areas of pharmaceutical science.

For future reference, two publications are reported which should aid the pharmaceutical scientist in surveying the literature. The initiation of a pharmaceutical abstracting journal was announced (3), and a list of the world's pharmacy periodicals was compiled (4).

GENERAL PHARMACY

This broad category encompasses those parts of the literature which are difficult to classify specifically. During 1963 considerable attention was focused on regulations for investigational drugs imposed by the Food and Drug Administration. As evidence, many publications were concerned with clarification of these regulations in light of the pharmaceutical industry's role (5–9). Kern emphasized that many requirements are in a continual state of change, thus making it impossible to be in full compliance with all of the regulations at all times (10). The adequacy of proposed clinical trials was discussed in two articles (11, 12). Palmer described the types of studies which may be used in testing the effectiveness of new drugs (13). Schreiner presented the liabilities of the clinical investigator in new drug testing (14), and the need for certain additional safeguards in the testing and handling of investigational drugs was discussed by Archambault (15). Detailed methods of drug evaluation in hospital pharmacy were reported (16). In a discourse on the hazards of new drugs, the scientific approach was advocated as being the safest and most effective (17).

Various aspects of ethical drug market research were presented by Hull (18), and the economics of pharmaceutical research in the United States was reviewed by Arnow (19). The complex and intricate steps essential to a good quality control system for the manufacture of drugs were described (20). A statistical approach to the design and analysis of experiments was disclosed (21). Shindell examined statistical probability (22) and the validity of conclusions (23). Possible errors in application of the Student *t* test in simple comparison studies were pointed out (24). King constructed a multiple-stage design for drug screening (25). Numerous references were presented in a survey of various types of designs which are useful in drug screening (26). Another investigator explored the use of the digital computer and its programming for the handling of complex data (27).

Preservatives.—A detailed review of preservatives in pharmacy, with 103 references, was presented by Nogueira (28). Parafinska and Zwolinska also presented an article, with many references, concerning the use of bacteriostatic and bactericidal agents in pharmacy (29). An extensive discussion of the properties and uses of a variety of antimicrobial agents in cosmetic preparations was also reported (30). In another publication, various preservatives in hydrogels, hydrosols, emulsions, and suspensions were studied (31). The adsorption of sorbic acid by plastic cellulose acetates was investigated (32).

Sixteen different 8-hydroxyquinoline salts were prepared and found to have strong fungistatic activity (33). The germ-inhibiting action of some 8-hydroxyquinoline salts was also examined (34). Hexachlorophene and its isomers were evaluated against a broad spectrum of bacteria, molds, and yeasts by Gump and Walter (35), who observed that maximum results occurred in products with bisphenols at pH 5.4 (36). Pyrocarbonic acid diethyl ester was investigated as a potential food preservative (37). Some other preservatives, effective *in vitro* against various bacteria and fungi, were studied by Gaind, *et al.* (38), and Judis conducted research on the mechanism of action of phenolic disinfectants (39). Myers and Sheahan demonstrated an adverse effect of calcium alginate on the antimicrobial activity of benzalkonium chloride (40). Another worker evaluated various antibacterial substances for enhancement of the bactericidal effect of steam (41).

The disappearance of preservative activity due to degradation in solution and absorption by rubber stoppers in multiple-dose vials was investigated (42, 43). Benzethonium chloride showed some antifungal activity but little antibacterial activity when tested as a preservative for poliomyelitis (Salk) vaccine (44). Kohn, *et al.*, developed methods of testing antibacterial effectiveness and evaluated 51 chemical substances for use in ophthalmic solutions against 13 different strains of *Pseudomonas aeruginosa* (45, 46).

Received from the Pharmaceutical Research Department, The Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

Data support the view that small amounts of certain perfume oils might be used effectively in the control of some dermatophytes (47). Fletcher and Norton studied the antioxidant effect of sodium metabisulfite in sulfacetamide eye drops (48). Schroeter found that many aqueous, oxygen-labile, pharmaceutical systems may be protected by the antioxidant action of sulfurous acid salts (49). Oxidative deterioration of cosmetics and its control have been discussed (50). Hydroquinone served as an effective antioxidant in the preservation of lard (51).

Flavor, Aroma, and Color.—Wesley surveyed recent trends in pharmaceutical flavors (52), and Moncrieff discussed the influence of taste and odor on flavor (53). A paper on the materials and factors which affect flavors was published (54). Production applications and stability tests were described for solid citrus flavors (55). Runti classified various synthetic sweetening compounds which have actual or potential application (56), and Brookes compared the sweetening effect of sodium cyclamate with that of sucrose and saccharin sodium in various preparations (57). Development of a stable, flavored, glyceryl guaiacolate cough syrup was reported (58).

The economic potential and future of the fragrance industry were analyzed by Vautin (59). In a discussion of odor mechanism theories, Moncrieff stated that evidence favoring the adsorption process is overwhelming (60). The scents of the fragrant flowers and leaves of the milkweed (61), the camellia (62), and the magnolia (63) families were reported in three manuscripts.

In a review on the psychophysical concept of color, several standard techniques for evaluating small color differences were presented (64). The importance of color and its psychological effect on the human appetite were studied by Birren (65). A current guide to color restrictions was presented (66); 47 references were cited in a review of natural dyes (67).

Adjuvants.—The chemical and physical characteristics and applications of several montmorillonite hydrocolloids were summarized by Barr (68). In a comparative study, two highly dispersed colloidal silicic acids were evaluated as adjuvants for pharmaceutical preparations (69). Molecular properties of Ghatti gum, a naturally occurring polyelectrolyte from *Anogeissus latifolia*, were investigated by Elworthy and George (70), and Faust discussed the properties and uses of modified starches in topical preparations (71).

Prescott, *et al.*, considered the chemical and pharmaceutical properties of poly(vinylpyrrolidone) from a cosmetic viewpoint (72, 73). Independent reports appeared on the cosmetic application of acetoglycerides (74) and various cellulose ethers (75). Five anionic certified dyes were adsorbed on various starches in accord with the Langmuir equation (76). Mandak used various condensation products of ethylene oxide to enhance the formulations of galenic preparations (77), and Ullmann demonstrated increased activity of several antibiotics with the aid of nonionic poly(oxyethylene) adducts (78).

The properties and applications of certain specialty polyols have been discussed (79). Hexadecyl alcohol, a new material for cosmetic formulations, was found to be essentially nontoxic and nonirritating (80). Allen reviewed the applications of some fatty alcohols and saturated fatty acids in pharmacy and cosmetics (81, 82). The alcoholic constituents of some modified wool fats were investigated (83).

Brown, *et al.*, suggested dimethyl sulfoxide as a useful solvent for chemical compounds under toxicological evaluation (84). Many references were cited by Spiegel and Noseworthy in a review of toxicity, chemical and physical properties, and applications of some nonaqueous solvents for parenteral products (85). The distribution of phenol in mixtures of water and organic solutions was explored in the presence of polysorbate 20 (86).

Stability.—Garrett reviewed the physicochemical and statistical procedures used to study the stability of drugs in solution (87). Statistical analysis was applied to short-term stability data for prediction of shelf life of pharmaceutical suspensions (88). In another paper, several factors to be considered in an accelerated stability-testing program were suggested (89). Awe and Kienert evaluated the effect of various oxidizing agents on phenylbutazones (90). A review of the literature concerning oil and fat oxidation and antioxidation was made by Froelich (91).

Nogami and associates, in a series of papers on drug stability in solution, studied the stabilization of methantheline bromide and choline derivatives (92) and investigated the hydrolysis of several sodium alkyl sulfates (93, 94). Other workers examined the influence of the state of aggregation on the specific acid-catalyzed hydrolysis of sodium dodecyl sulfate (95). Equilibrium moisture contents for a number of representative pharmaceutical materials were determined by Scott, *et al.*, who suggested that

such data are useful in predicting the effects of storage humidity on moisture sorption (96). Experiments for testing the stability of aspirin mixtures were described (97). In another paper, stability studies of aspirin-dried aluminum hydroxide gel tablets were reviewed (98). Guven observed an incompatibility of quinine sulfate or hydrochloride with acetylsalicylic or salicylic acids in tablets and powders (99).

An investigation was conducted on the effects of various granulating processes in decomposition of primary digitalis glycosides (100). Schou examined reactivity in various solid pharmaceuticals and showed that copper contamination from equipment and materials may be important in the catalysis of auto-oxidation (101). The effect of ball-milling on the stability of vateritic calcium carbonate was investigated (102). Everhard and Goodhart demonstrated that fading of FD&C Red No. 3 in tablets follows first-order rate kinetics (103). *In vitro* data have been presented which might explain clinically inactive prednisone tablets U.S.P. XVI (104). A brown discoloration occurring in some pharmaceutical granules was attributed to the reaction between glucose and sodium dihydroxyaluminum carbonate (105). Burkman ascribed the loss of biological activity of apomorphine to auto-oxidation (106). Changes in alkaloid content of ergot (107) and volatile oil content of nine drugs (108) during storage were studied.

From stability data on basic ethers of the diphenhydramine type in acid medium, it was predicted that no noticeable decomposition would occur in the stomach after oral administration (109). The hydrolysis of succinic and tetramethyl succinic anhydrides in water, dilute acids, and aqueous electrolytes was disclosed by Bunton, *et al.* (110). Heparin solutions were stabilized by the addition of 0.1% of sodium metabisulfite and adjustment of the pH to 4–5 (111). Optimum pH values for stability of aqueous physostigmine (112) and fructose solutions (113) were 2 to 4.5 and 4.0, respectively.

The interaction of papaverine hydrochloride with numerous alkaline substances in aqueous solution was investigated by Ryabukhina (114). Stainier, *et al.*, concluded that stability of barbiturates in the presence of sodium hydroxide is inversely related to the number of methyl groups (115). In another monograph, stability of acetazolamide and methazolamide in alkaline solution was evaluated (116). Other researchers found that aminoethylnitrate and some related compounds decompose *via* an imine mechanism down to pH 4.0 (117) and that radioactive 3-acetoxymercuri-2-methoxy-propylurea was stable for 60 days in dilute basic solution (118).

Preliminary data indicated that discoloration of 30% sodium sulfacetamide solutions was eliminated by addition of 0.01% sodium edetate or 0.1% sodium thiosulfate (119). A note was presented on the stability of pilocarpine hydrochloride ophthalmic solutions in two types of containers (120). Brownley and Lachman examined the influence of lactose on the stability of certified dyes in aqueous solution (121). Removal of iodine from aqueous solutions of hydriodic acid was described by Davidson and Jameson (122). Procedure modification enhanced the stability of sweet spirit of nitre (123). In aqueous solution, the rate of decomposition of calcium acetylsalicylate was claimed to be temperature dependent (124). Mitchell studied the hydrolysis of propyl benzoate in aqueous solutions of a nonionic surfactant (125). Blazek presented a review, with 30 references, of the chromatography and stability of some phenothiazine derivatives (126).

The controlled hydrolysis of some antibiotics and their decomposition products in the presence of cation-exchange resins was effected without complete degradation (127). In mixtures, dibazol was found to decompose in the presence of oxygen and moisture after long-term storage (128). Nakatani examined the stability of orotic acid and its amine salts in aqueous solution (129), and Sano, *et al.*, studied the stability of *d*-glucuronamide in the presence of various compounds (130). It has been suggested that polysorbate 80, a nonionic surfactant, might have considerable influence on the stability and availability of ionic drugs in pharmaceutical formulations (131). Forty-nine references were cited in a review of the stability and stabilization of streptomycin (132).

Photodecomposition of acid-aqueous thioproperazine 5-oxide occurred by irradiation with fluorescent light under anaerobic conditions (133). Kitahara and Tanaka studied the behavior of unsaturated fatty acids and liquid organic compounds in the gamma-ray field (134). Experimental work by Luduena, *et al.*, suggested the formation of adrenaline in aqueous solutions of phenylephrine after ultraviolet irradiation (135). The effects of ionizing radiation on two gelatin fractions were reported (136). Fenn and Belcastro examined the effect of ultrasonic waves on the stability of selected surface-active agents, sulfonamides, and *p*-aminobenzoic acid (137).

Stability Kinetics.—Conine demonstrated the application of stability data to the prediction

of the shelf life of pharmaceutical products (138). Applications and precautions in using the Arrhenius plot and statistical procedures for accelerated stability testing of pharmaceutical products were considered (139). Guttman discussed the use of kinetic principles and reaction mechanisms in studying drug stability (140). Reaction kinetics were stressed in a survey of the stability of drug solutions (141). A general method for determining the order of a reaction was reported (142). Rogers disclosed an accelerated storage test with programmed temperature rise (143), and Barker applied the Arrhenius equation to calculate the shelf life of atropine eye drops (144). The decomposition of chloral hydrate in the presence of phenobarbital sodium was reported to follow the kinetics of bimolecular reactions (145).

DeLuca discussed the photobinding and photoreactivity of riboflavin in the presence of macromolecules (146). The stabilization of vitamin B_{12} in liquid multiple-vitamin products was demonstrated by Conine and Zuck by the addition of alphahydroxynitriles or their esters (147). These investigators also found potassium ferrocyanide to be superior to iron salts for stabilization of vitamin B_{12} in multiple-vitamin preparations (148). Several compounds were evaluated for their effect on the rate of thiamine hydrolysis (149), and kinetic data were used in predicting the shelf life of vitamins B_1 and C in oral liquid formulations (150). A pH-rate profile of the anaerobic degradation of ascorbic acid in aqueous solution was published (151). The stability of ascorbic acid was also studied in a liquid multivitamin emulsion containing sodium fluoride (152).

Research on the solid-state decarboxylation of *p*-aminosalicylic acid was conducted under various conditions (153). Schroeter presented experimental data to corroborate the Abel theoretical equations and radical mechanism for sulfite oxidation (154). Tanaka, *et al.*, reported on kinetics of color changes of fradiomycin sulfate (155). Kinetic investigations were published on the hydrolysis of thioacetazone and its related compounds (156), trimethadione (157), some tropine esters (158), and some carbamate and carbonate esters (159). The inactivation of phenoxymethylpenicillin V and benzylpenicillin G in aqueous solutions above pH 7.0 was explored by Rozenberg (160). Inactivation rates of aqueous, buffered sodium penicillin G were investigated by Ullmann, *et al.*, in the presence of ionic surfactants, organic gel formers, and preservatives (161).

Vitamin Stability.—Vitamin degradation in liquid multivitamin preparations was accelerated by the routine of daily dosage-removal (162). Various antioxidants were evaluated for their stabilizing action of vitamin A in ethyl oleate solutions (163). Nordfeldt and Olsson determined the stability of vitamin A and tocopherol in a mixture of fish liver-wheat germ oils (164), and Christensen, *et al.*, discussed the influence of pH on the stability of vitamin B parenterals (165). The influence of sterilizing temperature on the decomposition rate of vitamin B_1 injectable solutions was also reported (166).

Microbiological analysis revealed two new compounds from the photochemical degradation of riboflavin; the major product was identified (167). A comparative study of gamma-ray decomposition and photodecomposition of aqueous solutions of riboflavin was conducted by Takahashi and Yamamoto (168). Results of a spectrophotometric analysis showed that vitamin B_{12} decomposition varied from 2.9% in presence of maltose to 23.5% in the presence of glucose (169). Thermostability and the moisture content of vitamin B_{12} preparations were examined by Bayer and Liptay (170). Degradation of vitamin B_{12} solutions by ionizing radiation was also reported (171).

Color changes of aqueous solutions of L-ascorbic acid during decomposition were investigated (172), and rubeanic acid was proposed as an effective stabilizer for aqueous solutions of L-ascorbic acid (173). In another study of vitamin C stability, ethylenediaminetetraacetic acid significantly stabilized an ascorbic acid syrup (174). Nogueira, *et al.*, reported that ampuls of sodium ascorbate of the Portuguese Pharmacopeia did not decompose on exposure to visible or ultraviolet light (175). Zwolinska reported that cysteine, propylene glycol, or disodium ethylenediaminetetraacetic acid improved the stability of vitamin C solution at pH 4.85 to 4.95 (176). The stability of ascorbic acid in various fruit juice vehicles was evaluated at elevated temperatures (177). Different decomposition products of rutin trisulfate were found at pH values of 3.2 and 8.0 (178). A variety of products was obtained by the oxidation of vitamin E with alkaline ferricyanide (179).

PHARMACEUTICAL TECHNOLOGY

Chalabala, *et al.*, surveyed 919 references in reporting progress (1959–1961) in the field of galenical preparations (180). The importance of galenic research and its implications in modern pharmaceutical techniques were discussed in another paper (181). Macek reviewed some of

the important physical and chemical factors in new product development and emphasized the magnitude of studies involved in new dosage forms (182). Five hundred and twenty-one references were cited in a comprehensive review of the use of sorbitol in medicine (183). Some aspects of controlled gelling and the use of thickening products were also reviewed (184). Magid summarized the pharmaceutical aspects of dextromethorphan hydrobromide (185).

A comparative evaluation was made of the preparation of tinctures by different methods (186). Ryabukhina studied the solubility of several alkaloids for the formulation of medicinal mixtures (187). Knowledge of dissociation and solubility constants was found useful to predict the salting-out incompatibility of certain pharmaceutical agents (188). Mirigian, *et al.*, formulated a potassium-ion replacement elixir (189); Letassy and Huyck modified the phenobarbital elixir U.S.P. XVI formula to produce a clear product without the time-consuming filtration procedure (190). Current knowledge of 23 commonly used Russian vitamin preparations was summarized (191).

Lach presented a theoretical dissertation on buffers, stressing the importance of these agents in pharmacy (192). A simple apparatus for rapid, aseptic filtration of eye drops was described in another publication (193). Wiegrebe discussed methods for calculating isotonic collyria and isohydric solutions—a table for a borate-acetate buffer system was included (194). The formulation and production of ophthalmic preparations have been reviewed (195).

A general review of the various types of sunscreens was published (196). Walker described the effects of organic sulfur hair tonic formulations (197); Barr discussed antacid properties and possible contaminants of various magnesium-containing substances (198). In a lengthy review of modern cough mixtures, composition and economic impact were examined (199). The effectiveness of keratinase as a depilatory was reported to be dependent upon its purity and the pH of the finished product (200).

The technique, theory, and applications of fluidization to pharmaceutical manufacturing were summarized (201). Hess and Lang described the preparation of granulations by spray-congealing (202). Other publications were found on factors affecting the uniformity of mixing (203) and theoretical and practical concepts of mixing solids (204). Particle size and tablet composition were shown to influence the antimicrobial effect of 5,7-dibromo-8-quinolinol *in vitro* (205). A comparative study of various U.S.S.R. diatomites as filtration material in the production of insulin was presented (206).

Parenterals.—Horsch reviewed the technology of injectables and eye drops in the German Democratic Republic (207). A survey of the literature concerning the use of demineralized water as a solvent for injectables was published (208). Gubitz considered the substitution of demineralized water for distilled water in pharmaceutical formulations (209). Methods for calculating isotonic drug concentrations were treated in two publications (210, 211); the latter contained a table of sodium chloride equivalents. Advantages of the hematocrit method over the hemolytic method in testing isotonicity of injectable solutions were presented (212).

Riffkin commented on potential incompatibilities due to mixing parenteral products (213). A procedure was recommended for the preparation of invert sugar solutions for intravenous use (214). Special precautions were necessary in the preparations of glyphylline injections to prevent extraction of silicon and borate from glass (215). Ilver and Jochumsen advocated more rigid testing for commercial nicotinamide used in injectable solutions (216). These investigators also proposed a formulation for decreasing the turbidity of nicotinic acid injections (217).

Macek illustrated some of the problems encountered in the manufacture and stability of parenteral dispersions (218). Aqueous suspensions of deoxycorticosterone trimethylacetate were prepared for injection (219). Revol commented on the pharmacology, accidents, contraindications, and precautions concerned with parenteral administration of lipid emulsions in one paper (220) and reported their preparation, composition, and stability in another (221). Sodium *p*-aminosalicylate solutions could be stabilized at pH 8 with disodium Versenate for sterilization and storage (222). Friebel and Patel attributed loss of action of local anesthetic drugs in glucose solution to the formation of glucosides (223). Studies were made on precipitation in injectable oily solutions due to temperature and impurities (224). Moldovan observed that carbonate and bicarbonate ions form precipitates of calcium carbonate due to the presence of calcium ion in glass containers (225). Several other publications dealt with preservation, stability, and preparation of parenteral products of adrenaline (226), helveticoside (227), morphine (228), procaine (229), and some sulfonamides (230).

Piersma discussed various aspects of numerous

biological products in the drug industry (231), and Gershenfeld reviewed the progress of recent developments in biological products and the importance of maintaining immunity against disease (232–234). The relative merits and disadvantages were compared for living and killed vaccines (235). Another publication was concerned with the principles of chemical preparation of formaldehyde-treated virus vaccines (236). The effect of pH on thermal stabilization of oral polio virus vaccine by magnesium chloride was investigated by Melnick and Wallis (237).

Sterility.—A general review of sterilization, disinfection, preservation, and terminology was published (238). Additional reviews on sterilization techniques (239) and pyrogens (240) were noted. Mathews reviewed the principles, sampling, and methodology of sterility testing of biologicals for the period 1957–1962 (241).

Various methods to obtain positive clarification and sterile filtration for pharmaceutical production were given by Stapowick (242). An apparatus for sterilizing heat-labile solutions (243) and a method for aseptic dialysis (244) were also presented. Using an evacuated bottle as the driving force, Minsley developed a simple "closed system" filtration process (245). Buffered glutaraldehyde, a new chemical sterilizing solution, did not have the undesirable properties of formaldehyde (246). The properties and applications of ethylene oxide in sterilization processes have been investigated (247, 248).

A filtration method was developed by Meinhard for the sterilization of urea solutions for intravenous use (249). Glucose degradation during sterilization in acid and alkaline media was discussed (250). Physical characteristics, method of preparation, and procedure for control were described for sterile mannitol solutions (251). Colchicine for injection was prepared by Smith, *et al.*, using sterilization by heat or filtration (252). Peroxides are decomposed by antimicrobial treatment by the Swiss Pharmacopeia VI method for oils used in parenteral injections (253).

Tablets.—Tablet manufacturing, including formulations and varieties of compressed tablets, was studied by Heinemann (254) and Burlinson (255). Two investigations were published concerning the instrumentation of tablet equipment for studying various forces operating during the compression cycle (256, 257). Windheuser, *et al.*, explored this problem with respect to pressure transmitted to the die wall during compression (258). Another paper was found which considered pressure distribution in tableting (259). In a preliminary report, Marshall described the application of electrical resistance measurements for investigating tablet compression (260). The advantages of direct compression of tablets (261) and a critical review of existing test methods, with description and proposal of improved test methods (262), were presented.

Engelbrecht presented a series of articles on the problems involved in the compression of granular aggregates and discussed the dynamics and "binding energy" of the compressed aggregates (263–265). Surveillance of the scientific advances and other areas in pharmaceutical research was made by Cooper (266). Successful application of dry granulations was reported for tablet manufacturing (267). Fluidized bed drying of tablet granulations was found to be 15 times faster than tray-drying procedures (268). Gunsel and Lachman presented a comparative evaluation of tablet formulations prepared from conventionally processed and spray-dried lactose (269). Severe powder-sticking to tablet punch surfaces during the production of an effervescent tablet was eliminated by punches tipped with poly(tetrafluoroethylene) (270).

A review of various lubricating agents in tableting was published (271), and methods for evaluating lubricants and tablet friability were established (272). Tawashi investigated the use of colloidal silica for enhancement of the flow properties of powder mixtures (273). The applications of microcrystalline cellulose as a filler, binder, disintegrating agent, and lubricant in tableting were discussed (274). Poly(vinylpyrrolidone) (275) and sodium carboxymethylcellulose gels (276) were evaluated as binding agents in tablets. Chewable vitamins (277) and the use of mannitol in such formulations (278) were discussed in separate papers.

The Pfizer hardness tester was evaluated with eight tablet formulations and found satisfactory (279). New equipment for testing tablet disintegration time has been developed (280, 281). Levy, *et al.*, studied the effect of granule size, starch concentration, and compression pressure on the dissolution rate of tablets (282). The influence of tablet lubricants and the mechanisms by which they modify the dissolution rates of pharmaceuticals in tablets were also examined (283). Nogami, *et al.*, conducted research on the physical properties of tablets; Washburn's equation was applicable for studying penetrating rate in tablet disintegration (284). Powdered corncobs, cellulose, and several starches and gums were evaluated as potential tablet disintegrating agents (285, 286). The disintegrating properties of kaolin in tablet formulations have been at-

tributed to their negative charge in the presence of moisture (287).

Muenzel cited 69 references in a review of the recent contributions to pharmaceutical coatings (288). New tablet shapes and sustained-release developments were also surveyed by this author (289). By combining spray techniques and drying into one operation, Rieckmann developed a new method for the automation of tablet coating (290). Rapid methods for the manufacture of various nonsugar, "film type," tablet coatings have been announced (291).

A procedure to detect core dislocation in compressed coated tablets was offered to determine the optimal granule-size distribution for optimal core concentration (292). Other investigators described the development of enteric coatings from methyl phthaloyl cellulose (293) and salol-shellac mixtures (294). Good color stability from water-soluble carotenoids was demonstrated in tablet coating (295). A simple procedure was developed by Bennett and Hess for the rapid detection of excess glucose in the coloring syrups used for tablet production (296).

In a study of complex tablets, it was found that compressibility and strength were dependent on the proportions of the individual components (297). The sublimation of iodochlorohydroxyquinoline was observed during the manufacture of tablets (298). Hadgraft and Smith examined the formulation and stability of effervescent potassium tablets (299). Sodium lauryl sulfate was found to be an effective foaming agent in the preparation of a new contraceptive tablet (300). Dosage variation was studied by Brochmann-Hanssen and Medina in various production-run tablets (301). Automated instrumentation was also used to study intertablet dosage variation (302).

Suspensions.—Finholt conducted a survey of pharmaceutical suspensions (303). Fundamentals, production, and testing were discussed in a paper on parenteral suspensions (304). Utilization of suspensions in hospital pharmacy has also been considered (305). The influence of various ingredients on the physical behavior of calamine suspensions was observed (306), and some of the properties of white lotion were examined (307).

During 1963, many references were found on various theoretical treatments of numerous suspension problems. Hiestand cited several important physicochemical factors in suspension formulation (308). The theoretical treatment of the properties of colloidal dispersions flocculated by polyelectrolytes was reviewed (309), and Higuchi, *et al.*, studied in detail the kinetics of rapid aggregation in suspensions (310). Caking and flocculation were investigated under various conditions for sulfamerazine suspensions (311). Theoretical calculation of sedimentation volume was considered for nonattracting spherical particles (312); the method was applied to data from the literature. Zacek described a sensitive torsion viscometer and its application to determine the viscosity of pharmaceutical suspensions (313). Rheological behavior, sedimentation rate, redispersibility, and microscopic appearance were evaluated for zinc oxide suspensions at varying concentrations (314).

Smith demonstrated some practical methods for converting powders from a nondispersible to a dispersible state after grinding (315); methods were also presented for stabilizing suspensions after dispersion. The dispersibility and adhesiveness of several powdered drugs were investigated in 1% aqueous polysorbate 80 solution (316). Zacek recommended the Grindometer for the quality control of pharmaceutical suspensions (317). Two methods were compared for measuring particle-size distribution of aqueous barium sulfate suspensions (318).

The applications and properties of clay suspending agents for cosmetic formulations were reviewed by Sperandio (319). Montmorillonite and kaolin gels were studied in relation to the "card-house" picture of flocculated suspensions (320). Granquist and McAtee considered the role of the dispersant in gelation of hydrocarbons by montmorillonite-organic complexes (321). Another researcher investigated the effects of dilution and addition of salts on the viscosity of a sodium-ion–containing montmorillonite suspension (322). Electrophoretic mobility of kaolinite gels was determined as a function of electrolyte concentration (323). The preparation and evaluation of a hexamethyltetracosane-organo-magnesium montmorillonite gel was described by Franke and Riley (324).

Emulsions.—Schneider discussed oil-in-water emulsions in a review of formulations (325). Two publications appeared concerning the application of ultrasonics in the preparation of emulsions (326, 327). Zettlemoyer, *et al.*, described a simple method of manufacturing reproducible water-in-oil emulsions and a shear method for rating stabilities (328). In another paper, the mechanical properties of emulsions were interpreted rheologically (329). A nonaqueous, immiscible system was used to evaluate the emulsifying effects of several ionic surfactants (330). Emulsions were formulated with a wool wax iso-

late and its ethoxylated derivative (331). Bogs and Naumann evaluated emulsifying agents employed in pharmaceuticals in terms of their emulsifying capacity (332). In a series of statistically designed experiments, the chemical and physical nature of four oils and 13 surfactants were evaluated (333).

Neumann discussed the mechanism of coalescence and compared the coalescence time of oil drops and water drops in various immiscible liquid systems (334). The parameters of emulsion stability were explored with respect to shelf life, creaming, or clearing—interacting forces involved in coalescence were also mentioned (335). In the presence of small amounts of surface-active agents, glycerol tristearate crystals stabilized water-in-oil emulsions (336). Research was conducted on the changes in rheological properties of emulsions on aging (337). Moore and Lemberger devised a procedure for the rapid counting and sizing of droplets as a function of time to study the sedimentation behavior of dispersions (338).

Ointments.—Higuchi has reviewed the theory of diffusion as applied to the transport of materials in ointment bases (339). Classification of various ointment bases was the subject of another review—new preservatives and antioxidants were also mentioned (340). In a series of publications, Allen reviewed various absorption bases, including hydrophilic petrolatums (341–343). Walker and Kennedy discussed the rational basis of dermatological formulations (344). The Grindometer was used to determine fineness of powdered drugs in various ointment bases (345); a statistical evaluation of the data was presented in a second paper (346). Hermetically sealed containers were advocated for water-in-oil ointments to prevent evaporation from their surfaces (347). Procedures and apparatus were described for the preparation of a new dosage form for ophthalmic ointments (348).

Suppositories.—Jernas derived a formula to calculate the amount of suppository base to use in formulation (349); Tuma discussed various hydrophobic, hydrophilic, and water-soluble bases for suppository preparation (350). The melt-process was applied in preparing suppositories with Lasupol G as the base (351). A simple four-step procedure was described for the preparation of a single shell which serves as mold and package for suppositories (352). Another paper dealt with the technology of various suppository bases for use in tropical climates (353). Benzalkonium chloride was formulated into a water-soluble, vaginal suppository (354). The liberation of water-soluble drugs from 24 different suppository bases was investigated by Krowczynski (355). The absorption rate of sulfonamides from suppository bases was related to the hydrophile-lipophile balance value of the surfactant employed (356). Simon and Slavin compared the stability of imitation cocoa butter and theobroma oil in terms of physical and chemical properties and storage requirements of the finished product (357). In an evaluation of physical properties of suppositories, maximum environmental temperatures were determined for 44 bases (358).

Packaging.—Discussions were noted concerning the need for standard specifications for eye drop bottles (359) and prescription containers (360). Consideration was also given to the applications and contraindications for various types of packaging materials and dispensing containers (361). Boerger, *et al.*, enumerated some problems on the new forms in drug packaging (362). An electronic control system was described for increased efficiency in pharmaceutical packaging (363).

Methods of evaluation were presented in a review of the effects of elastomer components on the composition, aging, and efficacy of drug products (364). Various opinions were stated concerning the testing of synthetic materials used in pharmaceutical packaging (365). In two separate publications, Beiersdorf discussed the testing of rubber stoppers used as closures for parenterals (366) and the selection of specific rubber stoppers for certain drug containers (367).

Aerosols.—Many comprehensive reviews were published in the field of aerosols in medicine. These covered a wide variety of subjects—from formulations, stability, particle-size measurement, and method of administration to propellants, containers, and packaging techniques (368–375). Methods of testing dip tubes were investigated in relation to stability in certain chemical environments (376).

Lin and deNavarre discussed the properties, viscosity, density of foam, and surface tension as they affect the wetting property of the aerosol foam (377). Over 400 surfactants were evaluated for use in aerosol foams with respect to their emulsion stability, viscosity, discharge characteristics, foam stiffness, stability, and density (378). The coagulation of a homogeneous, slightly charged aerosol of dioctyl phthalate was studied (379). Blaug, *et al.*, formulated a medicated aerosol foam for otic application (380). Electron diffraction was used to measure the particle size of a zinc oxide aerosol (381).

EQUIPMENT

An all-glass rotary evaporator for vacuum distillation was described by Naff and Spector (382). A new fractionating molecular still has also been designed (383). A laboratory pressure-filter apparatus was described for use in the pharmacy (384). Schroeter and Hamlin developed an automated apparatus for the determination of dissolution rates of capsules and tablets (385), and the design and operation of a programmed automated film-coating technique was illustrated by Lachman and Cooper (386).

For small-scale mixing, a modified 10-ml. syringe with exchangeable cylinders was proposed (387). The design and technical performance of a small, very simple, fast laboratory mixer was given by Smith (388); Albus discussed the principles of the jet mill and various types of nozzles (389). Equations have been derived to express roll-speed ratio, throughput, and efficiency of the three-roll mill (390). A laboratory colloid mill was developed for the emulsification of flavoring oils in the preparation of locked-in fruit flavor (391). Gelperin, *et al.*, used a continuous dryer for the rapid drying of suspended tetracycline (392). A small air-pressure pump for use in the pharmacy has also been described (393).

Application of a portable hand refractometer to normal control procedures was discussed in relation to bulk compounding and manufacturing (394). With the aid of a digital computer, ultracentrifuge data can be used to determine apparent molecular weight (395). The Severs rheometer was applied to rheological problems of lotions and semisolids by Wood, *et al.* (396), who also converted a Brookfield viscometer to an absolute rheometer for use in pharmaceutical rheology (397). For measurement of water diffusion through skin, Isherwood constructed a modified diffusion cell (398). A dielectric moisture meter was described for the determination of moisture in solid dosage forms (399).

Lukoyanov, *et al.*, developed a film evaporator for use in concentrating antibiotic solutions without the aid of mechanical agitation (400). A simple evaporator requiring no rotating joint was designed for flasks under reduced pressure (401). West also demonstrated a simple rotary film evaporator for operation under reduced pressure (402).

A small-scale apparatus was disclosed for the preparation of deionized water (403). Bonnet commented on industrial preparation of water for injection and advocated the use of the distillation technique (404). Water distillation and demineralization equipment were described in two publications: potential application for preparation of water for injection was considered in the first (405); an evaluation of the quality of water produced was presented in the second (406). Fifty-eight references were cited in a review of the preparation of demineralized water for pharmaceutical use (407).

The principles of operation and applications of the Coulter Counter were discussed by Cartmel and Gerrard (408). Size analysis of insoluble drugs has been facilitated by programming an electronic computer to handle Coulter Counter data (409). An automatic apparatus for measuring the specific surface of powders in production operations was reported by Papadakis (410).

The equipment and procedure were described for filling liquid smallpox vaccine into plastic ampuls (411). Two papers were concerned with the application of ultrasound equipment to the washing of ampuls (412) and glass tubing (413). Frost discussed a semiautomatic measuring device and its application to liquid filling operations (414).

PHYSICAL PHARMACY

Physical pharmacy continues to occupy a position of prominence in pharmaceutical science. This field is concerned with solutions to pharmaceutical problems on a physicochemical basis. Numerous papers on this subject appeared in the literature during 1963.

The freezing points of several pharmaceutical preparations not readily found in the literature were tabulated by Schmidt (415). Freezing-point observations were also reported for the micellar solutions of *p*-methylphenylglucose and *p*-butylphenylglucose solutions (416). McKay and Miles verified the thermodynamic law for solute partition by the independent determinations of the activities in the two phases (417). It was stated in a paper by Veis that entropy factors provide the driving force for the phase separation in gelatin coacervation systems (418). Equilibrium phase relationships for the ternary system sucrose-water-isobutanol were examined (419). Some observations were published on the physical and pharmacological properties of picrotoxin solutions (420).

Size analysis of insoluble drugs has been discussed (421). Heywood published a detailed treatment of the characteristics of size, shape, density, and surface of fine particles (422). A critical survey of the methods and their relative merits was presented by Connor for automatic counting and sizing of particles (423). The adhesion properties of particles less than 50 μ in

diameter were used for particle-size classification (424). In a brief note, a simple method was revealed for particle-size determination by turbidity measurements (425). Experiments have been carried out to study the influence of particle size on the mixing of powders—mixing time varied directly with particle-size distribution (426). Using the method of Derjagin, Okada and Abe measured the coefficient of friction and adhesion power of various drug powders (427).

Ionization.—A viscometric method was illustrated for the determination of the isoelectric point of a protein (428). With electromotive force measurements, Shedlovsky, *et al.*, examined the behavior of aqueous solutions of mixed colloidal electrolytes (429). A study of charged carboxylate bases in dilute acid solutions was published (430). Another presentation was concerned with the theoretical interpretation of the "weakly acid" properties of boric acid (431). Conductance measurements were employed in determining the dissociation constants for acetic, propionic, *n*-butyric, and benzoic acids in water between 25 and 225° (432). The ionization constants of some penicillins and their alkaline hydrolysis and penicillinase hydrolysis products were also determined (433). Bates, *et al.*, developed a suitable scale of pH for alcohol-water solvents (434). Experimental work on the effect of molecular complexing of riboflavin showed that pKa values may be modified if molecular complexation takes place (435). Random and systematic errors in the determination of association constants were considered by Pasternak and Brady (436). A simple method was developed for the determination of ionization constants by paper chromatography (437). Hepler evaluated the effects of various substituents on the acidities of organic acids in water with respect to the thermodynamic theory of the Hammet equation (438).

Diffusion.—Knuth announced a unified kinetic theory for description of transport phenomena (439). Schumaker proposed a theory for measuring weight-average diffusion coefficients in single-component and multiple-component systems from ultracentrifuge experiments (440). *In vitro* methods were used to measure the continuous availability of sodium salicylate from tablet bases (441). In another report the effect of polyethylene glycol lauryl ether on the permeation of chlorobutanol was studied kinetically by the partial-dialysis method (442). The permeability of organic amines through collodion membrane was measured by the membrane-electrode method (443). Saunders used a capillary cell and a conductivity method in studying the rates of diffusion of various salts through an interface between sols of lipid and protein (444).

Solubility.—Extensive research is being conducted to elucidate the numerous physical phenomena which enter into solubility and solubilization. The thermodynamic relationships involving polymorphism and solubility were investigated in a study of the physicochemical aspects of solid-solution behavior (445). Three papers on dissolution rates were published: in two papers, Niebergal and associates developed a continuous-recording technique for studying dissolution rates (446) and investigated the dissolution of particles under conditions of rapid agitation (447); the third paper, by Smith, described a new spectrophotometric method for determining dissolution rates (448). Higuchi and Hiestand explored the effect of particle-size distribution in a diffusion-controlled process and derived an equation for the dissolution rate (449). In a study of the dissolution rate of micronized methylprednisolone, diffusion of the drug in the aqueous phase was the rate-limiting step (450). The rate of release of benzphetamine pamoate from a self-coating pellet surface was expressed mathematically (451). Schematic models were used in discussing hydrogen-bonding systems as media for chemical mass transport (452).

Wahlgren tested solubility of 38 drugs in polyethylene glycol at 20 and 60° (453), and Kuttel noted the solubilizing effect of polyethylene glycols and their esters with 22 hydrophobic pharmaceuticals (454). The solubility and stability of oleovitamin A were determined in a polyoxyethylene sorbitan ester-polyol-water system (455). Several articles were also published on the solubility and solubilization of acetylsalicylic acid (456), cinchocaine (457), orotic acid and its derivatives (458), various sulfonamides (459), and some *N*-7 theophylline derivatives (460).

Shefter and Higuchi investigated the dissolution behavior of crystalline solvated and nonsolvated forms of some pharmaceuticals (461). The influence of hydrates and solvate formation on the rate of solution and the solubility of crystalline drugs was also discussed (462). The thermodynamic relationships involving polymorphism and solubility were examined and applied by Higuchi, *et al.*, to experimental results from a methylprednisolone system (463). A series of publications dealt with the theoretical and experimental techniques in the study of hygroscopicity of water-soluble, crystalline drugs (464–470).

A new type of ion-pairing was discussed in an

investigation of the aqueous solution behavior of large univalent ions (471). The significance of new ionic-radii values to solvation phenomena in aqueous solution was disclosed by Blandamer and Symons (472). In a study of ion-solvation, the energy transfer of halogen acids and alkali chlorides from water to methanol-water mixtures was explored (473). Frank proposed a return to the ascription of physical significance to single-ion activities in electrolyte solutions (474). True partition coefficients and acidity constants for chlorpromazine and promethazine were determined by Angadji and Colleter using a graphic method (475). It has been demonstrated that osmotic coefficients obtained by the thermoelectric differential vapor-pressure method were valid for a wide variety of electrolytes in moderate concentrations (476). Dielectric constants of water-ethanol-glycerin and water-ethanol-propylene glycol systems were experimentally determined (477).

The solubilization of various drugs with different concentrations of polysorbates (478) and the development of a method and formula for determining the type and amount of polysorbate to solubilize some hydrophobic drugs were presented (479). Solubility titration was used by Hall to evaluate the solubilization of salicylic acid with polysorbate 80 (480). Swarbrick and Carless examined phase equilibria in some betaine-benzaldehyde-water systems (481). Several amines and amides were examined as potential solubilizing agents of orotic acid (482).

Johnson, *et al.*, discussed equilibrium constants for water-alcohol mixtures (483). Methods were developed for estimating oil-water distribution coefficients of glyceryl trinitrate and two similar nitrate esters (484). Higuchi and Hom conducted a phase-solubility study of solid species formed by magnesium aluminate from aqueous solutions containing sulfate ions (485). Tables of specific gravity and refractive index were offered by Reuter and Biegel for the physical testing of stock solution concentrations (486). A simplified method was presented for determining densities of aqueous solutions of some organic compounds (487).

Complexation.—Solubility studies were used to follow the interaction of urea and thiourea with benzoic and salicylic acids (488). Graham and Baker discovered that antihistamines form insoluble complexes with carrageenan and other sulfated colloids (489). In another publication, these studies were expanded to include complex formations with various tranquilizers and hypotensive agents (490). A definite interaction has been demonstrated between hydroxybenzoic acids and *p*-hydroxybenzoates with Schardinger dextrins (491); also investigated was the effect of cyclodextrin on water solubility of selected drugs (492). Two papers were concerned with the interactions of dyes and surfactants (493, 494). Phase solubility studies enabled Pisano to determine the extent of complexation between some aromatic carboxylic acids and certain pharmaceuticals in aqueous solutions (495). It was reported that L-ascorbic acid in aqueous solution was stabilized through complexation with dehydroacetic acid (496).

Through a study of physical properties, a correlation was established between the extent of association and the molecular structure of various alcohols (497). Matsushima extended Schubert's method for the determination of complex stability constants by an ion-exchange method to a lower pH region (498). Ion-exchange methods were also used without radio-tracers to study magnesium citrate complexation (499). The properties of biguanide and the formation of its metallic complexes were investigated (500).

In another paper a soluble buffer antacid, sodium gluconatodihydroxoaluminate complex, was compared with some existing preparations by *in vitro* tests (501). George investigated the nature of chelates formed by boric acid in aqueous solution (502); an experiment by Halmekoski indicated that a corbadrine-molybdate chelate is more stable than molybdate chelates of adrenaline and noradrenaline (503). Complex formation between iodine and surfactants was suggested as a possible mechanism of iodine solubilization (504). Complexation was also used to prepare a slightly soluble product of copper and barbital (505). Thirty-nine liquid chelating agents were tested by Nielson for their ability to form dental root-filling cements with metal oxides (506).

The hydrolytic tendencies of several ferric chelates (507) and the stability constants of iron sulfate and chloride complexes (508) have been determined. Sakai prepared crystalline ferric complexes with some higher fatty hydroxamic acids and investigated their properties—molar ratios of 2:1 and 3:1 were found (509). A stable sodium ferric gluconate complex was synthesized by Tanabe and Okada (510). These investigators also examined the properties of some ferric gluconate complexes by means of paper electrophoresis (511).

Surface Phenomena.—Mattoon reviewed film formation, structure, and properties as applied to the pharmaceutical industry (512). The linear expansion of gelatin films by moisture

sorption (513) and a comparative study of liquid and vapor permeation through various polymer films (514) have been reported. James, *et al.*, commented on the enhancement of evaporation by protein films as compared with solutions of certain surfactants (515). A new interface balance for studying films at the oil-water interface was described (516). Equations were presented in a discussion of the computation of surface tension and contact angle by the sessile-drop method (517).

A general discussion of the potential theory of adsorption was published (518). Three papers dealt with methodology applicable to the study of adsorption: one discussed the measurement of contact angles and surfactant adsorption in three-phase systems (519); another was concerned with the use of a rapid-flow method for determining adsorption isotherms (520); the third reported a spectral reflectance method for solid-solid interaction studies (521). Fujiwara, *et al.*, employed a colorimetric method for investigation of barium sulfate adsorption of sodium carboxymethylcellulose in aqueous suspension (522). Adsorption by two forms of carbon has been studied (523, 524). MacRitchie and Alexander published extensively on the kinetics of adsorption of proteins at interfaces (525–527). A new experimental approach was presented for evaluating the protein-binding properties of penicillins (528).

Anderson and Plein continued an investigation of certain bentonites by determining cation-exchange properties (529). It was suggested that considerable energy change is involved in selective adsorption of organic ions by ion-exchange resins (530). Experimental results were published concerning the influence of pH on adsorption of amines by synthetic aluminum silicate gel (531). Vermiculite has also been used for the adsorption of alkylamine ions (532). Slabaugh and Kennedy measured adsorption isotherms in a study of amine complexes of montmorillonite (533), and phenol adsorption by quaternary ammonium derivatives of montmorillonite was investigated by Street and White (534). Another worker reported on the adsorption of polyvinyl alcohols by montmorillonite (535).

The Ferguson principle was suggested as a means of correlating thermodynamic activity with critical micelle concentration for a series of quaternary ammonium salts (536). Independent studies of the thermodynamics of micelle formation in nonionic detergents were made by Schick (537) and Elworthy and Florence (538). A simple dilatometric method was used to determine partial molal volumes of surface-active agents in micellar, singly dispersed, and hydrated solid states (539). Mysels, *et al.*, developed a rocking dialysis cell to demonstrate the activity of association colloids above the critical micelle concentration (540).

Experimental work was published on micelle formation by surfactants and its importance in the solubilization of drugs (541, 542). Several investigators reported on micelle formation and critical micelle concentration (CMC) of some betaine derivatives under a variety of conditions (543–546). Kato revealed the formation of micelles by glycols of the 1,4-hexanediol type (547). A conductivity method was applied in a study of the CMC of some primary and quaternary ammonium dodecyl sulfates (548). Becher commented on the effect of different sucrose concentrations on the CMC of aqueous surfactant solutions (549). Dissolved urea was proposed as a probe for studying micelle formation and hydrophobic bonding in aqueous solutions (550).

Gorman and Hall suggested the dielectric system as a method of classifying surfactants (551). Another publication was concerned with physical chemistry of detergents—including strong electrolytes, simple solutes, and soluble macromolecular colloids (552). Other investigators have pointed out that surfactant structure is not necessarily related to reactivity toward acids and bases (553). A procedure for determining the wetting rate of surface-active substances was presented (554), and Becher determined the interfacial tensions of solutions of nonionic surfactants (555). Surface tension measurements demonstrated pronounced surface-active properties of aqueous sols of lysophosphatidylethanolamine (556). Electric resistance–temperature curves and hydrophile-lipophile balance were reported for some nonionic surfactants (557). Sebba showed that ionic surfactant foams can be destroyed by addition of a surfactant of opposite charge (558).

Crystallization.—The physical techniques and measurements required in the application of vapor pressure phenomena to the study of crystallization were discussed at length by Powers (559). Other publications by this investigator dealt with the phenomena associated with initial crystal formation and early growth of the nucleus (560) and the macroscopic growth of crystals (561). Various standard, ionic-crystal structures have been illustrated and described (562). Crystallization of silver bromide was described in terms of the ideal size distribution and growth rate for microcrystalline precipitates (563). With the

aid of X-ray diffraction and infrared spectra, Biles identified four crystal modifications of tertiary butylacetates of prednisolone and hydrocortisone (564). Another study revealed the crystallization conditions in the hydrothermal synthesis of crystalline aluminosilicates (565).

In a review of X-ray and crystallographic applications to pharmaceutical research, Shell described quantitative X-ray diffraction with illustrations (566). A method was also presented for quantitating the crystal habit of given compounds and its use as a control procedure for tableting (567). The crystal structure of a calciferol derivative was solved by the heavy atom method (568). Other researchers established new types of melting-point diagrams for a large number of barbituric acid derivatives (569) and compared isomorphs of these derivatives (570).

Rheology.—In a review covering nomenclature and instrumentation, shear rate–viscosity curves of various systems were employed for microrheological classification (571). The logarithmic rheogram was proposed for treating non-Newtonian data (572). McVean developed a rising cylinder rheometer for low shear viscometry (573), and Rutgers presented an extensive review of equations describing the relationship between relative viscosity and concentration of dispersions (574). A viscometer was proposed for determining the intrinsic viscosity of macromolecular solutions at zero rate of shear (575). An evaluation of viscosity constants was also published (576).

Christoff used a rotational viscometer to measure the hysteresis loop of an emulsion ointment base (577). In a series of papers, Schulte and Kassem presented a comparative rheological study of some ointment gels (578) and reported the rheological investigation of a polyethylene–liquid petrolatum gel system (579) and of hydrocarbon mixtures (580). Thixotropic properties were determined for eight types of petrolatum (581). Gstirner and Bodenbach, in a series on the rheology of ointment bases, explored the change in viscosity and thixotropy of 12 lipogels with time (582) and used the electron microscope to study a thixotropic mixture of peanut oil and colloidal silica (583). Also investigated were the viscosity and crystal formations of lipogels (584) and the rheological properties of lipogels after heat treatment (585). The rheological properties of a few suppository bases have also been reported (586).

In a rheological study of aging, aqueous Veegum suspensions were found to be pseudoplastic for all temperatures of preparation and storage (587). Other rheological experiments with Veegum dispersions indicated that sedimentation can be reduced by addition of an electrolyte (588). The viscoelastic properties of bentonite pastes in various media were examined with a cone-disk rheometer (589). Hunter and Alexander considered the influence of physical and chemical conditions on the flow of kaolinite sols through silica columns (590). Rheograms and sedimentation volumes were obtained in an investigation of the rheological effects of some high polymers on barium sulfate particles (591).

Viscosity changes produced by thermal aging of gelatin sols were measured with a concentric cylinder viscometer (592). Gelatin was also found suitable for the demonstration of viscoelastic solution behavior (Weissenberg effect) (593). Nakagaki and Nishino determined the structural viscosity of aluminum distearate in benzene with the capillary viscometer under a variety of conditions (594–596). Changes in specific volume were used to follow the kinetics of solid-liquid phase transformations of methyl stearate (597). In another paper, by Hermans, the viscosity and sedimentation of cellulosic macromolecules in dilute solutions were reported (598).

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Nonclassical Antimetabolites XV

Bridge Principle of Specificity with Active-Site-Directed Irreversible Inhibitors VI. Highly Selective Irreversible Inhibition of Lactic Dehydrogenase

By B. R. BAKER and R. P. PATEL

Eleven compounds, all of which were reversible inhibitors of both heart and skeletal muscle lactic dehydrogenase, were investigated as irreversible inhibitors of the two enzymes. 4- and 5-(Iodoacetylglycylamido)salicylic acids (V and VI) irreversibly inhibited both enzymes. However, specificity was shown with 4- and 5-(iodoacetamido)salicylic acids (I and II), which irreversibly inhibited skeletal muscle lactic dehydrogenase, but not heart lactic dehydrogenase. A crossover specificity was noted with 5-(carbophenoxyamino)salicylic acid (IV); this compound irreversibly inhibited heart lactic dehydrogenase, but not skeletal muscle lactic dehydrogenase. It is proposed that these specific irreversible inhibitions are due to exploitable differences in the secondary-tertiary structure of these two substrate-identical enzymes from different tissues.

CLASSICAL ANTIMETABOLITES having only a small change in structure compared to the substrate have such a limitation in the structural changes that can be made (2) that utility is greatly limited. In contrast, nonclassical antimetabolites, defined as inhibitors that have large structural changes compared to the substrate (1), have much wider application. For example, the concept of irreversible inhibition by the exoalkylation mechanism with nonclassical antimetabolites was proposed (1). A properly designed inhibitor such as 4-(iodoacetamido)-salicylic acid (I) can reversibly complex with an enzyme such as GDH,[1] then become irreversibly bound within the complex by alkylation of the enzyme adjacent to the active-site; an expression more general than exoalkylation is active-site-directed irreversible inhibition.[2] In support of this phenomenon, strong experimental evidence has been presented (3, 4). Further evidence for active-site-directed irreversible inhibition requiring initial reversible complexing with the enzyme has been presented more recently; compounds with a lower reversible dissociation constant (K_I) between enzyme and inhibitor give about the same speed or irreversible inhibition at a lower concentration dependent upon the relative amount of reversible binding of the compounds to the enzyme (10). Five other laboratories have subsequently and independently made observations pertinent to active-site-directed irreversible inhibition with chymotrypsin (16, 18, 22, 23) and in the field of hapten immunochemistry (25).

Our definition of exoalkylation (1) and its experimental support (3, 4, 10) led to the concept of (4) and experimental evidence (5–7, 11) for the bridge principle of specificity, which is now restated to replace the word "exoalkylation."

Compared to a reversible inhibitor, the active-site-directed type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly bound inhibitor to bridge to and form a covalent bond with a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being covalently linked.

With the mechanistically closely related enzymes, LDH[1] and GDH, experimental evidence in support of both facets of the bridge principle has been observed. Specificity of irreversible

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For previous paper in this series see *Reference 10*.

[1] The following abbreviations are used: LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; tris, tris-(hydroxymethyl)aminomethane hydrochloride buffer; 4-ISA, 4-(iodoacetamido)salicylic acid (I); 4-IGSA, 4-(iodoacetylglycylamido)salicylic acid (V).

[2] The expression "exoalkylation" is not strictly correct for a carbophenoxy compound such as IV; the covalent linkage between the reversible bound inhibitor and the enzyme occurs in this case by acylation, but the principle is the same. The more general expression "site-directed irreversible inhibition" is preferable.

inhibition of GDH, but not LDH, or *vice versa* by inhibitors related to 4-ISA[1] (I) was attributed to differences in the spatial relationship of the reversible binding sites and the nucleophilic sites of the two enzymes (5, 6). The differences in nucleophilicity of the enzymic groups being covalently linked in LDH and GDH was demonstrated with compounds related to 5-(carbophenoxyamino)salicylic acid (IV), whereby GDH was irreversibly inhibited, but not LDH (7, 11).[2]

Substrate-identical enzymes from different tissues frequently give little or no cross-reaction with specific antisera (14, 15, 17), thus showing differences in the secondary or tertiary structure of the protein *other than the active-site* (6); we suggested (6) that—*via* the bridge principle—it may be possible to obtain highly selective irreversible inhibitors of the substrate-identical enzyme from two different tissues. Such specific irreversible inhibition of LDH from heart and skeletal muscle has recently been reported (12) wherein 4-(iodoacetylglycylamido)salicylic acid (V) could irreversibly inhibit LDH from either heart or skeletal muscle, but 4-ISA (I) irreversibly inhibited only the skeletal muscle LDH and not heart LDH. Further observations on selective irreversible inhibition of these two enzymes is the subject of this paper.

MATERIALS AND METHODS

Reagents.—Sodium pyruvate and DPNH[1] were purchased from the Nutritional Biochemical Corp. Heart LDH, skeletal muscle LDH, tris,[1] and tris hydrochloride were purchased from the Sigma Chemical Co.; heart LDH was the crystalline enzyme isolated from beef heart, and skeletal muscle LDH was the crystalline enzyme isolated from rabbit skeletal muscle.

Reversible Binding of Inhibitors to LDH.—The concentration for 50% inhibition by compounds I–XI was determined for heart LDH in the presence of 0.3 m*M* pyruvate and 0.23 m*M* DPNH in 0.05 *M* tris buffer (pH 7.4) by observing the change in optical density per minute at 340 mμ; sufficient enzyme was employed to give a change of about one optical density unit per minute with no inhibitor. The 50% inhibition values for skeletal muscle LDH were done similarly in the presence of 1 m*M* pyruvate and 0.6 m*M* DPNH (2). These 50% inhibition values were then converted to the apparent K_I values in Table I described under *Results*.

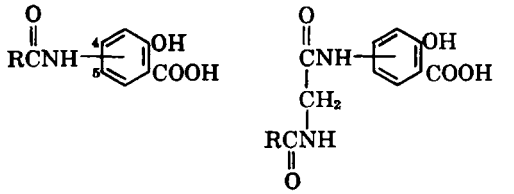
Enzyme Inactivation Procedure.—The inactivation procedure for skeletal muscle LDH has been previously described in detail (4, 6). The same procedure was used for heart LDH, except that the incubation time at 37° was increased from 60 minutes to 120 minutes. In all incubations for determination of irreversible inhibition of heart LDH, incubation solutions containing 2.3 m*M* DPNH and sufficient enzyme to give 9–11 optical density units change/minute/ml. of tris buffer were run simultaneously; the solutions were made from the same master enzyme-DPNH solution (4, 6): (*a*) the test compound in a concentration determined by the relative apparent K_I, (*b*) a standard of 4 m*M* 4-IGSA,[1] and (*c*) an enzyme control with no inhibitor. An 0.4-ml. aliquot was removed at the proper time interval, and the irreversible reaction was quenched by placement of the aliquot in an ice-bath. When time allowed, 0.30 ml. of the aliquot was added to 2.7 ml. of tris buffer (pH 7.4) in a cell. Then 0.10 ml. of 9.3 m*M* pyruvate was added, and the rate of oxidation of DPNH was followed at 340 mμ in a Cary 11 recording spectrophotometer. Under these conditions the change in optical density per minute was directly proportional to the remaining enzyme concentration in the aliquot. Since the concentration of inhibitor is in excess and does not change, the reaction is unimolecular with respect to enzyme concentration; therefore, the enzyme concentration (expressed in optical density units) is plotted on a log scale in Figs. 2–4. All runs were duplicated at least twice. An occasional bad run was readily eliminated if the control or standard did not behave properly.

When two concentrations of the same inhibitor were compared, the experiments were run similarly, except that (*b*) was a solution of the test compound at one-half the concentration of (*a*).

Considerable care, as previously described (11), must be taken in making up solutions of the carbophenoxy compounds listed in Table I so that the base-labile phenyl ester group does not hydrolyze.

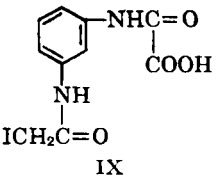
CHEMISTRY

Of the 11 compounds in Table I studied as irreversible inhibitors of heart LDH, nine have previously been studied as inhibitors of skeletal muscle LDH. The synthesis of compounds I, II, V, IX, and X have been previously described (8), as have their effects on skeletal muscle LDH (6). The synthesis and effects of compounds III, IV, and VII on skeletal muscle LDH have also been described (11), as have similar data on compound XI (10). The synthesis of the remaining two compounds (VI, VIII) were carried out as follows:

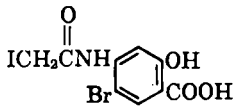


I, R = 4—ICH_2—
II, R = 5—ICH_2—
III, R = 4—C_6H_5O—
IV, R = 5—C_6H_5O—

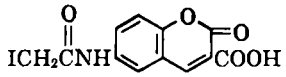
V, R = 4—ICH_2—
VI, R = 5—ICH_2—
VII, R = 4—C_6H_5O—
VIII, R = 5—C_6H_5O—



IX

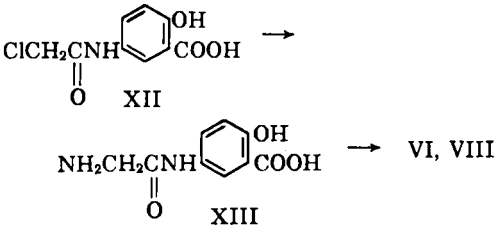


X



XI

5-(Chloroacetamido)salicylic acid (XII) (20) was prepared in 92% yield from 5-aminosalicylic acid and chloroacetyl chloride in aqueous sodium bicarbonate as previously described for related compounds (8). When XII was allowed to react with concentrated ammonium hydroxide, the amino acid (XIII) was obtained in 54% yield; the infrared spectrum of XIII indicated that it was not a zwitterion, in contrast to 4-(glycylamido)salicylic acid (8). Reaction of XIII with iodoacetyl chloride or phenyl chloroformate in cold aqueous sodium bicarbonate afforded VI and VIII, respectively, in good yield.



XII

XIII

TABLE I.—REVERSIBLE AND IRREVERSIBLE INHIBITION OF LDH FROM HEART AND SKELETAL MUSCLE

Compd.[a]	Skeletal Muscle LDH[b] Apparent[a] $K_I \times 10^4$	Skeletal Muscle LDH[b] Rate of Inactivation mM Concn.	Skeletal Muscle LDH[b] Rate of Inactivation Relative Rate	Heart LDH Apparent[a] $K_I \times 10^4$	Heart LDH Rate of Inactivation mM Concn.	Heart LDH Rate of Inactivation Relative Rate
4-ICH$_2$CO—ASA (I)	17	2	1.0[c]	45	2 4	0 0.2[d]
4-ICH$_2$CO—GSA (V)	4.0	2	0.87	25	4	1.0[c,e,f]
5-ICH$_2$CO—ASA (II)	5.0	2	0.78	19	2	0[f]
4-C$_6$H$_5$OCO—ASA (III)	11	2	0.98[g]	19	4	0
5-C$_6$H$_5$OCO—ASA (IV)	15	2	0	14	3	0.58[h]
5-ICH$_2$CO—GSA (VI)	23	2	1.9	34	4	1.0
4-C$_6$H$_5$OCO—GSA (VII)	16	2	0	17	4	0
5-C$_6$H$_5$OCO—GSA (VIII)	4.6	2	0	18	2	0
ICH$_2$CO—AOA (IX)	1.3	2	0	1.2	2	0[f]
5-Br-4-ICH$_2$CO—ASA (X)	7.3	2	1.6	28	2	0[f]
ICH$_2$CO—ACC (XI)	3.1	0.4	0.52	9.6[i]	1	0.67

[a] Abbreviations used: ASA, aminosalicylic acid; AOA, *m*-aminoxanilic acid; GSA, glycylaminosalicylic acid; ACC, 6-aminocoumarin-3-carboxylic acid. The units of the apparent K_I values are moles/liter. [b] Data taken from *References 6, 10,* and *11*, except for new data on compounds VI and VIII. [c] Arbitrary assignment of rate for relative purposes; the absolute values for the two LDH's are not the same. [d] Since no irreversible inhibition was detectable at 2 m*M* and slight irreversible inhibition was detectable at 4 m*M*, it is probable that the slight amount of irreversible inhibition at 4 m*M* is due to a small amount of bimolecular inactivation (tail-alkylation) of the type previously observed with iodoacetamide on skeletal muscle LDH (4). Even if this small amount of inactivation could be experimentally established as occurring in the same manner as inactivation by 4-IGSA, this amount of inactivation is on the border of the experimental error; any value in the table listed as 0 is actually less than 0.1, the limits of detection in this experimental design. Therefore, this compound (I) is considered to give negligible irreversible inhibition under these conditions. [e] The relative rates of inactivation in two runs when 4 m*M* V was compared to 2 m*M* V were 1.5 and 1.6; Calcd.: 1.4. (See Fig. 2.) [f] Data taken from *Reference 12*. [g] Comparison of the relative rates of inactivation of 4 m*M versus* 2 m*M* of compound III did not give a ratio less than 2, in disagreement with the ratio calculated from the ratio of the amounts of enzyme-inhibitor complex; see text of *Results* and *Reference 11*. [h] Showed relative rates of inactivation of 1.4 and 1.4 in two runs when 4 m*M* IV was compared with 2 m*M* IV; Calcd.: 1.3. [i] Estimated from 20% inhibition obtained from a saturated solution and may have a larger error than other apparent K_I's; see *Reference 10*.

EXPERIMENTAL

Melting points were taken in capillary tubes with a Mel-temp block and are uncorrected. Infrared spectra were determined with a Perkin-Elmer model 137B spectrophotometer.

5-(Glycylamido)salicylic Acid (XIII).—Reaction of 5-aminosalicylic acid with a 50% excess of chloroacetyl chloride in aqueous sodium bicarbonate by the previously described general method (8), gave a 92% yield crude product (XII), m.p. 236–238° (Raiziss and Clemence (20) recorded 242–244°), that was suitable for the next step.

A solution of 5.0 Gm. (0.0218 mole) of XII in 50 ml. of concentrated ammonia water was allowed to stand overnight. The solution was spin evaporated to dryness *in vacuo* and the residue triturated with 10 ml. of cold water; yield, 2.7 Gm. (54%), m.p. 240°. Recrystallization from water gave buff-colored crystals of unchanged melting point; $\nu_{max.}^{KBr}$ 3625, 3140 (NH, OH); 1687, 1650 cm.$^{-1}$ (C=O).

Anal.—Calcd. from $C_9H_{10}N_2O_4 \cdot H_2O$: C, 47.4; H, 5.26; N, 12.3. Found: C, 47.6; H, 5.35; N, 12.6.

5-(Iodoacetylglycylamido)salicylic Acid (VI).—To a stirred solution of 2.28 Gm. (10 mmoles) of XIII in 40 ml. of water containing 3.4 Gm. (40 mmoles) of sodium bicarbonate and cooled in an ice-bath, was added 4.08 Gm. (20 mmoles) of iodoacetyl chloride. After being stirred for 75 minutes, the solution was clarified by filtration, and the filtrate was acidified to about pH 3; yield, 3.0 Gm. (79%), m.p. 185–190°. Several recrystallizations from ethanol-ethyl acetate gave the pure material, m.p. 214°; $\nu_{max.}^{KBr}$ 3290, 3100 (NH, OH); 1675, 1638 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{11}H_{11}N_2O_5$: C, 34.9; H, 2.91; N, 7.40. Found: C, 35.0; H, 3.03; N, 7.22.

5 - (Carbophenoxyglycylamido)salicylic Acid (VIII). This was prepared similarly from 2.28 Gm. of XIII and 2.34 Gm. of phenyl chloroformate; yield, 2.7 Gm. (82%), m.p. 207–209°. Three recrystallizations from ethyl acetate gave the pure compound, m.p. 217–219°; $\nu_{max.}^{KBr}$ 3300 (NH, OH); 1735, 1710, 1670 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{16}H_{14}N_2O_6$: C, 58.2; H, 4.24; N, 8.48. Found: C, 58.4; H, 4.13; N, 8.30.

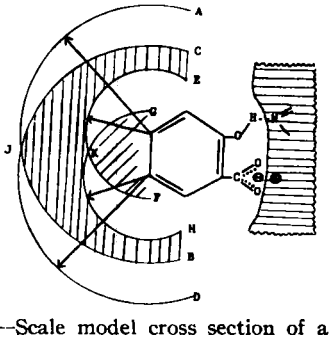


Fig. 1.—Scale model cross section of a possible relationship of the active site to the enzymic nucleophilic site. The horizontal cross-hatch represents the part of the enzyme with the active-site. See *Discussion* for the significance of the vertical and angular cross-hatch. The short arrow represents the most extended conformation of the —CH_2-CONH— group and is 3.7Å. long from the center of the methylene carbon to the *N*-phenyl bond; the long arrow represents the —$CH_2CONHCH_2$-CONH— group which is 7.7Å. long when fully extended. The measurements were made with Leybold models.

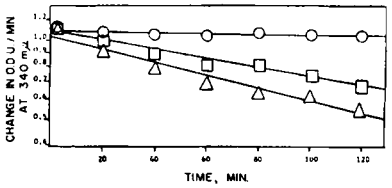


Fig. 2.—Effect of inhibitor concentration on the rate of inactivation of heart LDH·DPNH by 4-IGSA (V). Key: ○, heart LDH·DPNH control; □, 2 m*M* 4-IGSA; △, 4 m*M* 4-IGSA. The change in O.D. units/minute is proportional to the remaining enzyme concentration. (See *Materials and Methods*.)

RESULTS

A plot of V_o/V against I for several concentrations of inhibitor was used (2) to determine the concentration of I necessary for 50% inhibition ($V_o/V = 2$); where V_o = velocity of the enzymic reaction with no inhibitor, V = velocity with inhibitor, and I = concentration of inhibitor. If possible, several of the points should be in the 30–70% inhibition range to avoid the higher errors in higher or lower inhibitions. A reasonable approximation of the reversible dissociation complex (apparent K_I) between enzyme and inhibitor could be calculated from the previously derived (11) expression:

$$K_I = Km \times I/S \qquad \text{(Eq. 1)}$$

where Km is the enzyme-substrate dissociation constant and I is the concentration of inhibitor necessary to give 50% inhibition in the presence of a concentration of substrate S three to four times Km (see *Methods*); K_I and Km are expressed in moles/liter. This derivation contains the assumption that a complex between enzyme and inhibitor or enzyme and substrate are mutually exclusive, and does not distinguish "competitive" from "noncompetitive" reversible inhibition. Reiner (21) has pointed out that "competitive" kinetics determined by the reciprocal plot method do not prove that the inhibitor combines at the actual site, a common misinterpretation, but that a complex between enzyme and inhibitor or enzyme and substrate are mutually exclusive, regardless of where the inhibitor complexes with the enzyme. Since neither the reciprocal plot method nor the I_{50} method can prove unequivocally that complexing occurs at the active site, Eq. 1 is therefore sufficiently accurate to indicate the relative magnitudes of enzyme-inhibitor (EI) complex formation. In our system, pyruvate had $K_m = 0.95 \times 10^{-4}$ for heart LDH and $K_m = 2.5 \times 10^{-4}$ for skeletal muscle LDH, determined by a plot of $1/V$ *versus* $1/S$.

The apparent K_I values are listed in Table I, as are the effects of the compounds as irreversible inhibitors on both types of LDH. It should be noted that there is no difficulty in separating reversible from irreversible inhibition; the reversible inhibition is determined in 1 minute at 25°, an interval in which no measurable irreversible inhibition occurs (see Figs. 2–4). All 11 compounds

in Table I reversibly inhibited both types of LDH. Of these 11 compounds in Table I, skeletal muscle LDH was not irreversibly inhibited by four, but was irreversibly inhibited by seven compounds (I, II, III, V, VI, X, XI). With heart LDH, seven of the 11 compounds gave no irreversible inhibition and four showed irreversible inhibition (IV, V, VI, XI).

By definition of site-directed irreversible inhibition (1, 4), a reversible complex (XV) between the inhibitor (XIV) and the active-site of the enzyme is an obligatory intermediate for the formation of a covalent bond (XVI) between the inhibitor and the enzyme resulting in inactivation as shown in

$$E\overset{H}{\diagup} + AB \longleftrightarrow \underset{\text{XIV}}{} E\overset{H}{\diagup}\cdots AB \underset{\text{XV}}{} \rightarrow E\cdots A + BH \underset{\text{XVI}}{} \quad \text{(Eq. 2)}$$

The rate of inactivation is therefore dependent upon the concentration of the enzyme-inhibitor (EI) complex (XV), and the concentration of EI is expressed (4)

$$[EI] = \frac{E_t}{(K_I/I) + 1} \quad \text{(Eq. 3)}$$

where E_t is the total enzyme concentration excluding that portion of the enzyme that has become inactivated.

If a compound such as 5-(carbophenoxyamino)-salicylic acid (IV) inactivates heart LDH by active-site-directed irreversible inhibition, then doubling the concentration of inhibitor should give less than a doubling of the inactivation rate, according to Eqs. 2 and 3. For example, IV has an apparent $K_I = 1.4 \times 10^{-3}$ for heart LDH; then when $[I] = 4$ mM, $[EI] = 0.74\ E_t$ and when $I = 2$ mM, $[EI] = 0.59\ E_t$. Therefore, increasing $[I]$ from 2 mM to 4 mM increases $[EI]$ by 0.74/0.59 or 1.3. The actual observed values in two runs (Table I) were 1.4 and 1.4 (Table I), the so-called "rate-saturation effect" on rate of inactivation (4).

If inactivation occurred by bimolecular collision (tail-alkylation) between enzyme and inhibitor as shown in Eq. 4, it was previously assumed (4, 7, 10–12) that doubling of the concentration should

$$E\overset{H}{\diagup} + AB \rightarrow \widehat{EA} + BH \quad \text{(Eq. 4)}$$

double the rate of inactivation. This assumption is only true if the compound shows no reversible inhibition. If a reversible inhibitor operates by bimolecular collision with the active-site, then the compound can only inactivate the enzyme when the active-site is unoccupied by inhibitor, that is, in addition to Eq. 4, the equilibrium part of Eq. 2 is operating. Stated in another way, the rate of bimolecular inactivation (Eq. 7) is dependent upon the concentration of free enzyme $[E]$ in Eq. 5

$$[E_t] = [E] + [EI] \quad \text{(Eq. 5)}$$

$$K_I = \frac{[E]\,[I]}{[EI]} \quad \text{(Eq. 6)}$$

$$\text{rate} = k'[E]\,[I] = \frac{k'}{K_I}\,[EI] = k''[EI] \quad \text{(Eq. 7)}$$

Equation 7 for bimolecular inactivation with self-protection is kinetically indistinguishable from Eq. 8, the rate for site-directed irreversible inhibition. When two concentrations of inhibitors are compared, the ratio of the rates cancels k or k'' from

$$\text{rate} = k\,(EI) \quad \text{(Eq. 8)}$$

the equation. Thus, the "rate-saturation" effect does not distinguish between the two mechanisms, but at best can only be taken as evidence that the active-site is involved in the inactivation.[3] However, consideration of structure-activity relationships make the bimolecular inactivation mechanism practically untenable (see *Discussion*).

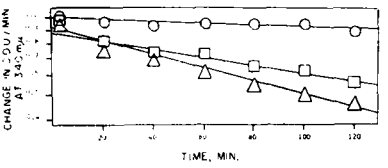


Fig. 3.—Comparative rate of irreversible inhibition of heart LDH·DPNH by 4-IGSA (V) and 5-(carbophenoxyamino)salicylic acid (IV). Key: O, heart LDH·DPNH control; □, 3 mM 5-(carbophenoxyamino)salicylic acid (IV); Δ, 4 mM 4-IGSA. The change in O.D. units/minute is proportional to the remaining enzyme concentration. (See *Materials and Methods.*)

DISCUSSION

Our earlier observation (12) that 4-(iodoacetylglycylamido)salicylic acid (V) could irreversibly inhibit LDH from either heart or skeletal muscle, but 4-(iodoacetamido)salicylic acid (I) showed irreversible inhibition of only the skeletal muscle LDH, has been further extended with six additional compounds. Of these six compounds, three (IV, VI, XI) showed irreversible inhibition of heart LDH.

Similar to the 4-series, 5-(iodoacetylglycylamido)-salicylic acid (VI) irreversibly inhibited both heart and skeletal muscle LDH (Table I), but in contrast, 5-(iodoacetamido)salicylic acid (II) showed irreversible inhibition only of the skeletal muscle LDH. The facts that (*a*) the two iodoacetyl compounds with a longer side chain (V, VI) showed irreversible inhibition of heart LDH, (*b*) the shorter side chain compounds (I, II) did not irreversibly inhibit heart LDH, but (*c*) all four compounds showed irreversible inhibition of skeletal muscle LDH, indicates that the position of the enzymatic nucleophilic group (that has been bridged to and alkylated) with respect to the binding points for the hydroxyl and carboxyl of the salicylate moiety is different in these two substrate-identical enzymes. Thus the 4- and 5-(iodoacetamido)-salicylic acids (I and II) show a specificity for irreversible inhibition of skeletal muscle LDH since the nucleophilic site is apparently closer to the binding site, but the nucleophilic site apparently cannot be bridged in heart LDH.

A working hypothesis of the differences in position of the nucleophilic site attacked by an iodoacetyl group can be gleaned by considering Fig. 1. The salicylate is probably ionically bound to the enzyme (horizontal crosshatch) by a cationic point and an imidazole NH on the enzyme surface (2). Within

[3] The authors thank Dr. David Pressman, Roswell Park Memorial Institute, for bringing this oversight to our attention.

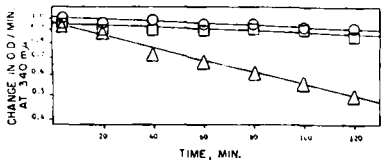


Fig. 4.—Comparative rate of irreversible inhibition of heart LDH·DPNH by 4-IGSA (V) and 4-(carbophenoxyglycylamido)salicylic acid (VII). Key: O, heart LDH·DPNH control; □, 4 m*M* 4-(carbophenoxyglycylamido)salicylic acid (VII); △, 4 m*M* 4-IGSA. The change in O.D. units/minute is proportional to the remaining enzyme concentration. (See *Materials and Methods.*)

the limits tolerated by the enzyme surface, the salicylate should be able to rotate in a cylinder on an axis which has as its radius a line segment connecting the two enzymic binding points. Thus, the enzyme and salicylic acid moiety in Fig. 1 would be a cross section of this cylinder. Attached to the salicylate at the 4- or 5-position can be a short or long side chain indicated by the short and long arrows. In its most extended conformation, the long side chain can rotate in a spherical segment with its center at the 4-position. Figure 1 then has one cross section of this spherical segment, *AJB*, which is in the plane of the benzene ring. *CJD* is then the cross-section circle segment for the 5-acetylglycylamido group of V, *EKF* the circle segment for the 4-acetamido group of I, and *GKH* the circle segment for the 5-acetamido group. The side chains can also fold to give a locus of spheres with the same center but shorter radii.

Since of the four compounds, only compounds V, VI, and XI can irreversibly inhibit heart LDH, the nucleophilic group most probably is in a three-dimensional figure with the cross section shown by the vertical hatch in Fig. 1 limited by the lines *EKH* and *CJB*. In contrast, the nucleophilic group of skeletal muscle LDH, which is attacked by both the long and short side chains of I, II, V, and VI most probably is inside a three-dimensional figure with a cross section limited to GDH on the outside and the benzene ring on the inside (shown as an angular cross-hatch); the longer side chains can fold back to the proper locus.

Another hypothesis is equally compatible with the current data. The relative position of the nucleophilic site could be identical in both enzymes, but a carbon chain may protrude from the heart LDH not present in skeletal muscle LDH that may require the side chain of the inhibitor to be sufficiently long to loop over the protruding group in heart LDH. Studies are being pursued to gain further evidence to differentiate between the two explanations or even give evidence for some yet unconceived explanation.

Although inactivation by bimolecular collision (tail-alkylation) cannot be eliminated unequivocally, considerations of structure-activity relationships make the bimolecular mechanism highly improbable. For example, *m*-(iodoacetamido)oxanilic acid (IX), an excellent reversible inhibitor of skeletal muscle LDH (Table I), shows no irreversible inhibition. Due to the increase in degrees of bond rotation in IX, compared to 4-ISA (I), there is no apparent reason why IX should not inactivate skeletal muscle LDH if I operates by bimolecular inactivation. A similar argument can be advanced against the bimolecular mechanism for inactivation of heart LDH. For example, 6-(iodoacetamido)-coumarin-3-carboxylic acid (XI) irreversibly inhibits heart LDH. Since XI is bulkier than I, there is no apparent reason why I should not inactivate heart LDH if XI had operated by the bimolecular mechanism.

A cross-over specificity was noted with 5-(carbophenoxyamino)salicylic acid (IV) (Fig. 3). *This compound irreversibly inhibited heart LDH, but showed no irreversible inhibition of skeletal muscle LDH* (Table I). That the active-site was most probably involved in this inactivation was shown by a "rate saturation effect"; doubling of the concentration from 2 m*M* to 4 m*M* increased the rate of inactivation by only 1.4-fold, compared to a "rate saturation" of 1.3 calculated from the apparent K_I for IV. In contrast (4-carbophenoxyamino)salicylic acid (III) irreversibly inhibited skeletal muscle LDH, but not heart LDH, even though a "rate saturation effect" was not observed.

It is clear that the enzymic nucleophilic group of heart LDH attacked by IV is most probably not the same as that attacked by V and VI. From Fig. 1, the carbophenoxy group has a locus of possible conformations limited to a sphere less than the cross-section line *GKH*, since only a two atom bridge is formed by IV and a three atom bridge by II. Since the carbophenoxy group can react only with an amino group of the enzyme surface, it is probable that this amino group reacts too slowly with I and II to be detected by our experimental design.

The carbophenoxy compounds with the longer side chains, VII and VIII, could theoretically assume conformations that place their urethane carbonyls in position to react with the same enzymic group of heart LDH attacked by the carbophenoxy group of IV. A possible explanation for the fact that VII (Fig. 4) and VIII do not irreversibly inhibit heart LDH is that the proper transition state cannot be assumed by the carbophenoxy groups of VII and VIII; the folding back of the side chain is apt to put the phenyl group in a position toward the nucleophilic site causing a hindrance by the phenyl group of the approach of the carbonyl to the enzymic nucleophilic site. Since 5-(carbophenoxyamino)salicylic acid (IV) irreversibly inhibits both heart LDH and GDH, it may be possible to place groups on the phenyl group which may sterically hinder the approach of the carbophenoxy carbonyl to the nucleophilic site of only one of these two enzymes; such a specificity for the transition state at the nucleophilic site is actively being sought in our laboratory.

Again the possibility that the carbophenoxyamino compounds operate by the bimolecular mechanism is remote. Since there would be less hindrance to the approach of the 4- and 5-(carbophenoxyglycylamino)salicylic acids (VI and VII) to a nucleophilic site for bimolecular reaction than IV or III, then VI and VII would be expected to inactivate heart or skeletal muscle LDH even more rapidly than IV or III; as stated before, VI and VII do not inactivate these two substrate-identical enzymes.

In conclusion, by use of both corollaries of the bridge principle of specificity, cross-over specificity

for selective irreversible inhibition of heart LDH, but not skeletal muscle LDH, and vice versa, has been found, which is attributed to exploitable differences in the secondary-tertiary structure of these two substrate-identical enzymes. Differences in secondary-tertiary structure of these two substrate-identical enzymes have previously been shown by amino acid analysis (24) and by antisera cross reactions (14, 24). The LDH from skeletal muscle and heart in the same animal are distinctly different, but the skeletal muscle LDH from different species are more similar; also, heart LDH from different species are more similar. Even though the studies in this paper were carried out with the LDH's from two distinct tissues of two species, these experiments give a first approximation of the selective inhibition that may be obtained in tissues of the same animal.

If this irreversible specificity for substrate-identical enzymes, which is presumably due to exploitable differences in the secondary-tertiary structure of the enzymes, can be carried over to such critical areas for cell division as (*a*) purine (19) or pyrimidine biosynthesis of (*b*) the folic cofactor area, the potential utility in chemotherapy would be obvious. Such studies in area (*a*) (13) and area (*b*) (9) are continuing in these laboratories.

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EVEN THOUGH plastics have made a great impact in the various facets of pharmacy and medicine, some problems have occurred which should indicate that perhaps there should be a slower pace to the introduction of these items to the health professions. This laboratory in the past has reported on some of these problems (1, 2). To the industrial and hospital pharmacist an appreciation and understanding of drug-plastic interactions would help minimize costly errors and at the same time provide the many advantages to be gained by the use of plastics.

The insoluble polyamides (various types of nylon) have a number of applications as parts or component parts in various apparatus where strength and resistance to thermal changes are needed. In pharmaceutical and medical applications these nylon parts[1] may come in contact with drug or biological products for varying periods of time. Depending upon a number of factors, selective drugs and chemical

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[1] For example, component parts of heart lung machines, containers, tubings, syringes, valve parts for aerosol bottles, etc.

for selective irreversible inhibition of heart LDH, but not skeletal muscle LDH, and vice versa, has been found, which is attributed to exploitable differences in the secondary-tertiary structure of these two substrate-identical enzymes. Differences in secondary-tertiary structure of these two substrate-identical enzymes have previously been shown by amino acid analysis (24) and by antisera cross reactions (14, 24). The LDH from skeletal muscle and heart in the same animal are distinctly different, but the skeletal muscle LDH from different species are more similar; also, heart LDH from different species are more similar. Even though the studies in this paper were carried out with the LDH's from two distinct tissues of two species, these experiments give a first approximation of the selective inhibition that may be obtained in tissues of the same animal.

If this irreversible specificity for substrate-identical enzymes, which is presumably due to exploitable differences in the secondary-tertiary structure of the enzymes, can be carried over to such critical areas for cell division as (*a*) purine (19) or pyrimidine biosynthesis of (*b*) the folic cofactor area, the potential utility in chemotherapy would be obvious. Such studies in area (*a*) (13) and area (*b*) (9) are continuing in these laboratories.

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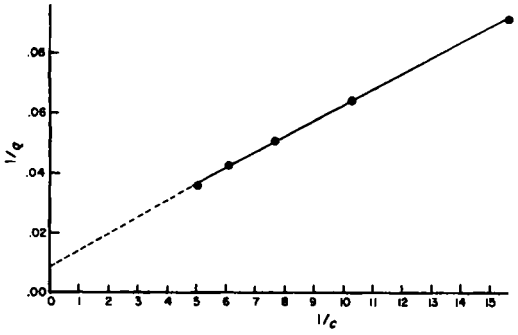


Fig. 1.—A Langmuir plot for the sorption of salicylic acid by nylon (41.5 C.°).

intervals aliquots of solution were withdrawn and the salicylic acid content remaining (in the solution) was determined by measuring the optical density of the sample at a wavelength of 296 mμ in a DU spectrophotometer, employing proper dilutions (using distilled water) and blanks. In all instances duplicate or triplicate tubes were run; the results reported in this paper are the average values of the two or three samples. The difference between the amount of salicylic acid remaining in the solution and the original concentration was then assumed to be the amount of the drug sorbed by the nylon.

Effect of Concentration on Equilibrium Sorption.—An experiment was conducted at a temperature of 41.5° to determine the equilibrium concentration of salicylic acid sorbed by nylon at different original concentrations. In all instances the strips of nylon were kept in contact with the solutions for sufficient periods of time to ensure that equilibrium had been reached.

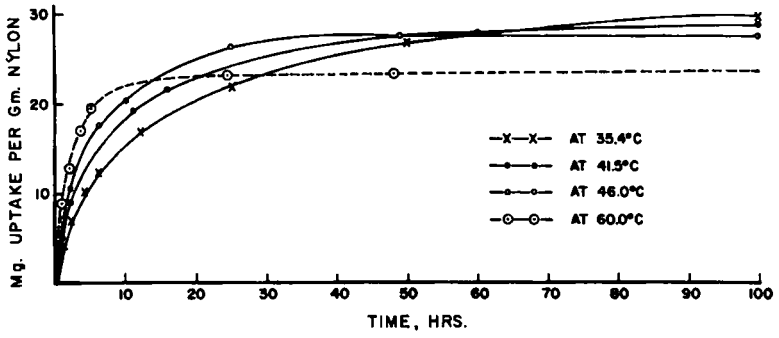


Fig. 2.—The effect of temperature on the sorption of salicylic acid.

agents might be sorbed by the nylon, reducing the concentration of the component in the solution.

Previous studies have revealed that nylon will sorb drugs and other agents having protonic groups (3, 4). This paper and others will present further information on drug-nylon interactions with the hope of relating structural configurations of solutes to their propensity to be sorbed by several types of nylon. This particular paper reviews the sorption of salicylic acid by nylon 66.

EXPERIMENTAL

General Procedures.—In all experiments reported in this paper, nylon 66 sheets[2] having a thickness of 0.012 in. were employed. The sheets were cut into strips measuring approximately 1 by 16 in., and soaked in a 50% alcohol-water solution for a period of 15 minutes, then repeatedly rinsed in distilled water to remove adhering contaminates. The strips were then dried to constant weight and placed into desiccators until ready for use. In any particular experiment, one or more strips were weighed accurately[3] and placed into a specially constructed glass tube. The particular solution (150 ml.) was then added and the tube closed by inserting a ground-glass stopper which was further secured to the tube by two metal springs. This tube and other tubes in the particular series were placed into a shaking device immersed in a constant temperature water bath. At various time

With Langmuir's equation in the following form

$$\frac{1}{q} = \frac{1}{KSC} + \frac{1}{S} \qquad \text{(Eq. 1)}$$

where q is the amount of salicylic acid sorbed at equilibrium in milligrams per gram of nylon, S is the saturation concentration when all sites in the nylon are occupied, C is the equilibrium concentration of the drug in solution (as grams per 150 ml.), and K a constant, a linear relationship was found when $1/q$ was plotted against $1/C$ for the range of concentrations employed in the experiment. From the intercept, the saturation value or S was calculated by the method of least squares and found to be equal to 117 mg./Gm. of nylon or 0.85 mole/Kg. (see Fig. 1).

Effect of Temperature on Sorption.—Nylon strips were kept in contact with 150 ml. of salicylic acid solution (0.15%), and at various time intervals the quantity of drug sorbed by the plastic was determined. This experiment was conducted at four different temperatures (35.4°, 41.5°, 46.0°, and 60.0°). A sorption isotherm is shown for each of these temperatures in Fig. 2, where milligrams of uptake per gram of nylon is plotted against time in hours.

Affinity and Heat of Sorption.—From equilibrium studies of sorption for salicylic acid by nylon, the standard affinity (5) was calculated using

$$-\Delta\mu^\circ = RT \ln C_s/C_l \qquad \text{(Eq. 2)}$$

where $-\Delta\mu^\circ$ is the difference in standard chemical potential between the solute in the liquid phase

[2] Polypenco Nylon 101, Polymer Corp. of Pennsylvania, Reading, Pa.
[3] Average weight of strips was 3.5 Gm.

(C_l) and the solute in the solid phase (C_s). The concentration terms for C_l and C_s are expressed for calculation purposes in moles per kilogram rather than in activities. For this specific nylon, standard affinities at several temperatures are shown in Table I.

From the relationship

$$-\frac{\Delta\mu^\circ}{T} = \frac{\Delta H^\circ}{T} + C \qquad \text{(Eq. 3)}$$

where $\Delta\mu^\circ$ is as previously defined, ΔH° is the standard heat of sorption, T is the absolute temperature, and C a constant, it is possible to evaluate ΔH° from the slope of the line when $-\Delta\mu^\circ/T$ is plotted against $1/T$. The heat of sorption was equal to -1.89 Kcal./mole in this case.

Diffusion of Salicylic Acid in Nylon.—To evaluate the apparent diffusion coefficient at one original concentration (0.15%) and at several temperatures, experiments were run as previously described where the uptake of a drug by the nylon was followed over a period of time at isothermal conditions. This type of experiment, where a limited volume of a solution is well agitated and in contact with a plane sheet, can be conveniently employed to accumulate data which can be treated by the method of Berthier (6, 7) using simple graphical procedures to evaluate the apparent diffusion coefficient.

The experimental data are plotted as M_t/M_∞ against the square root of time ($\sqrt{t}$) for each temperature as shown in Fig. 3. Correspondingly, for each temperature (each temperature has a different total uptake), a theoretical curve is plotted using the values of M_t/M_∞ (fractional uptake) *versus* $\sqrt{K}$ from Berthier's table (6). An example of this type of curve is shown in Fig. 4 for a total per cent uptake of 45.83 (at 35.5°). Clearly, it can be seen that at a particular M_t/M_∞ value from the experimental curve, a corresponding time (t) can be deduced and similarly at the same M_t/M_∞ value on the theoretical curve, a corresponding K can be found. Now since $K = Dt/l^2$ and since K, t, and l are known, it is a simple matter to calculate the diffusion coefficient D.

A second method which appears to give more accurate values for the apparent D is to determine the slopes of both the experimental and theoretical curves by the method of least squares. In this method the experimental curve may be expressed

$$M_t/M_\infty = S_e t^{1/2} \qquad \text{(Eq. 4)}$$

where M_t/M_∞ and t are the same as previously defined, and S_e is the slope of the line. Correspondingly, the theoretical curve may be expressed

$$M_t/M_\infty = S_t K^{1/2} \qquad \text{(Eq. 5)}$$

where S_t is the slope of the line and K is the same constant as defined before. When

TABLE I.—STANDARD AFFINITIES ($-\Delta\mu^\circ$) FOR SALICYLIC ACID AT SEVERAL TEMPERATURES[a]

Temp., °C.	$-\Delta\mu^\circ$ (Kcal./mole)
35.4	1.863
41.5	1.860
54.0	1.833
60.0	1.836

[a] Original concentration of solution: 225 mg. in 150 ml. Plastic sample: nylon 66.

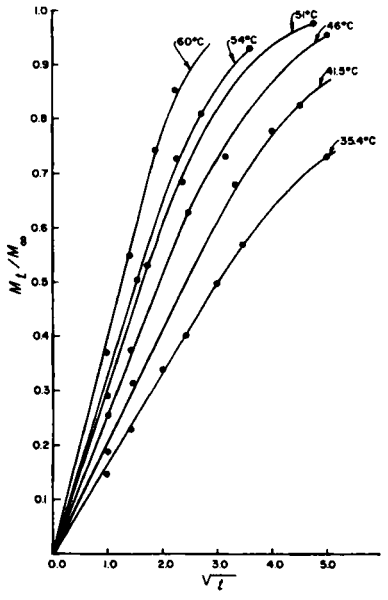


Fig. 3.—Fractional uptake of salicylic acid by nylon *vs.* square root of the time (in hours) at different temperatures.

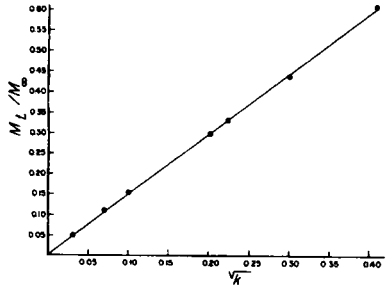


Fig. 4.—Theoretical plot of fractional uptake *vs.* K. From Berthier table (at total uptake of 45.83%).

$$M_t/M_\infty \text{ (experimental)} = M_t/M_\infty \text{ (theoretical)} \qquad \text{(Eq. 6)}$$

$$S_e t^{1/2} = S_t K^{1/2}$$

and since K is equal to Dt/l^2

$$S_e t^{1/2} = S_t (Dt/l^2)^{1/2} \qquad \text{(Eq. 7)}$$

or finally

$$D = \frac{S_e^2 l^2}{S_t^2} \qquad \text{(Eq. 8)}$$

Table II includes the data from the diffusion experiments with calculated apparent diffusion constants (by the slope method) for each temperature.

The influence of temperature may be noted by referring to Fig. 5. It follows a typical Arrhenius relationship which may be written

$$\log D = \log D_o + \Delta E/2.303\,RT \qquad \text{(Eq. 9)}$$

where D_o is a pre-exponential factor indicating the value of D at infinite temperature, ΔE the activation energy for diffusion, R the appropriate gas con-

TABLE II.—SORPTION DATA OF SALICYLIC ACID BY NYLON AS A FUNCTION OF TIME AND TEMPERATURE

Temp., °C.	t, Time in Hrs.	$\sqrt{t}$	M_t, mg./ Sample of Nylon	M_t/M_∞[c]	q, Mg. Sorbed per Gm. Nylon	S_e, Slope (Exptl.)	S_t, Slope (Theoret.)	D, Cm.²/Sec.
35.4	1	1.000	14.88	0.141	4.303	0.1701	1.443	...
	2	1.414	23.47	0.227	6.786	...	...	...
	4	2.000	35.21	0.341	10.178	...	...	...
	6	2.449	42.08	0.408	12.16	...	...	...
	9	3.000	50.95	0.494	14.79	...	...	8.97×10^{-10}
	12	3.464	58.68	0.569	16.96	...	...	...
	25	5.000	74.99	0.727	21.68	...	...	...
	50	7.071	92.02	0.892	26.60	...	...	...
	122.5	11.06	103.11[b]	...	29.58	...	...	...
	Total % Uptake (Amt. Removed from Soln. at Equilibrium) = 45.83							
41.5	1	1.000	18.89	0.190	5.296	0.2024	0.0409	...
	2	1.414	31.48	0.316	9.049	...	...	...
	11	3.317	66.94	0.674	19.23	...	...	...
	16	4.000	75.53	0.760	21.71	...	...	1.26×10^{-9}
	20	4.472	81.87	0.823	23.58	...	...	...
	84	9.165	99.34[b]	...	27.99	...	...	...
	Total % Uptake (Amt. Removed from Soln. at Equilibrium) = 44.17							
46.0	1	1.000	24.04	0.250	7.018	0.2575	0.0663	...
	2	1.414	36.07	0.376	10.42	...	...	...
	6	2.449	60.37	0.629	17.45	...	...	...
	10	3.162	64.56	0.725	20.10	...	...	2.08×10^{-9}
	25	5.000	91.31	0.952	27.57	...	...	...
	49	7.000	95.85[b]	...	...	...	...	...
	Total % Uptake (Amt. Removed from Soln. at Equilibrium) = 42.60							...
51.0	1	1.000	25.60	0.289	7.26	0.2986	1.418	...
	3	1.732	46.77	0.529	13.26	...	...	...
	5.50	2.345	60.14	0.681	17.05	...	...	2.73×10^{-9}
	22.5	4.761	84.44	0.956	23.94	...	...	...
	41.5	6.442	88.31[b]	...	24.49	...	...	...
	Total % Uptake (Amt. Removed from Soln. at Equilibrium) = 39.25							
54.0	2.5	1.581	45.67	0.500	12.47	0.3469	1.418	...
	5.0	2.236	66.51	0.728	18.17	...	...	...
	7.5	2.738	73.53	0.804	20.09	...	...	...
	13.0	3.605	84.78	0.928	23.16	...	...	3.86×10^{-9}
	25.0	5.000	88.08	0.964	24.06	...	...	...
	42.0	6.480	91.35[b]	...	24.36	...	...	...
	Total % Uptake (Amt. Removed from Soln. at Equilibrium) = 40.60							...
60.0	1.0	1.000	30.08	0.370	8.578	0.4213	0.1775	...
	2.0	1.414	44.55	0.549	12.70	...	...	...
	3.5	1.870	60.12	0.741	17.14	...	...	...
	5.0	2.236	69.05	0.851	19.68	...	...	5.90×10^{-9}
	24.0	4.899	81.13[b]	...	23.38	...	...	...
	Total % Uptake (Amt. Removed from Soln. at Equilibrium) = 36.14							

[a] M_t/M_∞ = Fractional uptake. [b] M_∞ = Final uptake at each temperature.

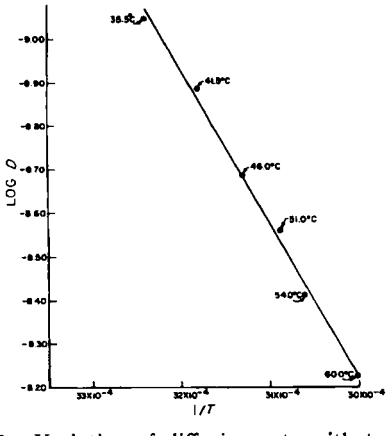


Fig. 5.—Variation of diffusion rate with temperature.

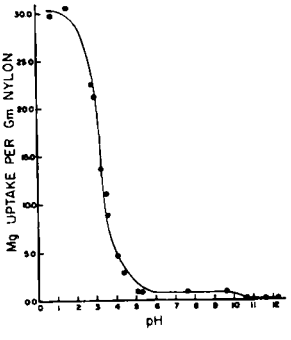


Fig. 6.—Effect of pH on the sorption of salicylic acid by nylon (41.5°C.).

stant, and T the absolute temperature. From the slope of the line ($\Delta E/2.303\ R$), ΔE may be calculated. In this case with the specific nylon samples

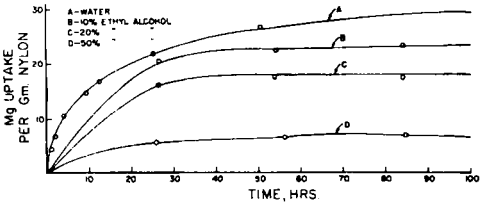


Fig. 7.—Effect of ethyl alcohol on the sorption of salicylic acid (0.15%) by nylon (41.5°C.).

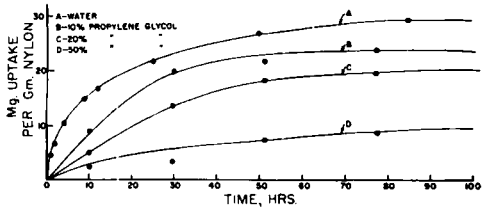


Fig. 8.—Effect of propylene glycol on the sorption of salicylic acid (0.15%) by nylon (41.5°C.).

used, the activation energy (ΔE) of diffusion was equal to 20.5 Kcal./mole.

Effect of pH on Sorption.—Salicylic acid solutions having an original concentration of 0.15% were adjusted to various hydrogen ion concentrations using hydrochloric acid at very low pH values and sodium hydroxide at the higher values. These were placed in contact with nylon strips as in the previous experiments. The tubes were placed in a constant temperature water bath having a temperature of 41.5° for a period of time to insure that equilibrium had been reached. The amount of salicylic acid which was sorbed by the nylon was then determined as well as the final pH of the solution. These data were plotted as milligrams uptake per gram of nylon against pH as shown in Fig. 6.

Effect of Solvents on Sorption.—Experiments were conducted to determine the effect several solvents might have on the uptake of salicylic acid. Several concentrations of alcohol–water, propylene glycol–water, glycerin–water, and polyethylene glycol 400–water were kept in contact with strips of nylon and the uptake of salicylic acid at various times observed. Sorption data of these results are shown in Figs. 7–10.

DISCUSSION

As has been demonstrated in the experiments reported in this paper, salicylic acid will be sorbed by nylon. The sorption of the drug may be thought of as an intermolecular reaction between binding sites in the polymer and the drug. An analysis of the chemical structure of nylon 66 reveals three polar sites which could attract solute particles. The structure of the plastic may be and shows a carboxylic group at one end and an amine group at the other end of the chain. In the chain proper, on every sixth carbon, an amide linkage is present. It may be seen clearly that the amide groups are far in excess of the terminal end groups. The binding of weak organic acids and phenols by both soluble and insoluble polyamides has been noted in the literature.

Much work has been performed on the dyeing of synthetic yarns with dyes which can be classified as organic acids. A nearly analogous situation may be drawn in the sorption of a drug by nylon as the dyeing of nylon yarn by a particular dye. Peters (8) has demonstrated both theoretically and experimentally that at pH values of 3 and above an ionic interaction takes place between the terminal amino group which in this pH range has a positive charge and the anion of the drug. The total amount of dye sorbed, however, is small and tends to be limiting. As the pH of the solution is decreased, a sudden surge of uptake or binding occurs; here the interaction is assumed to occur on the very weakly basic nitrogen in the amide which can take on a hydrogen atom (at very low pH

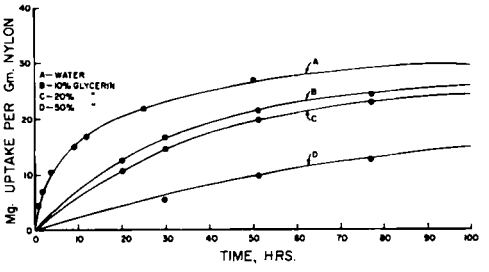


Fig. 9.—Effect of glycerin on the sorption of salicylic acid (0.15%) by nylon (41.5°C.).

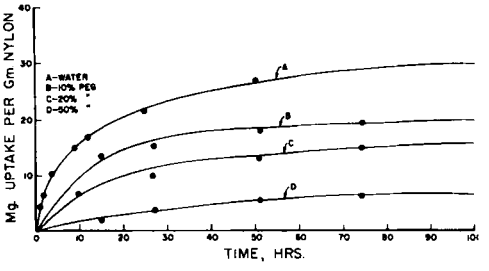
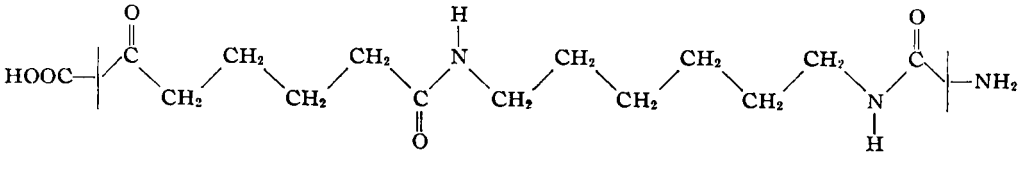


Fig. 10.—Effect of polyethylene glycol 400 on the sorption of salicylic acid (0.15%) by nylon (41.5°C.).

values). This positive site then attracts the negatively charged dye anion. In a later paper, O'Briain and Peters (9) indicated that high uptake at low pH may also be due to hydrolysis of the nylon permitting more binding sites than originally present.

In the case of salicylic acid the over-all pattern is the same, as may be noted by referring to Fig. 6. At high pH values little or no interaction is seen, but as the pH is decreased, the degree of binding



increases greatly. Here it may be assumed that in the pH range of 6 to 9, most of the binding is occurring at the amine end groups. The plateau effect indicates that all the available sites are filled and that the drug is bound as the salicylate ion. Below pH of 6.0 the large uptake of the drug may be ascribed to the binding of the drug in the unionized form through hydrogen bonding with the carbonyl group in the numerous amide linkages. Even at the lower pH values (below 3.0) this mechanism is probably still operating, and in this instance the mechanism differs from the case of the dye. At the very low pH values all of the drug is in the unionized form and it would seem difficult to vision an interaction of a solute having protonic characteristics to be attracted to a positive site (on the amide nitrogen). The organic dyes even at low pH values are dissociated to a significant degree and can thus appear as anions. In fact, Cannon (10) has excluded the possibility of phenolic compounds interacting with the amide nitrogen and postulates hydrogen bond formation with carbonyl groups. It is interesting to note that above a pH of 9.5 little sorption is detected, and this may be attributed to the loss of positive charges on the end terminal amines.

In the pH region of high uptake, one must consider that secondary valence forces are probably operating in stabilizing the interaction. This reasoning has already been demonstrated by other workers who have experimentally proved that the hydrophobic characteristics of the drug or agent can materially influence the degree of binding (11, 12).

What has been elucidated above is most likely an oversimplification of a highly complex mechanism. Obviously, other factors either recognized or completely obscured are surely influencing the uptake.

As might be expected (Table I) the standard affinity tends to decrease as the temperature is increased. This type of occurrence has been noted for other polymer-solute interactions and can be viewed as meaning that at the higher temperature the kinetic energy of the molecules of both the solute and the polymer is greater than at lower temperatures which in turn prevents proper orientation of the solute to the binding site and eventual loss of the solute from the particular site.

The heat of sorption (-1.89 Kcal./mole) found for salicylic acid (with the specific nylon 66 used in the experiment) indicates the low energy requirement for binding and supports the contention that dipole-dipole interactions (hydrogen bonds) with secondary valence forces are in operation.

The apparent diffusion values, as predicted by theory, increase as the temperature is increased (Table II). The diffusion values, as stated, are apparent values since a number of assumptions have been made (*i.e.*, diffusion in only one direction, constant D as diffusion takes place in the plastic, etc.).

From the apparent diffusion coefficient data at several temperatures it was possible to calculate the activation energy of diffusion which was found to be 20.5 Kcal./mole. The activation energy may be looked upon as the energy necessary for the solute to overcome the cohesive force existing between the polymers and other obstacles to migration.

The effect of concentration on sorption (equilibrium) which is shown as a Langmuir plot in Fig. 1 reveals a linear relationship. It should not be assumed that this linear relationship would continue had other concentrations been employed. Previous work in this laboratory has demonstrated that anomalous isothermal curves can result in different concentration zones. This point needs more attention both experimentally and theoretically.

The replacement of water with less polar solvents such as alcohol, glycerin, propylene glycol, and PEG 400 decreased the sorption of salicylic acid at the 20 and 50% levels. With hydrophilic plastics such as nylon, the water will have a great degree of attraction to the polymer and will in a sense help plasticize the nylon permitting greater ease of the solute to diffuse within the plastic.

SUMMARY

The sorption of salicylic acid by nylon 66 was studied at a number of conditions. Physical constants such as saturation value (S), standard affinity ($-\Delta\mu°$), standard heat of sorption ($\Delta H°$), apparent diffusion coefficients (D), and activation energy of diffusion (ΔE) were evaluated. Results of the various experiments indicate that the rate-determining step in the uptake of salicylic acid by nylon 66 is the diffusion of the solute within the polymeric structure of the plastic. The low energy requirement of sorption and the results from the pH experiments support the contention that the salicylic acid is most likely interacting in the undissociated state with basic groups in the nylon (amides) through hydrogen bonding. Secondary valence forces appear to stabilize the interaction. Finally, a decrease in the polarity of the solvent system decreases the extent of sorption.

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Influence of Chemical and Physical Factors on Biological Responses to Neomycins B and C

By W. T. SOKOLSKI, C. G. CHIDESTER, and D. G. KAISER

The two known components of the neomycin complex—neomycins B and C—have been shown to respond differently under the usual conditions of bioassay. Potassium phosphate, commonly used as a diluent, antagonizes both the activities of the neomycins and the growth of *Staphylococcus aureus*. The antagonism is greater against neomycin C than against neomycin B. The same effect occurs with potassium chloride. Both components adsorb to agar and are released by salts. There is an apparent difference in binding to Bacto agar compared to Noble agar with binding of B and C less on Noble agar. The potency values in neomycin bioassay will vary depending on the B:C ratios of the test and standard preparations and the method used for assay. This is probably the cause of many lab-to-lab differences in potency results. A critical analysis of existing methods and standards and some agreement on the method and the definition of neomycin standards is recommended.

THE TERM "NEOMYCIN" usually refers to mixtures of neomycin B and neomycin C. The "United States Pharmacopeia" (1) makes no direct mention of components or relative amounts of B and C that may be present. Kaiser (2) has shown that production lots from various sources which he examined varied in neomycin C content from 6.2 to 28.5%. The differences in the B:C ratio between neomycin preparations may be a factor in assay problems of day-to-day, lab-to-lab, and method-to-method variations.

The structures of neomycins B and C have been reported by Rinehart, *et al.* (3, 4), as 4-ring compounds; the only difference between the two components, excluding glycosidic linkages which have not all been established, is the optical configuration of an aminomethyl group in a pyranose sugar. As similar as the two structures are, however, the biological activities in broth (5–7) and in agar (7–9) are sometimes markedly different. This paper is a report on some factors which influence the responses to neomycins B and C in liquid and agar media.

METHODS AND RESULTS

Two lots of each component, neomycins B and C, were used in these studies. The neomycin C preparations, 2733-R2 and 2569-MEB, were found to be free (<1%) of neomycin B in a radioisotopic assay (2). The neomycin B preparations, 2732-R2 and 2689-MEB, contained approximately 4 and 11% neomycin C, respectively. Chemical and physical properties of the MEB preparations were described by Ford and co-workers (10).

Effect of Potassium Phosphate on Neomycin Activity.—Salts have been reported to antagonize the antibacterial activity of neomycin (11, 12). Since phosphate buffer is commonly used as a diluent in assay procedures (8, 13–15), the effect of phosphate on the activity of neomycin may be a factor in the assay response.

Minimum inhibitory concentration (MIC) end points were determined for neomycin B and neomycin C in broth, designated SAB, consisting of 0.15% beef extract, 0.3% yeasts extract, and 0.6% peptone adjusted to pH 7.9 before autoclaving. Sterile solutions of potassium chloride and phosphate at pH 7.9 were added to the media after autoclaving for final concentrations of 0.1 *M*. Readings of growth or no growth were made after 24 hours incubation at 32°. Table I shows the greater antagonism of both salts to neomycin C than to B and the greater antagonism to both neomycins with potassium phosphate than with potassium chloride.

Effect of Potassium Phosphate on the Growth of S. aureus.—*Staphylococcus aureus* FDA 209-P is the test organism of choice for neomycin assays in the U. S. Food and Drug Administration laboratories (14). The effect of potassium phosphate on *S. aureus* was examined because any influence on the test organism could be a factor in the antibiotic response in the assay system. The growth with phosphate buffer was compared with the growth with trishydroxymethylamino methane (tris) buffer.

Cell suspensions of *S. aureus* were prepared by diluting a culture grown in SAB 1:1000 with water to approximately the numbers of organisms encountered in an assay plate. Further dilutions and viable counts were made with the usual poured-agar-plate technique. One milliliter of diluted cell suspension was added to one end of the plate, and 1 ml. of water or 10× strength pH 7.9 phosphate or tris buffer was added to the other end. Care was taken so that the buffer did not contact the organisms before the agar was added. Eight milliliters of 10/8 strength Bacto streptomycin assay agar (SAA) or neomycin assay agar (NAA) were added to each plate, and the entire contents was mixed. All plates were incubated for 24 hours at 37°. Three experiments were conducted with results indicating that 0.1 *M* potassium phosphate antagonized the growth of *S. aureus* (Table II), while 0.1 *M* tris buffer did not.

Adsorption of Neomycin on Agar.—Adsorption tests were conducted by adding antibiotic to a 1.5%

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TABLE I.—EFFECT OF SALTS ON THE ACTIVITY OF NEOMYCIN AGAINST *S. aureus* IN pH 7.9 BROTH

Salt	Minimum Inhibitory Concn., mcg./ml.[a] — Neomycin B — Expt. 1	Expt. 2	Expt. 3	Expt. 4	Neomycin C — Expt. 1	Expt. 2
No salt control	0.2 (>0.1)	0.15 (>0.1)	0.1 (>0.05)	0.2 (>0.1)	0.2 (>0.1)	0.5 (>0.2)
0.1 *M* potassium chloride	1.0 (>0.5)	0.75 (>0.5)	0.5 (>0.2)	1.0 (>0.5)	5 (>2)	5 (>2)
0.1 *M* potassium phosphate	10 (>5)	7.5 (>5)	5 (>2)	10 (>5)	50 (>20)	75 (>50)

[a] Numbers in parentheses are the highest concentrations tested in which growth occurred.

TABLE II.—VIABLE COUNTS OF *S. aureus* SUSPENSIONS IN BUFFERED MEDIA

Expt.	Medium	Concn. of Buffer	Viable Count[a]
1	NAA	Water control	6.9×10^5
	NAA	0.05 *M* phosphate	3.7×10^5
	NAA	0.1 *M* phosphate	1.2×10^5
	SAA	Water control	7.0×10^5
	SAA	0.05 *M* phosphate	3.5×10^5
	SAA	0.1 *M* phosphate	1.9×10^5
2	NAA	Water control	4×10^6
	NAA	0.1 *M* phosphate	3×10^5
	SAA	Water control	2.5×10^6
	SAA	0.01 *M* phosphate	2.5×10^6
	SAA	0.05 *M* phosphate	5×10^5
	SAA	0.1 *M* phosphate	3×10^6
3	NAA	Water control	4×10^5
	NAA	0.05 *M* phosphate	2.8×10^5
	NAA	0.1 *M* phosphate	3.2×10^6
	NAA	0.05 *M* tris	4×10^6
	NAA	0.1 *M* tris	4×10^6

[a] On agar containing phosphate, the colonies were appreciably smaller than on agar without buffer. The colonies looked normal on agar with tris buffer.

agar solution at 50°, allowing the agar to gel, spinning the gel in an ultracentrifuge with a force of 50,000 × g for 2 hours, and assaying the supernatant liquid. The radioisotopic assay procedure reported by Kaiser (2) was used for the 1 mg./ml. or higher concentrations. Bioassays were used for the lower concentrations. In the bioassay procedure, standard and test solutions were diluted with supernatant liquid removed from agar gel in order to keep the solutions consistent. Potencies of the supernatant test solutions were estimated from standard curves using neomycin B as standard for B solutions and neomycin C for C solutions. Per cent binding was calculated by subtracting the per cent antibiotic remaining in the supernatant liquid after centrifugation from 100.

Table III shows that neomycin components were less adsorbed to Noble than to Bacto agar. When 3% potassium chloride was added to the system, no adsorption of neomycin B or C was detected.

Effect of the Agar on Neomycin Dose-Responses.—Because of the apparent difference in binding between Bacto and Noble agar, dose responses were obtained with neomycins B and C in media comparing the two agars. Streptomycin assay broth was prepared and adjusted to pH 7.9. Each agar was added to an aliquot of the broth for 1.5% solutions, and both media preparations were autoclaved. Ten-milliliter single layer test plates were made with agar seeded with 1% of a *S. aureus* culture incubated overnight at 32° in SAB. Antibiotic solutions were made in water. Each solution was added to four cylinders, one on each of four plates. The plates were incubated for 18 hours at 32°, and zone diameters were averaged for each solution. (Figure 1 indicates greater sensitivity with medium containing Noble agar than with medium containing Bacto agar.) The responses from neomycin B are almost identical to those from neomycin C.

Effect of Potassium Phosphate on the Response to Neomycin B in an Agar-Diffusion System.—Dose-responses were obtained comparing neomycin B in 0.1 *M* pH 7.9 potassium phosphate buffer to neomycin B in 0.1 *M* pH 7.9 tris buffer. The test plates were made as described in the preceding section, but only with the medium prepared with Noble agar. Figure 2 indicates lesser sensitivity but a slightly steeper slope with tris buffer.

Effect of Salt on the Activity of Neomycin B and Neomycin C.—Neomycin activity is known to be antagonized by salts (11, 12). The activity of neomycin C was reported (7) to be markedly suppressed by potassium chloride, more so than B, in

TABLE III.—ADSORPTION OF NEOMYCIN TO AGAR

Antibiotic	Concn. Antibiotic in Agar Gel, mg./ml.	% Bound[a] — Bacto Agar	Noble Agar
Neomycin B	0.05	...	56, 73
	0.20	...	62
	0.50	...	30, 40
	1.0	58, 67	35
	2.0	...	25
	10.0	...	0
Neomycin C	0.05	...	70, 80
	0.20	...	65
	0.50	...	36, 45
	1.0	43, 46, 53	25, 32
	2.0	...	18
	10.0	...	...

[a] Multiple numbers indicate results of different experiments.

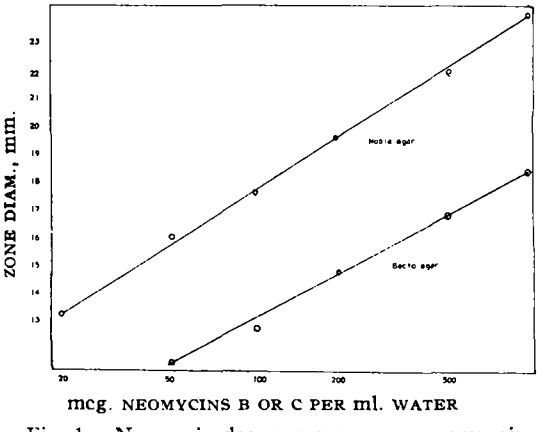


Fig. 1.—Neomycin dose-response curves comparing agars.

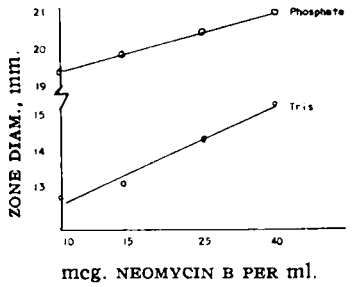


Fig. 2.—Neomycin standard dose-response curves comparing buffers.

an agar-diffusion system. This system was used to assay neomycin B in the presence of neomycin C (8). The difference in response between neomycin B and neomycin C in an agar diffusion system suggested studies on the effect of salt on the activity of each component in broth and agar systems.

Minimum inhibitory concentrations in SAB with and without 3% potassium chloride were determined for neomycin B and neomycin C against *S. aureus*. Each tube was inoculated with 0.1 ml. of a 1:100 dilution of a culture in SAB incubated overnight at 32°. The MIC end points shown in Table IV indicate that the salt antagonized neomycin C activity to a greater extent than it did B.

Since the antagonistic effect of salt may be different in agar and broth systems, a more intensive study was made of antagonism in agar against two species of bacteria reportedly used in neomycin assays, *S. aureus* FDA 209 (1–14) and *Bacillus subtilis* UC564 (8, 13–15). In a series of eight experiments, gradient plates were prepared according to the method described by Szybalski (16) with a 10-ml. SAA lower layer without antibiotic and a 10-ml. upper layer with antibiotic. In the plates with salt, 3% potassium chloride was added to both layers. The concentrations of antibiotic in the upper layer for plates without salt were 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 mcg./ml.; the concentrations for plates with salt were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mcg./ml. Each concentration was tested in duplicate plates. Cell suspensions of approximately 10 (7) cells per milliliter were streaked across the gradient with cotton swabs, and the plates were incubated at 32° for 24 hours. The MIC for each plate was determined by multiplying the fractional length of the streak by the antibiotic concentration in the top agar layer. Fractions of one-tenth or less and nine-tenths or more of the streaks were not used in the determinations. The results in Table V indicate a large day-to-day variation in MIC end points, but an analysis of salt antagonism ratios indicates that salt antagonizes neomycin C more than it does B. Also, the extent of antagonism differs with the two test organisms.

DISCUSSION

Many factors contribute to the responses obtained in agar-diffusion assays. To minimize the inescapable day-to-day variations in bioassays, the analyst attempts to standardize those factors which influence the response of the active agent. For some agents, designing an assay is routine; a buffered diluent, a nutrient medium, a susceptible test organism, an optimum temperature for growth, and the usual standardization of steps in the procedure. For many agents, the problems in developing an assay lead to "monumental headaches." The headaches become astronomical when the agent is a complex whose individual components react differently in the commonly accepted assay systems, particularly when the components are difficult to separate and the content ratios vary between test and standard preparations. Such is the case with neomycin.

Phosphate buffer, used as a diluent, is probably a cause of some of the day-to-day variation in neomycin assays. It antagonizes the activity of neomycin, more so than potassium chloride (Table I), but more important, it antagonizes the growth of the commonly used test organism, *S. aureus* (Table II). This is supported by the greater sensitivity to neomycin in phosphate buffer than in tris buffer (Fig. 2). The flatter slope obtained with phosphate buffer may be caused by the antagonism of the phosphate to the growth of the test organism, allowing concentrations of neomycin to be inhibitory which would be subinhibitory without phosphate.

Consider that potassium phosphate and neomycin are diffusing into the agar independently of each other. This is a situation in which two substances influence an assay response and only one is supposedly measured.

Neomycins B and C are both adsorbed to agar. The adsorption appears to be less on Noble agar than on Bacto agar (Table III). This is supported by the increased activity of neomycin in medium with

TABLE IV.—EFFECT OF 3% POTASSIUM CHLORIDE ON THE ACTIVITY OF NEOMYCIN AGAINST *S. aureus* IN BROTH

Concn. Antibiotic, mcg./ml.[a]	Neomycin B[b]				Neomycin C[b]			
	No salt		With KCl		No salt		With KCl	
	35 hrs.	72 hrs.	35 hrs.	72 hrs.	35 hrs.	72 hrs.	35 hrs.	72 hrs.
0	++	++	++	++	++	++	++	++
0.05	+±	+±	...	...	++	++	...	...
0.1	−±	±±	...	...	−−	++	...	...
0.2	−−	−−	++	++	−−	−−	++	++
0.5	−−	...	++	++	−−	−−	++	++
1.0	−−	...	++	++	−−	−−	++	++
2.0	−−	...	++	++	−−	−−	++	++
5.0	...	...	−−	−−	...	...	++	++
10.0	...	...	−−	−−	...	...	+−	++
20.0	...	...	−−	−−	...	...	−−	−−

[a] Each concentration was tested in duplicate. [b] Each sign represents a growth response in one tube. +, Good growth; ±, slight growth; and −, no growth.

TABLE V.—EFFECT OF 3% POTASSIUM CHLORIDE IN AGAR ON THE ACTIVITY OF NEOMYCIN

	Minimum Inhibitory Concn., mcg./ml.							
	B. subtilis				*S. aureus*			
	Neomycin B		Neomycin C		Neomycin B		Neomycin C	
Expt.	No Salt	With Salt	No Salt	With Salt	No Salt	With Salt	No Salt	With Salt
1	0.005	0.09	0.01	1.00	0.02	0.40	0.03	1.80
2	0.01	0.16	0.03	2.70	0.02	0.60	0.05	2.80
3	...	...	...	...	0.02	0.70	0.03	3.20
4	0.01	0.05	0.02	0.34	0.02	0.12	0.03	0.48
5	0.02	0.03	0.03	0.62	0.02	0.16	0.02	0.65
6	0.01	0.08	0.05	1.20	0.02	0.17	0.04	1.00
7	0.01	0.2	0.02	3.20	0.02	0.62	0.02	2.40
8	0.01	0.1	0.03	3.00	0.02	0.52	0.04	2.40
Av.	0.01	0.11	0.03	1.72	0.02	0.41	0.03	1.84
MIC Salt / MIC No salt	11		57		20		61	
C MIC Salt / MIC No salt / B MIC Salt / MIC No salt	5.4 ± 1.4 (95% Conf.)				3 ± 0.5 (95% Conf.)			

Noble agar (Fig. 1). Since salt releases the bound neomycin, a plausible explanation for the change in slope in the presence of salt as reported by Sokolski and Carpenter (8) is that neomycin is not bound in the salt agar and diffuses more freely. Noble agar with the lesser binding of neomycin may be a better agar to use for a lower day-to-day variation in the assay.

Salts appear to antagonize the activity of neomycin C more than B (Tables I and V). It is evident then that the ionic strength of the medium is an important factor for standardizing the assay procedure.

We have attempted to point out some of the factors that affect the responses of neomycins B and C in bioassay systems. Standardization of the neomycin assay necessitates an understanding of these factors.

Since the neomycin C content varies from lot-to-lot, the potency of one particular preparation will depend on the B:C ratio in the test and standard preparations. The variation of assay results between laboratories will depend on these ratios plus the differences in assay systems and how much of the C responds in these systems.

If neomycin C is included in the definition of neomycin, as it apparently is, then the present day assay methods should be made to assay the B and C additively. The Food and Drug Administration assay (14) performed in our laboratories measures only 50% of the C. Since all of the neomycin preparations tested in our laboratories contain some neomycin C, the problem becomes one of definition of potency. If the present concept of neomycin potency is retained, then rigid policies establishing universal standards and assay methods should be adopted. If neomycin C is to be considered as neomycin, then an assay design for the additive response to B and C would be more realistic than the existing methods.

SUMMARY

Salts antagonize the response of neomycin in agar-diffusion assay systems. Potassium phosphate is more antagonistic than is potassium chloride, and both salts antagonize neomycin C more than B.

The effect of 0.1 *M* buffers on *S. aureus*, a commonly used assay organism, indicated that potassium phosphate antagonized growth, while tris did not.

Neomycins B and C are both adsorbed to agar gel and desorbed with potassium chloride. The adsorption phenomenon slows neomycins diffusion in agar. Adsorption is less on Noble agar than on crude agar.

The extent of antagonism in an agar-diffusion system is more pronounced against *S. aureus* than against *B. subtilis*, with neomycin C being more antagonized than B. This emphasizes the importance of choice of test organisms for assays on preparations containing neomycin C.

Agreement on assay methods and standards are recommended for elimination of lab-to-lab differences in neomycin potency estimates.

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Lawsone Derivatives II

Sulfonium Mustard Precursors and 1-Naphthylisocyanate Complexes

By H. MACHATZKE and W. R. VAUGHAN

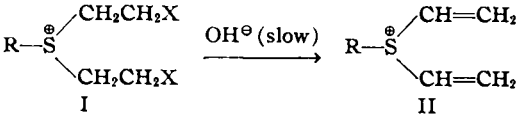
An approach to synthesis of derivatives of 2-hydroxy-1,4-naphthoquinone (lawsone) bearing the bis-β-chloroethylsulfonium group in a 3-alkyl side chain is described along with incidental formation of a related hemi sulfur mustard. In addition, the unexpected formation of what appear to be charge-transfer complexes of lawsone derivatives with 1-naphthylisocyanate is reported for the first time. The free 2-hydroxyl of lawsone appears to be essential for complexation, since in its absence insoluble complexes do not form. The presence of a 3-alkyl substituent also appears to be necessary since lawsone itself does not afford an insoluble complex.

ALTHOUGH IT IS not the purpose of this paper to report on or suggest the existence of significant physiological properties in the various compounds described but rather to delineate problems encountered in the course of a specific type of synthesis which may be of use in other contexts, it nevertheless seems appropriate to describe the rationale for undertaking the synthesis considered here.

A previous paper concerned with the potential use of lawsone derivatives bearing "alkylating" functions in a 3-alkyl side chain, pointed out that initial attempts afforded only very unstable products owing to facile reversal of the Mannich reaction whereby they were produced. Hence, it was desirable to prepare higher homologs wherein the alkylating group was farther removed from the quinone nucleus. To this end the preparation of ω-substituted 3-polymethylene lawsones was investigated with the replacement of the terminal substituent by the bis-β-chloroethylamino group as the objective. However, the chemistry of such compounds proved to be unexpectedly complex owing to a surprising tendency for even decamethylene groups with terminal ester or halogen substituents to cyclize both inter- and intramolecularly under the influence of diethanolamine (1).

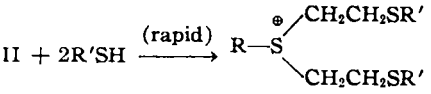
Since these anomalous reactions appear to depend upon the basic character of diethanolamine, attention was directed toward introduction of the sulfonium mustard system. Sulfonium salts of structure I are known to undergo a characteristic *elimination* in a basic medium (Scheme I) (2).

The reactivity of bis-vinyl sulfonium ions such as II toward nucleophiles, especially mercaptans at physiological pH (Scheme II) has been demonstrated (3), and sulfonium salts such as I (X = bromine or chlorine) have been shown as a consequence to be cytostatically active (3–5). Thus, synthesis of a number of compounds of type I, wherein R = (2 - hydroxy - 1,4 - naphthoquinon - 3 - yl)polymethylenyl, was undertaken.



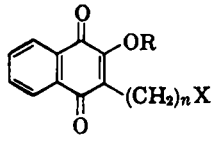
I II

Scheme I



Scheme II

In each case a 3-ω-halopolymethylenelawsone (III) was used to alkylate bis-β-hydroxyethylsulfide (IV) in refluxing ethanol.

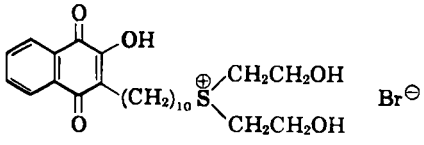


III

(*a*) R = H, *n* = 3, X = Cl
(*b*) R = CH_3, *n* = 3, X = Cl
(*c*) R = H, *n* = 10, X = Br

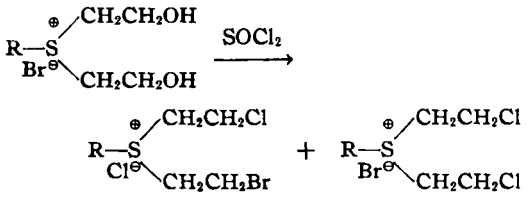
The reaction of III*a* (6) with IV was abortive, with only starting materials being recovered, and the reaction of III*b* afforded cyclized materials (6) as in the diethanolamine reactions cited above. Consequently, attention was focussed on III*c* (7) as the alkylating agent for IV.

This reaction was successful; 60% of the desired product, bis-β-hydroxyethyl-10(2-hydroxy-1,4-naphthoquinon-3-yl) decylsulfonium bromide (V), was obtained.



In addition, a by-product, 2-hydroxy-3(10-hydroxydecyl)-1,4-naphthoquinone (VI), was obtained, presumably from V by an SN reaction with adequate precedence (8).

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See Vaughan, W. R., Habib, M. S., McElhinney, R. S., Takahashi, N, and Waters, J. A., *J. Org. Chem.*, **26**, 2392 (1961).
This investigation was supported by Grant CY-2961 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.



Scheme III

Since previous work (9) had shown that thionyl chloride reacts with compounds such as I (R = OH, anion = $Br^{\ominus}$) with appreciable exchange of ionic bromide and covalent chloride in the product (Scheme III) and since V may well exist, at least in part, as the thetine and hydrogen bromide owing to the pronounced acidity of the 2-hydroxyl of the lawsone system, a model compound, bis-β-hydroxyethyl-10-carboxydecylsulfonium bromide (VII), was prepared for study by alkylating IV with 10-bromoundecanoic acid (10), a reaction which produced tris-β-hydroxyethylsulfonium bromide as a by-product. Treatment of VII with thionyl chloride at 0–5° in chloroform, followed by treatment of the resultant colorless oil with perchloric acid afforded an oily product (VIII) whose composition most nearly corresponds to that of a 3:1 mixture of the thetine, β-bromoethyl-β-chloroethyldecylsulfonium-10-carboxylate and its hydroperchlorate.

This result was unpromising for a similar conversion of V, but since the reaction represented by Scheme I may also involve X=OCOCH$_3$, conversion of VII to the diacetyl fluoborate (IX) and bromide (X) by treatment with boron trifluoride etherate in acetic anhydride (11) (for IX) followed by ethereal hydrogen bromide addition to methanolic IX (for X) was carried out. Unlike the colorless crystalline IX, bis-β-acetoxyethyl-10(2-hydroxy-(XI), 1,4-naph-thoquinon-3-yl)decylsulfonium fluoborate prepared analogously from V, was a brown-yellow oil.

The reaction of V with thionyl chloride proved as complex as expected, and in only one instance was a readily characterizable unique product obtained; at 60° the expected mixed bis-β-haloethylsulfonium halide (XII) decomposed to give a small quantity of β-chloroethyl-10(2-hydroxy-1,4-naphthoquinon-3-yl)decyl sulfide (XIII). At the recommended lower reaction temperature (4) XII showed considerable instability; hence conversion to a perchlorate was attempted (XIV). The infrared spectrum of XIV does not show the characteristic hydroxyl of the lawsone system, but the microanalysis is not far out of line for $C_{24}H_{32}Cl_2O_8S \cdot HClO_4$.

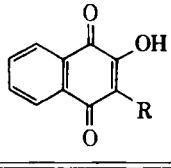
Since the initial reaction product of V with thionyl chloride was too unstable to permit analysis, an attempt was made to effect reaction of its 2-hydroxyl group with 1-naphthylisocyanate (XV) to provide a more tractable and crystalline product. However, on treatment of XII with XV in chloroform, another unstable oil was obtained (XVI). Examination of the infrared spectrum of XVI showed a strong band at 2260 cm.$^{-1}$ due to isocyanate as well as a broad band at 3220 cm.$^{-1}$, neither of which could be removed by repeated treatment with chloroform. Furthermore, the carbonyl band at 1640 cm.$^{-1}$ was appreciably less intense than in the spectrum of XII, and there was a slightly greater than 10% increase in absorbance for the ultraviolet band at 3270 Å.

These observations led us to suspect the formation of a complex with XV rather than urethane formation. And since both XII and XIV appeared to be too unstable for satisfactory study, V (the precursor of XII) was investigated. Similar treatment with XV afforded a yellow crystalline precipitate (XVII) as did III*c* (XVIII) and 6-(2-hydroxy-1,4-naphthoquinon-3-yl)hexanoic acid (XIX) which afforded XX.

The acetyl derivatives of XIX (XXI) as well as lawsone itself, 2-chloro-1,4-naphthoquinone, and 2-methoxy-3(3-chloropropyl)-1,4-naphthoquinone do not afford insoluble complexes with XV under similar conditions.

Analyses indicate that the complexes (XVII, XVIII, XX) comprise two molecules of the lawsone moiety and one of 1-naphthylisocyanate. Each of them shows the characteristic isocyanate band (2260 cm.$^{-1}$) in the infrared spectrum in addition to one hydroxyl band, which is shifted from 3310–3315 cm.$^{-1}$ (sharp) to lower frequency and broadened (3220 cm.$^{-1}$). Table I presents the significant infrared data, and except for the bands listed, the spectra are essentially unchanged from

TABLE I.—PRINCIPAL INFRARED BANDS IN FREE LAWSONE DERIVATIVES AND THEIR 1-NAPHTHYLISOCYANATE COMPLEXES



R		OH (cm.$^{-1}$)	C=O (cm.$^{-1}$) α	C=O (cm.$^{-1}$) β	N=C=O (cm.$^{-1}$)
$(CH_2)_5CO_2H$	Free	3310 (Sharp)	1660 (sh.)	1640	2260
	Complex	3220 (Broad)	1670	1645[b]	2260
$(CH_2)_{10}Br$	Free	3315 (Sharp)	1660 (sh.)	1645	...
	Complex	3220 (Broad)	1670	1645[b]	2260
$(CH_2)_{10}\overset{\oplus}{S}(CH_2CH_2OH)_2Br^{\ominus}$	Free	3220[a]	1670	1640	...
	Complex	...	1670	1640[b]	2260
1-Naphthylisocyanate	...	...	...	...	2260

[a] The shift of the hydroxyl frequency is not visible because of the additional hydroxyl groups in the side chain. [b] Less intense than in the free quinone.

those of the constituent parts (12) which are characteristic for the quinone system and the 1-naphthylisocyanate.

Infrared studies have shown two carbonyl bands: one (α) "normal" at about 1670 cm.$^{-1}$, and one shifted and weakened due to hydrogen bonding (β) (13–15). In the lawsone complexes a decrease in absorption band β and an increase in absorption band α is apparent. Consequently, it would appear that there may be less hydrogen bonding to the carbonyl groups of the quinone moieties, in spite of an increase in total hydrogen bonding shown by the more pronounced shift in the hydroxyl absorption. Failure of lawsone derivatives lacking the free hydroxyl to form complexes suggests that this hydrogen bonding is essential but not necessarily sufficient for complex formation.

The visible-ultraviolet spectra of the complexes compared with those of the quinone constituents show changes characteristic of charge-transfer or π-complexes (16). In acetone solution two of the complexes show a strong absorption at 3270 Å., compared to absorption at about 3310 Å. for the free quinones, with marked increase in the absorbances of the former. Pertinent data are collected in Table II.

Whether the complexes are of a "sandwich" type with one lawsone derivative bonded to the isocyanate nitrogen by a hydrogen bridge and the other to the opposite face of the isocyanate by charge-transfer attraction cannot be stated with the evidence at hand; but the possibility is attractive and accounts for the spectrographic phenomena as well as for the failure to detect complexes where the lawsone hydroxyl is masked. The role of the 3-substituent is not clear, but failure to obtain a complex with lawsone itself implies a contributory factor which is not associated with the ability of the system to hydrogen bond, since all of the lawsone derivatives examined are necessarily weaker acids than lawsone itself. It should be noted that whatever the structure of the complex may prove to be, the charge-transfer character has but a single precedent in which the quinone is the electron donor; phloroglucinol with two benzoquinones (17).

EXPERIMENTAL[1]

2 - Methoxy - 3 - (3 - chloropropyl) - 1,4 - naphthoquinone (III*b*).—The diacyl peroxide of 4-chlorobutyric acid was obtained in 46% yield by the hydrogen peroxide method (18). The alkylation of lawsone was effected by the "flash-off" method (18). Fractional crystallization from methanol or methanol-water afforded the quinone (III*b*) in 45% yield (based on recovered lawsone), m.p. 67–68°.

Anal.—Calcd. for $C_{14}H_{13}ClO_3$: C, 63.50; H, 4.95; Cl, 13.40. Found: C, 63.72; H, 4.91; Cl, 13.29.

Bis - β - hydroxyethyl - 10(2 - hydroxy - 1,4-naphthoquinon-3-yl)decylsulfonium Bromide (V).—A solution of 5.1 Gm. (0.013 mole) of 2-hydroxy-3(10 - bromodecyl) - 1,4 - naphthoquinone (III*c*) (8) and 6.0 Gm. (0.05 mole) of bis-β-hydroxyethyl sulfide (IV) in 50 ml. of 90% ethanol was refluxed for 2 days. Next the solvent was removed on the steam bath and ether was added; the resultant oily precipitate was repeatedly recrystallized by solution in methanol or methanol-acetone followed by addition of ether. The product was a yellow, crystalline substance which dissolves in water to give a red solution; 3.5 Gm. (53%), m.p. 87–88°*k*.

Anal.—Calcd. for $C_{24}H_{35}BrO_5S$: C, 56.00; H, 6.83; Br, 15.50; S, 6.23. Found: C, 55.74; H, 6.84; Br, 16.05; S, 6.45.

2 - Hydroxy - 3(10 - hydroxydecyl) - 1,4 - naphthoquinone (VI).—The ether-soluble residues from several preparations of V were triturated with petroleum ether (40–60°), and upon evaporation of the solvent there remained a yellow crystalline product which was recrystallized once from methanol and twice from acetone, m.p. 80–81°*k*. Approximately 10–20% (based on III*c*) was recovered in this manner.

Anal.—Calcd. for $C_{20}H_{28}O_4$: C, 72.70; H, 7.93. Found: C, 72.80; H, 8.26.

Bis - β - Hydroxyethyl - 10 - carboxydecylsulfonium Bromide (VII).—A solution of 7.0 Gm. (0.026 mole) of 10-bromoundecanoic acid (10) and 3.7 Gm. (0.03 mole) of bis-β-hydroxyethyl sulfide (IV) in 50 ml. of 80% ethanol was refluxed for 3 days. The solvent was then removed *in vacuo* and the oily residue taken up in methanol. Upon addition of ether there precipitated white crystals which were recrystallized three times from methanol to which ether was added to effect reprecipitation; 4.5 Gm. (45%), m.p. 110–111°*k*.

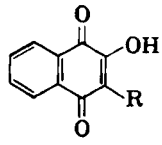
Anal.—Calcd. for $C_{16}H_{31}BrOS$: C, 46.60; H, 8.08. Found: C, 46.87; H, 8.11.

tris - β - Hydroxyethylsulfonium Bromide.—When a large excess of IV was used in the preceding preparation, the product was rather difficult to purify and under these circumstances fractional recrystallization afforded *tris*-β-hydroxyethylsulfonium bromide (19) in substantial amounts, m.p. 96–97°*k*.

Anal.—Calcd. for $C_6H_{15}BrO_3S$: C, 29.20; H, 6.13; Br, 32.40; S, 12.93. Found: C, 29.70; H, 6.20; Br, 31.73; S, 12.98.

Reaction of VII with Thionyl Chloride Followed with Perchloric Acid (VIII).—To a solution of 3.9

TABLE II.—SIGNIFICANT ULTRAVIOLET ABSORPTION BANDS IN FREE LAWSONE DERIVATIVES AND THEIR 1-NAPHTHYLISOCYANATE COMPLEXES IN ACETONE SOLUTION



R	Free Quinone λ max.	Free Quinone ϵ^a	Complex λ max.	Complex ϵ^a
$(CH_2)_5CO_2H$	3310	2800	3270	5900
$(CH_2)_{10}Br$	3310	2900	3270	5400
$(CH_2)_{10}\overset{\oplus}{S}(CH_2CH_2OH)_2Br^{\ominus}$	3270	3000	3270	3400

[a] Molecular absorbance. Complex calculated for two quinones plus one isocyanate.

[1] Melting points are uncorrected; Kofler block indicated by superscript *k* where used. Microanalyses by Spang Microanalytical Laboratory, Ann Arbor, Mich. Infrared spectra of Nujol mulls obtained with Perkin-Elmer model 21 double beam recording spectrometer. Ultraviolet spectra obtained with Cary model II double beam spectrometer. The authors are indebted to Mr. B. E. Wenzel for preparing certain of these spectra.

TABLE III.—ANALYTICAL DATA FOR 1-NAPHTHYLISOCYANATE COMPLEXES

Complex No.	M.p., °C.	Quinone	Compn. (2Q·XV)		Anal. C	H	Br	N	S
XVI	Oil	XII					Unstable		
XVII	106°	V	$C_{59}H_{77}Br_2NO_{11}S_2$	Calcd.	59.10	6.47	13.20	1.17	5.35
				Found	59.02	6.50	13.30	1.40	5.44
XVIII	70°	IIIc	$C_{51}H_{67}Br_2NO_7$	Calcd.	64.10	6.02	8.40	1.46	...
				Found	64.01	6.18	8.69	1.40	...
XX	117°	XIX	$C_{43}H_{39}NO_{11}$	Calcd.	69.20	5.27	...	1.88	...
				Found	69.50	5.54	...	1.74	...

Gm. (0.01 mole) of VII in 50 ml. of chloroform at 0° (ice-bath) was added slowly and with magnetic stirring 6.0 Gm. (0.05 mole) of purified thionyl chloride. After 2 hours the reaction was allowed to come to room temperature and stirring was continued for 3 additional hours. Addition of dry ether caused a colorless oil to separate, but the latter could not be crystallized. Consequently, an attempt was made to convert it to the perchlorate by solution in water and dropwise addition of perchloric acid until no further precipitate was formed. The resultant oil was dried *in vacuo* for analysis (2.5 Gm.).

Anal.—Calcd. for $C_{15}H_{28}BrClO_2S$ (A) (thetine): C, 46.40; H, 7.27; Hal (Cl), 18.30; S, 8.25.

Calcd. for $C_{15}H_{29}BrClO_6S$ (B) (thetine hydroperchlorate): C, 37.00; H, 6.00; Hal (Cl), 21.80; S, 6.57.

Calcd. for 3A + 1B: C, 43.50; H, 6.90; Hal (Cl), 19.40; S, 7.75. Found: C, 43.10; H, 7.12; Hal (Cl), 20.80; S, 7.40.

Bis - acetoxyethyl - 10 - carboxydecylsulfonium Fluoborate (IX).—A 0.01-mole sample of VII in 10 ml. of acetic anhydride was treated dropwise with 4 ml. of redistilled boron trifluoride etherate (11) and then allowed to stand at room temperature for 24 hours. Addition of dry ether afforded a colorless crystalline substance which was recrystallized from absolute methanol, precipitation being effected by addition of dry ether; m.p. 85–86°, yield 62%.

Anal.—Calcd. for $C_{19}H_{35}BF_4O_6S$: C, 48.60; H, 7.55; S, 6.85. Found: C, 48.38; H, 7.45; S, 6.56.

Bis - β - acetoxyethyl - 10 - carboxydecylsulfonium Bromide (X).—A 2-Gm. sample of IX was dissolved in 5 ml. of absolute methanol, and a dry ether solution of hydrogen bromide was added. The treatment was repeated, after which the product was repeatedly washed with dry ether, then dried *in vacuo* over both phosphorus pentoxide and sodium hydroxide; 76%, m.p. 85–86°[k].

Anal.—Calcd. for $C_{19}H_{35}BrO_6S$: C, 48.40; H, 7.50; Br, 16.80. Found: C, 48.51; H, 7.63; Br, 16.40.

Bis - β - acetoxyethyl - 10(2 - acetoxy - 1,4-naphthoquinon - 3 - yl)decylsulfonium Fluoborate (XI).—A 2.5-Gm. (0.01 mole) sample of V was treated in acetic acid with redistilled boron trifluoride etherate as for VII. The solution became red, but addition of the dry ether produced a brown-yellow oil which was "recrystallized" as for IX; 2.5 Gm. (92%).

Anal.—Calcd. for $C_{30}H_{41}BF_4O_8S$: C, 55.50; H, 6.38; S, 4.96. Found: C, 55.32; H, 6.33; S, 4.96.

Reaction of V with Thionyl Chloride (XII).—A 2.6-Gm. sample of analytically pure V in 20 ml. of chloroform was cooled in an ice-bath and treated dropwise with purified thionyl chloride while being magnetically stirred. Subsequent work-up was the same as for VIII. The product, a yellow oil, was reprecipitated three times from absolute methanol by addition of dry ether, but upon drying *in vacuo* it was apparent that decomposition was setting in. Consequently, conversion to the perchlorate (XIV below) was attempted. Infrared and ultraviolet spectra were obtained on freshly prepared samples.

β - Chloroethyl - 10 - (2 - hydroxy - 1,4 - naphthoquinon - 3 - yl)decylsulfide (XIII).—In a similar reaction to the preceding the temperature was allowed to reach 60°. The product was extracted with petroleum ether and the extract then recrystallized three times from petroleum ether to give yellow crystals, m.p. 64–65°[k].

Anal.—Calcd. for $C_{22}H_{29}ClO_3S$: C, 64.50; H, 7.13; Cl, 8.68; S, 7.82. Found: C, 64.63; H, 7.03; Cl, 8.96; S, 7.56.

The infrared spectrum showed the characteristic hydroxyl band of the lawsone system.

Reaction of XII with Perchloric Acid (XIV).—The brown-yellow oil (XII, above) was dissolved in 20 ml. of water and treated dropwise with concentrated perchloric acid until no further precipitation occurred. After refrigeration the water was decanted and the oily residue washed twice with distilled water and then twice "recrystallized" from methanol-ether. The resultant very viscous oil was dried *in vacuo* over phosphorus pentoxide.

Anal.—Found: C, 49.40; H, 5.60; Hal (Cl), 22.30; S, 6.25.

Ethyl 6 - (2 - Hydroxy - 1,4 - naphthoquinon-3-yl)hexanoate (XIX Ethyl Ester).—This compound was prepared from lawsone and the peroxide obtained from the acid chloride of ethyl hydrogen pimelate by the Fieser procedure (20). The product was taken up in ether and washed with potassium bicarbonate until the extracts were nearly colorless; recrystallized from petroleum ether (90–100°), 69%, m.p. 72–74°.

Anal.—Calcd. for $C_{18}H_{20}O_5$: C, 68.40; H, 6.33. Found: C, 68.34; H, 6.24.

6 - (2 - Hydroxy - 1,4 - naphthoquinon - 3 - yl)-hexanoic Acid (XIX).—A solution of 16 Gm. of the ethyl ester of XIX in 10 ml. of methanol was dropped into 300 ml. of a 30% aqueous potassium hydroxide solution containing 20 Gm. of sodium hydrosulfite (20), and the resultant solution was refluxed for 2 hours, then allowed to stand overnight at room temperature, after which it was acidified with hydrochloric acid and extracted with ether. The ether extract was then washed thoroughly with water and dried over magnesium sulfate. Evaporation afforded the quinone which recrystal-

lized from methanol-water (three times); 9.5 Gm. (65%), m.p. 113–114°.

Anal.—Calcd. for $C_{16}H_{16}O_5$: C, 66.65; H, 5.61. Found: C, 66.50; H, 5.80.

6 - (2 - Acetoxy - 1,4 - naphthoquinon - 3 - yl)-hexanoic Acid (XXI).—The boron trifluoride procedure described for IX and XI (11) was applied to 15 Gm. of XIX in 150 ml. of glacial acetic acid. After 2 hours at room temperature, water was carefully added and the yellow crystalline precipitate was recrystallized from methanol-water; 9.0 Gm. (53%), m.p. 85–86°.

Anal.—Calcd. for $C_{18}H_{18}O_6$: C, 65.50; H, 5.50. Found: C, 65.58; H, 5.31.

Complexes with 1-Naphthylisocyanate (XV): XVI, XVII, XVIII, XX.—The following procedure (XV + V) was also applied to XV and III*c*, XII, and XIX. A solution of 2 Gm. of V in 10 ml. of absolute chloroform was prepared by gentle warming, and 2–3 ml. of XV was added dropwise, whereupon yellow crystals immediately precipitated. Upon cooling the product was filtered off and an infrared spectrum taken. It was then washed 10 times with absolute ether and dried over phosphorus pentoxide. No change in the intensity of the 2260 cm.$^{-1}$ band in the spectrum was observed after washing; 1.8 Gm. (77%), m.p. 106°.

The same complex was obtained by refluxing a chloroform solution of V and XV for 5 hours. Neither the lawsone nor the primary alcohol groups in the side chain were affected.

When the complex was dissolved in absolute ethanol, the ethyl urethane was readily formed, and the quinone moiety could be recovered by addition of ether to the reaction mixture.

Analytical data for the complexes are collected in Table III.

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Munz recognizes 67 species of *Aquilegia* (5) distributed throughout the world. Of these, there are approximately 24 species in North America.

It is readily apparent when examining monographs on *Aquilegia* (5, 6) that most species have several synonymous names which would lead one to believe that a larger number of species must have been considered to exist prior to the thorough study of the genus. *Aquilegias* are found throughout the entire United States and were considered at one time for the title of

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lized from methanol-water (three times); 9.5 Gm. (65%), m.p. 113–114°.

Anal.—Calcd. for $C_{16}H_{16}O_5$: C, 66.65; H, 5.61. Found: C, 66.50; H, 5.80.

6 - (2 - Acetoxy - 1,4 - naphthoquinon - 3 - yl)-hexanoic Acid (XXI).—The boron trifluoride procedure described for IX and XI (11) was applied to 15 Gm. of XIX in 150 ml. of glacial acetic acid. After 2 hours at room temperature, water was carefully added and the yellow crystalline precipitate was recrystallized from methanol-water; 9.0 Gm. (53%), m.p. 85–86°.

Anal.—Calcd. for $C_{18}H_{18}O_6$: C, 65.50; H, 5.50. Found: C, 65.58; H, 5.31.

Complexes with 1-Naphthylisocyanate (XV): XVI, XVII, XVIII, XX.—The following procedure (XV + V) was also applied to XV and III*c*, XII, and XIX. A solution of 2 Gm. of V in 10 ml. of absolute chloroform was prepared by gentle warming, and 2–3 ml. of XV was added dropwise, whereupon yellow crystals immediately precipitated. Upon cooling the product was filtered off and an infrared spectrum taken. It was then washed 10 times with absolute ether and dried over phosphorus pentoxide. No change in the intensity of the 2260 cm.$^{-1}$ band in the spectrum was observed after washing; 1.8 Gm. (77%), m.p. 106°.

The same complex was obtained by refluxing a chloroform solution of V and XV for 5 hours. Neither the lawsone nor the primary alcohol groups in the side chain were affected.

When the complex was dissolved in absolute ethanol, the ethyl urethane was readily formed, and the quinone moiety could be recovered by addition of ether to the reaction mixture.

Analytical data for the complexes are collected in Table III.

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TABLE I.—SPECIES OF *Aquilegia* SCREENED

Species	Source	No. Plants	Av. Dried Wt. per Root, Gm.	Methanol Extractable Material in Dried Roots %
A. canadensis	Gardens of the Blue Ridge, Ashford, N. C.	50	8.34	18
A. vulgaris erecta edelweise	American Perennial Gardens, Garden City, Mich.	25	7.32	36
A. skinneri	American Perennial Gardens, Garden City, Mich.	25	3.64	21
A. oxysepala	American Perennial Gardens, Garden City, Mich.	15	1.67	10
A. Jonesii	American Perennial Gardens, Garden City, Mich.	15	0.20	..
A. canadensis nana	American Perennial Gardens, Garden City, Mich.	15	18.2	40
A. chrysantha	Springbrook Gardens, Mentor, Ohio	50	9.84	44
A. chrysantha alba	Springbrook Gardens, Mentor, Ohio	50	6.56	25
A. McKana hybrid	Springbrook Gardens, Mentor, Ohio	50	10.4	40
A. caerulea	Wayside Gardens, Mentor, Ohio	100	9.83	40

national flower (7). As with many plants, *Aquilegia* has acquired various common names. In many sections of the United States it is known incorrectly as honeysuckle (8, 9). Probably the most widespread common name of *Aquilegia* is columbine. Two references to the medicinal use of the genus *Aquilegia* have been encountered. Culpeper (10) wrote in 1814 that the leaves of columbine were commonly used in lotions for the treatment of sore mouths and throats, and that seeds of the plant were used in treatment of jaundice and as an oxytocic. He reported also that Spaniards used the root to treat kidney stones. Felter and Lloyd reported (11) in 1884 that columbine possessed antiscorbutic, diuretic, and diaphoretic properties. They also wrote that Linnaeus regarded the plant as toxic to children.

The alkaloids magnoflorine, berberine, and aquileginine have been isolated from *Aquilegia hybrida* var. Scott-Elliott (16).

Extracts of *A. hybrida* var. Scott-Elliott and authentic samples of magnoflorine chloride and

TABLE II.—SUMMARY OF R_f VALUES OF *Aquilegia* SPECIES QUATERNARY FRACTION

Species	Solvent System	No. of Alkaloidal[a] Spots	Fluorescence, U.V.	R_f
A. hybrida, Scott-Elliott	PAW[c]	1	Blue (magnoflorine)	0.54
	BAW[d]	1	Blue (magnoflorine)	0.55
	PEW[e]	1	Blue (magnoflorine)	0.26
A. canadensis	PAW	2	Blue	0.55
			Nonfluorescent	0.83
	BAW	2	Blue	0.55
			Nonfluorescent	0.85
	PEW	2	Blue	0.26
			Nonfluorescent	0.63
A. canadensis nana	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. vulgaris erecta edelweise	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. skinneri	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. oxysepala[b]	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. Jonesii[b]	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. chrysantha	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. chrysantha alba	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. McKana hybrid	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26
A. caerulea	PAW	1	Blue	0.55
	BAW	1	Blue	0.55
	PEW	1	Blue	0.26

[a] Stained with Dragendorff's reagent. [b] Represents total extractive. [c] Propanol, ammonium hydroxide, water; 2:1:1. Butanol, acetic acid, water; 5:1:4 (top phase, ascending). [e] Pyridine, ethyl acetate, water; 310:750:165.

TABLE III.—SUMMARY OF R_f VALUES OF *Aquilegia* SPECIES TREATED CHLOROFORM FRACTION

Species	Solvent System	No. of Alkaloidal[a] Spots	Fluorescence, U.V.	R_f
A. hybrida Scott-Elliott	PAW[b]	2	Yellow (berberine)	0.58
			Nonfluorescent[e]	0.85
	BAW[c]	2	Yellow (berberine)	0.41
			Nonfluorescent[e]	0.84
	PEW[d]	2	Yellow (berberine)	0.35
			Nonfluorescent[e]	0.54
A. McKana hybrid	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54
A. chrysantha	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0 35
			Nonfluorescent	0.54
A. chrysantha alba	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54
A. canadensis	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54
A. vulgaris erecta edelweise	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54
A. skinneri	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54
A. canadensis nana	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54
A. caerulea	PAW	2	Yellow	0.58
			Nonfluorescent	0.85
	BAW	2	Yellow	0.41
			Nonfluorescent	0.84
	PEW	2	Yellow	0.35
			Nonfluorescent	0.54

[a] Stained with Dragendorff's reagent. [b] Propanol, ammonium hydroxide, water; 2:1:1. [c] Butanol, acetic acid, water; 5:1:4 (top phase, ascending). [d] Pyridine, ethyl acetate, water; 310:750:165. [e] Identical with the R_f value of the nonfluorescent compound, aquileginine, isolated from *A. hybrida* var. Scott-Elliott.

berberine chloride were used as reference samples in this study, the object of which was the screening of ten additional species of *Aquilegia* for the presence or absence of the alkaloids present in *A. hybrida* var. Scott-Elliott.

EXPERIMENTAL

Procurement of Plant Material and Extraction of Roots.—Whole plants of the *Aquilegia* species listed in Table I were obtained as indicated by the source of supply.[1] The plants were dried in an oven at 60° and the aerial portions separated from the roots. The dried roots were ground to a coarse powder in a Wiley mill. The number of plants obtained and the resulting average dry weight per root is reported for each species in Table I. This information is given to afford an appreciation of the variance in plant size of the species under investigation. One-hundred grams of each species (except for *A. Jonesii*, *A. skinneri*, and *A. oxysepala* which yielded lower weights as indicated in Table I) were extracted in a Soxhlet apparatus with methanol. Extraction was carried to exhaustion as indicated by a negative Valser's test. The extracts were concentrated to

[1] A plant of each species is being grown to serve as a herbarium specimen.

dryness and the weights recorded (Table I). Each total extract was dissolved in dilute acid (aqueous hydrochloric, pH 2), made alkaline with ammonium hydroxide solution, and extracted with chloroform in a rotary film extractor.[2] This procedure yielded two fractions: an aqueous alkaline mother liquor (quaternary fraction) and a chloroform fraction.

Paper Chromatography.—The descending technique was utilized (except with one solvent system where the ascending technique was used) as indicated in Tables II and III. Sheets of Whatman No. 1 paper were employed. Extracts were chromatographed simultaneously with reference extracts and authentic alkaloids. The spotted paper sheets were equilibrated for several hours prior to development. Developed, air-dried chromatograms were viewed under long wave ultraviolet light to mark fluorescent spots and were then sprayed with Dragendorff's reagent (13) to reveal positive alkaloidal spots. All R_f values were calculated from measurements taken at the center of stained spots.

Quaternary Fractions.—The aqueous alkaline mother liquor from each species was made acid (pH 4) with 10% HCl, and the quaternary bases were precipitated with ammonium reineckate after the procedure of Panouse (14) as modified by Hogg (15). The alkaloidal reineckates were converted to the corresponding alkaloidal chlorides *via* the method of Kampfhammer (16). The resulting alkaloidal chlorides were dissolved in alcohol and chromatographed simultaneously with the corresponding fraction from *Aquilegia hybrida*, var. Scott-Elliott and with an authentic sample of magnoflorine chloride. The results are indicated in Table II.

Chloroform Fractions.—The chloroform fraction from each species was concentrated and chromatographed along with the corresponding fraction from the Scott-Elliott variety. However, because of extraneous matter, the chloroform fractions yielded brown areas on developed chromatograms which made positive alkaloidal spots difficult to discern with Dragendorff's reagent. It was then found that the alkaloids from this fraction could be extracted in purer form from an acid solution with chloroform. Therefore, each chloroform fraction was concentrated to dryness, and the residue was dissolved in dilute hydrochloric acid (10%) and again extracted with chloroform in a rotary film extractor. The resulting clear brown chloroform fractions were then chromatographed simultaneously with a known sample of berberine chloride and the corresponding chloroform fraction from *A. hybrida* var. Scott-Elliott as references. The results are listed in Table III. Because of a limited supply of plant material, a chloroform fraction was not prepared from *A. oxysepala* and *A. Jonesii*.

DISCUSSION

All ten species of *Aquilegia* examined contained alkaloids in the roots. On the basis of paper chromatographic data obtained with three solvent systems (Tables II and III), a total of only four different alkaloids appeared to be present in the entire group. Three of these compounds were chromatographically identical with the three alkaloids isolated from *Aquilegia hybrida* var. Scott-Elliott—namely, berberine, magnoflorine, and aquileginine (12).

Magnoflorine was present in every species screened in this study. In the case of three species (*A. Skinneri*, *A. McKana*, and *A. canadensis*), its identity was confirmed by the actual isolation of crystalline magnoflorine chloride (15). In the case of two species (*A. Jonesii* and *A. oxysepala*) magnoflorine was the sole alkaloid detected; however, the apparent absence of other alkaloids may be caused by the very limited amount of plant material available in these two instances.

All species investigated (except *A. Jonesii* and *A. oxysepala*) appeared to contain berberine. Since the completion of this work, however, we have been forced to the conclusion that berberine and the closely related palmatine cannot be distinguished satisfactorily by paper chromatography (17). In view of this fact, the possibility that palmatine may occur in *Aquilegia* species cannot be discounted at this time.

Every species containing berberine also contained a third alkaloid apparently identical with aquileginine, a new crystalline quaternary alkaloid found in *A. hybrida* var. Scott-Elliott (12). In addition, a fourth alkaloid, not yet characterized, was detected only in *A. canadensis*. Both the nature of this fourth alkaloid and the constitution of aquileginine are under continued investigation.

[2] Rinco liquid-liquid extractor, Scientific Glass Apparatus Co.

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Immunoassay for Extracted Insulin

By W. E. BAUM*, W. F. BROWN, and R. E. CRABTREE

A rapid, accurate, and reproducible assay has been developed for commercial insulin. The assay is based on the competition between the insulin sample to be analyzed and I^{131}-labeled insulin for binding to guinea pig anti-insulin antibody. Antibody bound insulin is separated from free insulin by the salt precipitation technique of Grodsky and Forsham. This procedure is described in detail together with the adjustment of variables that must be made. Examples are given to show the influence of the purity of the samples, species differences, statistical variation, and specificity of the assay. Comparisons with the mouse convulsion and U.S.P. rabbit assays show that this immunological approach is valid and has many advantages over the *in vivo* assays.

FOR MANY YEARS a method has been sought to supplant the small animal assays used to control the development and production of insulin. The requirements for a new assay were (*a*) that it not be subject to the biological variations exhibited in live animals, (*b*) that it yield results in a few hours, (*c*) that it be relatively inexpensive to operate, and (*d*) that it be sensitive to hormonal activity in preparations having specific activities from 0.5 to 26.0 u./mg. and yet not be too sensitive to operate in areas where insulin contamination is a possibility.

The introduction of the immunochemical assay of Grodsky and Forsham (1), which uses salt precipitation of antibody bound insulin-I^{131}, provided for the first time a procedure which met these requirements.

The present study was directed at determining the capability of the assay with regard to the number of samples that could be assayed, its ability to assay low purity samples, its specificity for hormonally active protein, its statistical reproducibility, and its ability to assay insulin from various species. In the process of the investigation, data were collected on technique and the effect of possible chemical interference.

We have made several studies comparing the immunochemical assay per Grodsky (1) with the mouse convulsion assay (2) and with the "twin-crossover" rabbit blood-sugar-lowering assay (3).

Though there are other aspects to investigate, it is our belief that this flexible and reliable assay procedure should be made available to others interested in the assay of insulin.

MATERIALS AND METHODS

Insulin Antibody Production.—A modification of the procedure of Morse (4) is used to prepare insulin in adjuvant. Crystalline zinc-insulin is dissolved in 37.0 ml. of insulin diluent (3) at pH 3.2. This solution is then mixed with 25.0 Gm. of heavy liquid petrolatum and 12.5 Gm. of Aquavor (Dukes Laboratories). The mixture is homogenized in a Virtis 45 homogenizer. Enough insulin is used to make a final concentration of about 0.5 mg./ml.

Young guinea pigs (250 Gm.) of either sex are given subcutaneous injections of 0.5 ml. of insulin in adjuvant. The injections are repeated 30 and 270 days later and 10 days before each blood collection. After each injection, the animals are observed for hypoglycemic convulsions for a 7-hour period. Convulsions are treated with 1 ml. of 50% (w/v) dextrose (subcutaneous injection preferably) as soon as the hypoglycemic response is recognized. Blood is withdrawn *via* heart puncture, and after coagulation in an ice bath, is centrifuged at 15°. Serum is held at −20°.

Pooled batches of serum are evaluated in the adjustment procedure below and diluted to the titer indicated with 5% albumin stock solution. Aliquots of the diluted antibody, sufficient for each half day of operation, are placed in plastic bottles and kept frozen until needed.

Standard Reagents.—All standard insulins used were compared in the rabbit bioassay (3) against U.S.P. zinc-insulin crystals reference standard "G." The radioactive insulin was obtained from Abbott Laboratories.[1] This form of iodo-insulin had full potency when tested in the mouse-convulsion test. This labeled material is diluted in 5% (w/v) human albumin stock solution; sufficient nonradioactive insulin is added to make a concentration of 100 microunits (μu.) and 0.04 μc. in 20 μl. of the iodo-insulin stock solution. For preparation of the albumin stock solution, a 25% (w/v) commercial solution of human serum albumin (Cutter Laboratories) was diluted to 5% with a 0.9% (w/v) NaCl solution containing 0.2% (w/v) phenol. The pH was adjusted to 8.5 with 40% (w/v) NaOH. This solution was stored at 5°. The 30% (w/v) urea solution in 5% albumin, as used by Grodsky, is always made daily. An ammonia-free reagent grade glycine is required for 0.2 *M* glycine solution in 5% albumin.

Inactivated Human Plasma.—Several bags of blood bank plasma (relatively free of hemoglobin) are pooled, centrifuged in the cold to remove residual blood cells, adjusted to pH 10.5 with 40% NaOH, and stored 24 hours at 5°. Following this aging, the pH is lowered to 7.4 with 10% HCl, the pooled

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[1] Marketed as Iodo-Insulin Sterile-I^{131}.

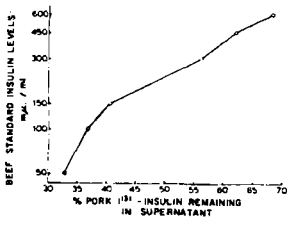


Fig. 1.—An extended standard curve to indicate the acceptable operating range, 150, 300, and 450 mu./ml., selected for one of our adjusted systems.

plasma is centrifuged in the cold, and the supernatant decanted from a slimy precipitate. After experimental determination of the dilution strength needed in the assay, the pooled plasma is divided into aliquots sufficient for each half day of operation and frozen until needed. Dilutions in this plasma are made immediately before use.

Assay System Adjustment.—Inactivated plasma and the antibody serum are diluted to concentrations of 1:3, 1:5, and 1:8 with 5% albumin stock solution. Beef insulin in insulin diluent (3) is diluted with 5% human albumin stock solution to 50, 100, and 150 milliunits (mu.)/ml. concentrations for use as a reference standard. A 27.32% (w/v) sodium sulfite solution in H_2O is prepared which will give an 18.5% (w/v) salt concentration after addition to the reaction mixture.

Four standard levels (one tube each of 0, 50, 100, and 150 mu.) are run with each possible combination of the serum and plasma dilutions. The set of conditions which gives the largest difference in supernatant radioactivity between the 50- and 150-mu. levels, while maintaining a low value for the 0-mu. level, is selected. In our hands, this difference is about 19%, and the low value for 0 mu. is about 20% of the initial radioactivity in the supernatant. A curve, as shown in Fig. 1, is plotted to show the linearity and the slope at these standard points.

With some antibody pools, pork and human insulins require 75, 150, and 225 mu./ml. standard levels to duplicate the competitive binding affinity of beef insulin for the same antibody. Assay system adjustment is usually required each time the albumin, serum, or plasma is changed.

Sample Preparation.—Micro dilutions of highly

TABLE I.—STATISTICAL DESIGN AND GENERALIZED FORMULAS FOR AN n POINT STANDARD CURVE AND m UNKNOWNS WITH K REPLICATION IN THE INSULIN IMMUNOASSAY

	Standards						Unknowns					
Concn.	Recovery						Potency	Recovery				
c_1	$S_{1,1}$	$S_{1,2}$	...	$S_{1,k}$	...	$S_{1,K}$	p_1	$U_{1,1}$	$U_{1,2}$	...	$U_{1,k}$	... $U_{1,K}$
.	.	.	.	.	.	.	.	.	.	.	.	. .
.	.	.	.	.	.	.	.	.	.	.	.	. .
.	.	.	.	.	.	.	.	.	.	.	.	. .
c_i	$S_{i,1}$	$S_{i,2}$	...	$S_{i,k}$	...	$S_{i,K}$	.	.	.	.	.	. .
							.	.	.	.	.	. .
							.	.	.	.	.	. .
c_{i+1}	$S_{i+1,1}$	$S_{i+1,2}$	...	$S_{i+1,k}$	...	$S_{i+1,K}$	.	.	.	.	.	. .
							.	.	.	.	.	. .
.	.	.	.	.	.	.	.	.	.	.	.	. .
.	.	.	.	.	.	.	p_j	$U_{j,1}$	$U_{j,2}$	...	$U_{j,k}$	... $U_{j,K}$
.	.	.	.	.	.	.	.	.	.	.	.	. .
c_n	$S_{n,1}$	$S_{n,2}$	...	$S_{n,k}$	...	$S_{n,K}$	.	.	.	.	.	. .
							.	.	.	.	.	. .
							p_m	$U_{m,1}$	$U_{m,2}$	...	$U_{m,K}$	... $U_{m,K}$

Situation	Formulae
$\bar{S}_{i-1} < \bar{U}_j < \bar{S}_i$	$\ln p_j = \ln c_i + \left[\frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}\right] \ln (c_{i-1}/c_i)$
when $(1 < i \leq n)$	$\text{var}(\ln p_j) = \frac{[\ln (c_{i-1}/c_i)]^2}{(\bar{S}_i - \bar{S}_{i-1})^4} \{(\bar{S}_i - \bar{U}_j)^2 \text{ var } \bar{S}_{i-1} + (\bar{U}_j - \bar{S}_{i-1})^2 \text{ var } \bar{S}_i + (\bar{S}_i - \bar{S}_{i-1})^2 \text{ var } \bar{U}_j\}$
$\bar{U}_j = \bar{S}_i$	$\ln p_j = \ln c_i$
when $(1 < i < n)$	$\text{var}(\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \left[\frac{1}{(\bar{S}_i - \bar{S}_{i-1})^2} + \frac{1}{(\bar{S}_{i+1} - \bar{S}_i)^2}\right] \text{var } \bar{S}_i$

where:

$$\bar{S}_i = \frac{1}{K} \sum_{k=1}^{K} S_{i,k} \qquad \bar{U}_j = \frac{1}{K} \sum_{k=1}^{K} U_{j,k}$$

$$\text{var } \bar{S}_i = \frac{K \sum_{k=1}^{K} (S_{i,k})^2 - \left(\sum_{k=1}^{K} S_{i,k}\right)^2}{K^2(K-1)}$$

$$\text{var } \bar{U}_j = \frac{K \sum_{k=1}^{K} (\bar{U}_{j,k})^2 - \left(\sum_{k=1}^{K} U_{i,k}\right)^2}{K^2(K-1)}$$

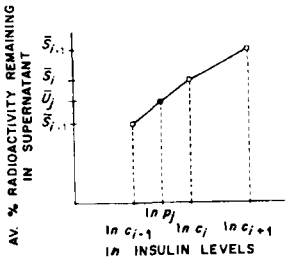


Fig. 2.—A sample graph to illustrate calculation of immunoassays for extracted insulin. Key: $\bar{U}_j$ = average response of the jth sample; $\bar{S}_i$ = average standard response at the ith dilution; p_j = insulin concentration of the jth sample; c_i = insulin concentration at the ith dilution.

concentrated insulin solutions are made with insulin diluent to about 50 u./ml., and the final dilution step is always made using 5% albumin stock solution. After final dilution, the samples are allowed to stand overnight at 5°.

Assay Procedure.—Six replicate tubes are run on each sample. Two patterns are used: eight samples per day in triplicate with two sets of 36 tubes. Fourteen samples per day can be run in duplicate with three sets of 36 tubes (only one "zero" tube is used with this pattern).

All glassware is cleaned with detergent, rinsed in 2% (w/v) HCl, water-rinsed, and oven-dried before use. A primary solution containing 38.0 ml. of 30% urea and 38.0 ml. of 0.2 *M* glycine solution is prepared for a set of 36 tubes. After removal of 2.04 ml. of the primary solution for a blank tube, the diluted antibody serum is added. The pH of this primary solution is adjusted to 8.5 with 10% NaOH, and the solution is stirred until it reaches room temperature. Twenty microliter-volumes of the diluted I^{131} insulin are measured into glass test tubes using a $^1/_4$-ml. syringe microburet (Micrometric Instruments Co.). The microburet, with a syringe for each dilution, is then used to measure 20 µl. aliquots of each of the standard dilutions and of the samples into their appropriate test tubes. Two zero tubes (no unlabeled insulin added) and one blank tube (no unlabeled insulin or antibody added) are included in each set with the eight-sample-per-day pattern. One control tube is made containing 20 µl. of the radioactive insulin stock solution. The volume differentials in the blank, zero, and control tubes are corrected with 5% albumin stock solution. Next, 2.04 ml. of the primary solution with antibody is rapidly injected sequentially into all the tubes except the blank. Glass rods are inserted in the tubes, and the set of tubes is placed on a rotating shaker (A. H. Thomas No. 3623) for 1 hour of gentle agitation at 24°. At the end of 1 hour, 0.8 ml. of the diluted inactivated plasma is injected sequentially into each of the tubes. Following the addition of plasma and a 15-minute agitation period on the rotating shaker, 6.0 ml. of 27.32% Na_2SO_3 is added to each of the tubes sequentially. This mixture is agitated on the rotary shaker for 1 hour at 24°. After the glass rods are removed, the tubes are centrifuged in a refrigerated

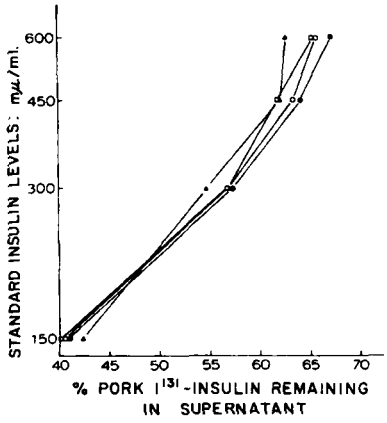


Fig. 3.—Responses of an antibody pool to insulins from different species. Rabbit assays (P = 0.95) on the insulins used are: W-3776, solution of U.S.P. reference standard at 24.0 u./mg.; 493-10GP-182—20.80 (22.63 to 19.12) u./mg.; ML 26—24.78 (26.01 to 23.61) u./mg.; PJ-5158—22.84 (24.75 to 21.07) u./mg. Key: ●—●, U.S.P. insulin, W3776; ▲—▲, human insulin, 493-10GP-182; ○—○, beef insulin, ML 26; and □—□, pork insulin, PJ5158.

TABLE II.—A SCHEMATIC INSULIN PURIFICATION PROCEDURE WITH THE IMMUNOASSAY RESULTS ON ONE LOT EXPRESSED AS A PER CENT OF TOTAL INITIAL PANCREAS EXTRACT ACTIVITY AT EACH ASSAY POINT

Active Portion	Discarded Portion
Pancreas Extract 100%	Meat Residue
↓	
Salt Precipitate 97.3%	Salt Filtrate 1.96%
↓	
1st Isoelectric Point Precipitate 82.8%	1st Isoelectric Point Filtrate 1.19%
↓	
2nd Isoelectric Point Precipitate 102.5%	2nd Isoelectric Point Filtrate 2.52%
↓	
3rd Isoelectric Point Precipitate 98.9%	3rd Isoelectric Point Filtrate (not assayed)
↓	
Zinc-Insulin Crystals 92.58%	
Amorphous Fraction 5.53%	
Soluble Insulin 2.03%	

TABLE III.—EFFECT OF PH AND VARIOUS IONS USED IN INSULIN PURIFICATION ON IMMUNOASSAY RESULTS[a]

Salt Soln.	Initial Biological Activity, %			
	pH 6.5 Soln.		pH 3.0 Soln.	
	Pork Insulin[b]	Beef Insulin[c]	Pork Insulin[b]	Beef Insulin[c]
0.5 *M* NH_4Cl	53.48(54.94–52.06)	38.80[e]	99.65(109.68–90.52)	114.41(118.68–110.28)
0.5 *M* $NH_4C_2H_3O_2$[d]	51.94(56.30–47.90)	41.40[e]	101.92(111.06–93.52)	113.21(122.60–104.52)
0.5 *M* $NaC_2H_3O_2$[d]	54.69(59.68–50.10)	40.60[e]	91.27(97.46–85.46)	110.43(117.94–103.38)
0.02 *M* Na_2HPO_4	56.66(58.98–54.42)	45.70[e]	107.41(112.76–103.02)	107.71(112.28–103.32)
0.05 *M* $Na_3C_6H_5O_7$	59.37(63.02–55.92)	48.90[e]	99.61(104.38–95.04)	104.60(115.44–94.76)
0.05 *M* $(NH_4)_2HC_6H_5O_7$	66.93(70.62–63.42)	74.46(78.70–70.44)	105.66(118.92–93.88)	110.94(120.94–101.76)
0.02 m*M* $ZnCl_2$	93.52(101.30–86.34)	95.38(102.88–88.42)	96.10(102.68–89.92)	101.04(109.04–93.62)
0.3 *M* NaCl	46.00[e]	85.07(88.96–81.34)	101.31(114.20–89.86)	106.13(112.50–100.10)

[a] Standard insulin solutions, 50 u./ml., were diluted to 5 u./ml. with the salt solution, followed by pH adjustment prior to final dilution with albumin stock solution. [b] Pork insulin lot was PJ-5158 (C-2598-9) which assayed 26.99 u./mg. (±7.87% $p = 0.95$) in 96 rabbits. [c] Beef insulin was ML 26 which assayed 24.8 u./mg. (±4.85% $p = 0.95$) in 192 rabbits. [d] pH adjustment was with the respective acid or base in all solutions, except those where H_2SO_4 was used. [e] Statistics used do not provide limits on values obtained by extrapolation from the standard curve which only extends from 50 to 150% of estimate.

angle head centrifuge at 15° at a relative centrifugal force of 2400 × g. for 45 minutes. Following centrifugation, 5 ml. of supernatant is carefully removed from each tube and placed in a plastic tube for counting.

Radioactivity Measurement.—A control tube is made up for counting with each set of samples containing 5 ml. of the diluted iodo-insulin solution (20 μl. diluted to 8.86 ml. with 5% albumin stock solution). Due to the short half-life of I^{131} the control count must be corrected for decay when calculating the result on each sample tube. Sets of samples are conveniently counted overnight in an automatic gamma spectrometer (Packard Instruments Co.) for the peak emission of I^{131}. A thallium activated sodium iodide crystal is used in the spectrometer. The usual counting time is 5 minutes per sample.

Calculation of Potency.—The statistical design of the assay procedure is presented in Table I. Total counts are corrected for backgrounds and reduced to counts per minute. The tube count is then divided by the control count (corrected for decay during counting) to obtain the per cent radioactivity remaining in the supernatant, S_i or U_j. A three point standard curve may be plotted on semilog graph paper as shown in Fig. 2. Sample potency can be estimated from this graph or calculated using Eqs. 1 or 2. With a point-to-point linear assumption (when $S_1 < \ldots < S_i < \ldots < S_n$), any one of $2n - 1$ situations may be encountered. For an n point standard curve with K sample replication, the generalized formulas are: when $\bar{S}_{i-1} < \bar{U}_j < \bar{S}_i \; (1 < i \leq n)$

$$\ln p_j = \ln c_i + \left[\frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}\right] \ln (c_{i-1}/c_i) \quad \text{(Eq. 1)}$$

where

$$\bar{S}_i = 1/K \sum_{k=1}^{K} S_{ik} \text{ and } \bar{U}_j = 1/K \sum_{k=1}^{K} U_{jk}.$$

When $\bar{U}_j = \bar{S}_i \; (1 \leq i \leq n)$,

$$\ln p_j = \ln c_i \quad \text{(Eq. 2)}$$

Variance of Sample Potency.—The variance of the ln p_j may be estimated by the formula for the propagation of error (5). This solution becomes asymptotic as the value of K increases. For the situation $\bar{S}_{i-1} < \bar{U}_j < \bar{S}_i \; (1 < i \leq n)$

$$\text{var } (\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \text{ var } \left[\frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}\right]$$

Let

$$y = \frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}$$

then

$$\text{var } (\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \left\{\left(\frac{\partial y}{\partial \bar{S}_{i-1}}\right)^2 \text{var } \bar{S}_{i-1} + \left(\frac{\partial y}{\partial \bar{S}_i}\right)^2 \text{var } \bar{S}_i + \left(\frac{\partial y}{\partial \bar{U}_j}\right)^2 \text{var } \bar{U}_j\right\}$$

By the usual methods of partial differentiation and variance determination of an independent variable

$$\text{var } \bar{S}_i = \frac{K \sum_{k=1}^{K} (S_{ik})^2 - \left(\sum_{k=1}^{K} S_{ik}\right)^2}{K^2(K-1)}$$

TABLE IV.—REPLICATE IMMUNOASSAYS ON DIFFERENT DAYS ON SAMPLES OF DIFFERENT SPECIFIC ACTIVITIES

Lot No.	Species	Specific Activity, u./mg.	Anticipated Potency Found ($p = 0.95$), % Day 1	Day 2	Day 3	Day 4	Day 5	Weighted Mean
813527-31	Beef	0.16	139.6(147.8–128.3)[a]	142.9(191.3–104.3)[b]	139.6(158.7–121.7)[a]	140.8(147.83–132.61)	129.1(137.0–120.0)	137.3
813527-31	Beef	11.47	110.1(121.1–100.1)	107.4(116.4–99.1)	103.0(107.9–97.4)	106.6(113.83–99.87)	101.0(105.8–96.6)	105.6
813527-31	Beef	24.22	115.1(127.4–104.3)	96.8(104.6–89.7)	105.4(113.2–98.3)	110.0(118.5–102.2)	95.0(101.6–88.9)	104.2
815271-5	Pork	0.08	93.76(98.7–88.3)	102.6(113.3–90.0)	97.5(110.0–83.3)[a]	87.0(92.7–81.3)	93.1(101.0–85.3)	94.6
815271-5	Pork	0.92	86.4(106.0–70.0)	75.2(80.4–70.3)[a]	70.5(77.6–63.9)[a]	75.2(83.2–67.9)	69.5(75.5–64.1)	75.1
815271-5	Pork	8.02	92.6(98.0–87.5)	99.9(107.6–92.8)	103.7(111.9–96.2)	98.0(106.8–90.0)	92.1(101.4–83.6)[a]	97.2
815271-5	Pork	15.26	99.1(106.3–92.4)	113.8(122.1–106.1)	99.7(110.6–90.1)	102.4(108.7–96.5)	94.5(100.7–88.6)	101.7
815271-5	Pork	17.55	105.8(115.7–96.8)	103.3(115.3–92.6)	94.0(101.6–87.5)	98.9(107.8–90.8)	93.8(100.5–87.5)	99.0
815271-5	Pork	20.42	106.2(113.0–99.9)	99.5(108.3–91.4)	94.3(104.5–85.3)	101.2(107.4–95.4)	92.7(96.6–88.9)	98.7

[a] Two of the six-tube results rejected as aberrant values. [b] Four of the six-tube results rejected as aberrant values.

$$\text{var } \bar{U}_j = \frac{K \sum_{k=1}^{K} (\bar{U}_{jk})^2 - \left(\sum_{k=1}^{K} \bar{U}_{jk}\right)^2}{K^2(K-1)}$$

the variance of the ln pj may be written as

$$\text{var } (\ln p_j) = \frac{[\ln (c_{i-1}/c_i)]^2}{(\bar{S}_i - \bar{S}_{i-1})^4} \{(\bar{S}_i - \bar{U}_j)^2 \text{ var } \bar{S}_{i-1} + (\bar{U}_j - \bar{S}_{i-1})^2 \text{ var } \bar{S}_i + (\bar{S}_i - \bar{S}_{i-1})^2 \text{ var } \bar{U}_j\} \quad \text{(Eq. 3)}$$

The variance, when ln p_j = ln c_i, must be estimated from the limits of Eq. 3 as $\bar{U}_j$ approaches $\bar{S}_i$ from both a positive and negative direction. By averaging (when $1 \leq i \leq n$)

$$\text{var } (\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \left[\frac{1}{(\bar{S}_i - \bar{S}_{i-1})^2} + \frac{1}{(\bar{S}_{i+1} - \bar{S}_i)^2}\right] \text{var } \bar{S}_i \quad \text{(Eq. 4)}$$

Reporting of Assay Results.—The logs of the results from each set of tubes are averaged. The antilog of this average coupled with the 95% confidence interval values is reported as the assayed potency of the sample dilution. A digital computer (IBM 1620) program is available to perform the necessary calculations.

RESULTS

Species Similarities in Competitive Insulin Binding Situations.—The data in Fig. 3 demonstrate the similarity in binding affinity for human, beef, and pork insulins, and U.S.P. reference standard in-

TABLE V.—COMPARISON OF PORK AND BEEF IODO-INSULIN IN LIKE ASSAY SYSTEMS[a]

Tube Description	I^{131} Remaining in the Supernatant, % Beef Iodo-Insulin A.M.	Beef Iodo-Insulin P.M.	Pork Iodo-Insulin A.M.	Pork Iodo-Insulin P.M.
Blank	82.53	85.12	84.10	78.86
Zero	22.35	21.71	21.90	19.15
50 mu./ml. W-3743 beef[b] standard	32.53	33.60	39.45	38.80
100 mu./ml. W-3743 beef[b] standard	49.13	49.78	55.98	54.38
150 mu./ml. W-3743 beef[b] standard	56.07	55.92	60.62	60.01
50 mu./ml. U.S.P. standard	29.87	31.05	38.21	37.29
100 mu./ml. U.S.P. standard	45.89	45.74	55.76	52.86
150 mu./ml. U.S.P. standard	51.71	50.66	57.50	58.95
100 mu./ml. W-3752 acidified U.S.P. protamine zinc insulin	44.97	45.40	51.72	52.73
100 mu./ml. W-3751 acidified beef NPH insulin	49.25	48.96	55.03	55.56
100 mu./ml. W-3750 acidified beef protamine zinc insulin	48.64	49.64	54.60	54.82
100 mu./ml. W-3751 supernatant	21.23	21.16	20.08	19.39
100 mu./ml. W-3750 supernatant	21.07	20.62	19.65	19.40

[a] Three-tube averages except on blank and zero. [b] Beef insulin used in W-3743, W-3750, and W-3751 was from ML 795372 which assayed 25.54 u./mg. in the U.S.P. rabbit test for insulin (368 rabbits).

TABLE VI.—ASSAYS OF PROTAMINE-INSULIN MIXTURES[a]

Insulin Soln.	Estimated Potency, u./ml.	Estimated Potency Found, % Iodo-Insulin Beef	Iodo-Insulin Pork
W-3752, U.S.P. protamine zinc insulin[b]	40.0	83.46(87.05–80.00)[d]	88.17(92.05–84.43)[d]
W-3751, Beef NPH insulin[b]	40.0	98.60(106.28–91.48)	102.31(108.48–96.48)
W-3750, Beef protamine zinc insulin[b]	40.0	98.68(103.23–94.33)	98.71(104.95–92.83)
W-3751, NPH supernatant[b]	1.0	29.60[c]	21.60[c]
W-3750, P.Z.I. supernatant[e]	1.0	29.80[c]	21.80[c]

[a] Results are calculated against the recoveries shown in Table V for beef standards. [b] Acidified with 30 μl. concentrated HCl/10 ml. of mixture for solution prior to dilution with albumin stock solution. [c] Values were extrapolated from standard curve; confidence limits not calculated. [d] Antibody pool used was demonstrating species specific properties; thus the pork insulin content of the U.S.P. reference standard insulin causes low results. [e] The result on this sample from a 24-rabbit U.S.P. assay was 0.09 (0.195–0.013) u./ml.

sulin at various dilution levels. Each of these samples has been carefully tested on the U.S.P. assay for insulin in rabbits (3). The actual rabbit assay results are given in the caption under Fig. 3. When antibody pools demonstrate differential competitive binding, it can be circumvented by either using the same species on both the standard and sample or by appropriate mixing of the standards from different species. Thus, reliable assay results can be obtained for an insulin solution of known species composition.

Total Competitive Binding Curve.—Figure 1 gives a typical total competitive binding curve which is shown to illustrate the portion selected for the performing of assays.

Assay of Low Purity Samples.—Low purity samples from the manufacturing process are normally not tested in rabbits because of the toxic effects caused by the impurities present. Table II illustrates an experiment to show that immunoassay results on low purity samples are within the range of expectation. Both the beef and pork insulin manufacturing processes were sampled at various stages of purification and material balance figures computed from the immunoassay results.

Acidification Before Dilution.—In an experiment to test for ionic interference with the immunoassay results, the apparent interference caused by some ions could be nullified by acidification of the sample prior to dilution in the 5% albumin stock solution. Table III illustrates the differences found with pH 6.5 and pH 3.0 solutions in the presence of various ions.

Day-to-Day Replications.—Replicate samples of two different production lots of insulin were prepared and frozen. A fresh sample was removed and assayed each day for a period of 5 days. The data in Table IV show the characteristic variations in results.

TABLE VII.—SAMPLES OF CRYSTALLINE INSULIN DILUTED TO ABOUT 80 u./ml. IN INSULIN DILUENT AT pH 3.0[a]

Boiling Time, Hr.	Immunoassay Results, u./ml. Beef Insulin	Pork Insulin
0	77.9 (87.4–69.3)[b]	75.2 (94.5–59.9)[c]
1	62.2 (71.8–53.9)	47.1 (56.5–39.3)
2	48.0 (52.2–44.1)	48.8 (59.5–40.0)
3	46.5 (53.6–40.4)	39.8 (47.5–33.4)
4	41.6 (46.7–37.0)	35.2 (42.1–29.4)
5	37.8 (42.4–33.7)	29.5 (37.2–23.5)
6	32.2 (35.7–29.1)[d]	32.5 (38.7–27.3)[e]

[a] Solutions gently refluxed at 100°C. [b] U.S.P. rabbit assay in 48 rabbits on pooled lots C2730, 1, 3, and 4 showed this to be 76.58 (93.89 to 62.46) u./ml. [c] U.S.P. rabbit assay in 240 rabbits on lot C2732X showed this to be 76.48 (80.80 to 72.38) u./ml. [d] U.S.P. rabbit assay in 96 rabbits showed this to be 23.11 (25.60 to 20.87) u./ml. [e] U.S.P. rabbit assay in 96 rabbits showed this to be 27.48 (30.38 to 24.86) u./ml.

Comparison of Pork and Beef I^{131} Labeled Insulin.—Duplicate assays on known standard material were run first using beef I^{131} insulin and then pork I^{131} insulin. The comparative results are shown in Table V.

Specificity for Biologically Active Insulin.—To demonstrate that the antibodies were specific for biologically active insulin and also to demonstrate that the assay could be used with long-acting forms of insulin, several experiments were seen to show this selective action. Table VI shows the final results obtained with acidified NPH and protamine zinc insulins run against their respective solutions of insulin crystals in insulin diluent (computed from the data in Table V). Table VII gives the results when solutions of insulin (either beef or pork) are boiled in acid solution. The data in Table VIII show a comparison between mouse, rabbit, and immunoassay. These data also illustrate the sensitivity of the immunoassay for that insulin which has not been attacked by trypsin. The mouse and rabbit data were previously published in Table I of (6).

Comparative Assay Results.—Table IX shows a collection of routine assays performed on the samples with both the U.S.P. rabbit assay (3) and this immunological technique.

Influence of Ethanol.—Table X shows how small amounts of ethanol present before dilution with albumin diluent affect the antibody.

DISCUSSION

Some of our antibody pools have demonstrated a species specificity such as indicated by Berson (7). Carefully standardized lots of different insulin would give curves, plotted as in Fig. 3, having different slopes. When differential binding is

TABLE VIII.—TRYPSIN HYDROLYZED BEEF INSULIN SAMPLES[a]

Hydrolysis Time, Min.	Biological Activity, u./mg. Immunoassay[b]	Mouse	Rabbit
0	22	23	25
5	17	17	18
11	15	14	...
19.5	12	9	10
40	4.6	3.4	...
80	1.8	0.7	...
160	0.8	...	0.3

[a] All hydrolyzed samples are presumed to contain insulin, desoctapeptide insulin, alanine, heptapeptide, trypsin, and soybean trypsin inhibitor. [b] These were three-tube assays.

TABLE IX.—IMMUNOASSAYS AND U.S.P. RABBIT ASSAYS ON SOME UNUSUAL AND ROUTINE SAMPLES

Lot and Description	Immunoassay Potency, u./ml.	Immunoassay Confidence Limits (p = 0.95), u./ml.	U.S.P. Rabbit Assay Potency, u./ml.	U.S.P. Rabbit Assay Confidence Limits (p = 0.95), u./ml.	No. Rabbits
Dealaninated[a] pork insulin CT-1530A	118.5	(128.4–109.4)	107.38	(116.50–98.97)	96
Human insulin 493-10GP-182	155.7	(162.9–148.7)	123.10	(133.93–113.16)	72
Acidified beef protamine zinc insulin					
190-14GP-21A	18.1	(19.3–17.1)	20.50	(25.02–17.12)	24
190-14GP-21B	9.2	(11.3–7.5)	11.18	(13.57–9.46)	24
Pork and beef insulin					
W-3775	37.9	(40.6–35.3)	40.69	(43.15–38.37)	240
W-3786	41.0	(45.1–37.2)	40.07	(42.41–37.86)	192
Beef insulin					
M.L. 31	36.0	(36.7–35.3)	39.47	(42.72–36.47)	96
M.L. 32	38.6	(39.5–37.6)	40.96	(44.43–37.76)	96
C-2695-96	41.7	(42.5–40.8)	36.72	(42.12–32.01)	48
C-2701 + 2706	38.2	(41.7–35.1)	41.79	(50.33–34.70)	48
C-2710-11 + 13-14	39.6	(43.3–36.2)	41.09	(46.06–36.65)	48
PJ-5504	41.0	(42.4–39.7)	42.82	(49.56–37.00)	96
Pork insulin					
C-2712 + 2715	39.1	(41.3–37.1)	37.70	(43.04–33.02)	48
C-2716-17	39.1	(41.2–37.0)	36.84	(40.54–33.48)	48
C-2732X	40.1	(43.3–37.2)	37.48	(39.60–35.47)	240
C-2730-31-33-34	40.2	(41.7–38.7)	36.87	(40.60–33.48)	96

[a] Dealaninated insulin, porcine insulin less the C-terminal alanine on the B-chain.

TABLE X.—IMMUNOASSAY TOLERANCE FOR ETHANOL IN SAMPLES FOR ASSAY[a]

Potency, u./ml.	Original Ethanol, %	Anticipated Activity Found, %
3	20	102.4
2	40	108.9
1	60	107.5

[a] Test solutions were made from M.L. 26 beef insulin.

observed, beef insulin is the most effective competitor for binding sites, followed in decreasing order by U.S.P. reference standard (beef and pork), pork, and human insulins. Errors in potency estimation show up as parallel lines in plots such as those shown in Fig. 3. A change in slope represents a proportionate difference and species specificity.

One of the questions which must be answered about any *in vitro* hormone assay is the specificity of the assay for the active hormone. We have attempted to supplement the work of Grodsky (8) by indirect activity correlation and some interference testing. Table II is an indirect correlation of crude extract assays and intermediate purity assays with final crystal results. One of the problems with prior *in vitro* or chemical techniques is an inability to assay insulin quantitatively in mixtures having a specific activity of less than 16 u./mg. We believe that this test gives adequate results down to 0.05 u./mg. specific activity.

The data in Table III resulted from an experiment to discover if the ions used in processing insulin would interfere with the assay. This is the first report showing that the pH of the sample must be below the isoelectric point of insulin prior to dilution with pH 8.5 albumin stock solution. This pH requirement holds for both beef and pork insulins. There are at least two explanations for the apparent loss of activity in alkaline solutions. First, insulin is lost through adsorption on glassware at higher pH's; or second, the insulin molecule is modified so that the antibody cannot bind it. We prefer the latter explanation since we found that acidification was necessary to give reliable results on NPH and PZI insulins which are originally nearly neutral. In Table IV the assay shows normal variation about a weighted mean for both beef and pork samples.

The experiments described in Tables V and VI were designed to show several points about and uses for the assay. Either the beef or pork section of Table V illustrates a complete set of converted data from an eight-sample assay pattern. Subset replication is indicated. The use of the assay with protamine containing samples both for acidified total potency and centrifuged supernatant assays, and the two forms of commercially available iodo-insulin were compared. We have concluded from the completed calculations in Table VI that there was no appreciable difference in the two tests due to changes in the species of iodo-insulin used.

The experiments described in Tables VII and VIII illustrate that the assay discriminates between natural and denatured forms of insulin. Neither heat nor enzyme denatured insulin are effective in the assay.

In Table IX we reported routine instances of direct comparison with some fairly precise U.S.P. rabbit assays. The high immunoassay results on the human insulin sample are possibly due to a low response on the standard (493-10GP-90) which could in turn be due to poor stability in albumin stock solution, statistical variation due to the relatively small samplings of the rabbit population when its potency was established in the U.S.P. test, or some other unrecognized variable.

The two protamine zinc insulin samples were of interest as 20-year-old room temperature samples which are still active (though weaker than they were at time of manufacture). Thus, a supposedly mildly inactivated portion of these samples does not affect the immunoassay.

Ethanol in samples, such as crude extracts, apparently does not inhibit the antibody as indicated in Table X.

Output quantity is a function of manpower, equipment available, and the specialization possible with larger numbers of samples. The following

sample patterns 8, 14, and 22, respectively, require about 1.5, 2.0, and 2.5 men per day. The assay takes a little longer than a mouse convulsion test, but is many times more accurate.

Since we were not assaying serum insulin we were able to increase volumes, standard concentrations, and I^{131}-insulin concentration to provide adequate counting quantities when low specific activity commercially available iodo-insulin was used. If high specific activity iodo-insulin is used, the sensitivity of the test could be increased by reverting to the original volumes used by Grodsky (1).

As suggested by Grodsky (1), we started using Na_2SO_3 in the salt-out step but changed to Na_2SO_4 for operation at 24°. Frictional heat arising during centrifugation led us to the use of a refrigerated centrifuge. However, since Na_2SO_4 crystallizes at 5–15°, we returned to Na_2SO_3 and 15° as our operating temperature.

We have found this test to be accurate, fast, inexpensive, and specific for natural insulin.

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Gas-Liquid Chromatography of Local Anesthetics and Related Compounds

By HENRY M. KOEHLER and JOHN J. HEFFERREN

Conditions for the gas chromatography of local anesthetics and related materials were investigated. Flexol on Chromosorb W and SE 30 on glass bead columns were preferred for the low and high melting compounds, respectively.

ANALYSES OF local anesthetic drugs in various dosage forms and tissue preparations have been carried out using ultraviolet, visible, and infrared spectrophotometric and titrimetric and gravimetric methods. The use of chromatographic methods for separation, identification, and quantitation of components of mixtures is well established in pharmaceutical analysis. Gas-liquid chromatography offers the advantage of rapidity combined with the sensitivity and versatility of other chromatographic techniques (1). This technique was applied to the detection and quantitation of local anesthetic bases, hydrochloride salts and degradation products. Kirk, describing the application of gas-liquid chromatography to criminalistic problems, included procaine hydrochloride among the drugs which could be chromatographed (2).

APPARATUS

The instrument used in this work was the Aerograph Hy-Fi model 600 fitted with a gold-plated hydrogen flame ionization detector and a Sargent model SR recorder with an input filter and a Disc integrator. Hydrogen, at a flow rate of 25 ml./minute, was provided for the flame ionization detector by the Aerograph hydrogen generator, model 650. Dry nitrogen was used as carrier gas at a flow rate of 30 ml./minute. Liquid phases used included Carbowax 400, Apiezon L, Ucon Polar, Flexol plasticizer 8N8, and silicone rubber SE 30. Solid supports used included 60–80 mesh firebrick, 100–120 mesh siliconized Chromosorb W, and 100–120 mesh siliconized glass beads. Five-foot columns of $^1/_8$ inch O.D. copper, aluminum, or stainless steel tubing were used.

The liquid phases were coated on the solid supports by evaporating with a Rinco rotary evaporator a slurry of the solid support and a dichloromethane solution of the liquid phase. The columns were packed with a vibrator and shaped into $2^1/_2$ inch diam. coils which were conditioned overnight in a slow stream of nitrogen at the expected operating temperature or at 200°, whichever was lower.

RESULTS AND DISCUSSION

The structural relationships of the local anesthetic agents used in this study are summarized in Table

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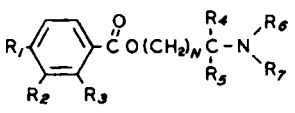
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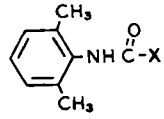
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TABLE I.—STRUCTURE OF LOCAL ANESTHETICS





	R_1	R_2	R_3	N	R_4	R_5	R_6	R_7
ESTER-TYPE LOCAL ANESTHETICS								
Procaine	NH_2	H	H	1	H	H	C_2H_5	C_2H_5
Chloroprocaine	NH_2	H	Cl	1	H	H	C_2H_5	C_2H_5
Butethamine	NH_2	H	H	1	H	H	H	$CH_2CH(CH_3)_2$
Meprylcaine	H	H	H	1	CH_3	CH_3	H	C_3H_7
Isobucaine	H	H	H	1	CH_3	CH_3	H	$CH_2CH(CH_3)_2$
Tetracaine	NHC_4H_9	H	H	1	H	H	CH_3	CH_3
Propoxycaine	NH_2	H	OC_3H_7	1	H	H	C_2H_5	C_2H_5
Naepaine	NH_2	H	H	1	H	H	H	C_5H_{11}
Parethoxycaine	OC_2H_5	H	H	1	H	H	C_2H_5	C_2H_5
Benoxinate	NH_2	OC_4H_7	H	1	H	H	C_2H_5	C_2H_5
Butacaine	NH_2	H	H	2	H	H	C_4H_7	C_4H_7
Metabutethamine	H	NH_2	H	1	H	H	H	$CH_2CH(CH_3)_2$
Metabutoxycaine	H	NH_2	OC_4H_7	1	H	H	C_2H_5	C_2H_5
Piperocaine	H	H	H	2	H	H	1-(2-methylpiperidyl)	
Benzocaine	NH_2	H	H	1	H	H_2	No aliphatic amine	
Butyl aminobenzoate	NH_2	H	H	3	H	H_2	No aliphatic amine	

	X
AMIDE-TYPE LOCAL ANESTHETICS	
Lidocaine	$CH_2N(C_2H_5)_2$
Mepivacaine	2-(1-methylpiperidyl)
Pyrrocaine	1-methylpyrrolidine

TABLE II.—RETENTION TIMES OF AMINOALCOHOLS

	Retention Time, seconds[a]		
	Column A	Column B	Column C
Ethanol	27	24	..
Aminoethanol	...	57	..
Dimethylaminoethanol	87	43	42
Diethylaminoethanol	187	43	42
Isobutylaminoethanol	246	48	50
Amylaminoethanol	489	58	68
Dibutylaminopropanol	..	66	..
2-Propylamino-2-methylpropanol	300	66	..
2-Isobutylamino-2-methylpropanol	333	48	..
N-(3-Hydroxypropyl)-α-pipecoline	108	57	..
2,6-Xylidene	...	37	..
N,N-Diethylaminoacetic acid	...	..	55

[a] Column A: 15% Flexol on 60–80 mesh firebrick, injection port temp. 185°, column temp. 170°; Column B: 15% Ucon Polar on 60–80 mesh firebrick, injection port temp. 200°, column temp. 185°; Column C: 0.1% SE 30 on 100–120 mesh glass beads, injection port temp. 150°, column temp. 100°.

I. The ester-type local anesthetics, such as procaine hydrochloride, undergo hydrolysis to the corresponding acid and aminoalcohol (3). The amide-type local anesthetics, such as lidocaine hydrochloride, are considerably more resistant to hydrolysis; however, some does occur (4).

The retention times of the aminoalcohols and other local anesthetic related compounds on three different columns are listed in Table II. The solid compounds were injected as alcoholic solutions. Normally 1–10 mcg. of the compounds gave an adequate peak. Most of these compounds contained trace amounts of the related alcohols which were easily separated. Although the Flexol column was generally the column of choice for the aminoalcohols, the over-all magnitude of the difference between the columns in Table II probably reflects the selection of the specific conditions rather than merely characteristics of the liquid phase. Identifiable peaks were observed with 1 μl. of 1% aqueous solutions of the aminoalcohols on a 15% Flexol column. Elution times generally corresponded to those seen when the pure substance was injected.

The free bases of local anesthetics are generally low melting, water insoluble solids. Usually column temperatures greater than 200° were required for the elution of these compounds (Table III). Because of relative insensitivity of the detector to carbon disulfide, this was a convenient solvent. However, reaction of the amino groups in the anesthetic molecule complicated its general use.

Local anesthetic hydrochloride salts have high melting points; for example, procaine hydrochloride melts at 153–156°. In order to minimize the injection port and column temperatures necessary to elute these high melting salts, coated glass-bead columns were used. Of the liquid phases stable at high temperatures, SE 30 proved to be the most useful. The retention times of local anesthetic salts

TABLE III.—LOCAL ANESTHETIC BASES IN CS_2[a]

	M.p., °C.	Retention Time, seconds
Mepivacaine	149–152	30
Lidocaine	66– 69	96
Procaine	60– 61	106
Pyrrocaine	82– 84	70
Tetracaine	41– 46	192

[a] Conditions: 0.1% SE 30 on 100–120 mesh glass beads, injection port temp. 265°, column temp. 210°, 0.1 μl. of 2% solution.

TABLE IV.—RETENTION TIMES OF LOCAL ANESTHETIC SALTS[a]

Local Anesthetics	M.p., °C.	Retention Time, seconds
Naepaine HCl	175–177	44
Isobucaine HCl	183–184	44
Dyclonine HCl	175–176	48
Parethoxycaine HCl	174–178	52
Lidocaine HCl	128–129	55
Piperocaine HCl	172–175	62
Pyrrocaine HCl	201–203	65
Procaine HCl	153–156	72
Mepivacaine HCl	255–262	78
Tetracaine HCl	147–150	87
Butethamine HCl	192–196	99
Chloroprocaine HCl	176–178	121
Metabutethamine HCl	116–118	133
Metabutoxycaine HCl	117–119	124
Benoxinate HCl	157–160	150
Butacaine Sulfate	100–103	192
Meprylcaine HCl	150–152	[b]
Propoxycaine HCl	148–150	[b]

[a] Conditions: injection port temp. 265°, column temp. 225°, 0.1% SE 30 on glass beads, 1 μl. 2% fresh aqueous solutions. [b] No detector response with up to 5 μl. of 2% solution at column temperatures from 100–225°.

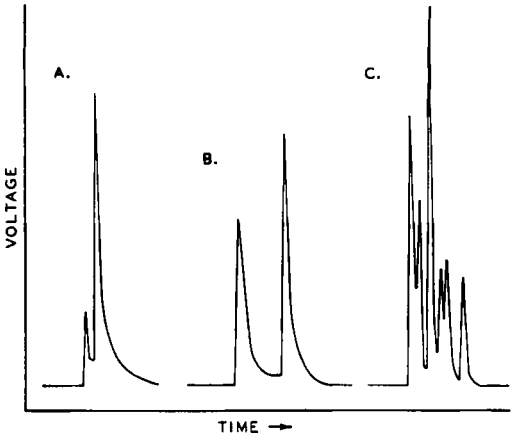


Fig. 1.—*A*, 0.1 μl. of 2% aqueous isobutylaminoethanol on 0.1% SE 30 glass-bead column; *B*, 1 μl. of 2% aqueous mepivacaine hydrochloride on 0.1% SE 30 glass-bead column; *C*, 0.5 μl. of a mixture containing 2% each of isobucaine HCl, lidocaine HCl, procaine HCl, mepivacaine HCl, and tetracaine HCl. Chart speed was 1 inch per minute.

are listed in Table IV. Columns of SE 30 on firebrick or Chromosorb W were also used; however, the results with these columns were generally less satisfactory.

Representative chromatograms of an aqueous solution of an aminoalcohol and of local anesthetic hydrochloride salts are illustrated in Fig. 1.

Several mineral acid salts of lidocaine including the hydrobromide, hydrochloride, nitrate, perchlorate, phosphate, and sulfate (5) were chromatographed on a 60–80 mesh firebrick column coated with 15% Flexol. Methanolic solutions of the base and the salts had similar retention times, suggesting that the observed peaks may be due to the free base.

Although the analyses of mixtures (6) of local anesthetics for parenteral administration, such as aqueous solutions of procaine hydrochloride with either propoxycaine (7) or tetracaine (8, 9) hydrochlorides, are possible with specific spectrophotometric procedures, gas chromatography offers a simple convenient method. Analysis of topical anesthetic mixtures, which may be aerosols, ointments, or solutions, are complicated by the frequent presence of two or more local anesthetics with quaternary ammonium compounds or other antibacterial agents in addition to pharmaceutical aids (6). The usual topical mixtures containing 2–4% lidocaine or 8–22% benzocaine with 2% tetracaine hydrochloride were readily assayed with a 0.1% SE 30 glass-bead column. A topical glycol solution of 14% benzocaine (ethyl aminobenzoate), 2% butyl aminobenzoate, and 2% tetracaine hydrochloride was assayed with the SE 30 glass-bead column. It is very difficult to quantitate each of the three local anesthetics in this mixture by any other analytical procedure.

SUMMARY

Liquid phases, solid supports, and tubing were investigated for use in the gas-liquid chromatography of aqueous and nonaqueous solutions of local anesthetics, their salts and related compounds. A Flexol on Chromosorb W column was preferred for the lower melting compounds such as the aminoalcohols, while a low load SE 30 on glass-bead column was preferred for higher melting local anesthetic bases and salts. Stainless steel tubing was preferred for the aqueous solutions of the higher melting compounds; however, no difficulties were encountered with columns prepared with copper or aluminum tubing.

The gas chromatographic procedures developed may be suitable for the determination of local anesthetics and other compounds in specific parenteral and topical local anesthetic mixtures which may not be readily assayed by the other methods.

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Drug-Induced Hyperthermia and Amphetamine Toxicity

By HAROLD H. WOLF and DAVID J. GEORGE*

Experiments were conducted to determine the relative importance of hyperthermia as a factor influencing the toxicity of amphetamine in aggregated and isolated mice. The ability of several agents to interfere with the hyperthermic response to amphetamine was compared with their ability to alter the incidence of lethality to this drug. It was evident that there was no direct relationship between the ability of the selected drugs to prevent the body temperature of mice from reaching the maximum temperature level of amphetamine-treated controls and their ability to protect mice from amphetamine-induced lethality. It was concluded that hyperthermia does not, in itself, appear to be the causative factor for the increased incidence of mortality to amphetamine observed in aggregated *versus* isolated mice.

MORE THAN 20 years have elapsed since Gunn and Gurd (1) first observed that amphetamine was several times more lethal to aggregated than to isolated mice. In recent years this phenomenon, known as the amphetamine aggregation effect, has served as a research tool in the search for new psychotherapeutic agents (2, 3). Although many potentially useful ataractics have been shown to protect against this phenomenon, the means whereby these agents alter the lethality to amphetamine remains unclear. In part this is due to a lack of insight concerning the mechanism whereby a heightened degree of social contact markedly enhances the toxicity of this drug.

It has been demonstrated that the amphetamine aggregation effect can be influenced by a large number of complex variables, including cage size (4, 5), body weight and degree of hydration (5, 6), environmental noise (6), animal strain (6, 7), ambient temperature (4, 6, 8), and exposure to stress (9). Swinyard, *et al.* (10), have suggested that the increased lethality seen with aggregation is related to anxiety or fear induced by a sudden change in the environment of the animal. Askew (11) has reported that the obvious increase in body temperature observed after the administration of amphetamine is also a major contributory factor to the aggregation effect. These findings contrast sharply with the earlier observations of Swinyard, *et al.* (10), who reported that alterations in body temperature cannot be considered a critical factor in the enhanced toxicity exhibited by amphetamine in aggregated animals.

In view of the above it appeared of interest to investigate in detail the relative importance of hyperthermia in the expression of the amphetamine aggregation phenomenon. The results obtained constitute the basis for this report.

METHODS

The experimental animals employed were novice, adult, male, albino mice of a random bred Swiss strain (Research Animal Associates, Columbus, Ohio). These animals, which ranged in weight from 16 to 26 Gm., were housed in groups of 25 in opaque plastic cages 45 × 24 × 12 cm. with wire mesh tops. They were maintained on Purina laboratory chow and had free access to food and water, except during the experimental test periods.

All temperature studies were conducted in both an aggregated and isolated environment. Aggregation was defined as placing three mice in a metal cage 7 × 7 × 7.5 cm. with a wire mesh bottom; isolation consisted of placing a solitary mouse in a similar cage. The room temperature in all instances was maintained at 24–26°.

Throughout this entire study racemic amphetamine sulfate[1] was administered intraperitoneally in a constant dose of 100 mg./Kg. With mice of the above described strain, this dose of amphetamine is lethal to approximately 30% of isolated and 60% of aggregated animals. Thus any drug-induced increment or decrement in amphetamine lethality could be observed. The following drugs were evaluated for their ability to alter amphetamine-induced lethality and hyperthermia: chlorpromazine,[2] acetylsalicylic acid, and phenoxybenzamine.[3] These drugs were all administered intraperitoneally in aqueous solution, except for acetylsalicylic acid, which was given orally in a 1% methylcellulose suspension. The concentration of the drug solution or suspension employed was such that the dose administered always represented 1 ml. per 100 Gm. body weight. The mean neurotoxic dose (TD_{50}) was determined for each drug by employing a modification of the method of Dunham and Miya (12). The end point for minimal neurotoxicity was muscular incoordination based on the inability of the mouse to remain for 1 minute, given three

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[1] Marketed as Benzedrine Sulfate by Smith Kline and French.
[2] Marketed as Thorazine Hydrochloride by Smith Kline and French.
[3] Marketed as Dibenzyline Hydrochloride by Smith Kline and French.

successive trials, on a horizontal rod 2 cm. in diameter, rotating at 8 r.p.m., and suspended 38 cm. above a flat surface. Groups of at least nine mice were given various doses of drug until a minimum of three points were established in the range between 0 and 100% minimal neurotoxicity. Observations were made every 30 minutes up to a period of 8 hours. The results obtained were then plotted on logarithmic probability paper and a regression line was fitted to the plotted points by eye. From this plot of the data the TD_{50} and 95% fiducial limits were calculated by the method of Litchfield and Wilcoxon (13). The time of peak activity for each drug was determined by inspection of the neurotoxicity *versus* time data, and thereafter all test procedures were designed to coincide with this time. To insure that comparisons were made on the basis of equipotent doses, all drugs were administered in amounts equivalent to similar fractions of their respective TD_{50}'s.

To determine the effects of the agents employed on body temperature, *per se*, a series of experiments was conducted in which rectal temperatures were recorded immediately prior to drug administration and hourly thereafter for a period of 3 hours. The temperature was taken using a Tele-Thermometer and thermocouple probe (Yellow Springs Instrument Co.) inserted 2 cm. into the rectum for a period of 30 seconds. The number of mice in each drug treatment group ranged from 12 to 15, and saline controls were included for both the aggregated and isolated situations.

TABLE I.—TIME OF PEAK ACTIVITY AND NEUROTOXICITY OF SOME SELECTED DRUGS IN MICE[a]

Drug	Time Peak Effect[b]	TD_{50}[c]
Acetylsalicylic acid	90	682 (627–744)
Chlorpromazine	60	4.3 (3.1–5.9)
Phenoxybenzamine	180	50 (40–62)

[a] Values in parenthesis are 95% fiducial limits. [b] Minutes. [c] mg./Kg.

To test the hypothesis that drugs which protect against amphetamine-induced lethality do so by preventing body temperature from reaching a critical maximum level, groups of at least 24 animals each were first pretreated with varying doses of chlorpromazine, acetylsalicylic acid, or phenoxybenzamine and later challenged during the time of peak drug activity with 100 mg./Kg. of amphetamine. Rectal temperatures and the incidence of lethality were recorded hourly for 3 hours following amphetamine administration. As in previous studies, the experiments were conducted under conditions of both isolation and aggregation and controls pretreated with a requisite volume of saline were included. Any mice which died during the course of the experiment were not removed from the metal cages. All lethality data were statistically analyzed by means of a chi square test; temperature data were compared on the basis of means and standard errors.

RESULTS

The time of peak effect and neurotoxicity of the compounds in mice are shown in Table I. The time of peak effect measured by the test for minimal neurotoxicity varied from 1 hour for chlorpromazine to 3 hours for phenoxybenzamine. Acetylsalicylic acid exhibited peak activity at 90 minutes. Acetylsalicylic acid, chlorpromazine, and phenoxybenzamine induced overt symptoms of neurotoxicity in 50% of the animals after doses of 682, 4.3, and 50 mg./Kg., respectively. All future doses of these drugs were given in quantities amounting to 1/4, 1/2, and 1 TD_{50}.

The effects of the drugs employed on the body temperature of mice maintained in both an isolated and aggregated situation are shown in Table II. These results demonstrate the influence of each drug when administered alone on body temperature.

As may be observed from Table II, amphetamine, at a dose level of 100 mg./Kg., was markedly hyperthermic; the peak response occurring 2 hours

TABLE II.—INFLUENCE OF SOME SELECTED DRUGS ON RECTAL TEMPERATURE OF MICE

Environmental Condition	Drug	Dose[a]	Mice, No.	Hr. after Drug Administration 0	1	2	3
Isolation	Saline (control)	...	72	38.2[b]	37.6	37.4	37.1
	Amphetamine	[c]	99	38.0	39.0[d]	39.7[d]	39.3[d]
	Chlorpromazine	1/4	12	38.6	38.0	37.8	38.2
		1/2	12	38.6	37.3	36.3	37.0
		1	12	38.4	35.4[d]	32.1[d]	31.9[d]
	Acetylsalicylic acid	1/4	12	38.1	37.5	37.4	36.9
		1/2	12	38.3	36.9	36.9	37.0
		1	12	38.9	38.0	37.5	37.7
	Phenoxybenzamine	1/4	12	38.6	36.3	35.3[d]	34.8[d]
		1/2	15	37.2	35.0[d]	33.6[d]	33.1[d]
		1	12	38.3	35.1[d]	33.8[d]	33.8[d]
Aggregation	Saline (control)	...	72	38.2	37.2	37.2	37.4
	Amphetamine	[c]	98	37.8	39.4[d]	39.9[d]	39.8[d]
	Chlorpromazine	1/4	12	38.5	37.8	37.4	37.8
		1/2	12	38.5	37.1	36.6	36.8
		1	12	38.6	37.0	35.8[d]	35.4[d]
	Acetylsalicylic acid	1/4	12	38.2	37.7	37.4	37.2
		1/2	12	37.9	37.2	37.0	37.0
		1	12	38.8	38.0	37.8	38.0
	Phenoxybenzamine	1/4	12	37.6	35.2[d]	34.3[d]	34.0[d]
		1/2	15	38.3	36.5	35.4[d]	35.5[d]
		1	12	38.3	34.9[d]	34.6[d]	34.2[d]

[a] Fractions of TD_{50}. [b] Average rectal temperature in °C. [c] 100 mg./Kg. [d] Significantly different from control ($p < .05$).

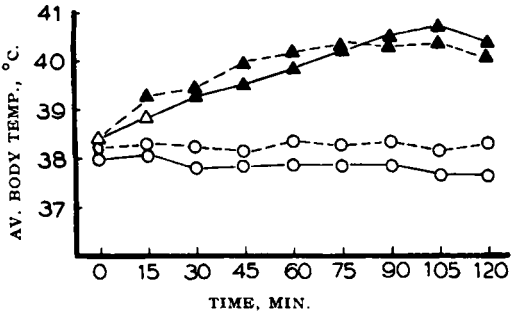


Fig. 1.—The influence of isolation and aggregation on amphetamine-induced hyperthermia. Key: O—O, isolated-saline (control); O- - -O, aggregated-saline (control); Δ—Δ, isolated-amphetamine, 100 mg./Kg.; Δ- - -Δ, aggregated-amphetamine, 100 mg./Kg.; and ▲, significantly different from control ($p < .05$).

after drug administration and amounting to 39.7° and 39.9° in the isolated and aggregated environments, respectively. It is also evident from Table II that, of the three agents under investigation, only phenoxybenzamine had a significant effect on body temperature at all dose levels employed. This agent demonstrated marked hypothermia under both environmental conditions, causing rectal temperature to fall to 33.1° 3 hours after administration of 1/2 TD_{50} followed by isolation and to 34.0° 3 hours after administration of 1/4 TD_{50} followed by aggregation. Chlorpromazine produced a hypothermic response only when given in doses equivalent to the full TD_{50}. Three hours after administration of this dose, body temperature fell to 31.9° and 35.4° in the isolated and aggregated environments, respectively. Acetylsalicylic acid in the dose levels employed appeared to have no appreciable effect on body temperature.

To explore more fully the hyperthermic response to amphetamine in an isolated *versus* aggregated environment, an experiment was carried out in which the rectal temperatures of 24 animals were recorded every 15 minutes for a 2-hour period following the administration of either 100 mg./Kg. of amphetamine or a requisite volume of saline (controls). The results of this study are shown in Fig. 1. As indicated in this figure, amphetamine was decidedly hyperthermic, causing body temperature to rise to a maximum of 40.7° in the isolated environment and 40.4° in the aggregated environment. It is also evident that this elevation in body temperature was quite rapid in its onset. However, it should be noted that essentially no difference in degree of hyperthermia was achieved in isolated *versus* aggregated animals.

The ability of the agents selected to alter amphetamine-induced hyperthermia and lethality can be observed in Table III. It is evident that the degree of hyperthermia achieved in the isolated saline-pretreated controls was not significantly different from that achieved by their aggregated counterparts. Pretreatment with chlorpromazine significantly interfered with amphetamine-induced hyperthermia when given to isolated animals in doses of 1/2 and 1 TD_{50}, whereas only the full TD_{50} was effective in the case of aggregation. On the other hand, pretreatment with acetylsalicylic acid (1/2 TD_{50}) altered amphetamine-induced hyperthermia in aggregated animals without significantly influencing the response in isolated mice. The rapid onset of lethality in animals which had been pretreated with 1/4 or 1 TD_{50} of acetylsalicylic acid and then subjected to amphetamine precludes an interpretation of temperature data for these dose levels. It is quite evident that, of the three agents evaluated, only phenoxybenzamine interfered with amphetamine-induced pyrexia in both environmental situations and at all dose levels employed.

The lethality data presented in Table III clearly demonstrate the existence of the amphetamine aggregation phenomenon in that 28.3% of the saline-pretreated control animals died in isolation, whereas 55.1% of a similarly treated group of animals succumbed to the combined effects of amphetamine and aggregation. Chlorpromazine may be observed to have decreased significantly the incidence of lethality to amphetamine in both environmental situations and at all dose levels used. On the other hand, acetylsalicylic acid afforded no protection against amphetamine-induced lethality and, indeed, seemed to potentiate the response. For example, it can be seen that when mice which had been pretreated with acetylsalicylic acid (1/4, 1/2, or 1 TD_{50}), followed by amphetamine were subjected to aggregation, the incidence of lethality rose to 100% in all cases. This same result was observed in isolated animals pretreated with the full TD_{50} of acetylsalicylic acid prior to the administration of amphetamine. Phenoxybenzamine appeared to be especially effective in abolishing the

TABLE III.—AVERAGE MAXIMUM TEMPERATURE AND LETHALITY OF MICE PRETREATED WITH SELECTED DRUGS AND ADMINISTERED AMPHETAMINE[a]

Drug	Dose Fractions of TD_{50}	Isolated Mice Temp., °C.[b]	Isolated Mice % Lethality[c]	Aggregated Mice Temp., °C.[b]	Aggregated Mice % Lethality[c]
Saline (control)	...	40.2 ± 0.8	28.3	40.6 ± 0.3	55.1
Chlorpromazine	1/4	39.8 ± 0.5	8.3[d]	40.2 ± 0.5	33.3[d]
	1/2	38.9 ± 0.4[d]	4.2[d]	40.2 ± 0.5	33.3[d]
	1	37.8 ± 0.3[d]	8.3[d]	38.9 ± 0.7[d]	30.6[d]
Acetylsalicylic acid	1/4	40.3 ± 0.6	33.3	[e]	100[d]
	1/2	40.1 ± 0.6	33.3	39.4 ± 0.7[d]	100[d]
	1	[e]	100[d]	[e]	100[d]
Phenoxybenzamine	1/4	36.5 ± 0.6[d]	4.2[d]	38.4 ± 0.4[d]	16.6[d]
	1/2	36.8 ± 0.8[d]	16.6	39.5 ± 0.4[d]	29.2[d]
	1	36.4 ± 1.1[d]	20.8	38.2 ± 0.7[d]	29.2[d]

[a] All mice received amphetamine, 100 mg./Kg. [b] Average of maximum rectal temperatures recorded during 3 hours. Values representing 95% confidence limits are included. [c] Three-hour observation period. [d] Significantly different from control ($p < .05$). [e] Lethality occurred before a significant number of rectal temperatures could be recorded.

amphetamine aggregation effect, in that all the dose levels employed in grouped mice significantly lowered the incidence of lethality to amphetamine. However, in an isolated situation, protection could only be achieved with a dose equivalent to $^1/_4$ TD_{50}.

DISCUSSION

The data presented indicate that the degree of hyperthermia induced by amphetamine cannot fully account for the increased incidence of lethality to this drug observed in aggregated *versus* isolated mice. This is evident from the results seen in Fig. 1 and Table III (saline-pretreated controls) which demonstrate that choice of environment did not significantly influence the hyperthermic response to amphetamine. The results also tend to refute the hypothesis that drugs which offer protection against the amphetamine aggregation phenomenon do so by means of a hypothermic mechanism. The information presented in Table II demonstrates that small doses of chlorpromazine, an agent well known to abolish the aggregation phenomenon (2, 3, 9, 10), were not hypothermic *per se* in this environmental situation.

Askew (11) has reported that the incidence of amphetamine-induced lethality in female Schofield albino mice can be predicted on the basis of body temperature. Mice whose body temperature exceeds a critical level (42.4°) will die, whereas those whose body temperature remains below a certain level (41.8°) will survive. In this same report, the author provides results which demonstrate that body temperature rises to a higher level in aggregated than in isolated mice, both receiving identical doses of amphetamine. On the basis of these data, the author postulates that a significant increase in body temperature in an aggregated *versus* isolated environment could account for the greater toxicity of amphetamine seen in the grouped mice. Recently Askew (14) has presented evidence to support the hypothesis that there is a direct relationship between the ability of drugs to reduce amphetamine-induced hyperthermia and their ability to protect against the amphetamine aggregation phenomenon. Table III presents a collection of data which do not support this hypothesis and Table IV summarizes these observations.

It is evident that there was no direct relationship between the ability of the selected drugs to prevent the body temperature of mice from reaching the maximum level of amphetamine-treated controls and their ability to protect mice from amphetamine-induced lethality. Chlorpromazine demonstrated protection in both isolated and aggregated mice at dose levels which had no significant effect on the degree of hyperthermia induced by amphetamine. Acetylsalicylic acid offered no protection at any dose employed in either the isolated or aggregated situation. Indeed, in all instances involving aggregation this agent was observed to increase the incidence of lethality. It is important to note that this occurred despite the observation that acetylsalicylic acid ($^1/_2$ TD_{50}) did significantly interfere with the hyperthermic response to amphetamine. Furthermore, it can be seen that phenoxybenzamine, which had been noted to interfere markedly with amphetamine-induced hyperthermia in both environmental situations, offered consistent protection against the lethal effects of the drug only in the aggregated environment. Thus, almost every combination of protection and temperature effect was observed.

There are several possible reasons for the discrepancy in results reported by Askew (14) and those presented above. The experimental methods employed in the two studies differed considerably with respect to parameters which have previously been demonstrated to influence markedly the amphetamine aggregation phenomenon, *e.g.*, cage size, degree of aggregation, drug dosage levels, and animal strain. Moreover, our experiments were terminated at the end of 3 hours, whereas Askew's were carried out over intervals of 6 and 7 hours; thus the results obtained may reflect "acute" *versus* "long term" effects of the drugs. It is also possible that the large amount of animal manipulation employed in Askew's procedure (rectal temperatures were taken every 20 minutes up to 2 hours and then every hour until termination of the experiment) may have contributed to the results reported.

It should be emphasized that since the work described herein was essentially complete at the time of Askew's recent publication (14), and since the experimental conditions were by no means analogous in the two situations, the results presented are not to be interpreted as an unsuccessful attempt to replicate that author's work. On the other hand, on the basis of our observations it would appear that, while amphetamine is indeed hyperthermic, a causal relationship does not always exist between this hyperthermia and the mechanism whereby an alteration in social environment can markedly increase the

TABLE IV.—SUMMARY OF EFFECTS OF SELECTED DRUGS ON AMPHETAMINE-INDUCED[a] LETHALITY AND HYPERTHERMIA

Drug	Dose[b]	Isolated Mice: Diminished Lethality[c]	Isolated Mice: Diminished Hyperthermia[d]	Aggregated Mice: Diminished Lethality[c]	Aggregated Mice: Diminished Hyperthermia[d]
Chlorpromazine	1/4	Yes	No	Yes	No
	1/2	Yes	Yes	Yes	No
	1	Yes	Yes	Yes	Yes
Acetylsalicylic acid	1/4	No	No	No	[e]
	1/2	No	No	No	Yes
	1	No	[e]	No	[e]
Phenoxybenzamine	1/4	Yes	Yes	Yes	Yes
	1/2	No	Yes	Yes	Yes
	1	No	Yes	Yes	Yes

[a] All mice received amphetamine, 100 mg./Kg. [b] Fractions of TD_{50}. [c] Significantly decreased amphetamine-induced lethality ($p < .05$). [d] Significantly interfered with amphetamine-induced hyperthermia ($p < .05$). [e] Data not available.

toxicity of this drug. Moreover, in view of two recent reports which indicate that magnitude of prior social experience (15) and quantity of body contact (16) represent important factors influencing the expression of the amphetamine aggregation effect, it is evident that this phenomenon represents the product of a complex interaction between several environmental, genetic, and pharmacological factors.

SUMMARY

A series of experiments was designed to test the hypothesis that hyperthermia is a major factor contributing to the increased toxicity of amphetamine observed in aggregated *versus* isolated mice. Incidence of lethality and rectal temperatures were recorded hourly over a 3-hour interval following the intraperitoneal administration of 100 mg./Kg. amphetamine to animals placed in either an aggregated (3 mice per 7 × 7 × 7.5 cm. cage) or isolated environment. The effect of pretreatment with several agents known to influence body temperature (chlorpromazine, acetylsalicylic acid, and phenoxybenzamine) was also determined. On the basis of the results obtained the following conclusions appear to be justified:

Although amphetamine is definitely more toxic in grouped animals (aggregation effect), the degree of hyperthermia induced may be essentially the same for both aggregated (40.6°) and isolated (40.2°) mice.

Drugs which have the ability to protect animals against the occurrence of the amphetamine aggregation phenomenon, *e.g.*, chlorpromazine, may do so at dose levels which are not hypothermic *per se* and which do not alter amphetamine-induced hyperthermia.

Phenoxybenzamine, which has the ability to depress markedly the hyperthermic response to amphetamine, does not consistently abolish the lethality of this drug in an isolated environment.

Pretreatment with acetylsalicylic acid using a dose level which decreases the magnitude of amphetamine-induced hyperthermia significantly increases its lethality.

A generalization indicating a causal relationship between hyperthermia and the amphetamine aggregation phenomenon may be unwarranted.

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Spectrophotometric Method for the Assay of Individual Nitroglycerin Tablets

By FREDERICK K. BELL

A spectrophotometric procedure for the microdetermination of glyceryl trinitrate through alkaline hydrolysis and subsequent determination of the nitrite formed through diazotization and coupling has been devised which avoids the serious interference in the presence of lactose. The principal feature of the method is based on the substitution of strontium hydroxide for the usual sodium hydroxide as the hydrolyzing alkali. Presumably the formation of the strontium salt of lactose removes this carbohydrate from the field of interference. The results of more than 500 assays of individual tablets of nitroglycerin representing four different sources of manufacture and five different dosages are reported.

PROBABLY BECAUSE of its high degree of sensitivity and relative simplicity, a preferred method for the microdetermination of glyceryl trinitrate is the one based on the alkaline hydrolysis (1) of the nitrate ester and determination of the nitrite formed through the customary diazotization and coupling reaction and subsequent spectrophotometric measurement of the dye formed. An obvious application of this procedure would be the assay of individual nitroglycerin tablets in which suitable aliquots of a simple aqueous solution of the tablet would be subjected to the procedure.

However, it is known that lactose, which appears to be the excipient of preference in the manufacture of these tablets and is present in gross excess, seriously interferes with this analytical procedure (2). In a consideration of possible methods of avoiding this source of inter-

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toxicity of this drug. Moreover, in view of two recent reports which indicate that magnitude of prior social experience (15) and quantity of body contact (16) represent important factors influencing the expression of the amphetamine aggregation effect, it is evident that this phenomenon represents the product of a complex interaction between several environmental, genetic, and pharmacological factors.

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Spectrophotometric Method for the Assay of Individual Nitroglycerin Tablets

By FREDERICK K. BELL

A spectrophotometric procedure for the microdetermination of glyceryl trinitrate through alkaline hydrolysis and subsequent determination of the nitrite formed through diazotization and coupling has been devised which avoids the serious interference in the presence of lactose. The principal feature of the method is based on the substitution of strontium hydroxide for the usual sodium hydroxide as the hydrolyzing alkali. Presumably the formation of the strontium salt of lactose removes this carbohydrate from the field of interference. The results of more than 500 assays of individual tablets of nitroglycerin representing four different sources of manufacture and five different dosages are reported.

PROBABLY BECAUSE of its high degree of sensitivity and relative simplicity, a preferred method for the microdetermination of glyceryl trinitrate is the one based on the alkaline hydrolysis (1) of the nitrate ester and determination of the nitrite formed through the customary diazotization and coupling reaction and subsequent spectrophotometric measurement of the dye formed. An obvious application of this procedure would be the assay of individual nitroglycerin tablets in which suitable aliquots of a simple aqueous solution of the tablet would be subjected to the procedure.

However, it is known that lactose, which appears to be the excipient of preference in the manufacture of these tablets and is present in gross excess, seriously interferes with this analytical procedure (2). In a consideration of possible methods of avoiding this source of inter-

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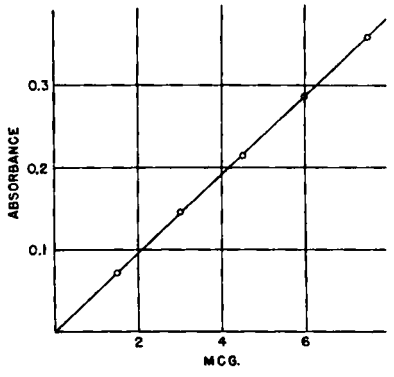


Fig. 1.--Standard recovery curve for glyceryl trinitrate.

sources of manufacture and in a number of different dosages.

MATERIALS

The assay values reported in this paper were obtained on commercial tablets purchased through the usual channels in bottles of 100 which originated from the following pharmaceutical firms: Abbott Laboratories, Eli Lilly and Co., Merck Sharp and Dohme, and Parke, Davis and Co. The dosage range included the following values: 1/100, 1/150, 1/200, 1/250, and 1/400 gr.[1]

The following reagents are required: procaine hydrochloride, 3 mg./ml.; hydrochloric acid, 4 *N*; *N*-(1 naphthyl) ethylenediamine dihydrochloride, 1 mg./ml.; and strontium hydroxide, octahydrate, 1 Gm./100 ml. It was somewhat disappointing that

TABLE I.—TYPICAL ASSAY DATA SHEET[a]

Tablet	Absorbance			Nitroglycerin	
	Observed	Av.	Corrected	Found, mcg.	Calcd., mg./Tablet
Control	0.016 0.018	0.017	...	...	...
1	0.275 0.283	0.279	0.262	5.5	0.69
2	0.292 0.291	0.292	0.275	5.8	0.73
3	0.262 0.258	0.260	0.243	5.1	0.64
4	0.214 0.211	0.213	0.196	4.1	0.51
5	0.252 0.255	0.254	0.237	5.0	0.63
6	0.275 0.274	0.275	0.258	5.4	0.68
7	0.264 0.265	0.265	0.248	5.2	0.65
8	0.280 0.280	0.280	0.263	5.5	0.69
9	0.290 0.298	0.294	0.277	5.8	0.73
10	0.276 0.277	0.277	0.260	5.45	0.68
				Av.	0.66

[a] Tablet X, dosage 1/100 gr., 0.2-ml. aliquots.

ference, the thought occurred that advantage might be taken of the well known fact that some carbohydrates, including lactose, form compounds with metallic ions, especially of the alkaline earth group. The stability of such a compound might be sufficient to reduce greatly or remove completely the lactose from the field of interference. Early preliminary experiments using barium hydroxide as the hydrolyzing alkali instead of the usual sodium hydroxide showed immediate promise. However, as the work progressed, strontium hydroxide was found to be considerably more efficient, both as the hydrolyzing alkali and as a supressor of the lactose interference.

This communication gives a detailed description of the method which has been devised and records the results obtained in the assay of commercial nitroglycerin tablets of four different

apparently the latter material is no longer readily available as a reagent chemical. The salt used in these studies was obtained from K & K Laboratories, Inc., Jamaica, N. Y., and the purity was indicated as 95–99%. Although appreciable turbidity in the 1% solution did not interfere with the procedure, the standard practice was to make up the day's requirement on the preceding day, thus providing a clear supernatant fluid for use in the assay. More prolonged storage of the solution was avoided.

METHOD

The assay experiment was set up to include the assay of ten individual tablets, the average value of which was taken as the final assay value for the tablet. The procedure is as follows.

Place one tablet in each of ten 50-ml. glass-stoppered centrifuge tubes (Pyrex No. 8424). The tablet may be crushed with a glass rod. After adding exactly 25 ml. of distilled water to each tube, stopper and shake vigorously by hand for some

[1] Grateful acknowledgment is made to these firms for supplying tablets for the preliminary studies.

seconds to complete solution. Place the ten tubes in a shaking machine and shake vigorously for 5 minutes. An appreciable turbidity in the solution at this point is, of itself, of no significance since the mixture will undergo at least a tenfold dilution before spectrophotometric analysis. Transfer duplicate aliquots from each tube to a pair of 15-ml. graduated glass-stoppered centrifuge tubes. The schedule of aliquot volume with respect to tablet dosage is as follows: 0.2 ml. for 1/100 gr., 0.3 ml. for 1/150 gr., 0.4 ml. for 1/200 gr., 0.5 ml. for 1/250 gr., and 0.8 ml. for 1/400 gr. Distilled water is added to each of the 20 tubes to the 1-ml. mark, and 1 ml. of water is added to two additional tubes which serve as controls. From this point on, the experiment should proceed at reasonable speed without interruption. Add to each of the tubes 4 ml. of 1% strontium hydroxide. Stopper and mix by inversion. The lightly stoppered 22 tubes are then placed in a suitable rack which is then suspended in a water bath at 50 ± 0.2° so that the water level is approximately at the 10-ml. mark of the tubes. After 15 minutes the

TABLE II.—SUMMARY OF ASSAY RESULTS[a]

Tablet	Dosage, gr.	Assay No.	Individual Tablet Assay Values, mg. × 100[b]										Av. mg.
			1	2	3	4	5	6	7	8	9	10	
A	1/100	1	69	55	80*	60	66	59	60	68	63	56	0.64
		2	67	58	62	59	66	75*	69	52	62	51*	0.62
		3	63	58	68	51*	70	63	50*	69	64	63	0.62
		4	55	54	66	76*	63	65	63	55	69	58	0.62
		5	68	68	69	55	66	54	63	51*	50*	68	0.61
B	1/100	6	75*	68	66	63	70	72	63	50*	58	65	0.65
		7	63	68	71	58	59	86*	64	75*	73	70	0.69
		8	74*	76*	67	50*	63	73	87*	73	69	59	0.69
		9	61	64	69	58	74*	78*	63	57	62	65	0.65
		10	65	48*	67	69	73	65	70	63	63	73	0.66
C	1/100	11	52	69	65	65	73	65	65	74	61	66	0.66
		12	63	65	63	67	74*	67	70	78*	63	70	0.68
		13	66	66	71	58	65	71	64	69	69	64	0.66
		14	59	68	62	69	67	69	68	68	63	71	0.66
		15	68	75*	66	71	73	66	66	75*	66	71	0.70
D	1/150	16	38	39	37	35	45	35	39	39	34	41	0.38
		17	41	39	44	43	38	38	38	43	46	42	0.41
		18	45	39	31*	39	40	37	33*	38	42	42	0.39
		19	41	42	47	41	35	47	39	43	49*	40	0.42
		20	49*	38	38	40	39	32	39	36	37	45	0.39
E	1/150	21	49*	45	46	44	47	50*	44	38	41	45	0.45
		22	39	35	48	37	49*	50*	44	42	42	44	0.43
		23	47	44	45	47	40	47	40	37	42	44	0.43
		24	47	43	44	43	50*	42	45	42	50*	50*	0.46
		25	40	41	47	43	41	48	39	45	47	43	0.43
F	1/150	26	40	45	42	50*	45	51*	47	42	36	42	0.44
		27	47	47	47	39	49*	42	42	47	48	47	0.46
		28	42	44	47	44	44	46	45	47	50*	50*	0.46
		29	33*	39	42	46	45	49*	47	44	42	44	0.43
		30	47	49*	47	46	40	46	44	43	45	49*	0.46
G	1/200	31	34	35	31	25*	34	28	28	35	40	32	0.32
		32	27	28	26	26	23*	33	27	33	36	29	0.29
		33	35	28	24*	22*	31	39*	27	35	23*	36	0.30
		34	35	21*	24*	41*	34	23*	28	28	35	33	0.30
		35	24*	48*	39*	35	35	24*	33	32	28	31	0.33
H	1/200	36	25*	29	25*	28	27	26	25*	28	28	28	0.27
		37	25*	27	29	26	29	29	26	26	27	29	0.27
		38	29	26	26	28	29	30	26	25*	25*	28	0.27
		39	29	31	29	28	28	28	28	25*	28	27	0.28
		40	25*	30	29	27	30	28	27	27	28	29	0.28
I	1/200	41	26	32	33	39*	36	38*	29	37*	30	34	0.33
		42	26	39*	35	38*	36	36	41*	37*	38*	39*	0.37
		43	35	31	40*	40*	26	29	38*	41*	27	30	0.34
		44	39*	34	36	37*	32	34	34	28	36	26	0.34
		45	31	31	41*	34	29	30	36	41*	41*	37*	0.35
J	1/250	46	24	27	24	24	25	25	21	23	23	26	0.24
		47	25	25	24	26	25	23	21	25	24	24	0.24
		48	21	25	25	23	24	24	24	25	21	24	0.24
		49	23	22	27	26	24	23	22	24	25	26	0.24
		50	28	21	25	26	25	22	22	24	22	27	0.24
K	1/400	51	19*	20*	18	18	18	12*	19*	21*	19*	15	0.18
		52	19*	16	18	20*	15	18	16	20*	21*	18	0.18
		53	15	16	18	17	17	19*	17	14	18	15	0.17
		54	20*	18	19*	21*	18	14	20*	17	14	20*	0.18
		55	21*	16	21*	20*	18	13	20*	18	14	18	0.18

[a] The tablets were carefully removed at random from original unopened bottles of 100. Visibly defective tablets were discarded when encountered. It appears reasonable to assume that the physical condition of these tablets at the time of assay approximates that which prevails at the time the patient receives them from the pharmacist. Individual weights of the tablets were not determined and therefore this factor, which is of considerable importance to the tablet manufacturer, is not included in the data. [b] Assay values with an asterisk indicate failure to meet U.S.P. requirements.

rack is removed from the bath, then immersed in cold tap water for approximately 5 minutes. The contents of each tube are treated in the following consecutive steps, mixing being accomplished after each step by inversion of the stoppered tube: (*a*) add 1 ml. procaine hydrochloride, 3 mg./ml.; (*b*) add 1 ml. hydrochloric acid, 4 *N*; (*c*) add 1 ml. *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 1 mg./ml., and distilled water to the 10-ml. mark.

Full color development is attained in approximately 5 minutes, after which time the absorbance of each of the 22 solutions is measured with a Beckman model DU spectrophotometer at a wavelength setting of 550 mμ and a slit width of approximately 0.025 mm. Absorption cells having a light path of 1 cm. were used; distilled water served as the reference standard. The paired values are averaged and then corrected for the control value. The corrected values are then converted into terms of nitroglycerin with a standard recovery curve.

The preparation of the standard recovery curve using the procedure detailed above is quite similar to that described in an earlier communication (1). A typical curve is shown in Fig. 1. The reproducibility is very satisfactory.

A typical assay data sheet is shown in Table I and a summarized tabulation of all the tablets assayed in this study is shown in Table II. The ten-tablet assay, as described, requires several hours in the hands of one operator. An obvious modification of the procedure is a multiple-tablet composite assay in which ten tablets are dissolved in 250 ml. of water and aliquots of the proper volume are subjected to analysis. Using triplicate aliquots, such an assay can be carried out in less than 1 hour. The results obtained from a considerable number of such assays indicate the method is quite satisfactory. There is no reason why this method could not be extended to the case of 20 tablets in 500 ml. water or even 40 tablets in 1 L. if desired.

DISCUSSION

Concerning lactose interference, recovery experiments indicated that 5 mcg. of nitroglycerin can be satisfactorily determined in the presence of 1.5 mg. of lactose. This lactose-nitroglycerin ratio of 300:1 is considerably in excess of the maximum value encountered in this study which, of course, would be in the case of the 1/400-gr. tablets. The same observation holds for β-lactose. Sucrose, in similar amount, was without effect, which is in accord with the observations of Hansen (3).

If the procaine was not added before acidification in the assay procedure following the alkaline hydrolysis step, erratic results were obtained. This indicates that at least an important part of the lactose interference takes place at the diazotization step and that the strontium salt of lactose is of sufficient stability to suppress this interference with the diazotization reaction which probably takes place very rapidly.

In considering the data presented in Table II, it is quite obvious that a rigid quantitive interpretation is not permissible, since, if for no other reason, there are probably at least five different samples of glyceryl trinitrate involved in this study. If, however, the validity of the method is accepted, then the assay data reveal some interesting information.

First, it will be noted that the average assay value for each of the 55 assay experiments is within the limits required by the U.S.P. XVI, *i.e.*, within 80 and 112% of the labeled dosage. Furthermore, there is good agreement among the five average values obtained for each type of tablet. This indicates, from this limited study, that the ten-tablet procedure might be adequate to establish the assay value for a given tablet preparation. It is to be noted also that the inherent accuracy of the method increases as the tablet dosage decreases.

Individual tablet assay values which are starred in Table II indicate failure to meet U.S.P. requirements. Among the 550 assays listed there are 101 such values, of which 78 show excessive dosage and 23 fall below the requirements.

The assay method presented in this study should be of immediate interest to the control laboratory for the manufacture of nitroglycerin tablets. It provides information concerning the uniformity of the tablet dosage and also a rapid (ten-tablet composite procedure) assay procedure. Based on a knowledge of complete composition of the tablet, rigid controls could be established and the standard recovery curve could be prepared from the sample of nitroglycerin that is used in the preparation of the tablets.

To determine whatever merit the procedure may have as an official assay method would doubtless require considerable collaborative study. A reference standard preparation of glyceryl trinitrate would probably be required and, in this connection, a simple aqueous solution of the nitrate ester (1 mg./ml.) was surprisingly stable over a period of many months. However, Hansen (4) reports that an aqueous solution of 0.12 mg./ml. showed a 10% loss in potency after 10.5 months' storage.

Additional study will also be required to determine if the method has application to other pharmaceutical preparations containing nitroglycerin.

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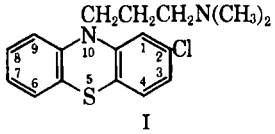
Photodegradation of Chlorpromazine Hydrochloride

By ALVIN FELMEISTER and CLARENCE A. DISCHER

The quantum efficiency of chlorpromazine hydrochloride (CLP HCl) at 253.5 mμ in aqueous media (pH 1.5) was 0.18 and 0.14 under normal and oxygen-free atmospheres, respectively. The quantum efficiency remained constant within the concentration range and periods of irradiation studied, but increased at pH values above 4.5. The photoreaction appears to follow zero-order kinetics. Reactivity toward acetic anhydride, partition studies, and infrared spectra of light degraded solutions of CLP HCl suggest ring hydroxylation occurs during irradiation. Spectral evidence indicated the formation of a semiquinone free radical intermediate on irradiation of aqueous solutions of CLP HCl at 253.5 mμ. Chlorpromazine-5-oxide hydrochloride (CLP-O HCl) forms by disproportionation of this free radical in aqueous media, both in the presence and absence of dissolved oxygen. The phenolic and other unidentified compounds formed on irradiation of CLP HCl apparently result from degradation of the photolabile CLP-O formed.

CHLORPROMAZINE, 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine (I) represents a large group of antipsychotic agents which are photosensitive. However, photolytic studies reported on this compound have been limited. Nakagawa, *et al.* (1), irradiated CLP under both normal and nitrogen atmospheres. They reported that degradation occurred under both conditions with very little apparent difference in the resultant products. Raven, *et al.* (2), measured manometrically the oxygen uptake of an aqueous solution of CLP HCl subjected to ultraviolet radiation. Under these conditions, after a short induction period, the oxygen uptake was linear with time, but stopped as soon as the source of radiation was removed. Turse (3) subjected CLP HCl in acidic medium to ultraviolet radiation. He noted that very little degradation occurred when the wavelength of irradiation employed was above 365 mμ. He also noted that CLP and CLP-O degraded on exposure to ultraviolet radiation; on long exposure, both compounds appeared to degrade to the same product. Forrest, *et al.* (4), detected free radicals by ESR measurements on exposure of chlorpromazine to ultraviolet radiation. Under these conditions the ultraviolet spectrum of this radical showed no characteristic peaks. Fels and Kaufman (5) confirmed the presence of free radicals under similar conditions. Borg and Cotzias (6) on extended ultraviolet irradiation of a solution of CLP detected a weak ESR peak and concluded that while radicals were produced they were not the typical semiquinone free radical produced on chemical oxidation of CLP. It should be noted that except for the work reported in *References 1* and *3*, no attempt was made to restrict the wavelength employed for irradiation. Ultraviolet lamps were employed without using filters or monochromators. None of this work served to establish a quantum efficiency or mechanism for the photodegradation.

The purpose of this work was to study photodecomposition of CLP HCl under carefully controlled conditions to evaluate the quantum efficiency at 253.5 mμ (the absorbance peak) and define the mechanism of this reaction better.



I

EXPERIMENTAL AND RESULTS

Instrumentation.—All solutions were analyzed using the Beckman model DU spectrophotometer. Ultraviolet absorption spectra were obtained using a Beckman ratio recording spectrophotometer model DK-2; infrared spectra were obtained using a Perkin-Elmer model 21 spectrophotometer. A Beckman model N pH meter was used for pH determinations.

The microirradiation studies, using concentrations in the range of 10^{-5} *M*, were conducted using the irradiation instrumentation described by Discher, *et al.* (7), except for a modification in the reaction box which permitted the use of standard size Beckman quartz cells. This consisted of a mercury arc light source operated from a voltage regulator. This light was passed through a Bausch & Lomb grating monochromator into the reaction box. Within the reaction box was a water coil connected to an externally placed constant temperature water bath, a thermopile, and a cell holder which accommodated three standard 1-cm. Beckman quartz cells. The cell holder was set so that two of the cells could be moved individually into the light beam. This permitted irradiation of the solvent and sample while a reference solution was maintained in the reaction box without being subjected to irradiation. The thermopile, which was positioned so that the light passing through the cell being irradiated would strike it, was attached through a direct current

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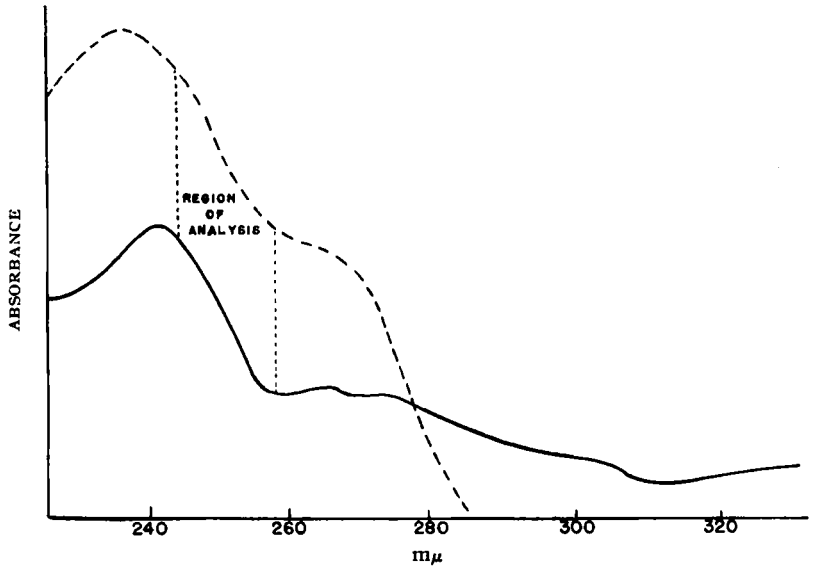


Fig. 1.—The ultraviolet spectra of compounds formed on irradiation of CLP HCl. Key: ———, spectrum obtained by a differential spectrophotometric technique; – – – –, spectrum obtained by an extraction procedure.

breaker amplifier to a direct current recording milliammeter. Thus, the incident radiant energy and that absorbed by the sample could be measured.

The macroirradiation studies, using concentrations in the range of 10^{-2} to 10^{-3} *M*, were conducted using a Hanovia ultraviolet lamp equipped with a heat filter and removable glass filter transmitting from 360 to 370 mμ. A 30-ml. capacity quartz cell was used in conjunction with this lamp. This portion of the work was qualitative in nature, and no attempt was made to determine the quantum efficiency or extent of degradation under these conditions.

Material.—The CLP HCl and CLP-O HCl were generously supplied by Smith Kline & French Laboratories. These compounds were used without further purification. All other chemicals were of reagent grade. The distilled water was ordinary distilled water, redistilled from permanganate and phosphoric acid using all glass equipment.

Preparation of Sample.—Solutions were prepared on the day they were to be irradiated. Hydrochloric acid, 0.05 *N*, was used as the solvent unless otherwise noted. When oxygen-free atmospheres were employed, the solutions were prepared using recently distilled nitrogen-deaerated water. The nitrogen used for the deaeration was first passed through 0.05 *N* hydrochloric acid to prevent loss of HCl from the solvent. In the microirradiation studies these deaerated samples were measured and transferred to Beckman 1-cm. ground glass-stoppered quartz cells under a nitrogen atmosphere. A positive nitrogen pressure was maintained in the irradiation reaction box and the cell chamber of the Beckman DU. In both oxygen-free and normal atmosphere runs, the irradiation sample and a reference sample were analyzed immediately before and after irradiation.

All glassware was rinsed three times with distilled water prior to use to minimize reaction with trace metal ions.

Assay and Proof of Specificity.—To permit the analysis of CLP HCl in the presence of the interfering compounds formed during the irradiation, a three point spectrophotometric assay similar to that used by Flanagan, *et al.* (8), and Smith Kline & French Laboratories (3) was employed. An additional modification involving the selection of wavelengths was made in this study, since preliminary work indicated this change would increase the accuracy of the assay in the presence of light degraded materials.

TABLE I.—ANALYSIS OF CLP HCl IN THE PRESENCE OF INTERFERING COMPOUNDS

10^5 × CLP HCl Concn., mg./ml.	10^5 × CLP-O HCl Concn., mg./ml.	10^5 × Calcd. CLP HCl Concn., mg./ml., by Eq. 1	% Error	% of Total Compn. Present as CLP-O HCl[a]
800	13	795	0.40	1.6
800	25	801	0.13	3.1
400	13	403	0.75	3.2
800	50	799	0.13	5.8
800	50	795	0.40	5.8
800	100	789	1.40	11.0
800	100	808	1.00	11.0
800	100	796	0.50	11.0
800	100	782	2.30	11.0
800	100	782	2.30	11.0
400	50	400	0.00	11.0
400	100	394	1.50	20.0
533	371	528	1.00	41.0
267	371	257	4.00	51.0
400	742	386	3.50	65.0
0	400	0	0.00	100.0

[a] Represents either pure or light degraded chlorpromazine-5-oxide hydrochloride.

This assay is specific for CLP HCl as long as interfering compounds (light degraded products) present absorb linearly within the wavelengths selected for the analysis (9). The linearity of the absorbance of degraded products was demonstrated by the following procedures: (*a*) a differential spectrophotometric technique whereby the nondegraded CLP HCl present in an irradiated solution was compensated for by including an indentical amount in the reference cell. The resultant spectrum represented only the degraded compounds (Fig. 1); (*b*) by an extraction procedure whereby the nondegraded CLP HCl was extracted with chloroform from an irradiated solution, pH of 5 (Fig. 1).

The assay was further tested by analyzing solutions of known concentrations of CLP HCl to which had been added known amounts of CLP-O HCl or light degraded CLP-O HCl (Table I).

Assay Procedure.—The absorbance of a solution to be analyzed was determined at 246.5, 254.5, and 259.5 mμ. The absorbances at 246.5 and 259.5 mμ were plotted *versus* wavelength and the two points connected with a straight line. The absorbance at 254.5 mμ was then read off this line (A_{base}). The difference between the observed absorbance at 254.5 mμ ($A_{max.}$) and (A_{base}), that is ($A_{max.} - A_{base}$), was proportional to the concentration of nondegraded CLP HCl within the range studied. Thus, the concentration, *c*, of CLP HCl may be calculated using

$$c = K(A_{max.} - A_{base}) \qquad \text{(Eq. 1)}$$

TABLE II.—EVALUATION OF THE PROPORTIONALITY CONSTANT K[a]

$10^5 \times$ CLP HCl Concn., mg./ml.	$A_{max.} - A_{base}$, Absorbance Units	$10^4 \times K$ Calcd. from Eq. 1
200	0.046	435
400	0.090	444
400	0.092	435
400	0.090	444
600	0.134	448
800	0.183	437
800	0.181	442
800	0.182	439
800	0.180	444
800	0.182	439
800	0.180	444
800	0.182	439
800	0.183	437
800	0.180	444
800	0.181	442
800	0.180	444
800	0.180	444
1000	0.225	444

[a] Average K = 0.0442 S.D. ±0.0005.

TABLE III.—QUANTUM EFFICIENCY AT 253.5 mμ UNDER NORMAL ATMOSPHERE AND pH 1.5

$10^5 \times$ Initial Concn. of CLP HCl, moles/L.	Time of Irradiation, Min.	Quantum Efficiency[a]
1.13	20	0.17
1.13	15	0.18
1.13	10	0.19
2.25	15	0.19
2.25	13	0.18
2.25	10	0.17
	Av.	0.18 ± 0.02

[a] The values in this column represent an average of at least three determinations.

TABLE IV.—QUANTUM EFFICIENCY AT 253.5 mμ UNDER OXYGEN-FREE ATMOSPHERE AND pH 1.5

$10^5 \times$ Initial Concn. of CLP HCl, moles/L.	Time of Irradiation, Min.	Quantum Efficiency[a]
1.13	20	0.15
1.13	15	0.14
2.25	30	0.16
2.25	20	0.13
2.25	10	0.14
	Av.	0.14 ± 0.02

[a] The values in this column represent an average of at least three determinations.

where K is a proportionality constant having the experimentally determined value of $4.42 \times 10^{-2} \pm 0.05 \times 10^{-2}$ mg./ml. (Table II).

Calculation of Quantum Efficiency.—The quantum efficiency, ϕ, may be defined

$$\phi = \frac{\text{No. of molecules decomposed}}{\text{No. of photons absorbed}} \qquad \text{(Eq. 2)}$$

The evaluation of this equation is described in detail by Discher, *et al.* (7). However, a brief discussion of the procedure will be covered here.

The numerator can be shown to be equal to

$$(M)(V)(N)(r) \qquad \text{(Eq. 3)}$$

where M is the molarity of the irradiated solution with respect to CLP HCl, V is the volume of the irradiated solution in liters, N is Avogadro's number (6.025×10^{23}), and r is the fraction of CLP HCl degraded during the irradiation.

To evaluate the denominator, the total radiant energy absorbed by the sample during the period of irradiation must first be determined.

The voltage output of the thermopile is proportional to the amount of radiation striking it per unit time. The recorder response is proportional to the voltage output of the thermopile. Thus, the radiant energy absorbed by the sample is proportional to the difference between the recorder response when a cell filled with solvent, I_o, is placed between the thermopile and the light source, and when a cell filled with a solution of the sample, I, is placed in this position, *i.e.*, $I_o - I$.

To calibrate the recorder response to irradiation in units of voltage, the recorder response, I_s, to a test signal of known voltage, s, is determined. Thus, the value of $I_o - I$ can be calculated in terms of volts.

The use of the thermopile constant, k, determined to be 0.0444 microvolts/microwatt/cm.^{2} (7) and the area of irradiation, A, determined to be 1.561 cm.^{2} (10) permit the calculation, in microwatts, of the radiant energy absorbed per second. The time of irradiation, t, in seconds permits calculation of the microwatts-seconds absorbed during the period of irradiation. Since 1 microwatt-second equals 10 ergs, the factor 10 is used to convert the energy to units of ergs. Thus, the total energy absorbed in ergs can be

$$\frac{10(I_o - I)(A)(t)(s)}{(I_s)(k)} \qquad \text{(Eq. 4)}$$

The energy, E, of a photon in ergs can be

$$E = hc/\tau \qquad \text{(Eq. 5)}$$

where h is Planck's constant (6.62×10^{-27} erg-sec.),

TABLE V.—SUMMARY OF EFFECT OF CHANGE IN pH ON QUANTUM EFFICIENCY AT 253.5 mμ UNDER NORMAL AND OXYGEN-FREE ATMOSPHERE

pH	Av. Quantum Efficiency Under Normal Atmosphere[a]	Av. Quantum Efficiency Under Oxygen-Free Atmosphere[a]
1.5	0.18	0.14
3.2	...	0.13
4.5	0.19	...
5.0	0.23	0.21
5.8	0.24	0.21
6.3	0.26	...
7.2	...	0.22

[a] The values in these columns represent an average of at least three determinations.

c is the velocity of light (3.00×10^{10} cm./sec.), and r is the wavelength in cm. The energy of the wavelength used for the determination of the quantum efficiency, 253.5 mμ, was calculated to be 7.82×10^{-12} ergs/photon using Eq. 5.

The number of photons absorbed can be calculated by dividing the total number of ergs absorbed (Eq. 4) by the energy per photon, 7.82×10^{-12} ergs/photon.

The quantum efficiency can now be

$$\phi = \frac{(M)(V)(N)(r)(I_s)(k)\ 7.82 \times 10^{-12}}{10\ (I_o - I)(A)(t)(s)} \quad \text{(Eq. 6)}$$

A typical calculation is $M = 1.13 \times 10^{-5}$ moles/L., $V = 3.00 \times 10^{-3}$ L., $r = 0.234$, $I_s = 35.4$ chart units, $I_o - I = 18.0$ chart units, $t = 1200$ seconds, and $s = 0.1$ microvolts. The other symbols represent constants with previously assigned values.

$$\phi = \frac{(1.13 \times 10^{-5})(3.00 \times 10^{-3})(6.03 \times 10^{23})}{10(18.0)(1.56)(1200)(0.1)} \frac{(0.234)(35.4)(0.0444)(7.82 \times 10^{-12})}{}$$

$$\phi = 0.177 = 0.18$$

Microirradiation—Determination of Quantum Efficiency.—A series of CLP HCl solutions was irradiated at 253.5 mμ for 10 to 30 minutes at selected pH values within the range of 1.5 to 7.2, under normal and oxygen-free atomspheres. The concentration range selected allowed both irradiation and analysis to be conducted in the same cell, eliminating the chemical degradation possible during transfer or dilution techniques.

At a pH of 1.5, the average quantum efficiency was 0.18 ±0.02 under a normal atmosphere (Table III), while under an oxygen-free atmosphere it was 0.14 ±0.02 (Table IV). The effect of changing pH under both normal and oxygen-free atmospheres is summarized in Table V. Typical examples of the change in absorbance taking place during irradiation are shown in Fig. 2. The spectrum obtained on irradiation under oxygen-free atmosphere is similar to that obtained during the early phase of irradiation under a normal atmosphere. Thus, the difference in the spectra in Fig. 2 is indicative of the more rapid rate of degradation which occurs in the presence of dissolved oxygen. This is also demonstrated by the determined quantum efficiencies.

Microirradiation—Detection of CLP Semiquinone Free Radical.—Since previous work indicated the semiquinone free radical of CLP was stabilized in 9 N sulfuric acid (11), CLP HCl dissolved in this solvent was irradiated for 2 hours at 253.5 mμ under a normal atmosphere in an attempt to stabilize any free radical produced as a result of irradiation. During the irradiation the solution turned pink, typical of the semiquinone free radical. The absorbance spectrum of the solution at the end of the irradiation differed markedly from that obtained on irradiation of CLP HCl in 0.05 N hydrochloric acid. Comparison with the spectrum of a 9 N sulfuric acid solution of a crystalline semiquinone free radical of CLP (compound R), prepared as described by Felmeister, Discher, and Merkle (11), indicated a semiquinone free radical of CLP was a product of the irradiation (Fig. 3).

Macroirradiation—Effect of Irradiation on pH.—A solution of CLP HCl ($\approx 3 \times 10^{-2}$ M) in distilled water was irradiated for 20 hours under a normal atmosphere. A significant reduction in pH was noted. The results are recorded in Table VI.

It appeared from the irradiations performed under an oxygen-free atmosphere that the water present as the solvent was involved in the formation of CLP-O. If this were the case, it appeared likely that two protons might be released for each molecule of CLP-O formed. This should manifest itself by a decrease in pH following irradiation.

Macroirradiation—Effect of Irradiation on Polarity and Reactivity.—The products formed on extensive irradiation of CLP HCl appeared to be more hydrophilic than CLP HCl itself. CLP-O HCl did not demonstrate this property, but the formation of phenolic compounds, which had been suggested as oxidation products of CLP (4), would be expected to increase the hydrophilic nature of the compound. Attempts to separate pure compounds from the

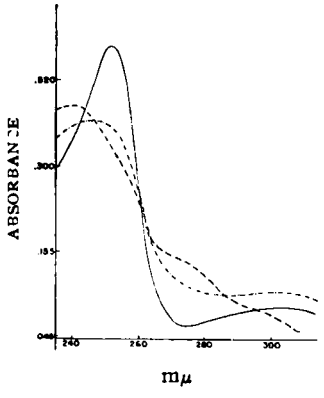


Fig. 2.—Ultraviolet absorbance spectrum of CLP HCl in 0.05 N HCl irradiated at 253.5 mμ for 4 hours. Key: ———, before irradiation; – – –, after irradiation under a normal atmosphere; –·–·–·–, after irradiation under an oxygen-free atmosphere.

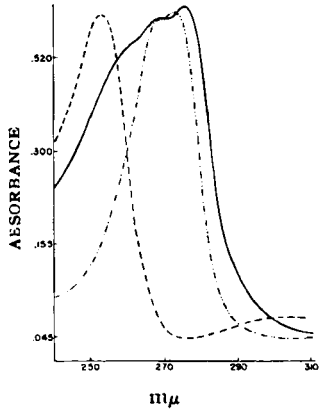


Fig. 3.—Ultraviolet absorbance spectra of a 9 N H_2SO_4 solution of CLP HCl before and after irradiation compared with a 9 N H_2SO_4 solution of compound R. Key: – – –, CLP HCl before irradiation; ———, CLP HCl after irradiation; –·–·–·–, compound R.

TABLE VI.—EFFECT OF IRRADIATION ON pH OF SOLUTION OF CLP HCl IN DISTILLED WATER

	pH of Soln. Before Irradiation	pH of Soln. After 20 Hr. Irradiation, 360 to 370 mμ
Run *1*	5.05	1.90
Run *2*	4.90	2.00

degraded mixtures were unsuccessful. The detection of phenols by use of Fe (III) was attempted, but interference from both the CLP-Fe (III) reaction and the color of the degraded solution made interpretation of the results doubtful.

Since the nitrogens in CLP are tertiary and would not react with acetic anhydride, it was felt that any reactivity toward this reagent should serve to indicate the presence of phenolic compounds.

A solution of CLP HCl ($\approx 3 \times 10^{-2}$ *M*) was irradiated for 3 hours under a normal atmosphere. A typical dark brown developed during the course of the irradiation. The pH of the irradiated solution was adjusted to about 5 with 0.1 *N* sodium hydroxide solution and extracted three times with chloroform. The ultraviolet absorbance spectrum of the material extracted into the chloroform layer was typical of nondegraded CLP HCl. The dark brown degraded material, which remained in the aqueous layer, was treated with acetic anhydride. After 20 minutes it was noted the dark brown material readily partitioned into chloroform, and was extracted by two separate portions of chloroform. The chloroform was evaporated at room temperature and the residue dissolved in methanol. To remove excess acetic anhydride or acetic acid, the dark brown material was precipitated from the methanol by the addition of ether; this process was repeated three times. The material was then dried in a vacuum desiccator for 48 hours. The infrared spectrum of the residue was determined. A strong peak at 5.97 μ was noted which was absent in the spectrum of CLP HCl or its 5-oxide.

Macroirradiation—Effect of Dissolved Oxygen.—To note the effect of dissolved oxygen in the absence of water, solutions of CLP HCl in anhydrous methanol were treated as follows. One 20-ml. portion was placed in the 30-ml. quartz cell and flushed with nitrogen for 1 hour. The cell was sealed and irradiated for 2 hours. During this irradiation the solution turned a pale yellow. A second 20-ml. portion of the initial solution was placed in the quartz cell under normal atmosphere and irradiated for 2 hours. During this irradiation the solution developed a deep brown. The ultraviolet absorbance spectra of these solutions after irradiation indicated the 5-oxide does not form under anhydrous conditions in the absence of oxygen (Fig. 4).

The slight shift in the absorbance peak after irradiation in the absence of water and oxygen could not be explained on the basis of this work. However, a reaction at the alkyl chain or the ring at positions other than the sulfur could conceivably result in such a shift.

Macroirradiation—Irradiation of CLP-O HCl.—To demonstrate the photosensitivity of CLP-O HCl and to show that the photodegradation of CLP HCl probably results in the formation of CLP-O HCl, a solution of CLP-O HCl was irradiated for 2 hours. The resultant spectrum (Fig. 5) strongly resembles that of photodegraded CLP HCl (Fig. 2).

DISCUSSION

Semiquinone free radicals which undergo disproportionation to yield their respective 5-oxides have been shown to be intermediates in the electrolytic oxidation of phenothiazine (12) and CLP HCl (13) and in the chemical oxidation of CLP HCl (6).

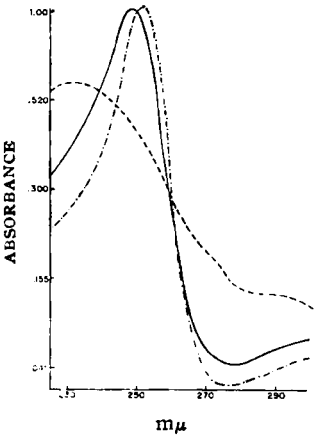


Fig. 4.—Ultraviolet absorbance spectra of anhydrous methanol solutions of CLP HCl irradiated for 2 hours under normal and oxygen-free atmospheres. Key: -·-·-·-, before irradiation; - - -, after irradiation under a normal atmosphere; ——, after irradiation under an oxygen-free atmosphere.

The formation of free radicals on photolysis of CLP has been demonstrated by electron spin resonance studies (4–6). However, identification of these radicals was not reported.

The ultraviolet absorbance spectrum which resulted on irradiation of CLP HCl in 9 *N* sulfuric acid at 253.5 mμ (Fig. 3) is quite similar to that of compound R, indicating that the intermediate formed on irradiation of CLP HCl is a semiquinone free radical. The observed difference in these spectra can be accounted for on the basis of the disproportionation of a small fraction of the photoproduced free radical, followed by photodegradation of the 5-oxide formed as a result of this disproportionation. The fact that the absorbance peak at 227 mμ, indicative of the semiquinone free radical, is not present in the spectrum of an irradiated solution of CLP HCl when 0.05 *N* hydrochloric acid is used as the solvent is not surprising (Fig. 2), since the semiquinone free radical of CLP HCl disproportionates rapidly at hydrogen ion concentrations below 4 *N* (11).

It appears from these data that ultraviolet irradiated CLP HCl degrades *via* a semiquinone free radical intermediate. Disproportionation of this free radical results in the formation of CLP-O HCl and apparently further changes result from degradation of this photolabile CLP-O (3) (Fig. 5) rather than from CLP itself.

The presence of phenolic compounds was demonstrated by Brown (14) in the reaction mixture of ultraviolet irradiated phenothiazine-5-oxide. The formation of similar compounds on extensive irradiation of CLP HCl, probably *via* the CLP-O HCl, are indicated in this work by (*a*) the increase in polar properties of CLP HCl after irradiation, demonstrated by the change in partitioning properties in a chloroform-acidic system, (*b*) the ability of the degraded compounds to undergo acetylation, (*c*) the

reduction of the polar properties as a result of this acetylation, (*d*) the appearance of an infrared absorbance peak at 5.97 μ indicative of a carbonyl group after acetylation.

It should be noted that the loss of an electron from a semiquinone free radical of phenothiazines, as results in disproportionation, yields first a phenazathionium ion (15), which on further reaction results in the 5-oxide. Work now in progress has shown that the semiquinone free radial of CLP disproportionates in aqueous media both in the absence and presence of dissolved oxygen, though the loss of free radical is slightly faster in the presence of oxygen. Thus, it would appear water and oxygen both may compete for the short lived CLP phenazathionium ion or the oxygen may react directly with the free radical, yielding the 5-oxide in both cases. Since the introduction of molecular oxygen into the system has only a minor effect on the rate of radical loss, it appears the water reaction is much more rapid than the oxygen reaction.

The significant increase in hydrogen ion concentration on irradiation of CLP HCl in water, shown in Table VI, can be explained on the basis of the reaction of the short lived phenazathionium ion with water to yield the 5-oxide, as shown below. It can be seen from this reaction mechanism that two hydrogen ions result for each molecule of 5-oxide formed. Thus, on the irradiation of a 10^{-2} *M* solution of CLP HCl (initial pH $\approx$ 5) a resultant pH of 2 is reasonable to expect.

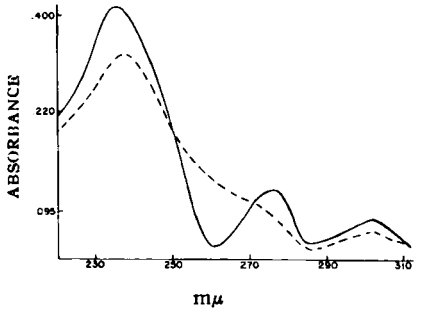


Fig. 5.—Ultraviolet absorbance spectrum of CLP-O HCl in 0.05 *N* HCl irradiated for 2 hours under a normal atmosphere. Key: ———, before irradiation; - - - - - -, after irradiation.

The fact that the irradiation of CLP HCl in the absence of water and dissolved oxygen did not yield the 5-oxide, but in the presence of either one of these species apparently did (Figs. 2 and 4), tends to support the postulation that water and/or oxygen are involved in the 5-oxide formation.

The increase in quantum efficiency with increase in pH as shown in Table V is not readily explained on the basis of this study, though it may be a measure of the greater instability of the free amine base (which increases in concentration with pH) compared to the amine salt.

If each photon absorbed resulted in the decomposition of a molecule of CLP HCl the quantum efficiency would have a value of 1. However, any process that tends to dissipate the energy of the absorbed photons before decomposition occurs will reduce the value of the quantum efficiency. The low quantum efficiency determined for this reaction no doubt is due to a number of such factors (*a*) fluorescence, the emission by the excited molecule at a higher wavelength of part of the absorbed energy, (*b*) loss of absorbed energy through collisions with solvent molecules, and (*c*) the gaining of an electron by the photo produced radical, resulting in reformation of CLP. However, the proposed disproportionation reaction would also favor a low quantum efficiency, since this tends to reproduce the starting compound continually.

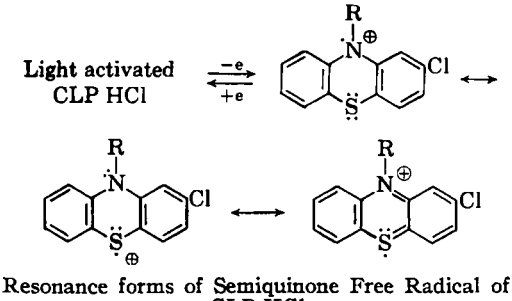
The small reduction in quantum efficiency on deaeration of the system demonstrated that the removal of oxygen is insufficient in itself to prevent the photodegradation of aqueous solutions of CLP HCl. This further supports the postulation that while apparently both water and/or oxygen take part in the reaction the water reaction proceeds at a more rapid rate.

From the quantum efficiency data it can be shown that the over-all photodegradation of aqueous solutions of CLP HCl follow zero-order kinetics as expressed by Eq. 7

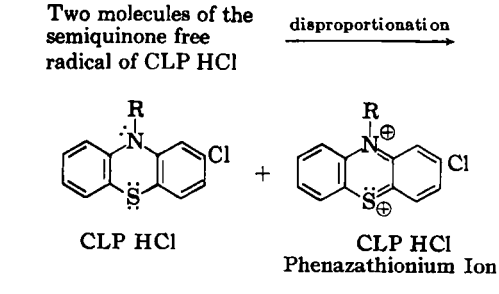
$$A = A_o - \phi I_a t \qquad \text{(Eq. 7)}$$

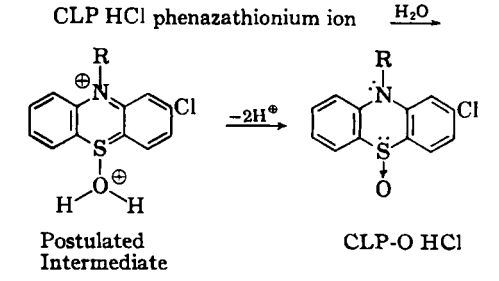
where A_o = the initial concentration of CLP HCl (molecules/ml.), A = the concentration of CLP at time t, t = the period of irradiation in seconds, I_a = the number of photons absorbed/ml. second, and ϕ = the quantum efficiency.

A plot of concentration of CLP HCl, *A*, *versus*



Resonance forms of Semiquinone Free Radical of CLP HCl





R = $CH_2CH_2CH_2N(CH_3)_2 \cdot HCl$

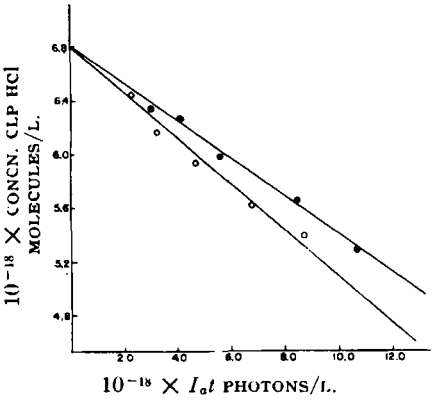


Fig. 6.—A plot of concentration of CLP HCl *vs.* number of photons/liter absorbed showing the zero-order dependency of the photoreaction. Key: ○, under a normal atmosphere, slope = 0.18; ●, under an oxygen-free atmosphere, slope = 0.14.

total photons absorbed per milliliter, $I_a t$, is included (Fig. 6).

SUMMARY

The presence of a semiquinone free radical as an intermediate in the ultraviolet photolysis of CLP HCl was demonstrated. Disproportionation of this free radical intermediate yields the photolabile CLP-O HCl, which apparently is the precursor to phenolic and other unidentified degradation products formed on irradiation.

Photodegradation of CLP HCl seemed to be more dependent on the presence of water than dissolved oxygen, and it would not be expected that elimination of oxygen alone would have a marked effect on the degradation of ultraviolet irradiated solutions of CLP HCl at 253.5 mμ.

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By VERNON A. GREEN

Mice pretreated with anticholinesterases showed enhanced stimulation in the presence of pentylenetetrazol and strychnine, a decreased lag period with anesthetic doses of phenobarbital, and a prolongation of anesthesia with pentothal. An attempt was made to correlate these changes in response with the depression of acetylcholine hydrolyzing enzymes.

BECAUSE THE brain has higher concentrations of acetylcholinesterase than other areas of the body (1), attempts to potentiate drug action through cholinesterase inhibition should be accomplished more readily with agents which primarily affect the central nervous system.

The findings of potentiation of the rate of diffusion of acid fuchsin (2) in frog and dog (3), trypan red (4) in dog, and the potentiation of morphine (5) in cats, streptomycin on *Escherichia coli* (6) and rats (7), and barbital (8) in mice by neostigmine or physostigmine were indications that possibly the activity of other drugs could be potentiated.

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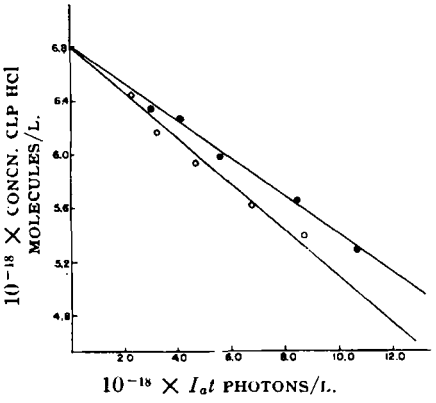


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TABLE I.—DETERMINATION OF CHOLINESTERASE INHIBITION IN MOUSE BRAIN AND BLOOD

Group	Units[a] Esterase Activity		Depression, %	
	Blood	Brain	Blood	Brain
Control	0.558	0.846	0	0
Physostigmine	0.495	0.781	11.3	7.6
Neostigmine	0.421	0.700	24.5	17.0
DFP	0.510	0.822	8.5	2.9

[a] A unit of cholinesterase for this study is defined as the number of milliliters of 0.1 *M* acetic acid derived from 3 ml. of 0.1 *M* acetylcholine bromide solution by the cholinesterase activity in 1 Gm. of mouse brain or 1 ml. of whole blood in 10 minutes at a constant pH of 8.

and in the case of CNS depressants—to decrease the anesthetic lag, to prolong the anesthetic time of a given dose, or to increase the anesthetic activity of a smaller dose.

EXPERIMENTAL AND DISCUSSION

Determination of Cholinesterase Inhibition

Eighty mice (28 to 40 Gm., no regard to sex or age) were divided into four groups of 20 each. Group *A* was injected with 0.0004 mg./Gm. physostigmine salicylate; group *B* received 0.0004 mg./Gm. neostigmine; group *C* received DFP (2 mg./Kg.); and group *D* was used as the control. The animals were etherized and then decapitated 15 minutes after the injection of the cholinesterase inhibitors. The brain and blood were removed for the determination of cholinesterase content.

The degree of cholinesterase inhibition was determined and reported in units per gram of brain and milliliters of whole blood as shown in Table I.

Strychnine Potentiation

The possible potentiation of strychnine sulfate (0.00025 mg./Gm.) by cholinesterase inhibitors was studied on mice with no regard to age or sex. The cholinesterase inhibitor was administered prior to the strychnine; any increase in the stimulating activity above that of the 0.00025 mg./Gm. controls was interpreted as potentiation.

Twenty mice injected subcutaneously with 0.0004 mg./Gm. of physostigmine salicylate had convulsions and died in an average of 40.35 seconds following 0.00025 mg./Gm. of strychnine sulfate intravenously (Fig. 1), but none of the controls showed visible signs of stimulation (Fig. 1).

Twenty mice injected subcutaneously with neostigmine methylsulfate (0.0004 mg./Gm.) 15 minutes before strychnine sulfate (0.00025 mg./Gm.) had convulsions and died (Fig. 1), while the same number of mice with 2 mg./Kg. of DFP 2.5 hours prior to strychnine showed only convulsions (Fig. 1).

Since cholinesterase inhibitors cause vasodilation either directly or indirectly through the accumulation of acetylcholine, an attempt was made in this study to produce vasodilation by the administration of acetylcholine. Of 23 mice injected intravenously with 0.05 mg./Kg. of acetylcholine bromide plus strychnine sulfate, all showed convulsive seizures of short duration with only one death (Fig. 1).

To study the effects of possible vasodilation on potentiation of strychnine further, attempts were made to prevent vasodilation with a parasympatholytic drug. Twenty mice were given the cholinesterase inhibitors with atropine sulfate (0.1 mg./Gm.) 15 minutes prior to the intravenous strychnine, with no appreciable change in the stimulating activity above or below that of the animals receiving inhibitor and strychnine alone (Fig. 1). Physostigmine prior to strychnine and acetylcholine combined showed erratic results (Fig. 1).

Schweitzer (10) reported that neostigmine and acetylcholine depressed spinal reflexes, thereby inhibiting strychnine induced convulsions. The opposite action was found in this study since a great potentiation of a subconvulsive dose of strychnine was noted by neostigmine and a small potentiation by acetylcholine. It is true, however, that large amounts of acetylcholine will decrease nerve transmission since it will keep nerve endings depolarized. Therefore, large doses of neostigmine or acetylcholine could depress spinal reflexes, but with the doses used in this study, there was potentiation of strychnine activity instead of depression.

Pentylenetetrazol Potentiation

The use of strychnine as a drug is almost purely academic, and since Nachmansohn (12) has reported strychnine as a cholinesterase inhibitor *in vitro*, it was decided to attempt potentiation of another

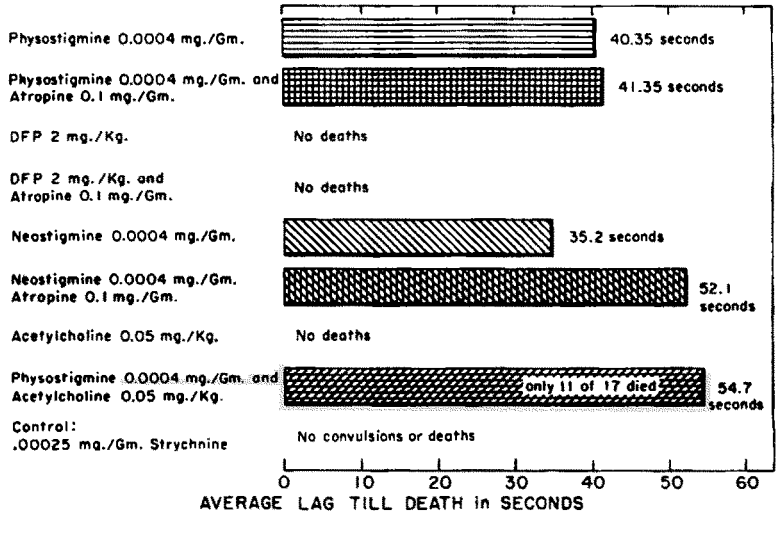


Fig. 1. — Effect of cholinesterase inhibitors, atropine, and acetylcholine on CNS stimulating activity of strychnine (0.00025 mg./Gm.) intravenously in mice.

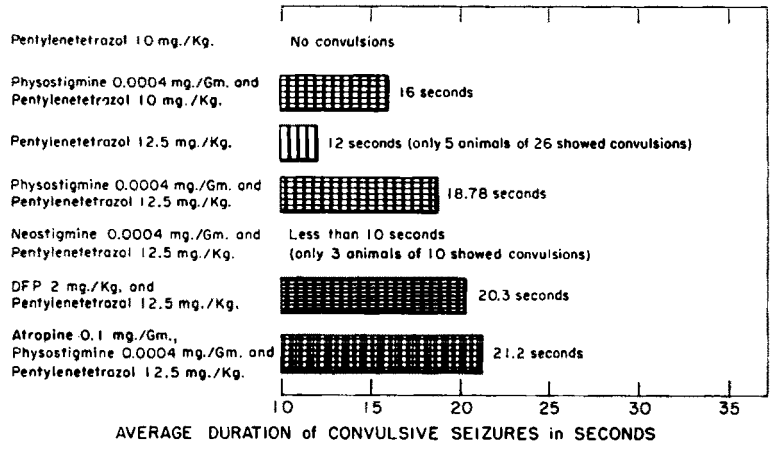


Fig. 2.— Effect of cholinesterase inhibitors and atropine on activity of pentylenetetrazol in mice.

CNS stimulant—one used medicinally today that is not thought to be a cholinesterase inhibitor.

Pentylenetetrazol was given to mice in doses of 10, 12.5, and 15 mg./Kg. intravenously to determine a minimal convulsive dose. It was found that 10 mg./Kg. was subconvulsive in all mice used in this study (Fig. 2). Five of 26 mice showed convulsive seizures of very short duration with 12.5 mg./Kg.; therefore, these two dose levels were selected for this study.

Nineteen of 20 mice receiving 0.0004 mg./Gm. of physostigmine 15 minutes prior to pentylenetetrazol intravenously, 10 mg./Kg., showed convulsive seizures of an average duration of 15.8 seconds (Fig. 2), while there were no convulsions in the 10 mg./Kg. controls. On using 12.5 mg./Kg. of pentylenetetrazol 15 minutes after physostigmine, all 28 mice showed convulsive seizures compared to five convulsive seizures in 26 mice used as 12.5 mg./Kg. controls (Fig. 2), but no deaths occurred.

Neostigmine, 0.0004 mg./Gm. of mouse, 15 minutes prior to pentylenetetrazol, 12.5 mg./Kg., gave convulsions in all 20 mice tried (Fig. 2), while DFP (2 mg./Kg.) 2.5 hours prior to pentylenetetrazol gave convulsions in only six mice of 20 tried and the seizures were like those of pentylenetetrazol alone, an average of 7 seconds.

In an attempt to rule out a vasodilating action of physostigmine and neostigmine, atropine sulfate (0.1 mg./Gm. of mouse) was administered subcutaneously with the cholinesterase inhibitor with no appreciable change in the percentage of seizures or duration of seizures above or below those occurring in mice which received only inhibitor and CNS stimulant (Fig. 2).

Alteration of Action of Phenobarbital Sodium

Greig and Mayberry (8) reported a decrease in the anesthetic lag time of barbital sodium in mice receiving prior medication with physostigmine. In addition, the above investigators reported an increase in the brain tissue level of the barbital following prior medication with physostigmine. More recently, in 1958, Rosenberg (11) found an increase in the sleeping time of hexobarbital following prior medication with some of the organic phosphates that are cholinesterase inhibitors.

Since the above-mentioned investigations reported potentiation of barbital and hexobarbital, this investigator attempted to study the effects of physostigmine, neostigmine, and DFP on the anesthetic lag time of phenobarbital sodium in mice, with no consideration of age, weight, or sex.

A dose of 160 mg./Kg. of phenobarbital sodium was given to 50 mice intravenously, and the average lag time from administration until onset, loss of righting reflex, was determined as 1147 seconds (Fig. 3). This average lag time was used as the

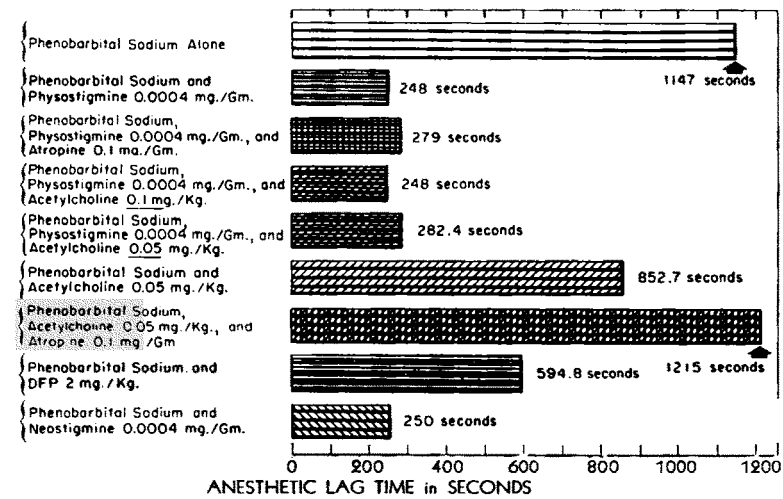


Fig. 3.—Effect of physostigmine, neostigmine, DFP, atropine, and acetylcholine on anesthetic lag of intravenous phenobarbital sodium (160 mg./Kg.) in mice.

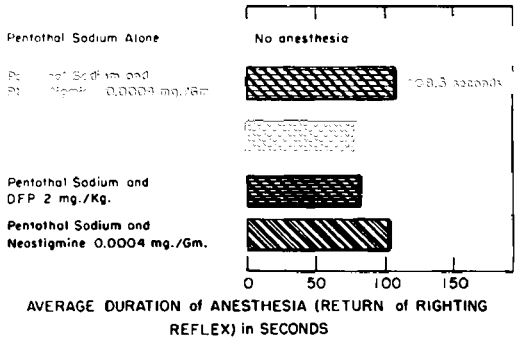


Fig. 4.—Effect of cholinesterase inhibitors and atropine on duration of anesthesia produced by 10 mg./Kg. of pentothal sodium intravenously in mice.

basis of comparison to determine any decrease in the lag time until onset of action that might be caused by prior medication with cholinesterase inhibitors.

Thirty mice receiving physostigmine salicylate (0.0004 mg./Gm.) subcutaneously 15 minutes prior to phenobarbital sodium (160 mg./Kg. intravenously) showed an average lag time until onset of 258.4 seconds (Fig. 3). This represents about a 75% decrease from the values for the controls receiving 160 mg./Kg. of phenobarbital alone.

DFP (2 mg./Kg.) subcutaneously as prior medication 2.5 hours previous to intravenous phenobarbital (160 mg./Kg.) gave an average lag time of 594.8 seconds (Fig. 3), while neostigmine (0.0004 mg./Gm.) subcutaneously 15 minutes prior to phenobarbital, gave an average lag time of 250.7 seconds (Fig. 3). This represents a 50% decrease for DFP and a 75% decrease for neostigmine below the lag time of the controls.

Other investigators reporting potentiation of barbiturate-induced sleeping time and decreased lags did not attempt to preclude the effects of vasodilation as a possible cause of the potentiation. It was decided that in this study the investigator would not only use a parasympatholytic in an attempt to block the acetylcholine vasodilation, but would also try to potentiate the barbiturate depression by acetylcholine-induced vasodilation. If the potentiation were the result of increased tissue uptake due to dilation of vessels, atropine should block it, and acetylcholine should produce similar potentiations.

Twenty mice receiving atropine sulfate (0.1 mg./Gm.) and physostigmine salicylate (0.0004 mg./Gm.) 15 minutes prior to phenobarbital gave an average lag time of 279 seconds. Twenty-four animals receiving physostigmine 15 minutes prior to acetylcholine bromide (0.05 mg./Kg.) plus phenobarbital sodium showed an average lag time of 282 seconds, while 20 animals receiving physostigmine 15 minutes prior to 0.1 mg./Kg. acetylcholine plus phenobarbital showed an average lag time of 248 seconds.

Acetylcholine bromide (0.05 mg./Kg.) plus 160 mg./Kg. of phenobarbital sodium intravenously showed an average lag time of 852.7 seconds (Fig. 3); and atropine sulfate (0.1 mg./Gm.) 15 minutes prior to acetylcholine (0.05 mg./Kg.) plus phenobarbital gave an average lag time of 1215 seconds.

These findings indicate that some method of potentiation other than vasodilation is involved in the potentiating action of these anticholinesterases. However, a vasodilating action could be involved in the mild potentiating action of the acetylcholine.

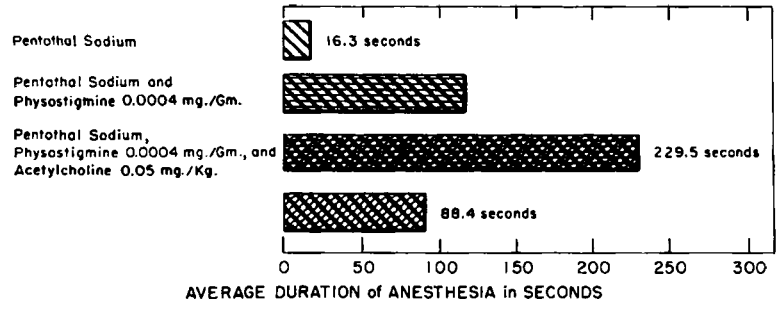
Pentothal Sodium Potentiation

Pentothal sodium was administered to mice in the amounts of 10 mg./Kg., 15 mg./Kg., and 35 mg./Kg. intravenously, and the anesthetic times or time lapses from loss of righting reflex until righting reflexes were regained were recorded. The time lapses from onset until recovery of walking ability was regained were also noted for comparison with controls.

Twenty mice injected with physostigmine salicylate 15 minutes prior to 10 mg./Kg. pentothal sodium gave an average anesthetic time of 108.5 seconds until recovery of righting reflex and 206.4 seconds until walking ability was regained (Fig. 4). Atropine sulfate (0.1 mg./Gm.) administered 5 minutes before the physostigmine in other mice (1) decreased the average anesthetic time to 79.1 seconds and the regaining of walking ability to 122 seconds. These average anesthetic times were in direct contrast to the controls, since in 20 control mice, 10 mg./Kg. of pentothal sodium produced no loss of righting reflex.

DFP and neostigmine produced average anesthetic times with 10 mg./Kg. of pentothal sodium of 82.7 and 102.2 seconds, respectively, while the time lags until walking was regained were 196.1 and 212.6 seconds.

The 15 mg./Kg. pentothal controls showed an average anesthetic time of 16.3 seconds and 30 seconds before walking ability was regained, but in 20 mice premedicated with physostigmine, the average anesthetic time was 116.2 seconds and 205 seconds until walking was regained (Fig. 5). When acetylcholine bromide (0.05 mg./Kg.) was given with 15 mg./Kg. pentothal sodium to physostigminized animals, the average anesthetic time increased to 229.5 seconds, and the recovery of walking ability to 326 seconds. Acetylcholine with pentothal sodium and no prior medication showed



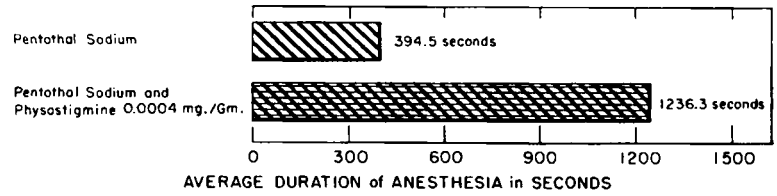


Fig. 6.—Effect of physostigmine on duration of anesthesia produced by 35 mg./Kg. of pentothal sodium intravenously in mice.

an average anesthetic time of 88.4 seconds and no prior medication showed an average anesthetic time of 88.4 seconds and 110.7 seconds until walking was recovered. These values were approximately the same as those for animals receiving DFP and pentothal.

Twenty physostigmine treated animals receiving 35 mg./Kg. of pentothal sodium gave an average anesthetic time of 1236.3 seconds (Fig. 6), contrasted with 394.5 seconds for the controls and 1412.7 seconds for recovery of walking ability compared to 471.9 seconds for the controls.

Potentiation of pentothal sodium by prior medication with physostigmine and neostigmine resulted in increasing the anesthetic activity of a non-anesthetic dose and prolonging the anesthetic time of an anesthetic dose. These potentiations by cholinesterase inhibitors could not be blocked by 0.1 mg./Gm. of atropine, and although acetylcholine enhanced the potentiation, it alone did not produce the potentiations equal to those of neostigmine and physostigmine.

The decreases in cholinesterase (Table I) activity brought about by the inhibitors used in this study correlate quite well with the increases in activity of the CNS stimulants and depressants. DFP showed the least inhibitory activity in the dose used and the least potentiating action, while neostigmine showed the most cholinesterase depression and the greatest potentiations.

SUMMARY

Physostigmine or neostigmine as prior medication caused potentiation in mice of the actions of strychnine and pentylenetetrazol. Subconvulsive doses were made convulsive and, in the case of strychnine, the subconvulsive dose was made lethal. In addition, the above cholinesterase inhibitors shortened the anesthetic lag time of intravenous phenobarbital sodium and increased the anesthetic activity of pentothal sodium, lengthening the anesthetic time. DFP was less effective in producing these potentiations.

Atropine sulfate (0.1 mg./Gm.) as prior medication did not block the potentiation; acetylcholine bromide (0.05 mg./Kg.) produced potentiations of much less magnitude than the reversible cholinesterase inhibitors. Thus, factors other than vasodilation must be of primary importance in these observed potentiations.

The per cent depression of cholinesterase activity by the inhibitors correlated with the degree of change in the activity of the CNS stimulants and depressants.

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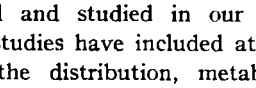
Determination of Sulfamylurea Hypoglycemic Agents and their Metabolites in Biological Fluids

By EDWARD H. WISEMAN, JOSEPHINE CHIAINI, and REX PINSON, JR.

Amine metabolites (basic) are separated from their parent sulfamylureas and sulfamide metabolites (both acidic) by extraction. Sulfamylureas are hydrolyzed by acid to the corresponding amine for assay, under which conditions sulfamides are unaffected. Sulfamides are similarly hydrolyzed to an amine under basic conditions, sulfamylureas being stable. Flourine-containing sulfamylureas were assayed by Schöniger combustion and determination of the resultant fluoride ion.

AS PART OF a continuing program on oral hypoglycemic agents, a series of sulfamylureas of the general formula (I) has been synthesized and studied in our laboratories (1). These studies have included attempts (2, 3) to relate the distribution, metabolism, and ex-

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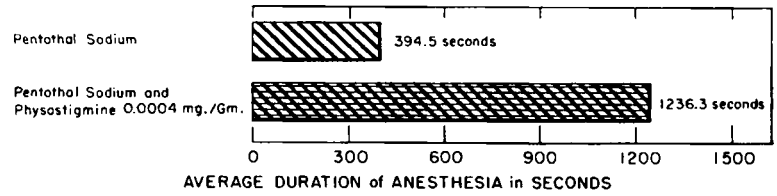


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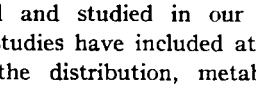
Determination of Sulfamylurea Hypoglycemic Agents and their Metabolites in Biological Fluids

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AS PART OF a continuing program on oral hypoglycemic agents, a series of sulfamylureas of the general formula (I) has been synthesized and studied in our laboratories (1). These studies have included attempts (2, 3) to relate the distribution, metabolism, and ex-

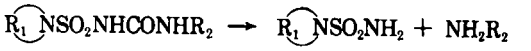
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cretion of these materials to various physical properties such as lipid/water distribution ratios, pKa, plasma binding, and solubility. This paper describes the development of analytical procedures required to determine these compounds and their metabolites in plasma and urine.

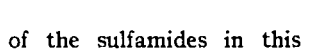
The major route of metabolism of the sulfamylureas is degradation to the sulfamide and a primary amine (2, 3)



Methods therefore had to be developed for the determination of the sulfamylurea, sulfamide, and amine either alone or in admixture with each other.

The sulfamylureas studied can be divided into two classes: those in which R_2 was either alkyl or cycloalkyl, and those in which R_2 was pentafluoropropyl. The former series were determined by a modification of the Spingler method (4). The sulfamylurea was extracted from the acidified biological sample into hexyl acetate and the hexyl acetate extract then heated to 130°. Under these conditions sulfamylureas are degraded to the corresponding cycloalkyl or alkyl amine, which can be determined as the yellow 2,4-dinitrophenyl derivative by reaction with 2,4-dinitrofluorobenzene (DNFB). The fluorine-containing sulfamylureas were extracted, and determined as fluoride ion after a Schöniger oxidation (5). There is no interference from metabolites in either assay procedure.

The sulfamylureas are stable in strongly basic solution at 100°, whereas the sulfamides are smoothly hydrolyzed to an amine



The determination of the sulfamides in this study was based on alkaline hydrolysis to the amine under conditions that did not affect the parent sulfamylurea. Interfering amines were first removed from the alkalinized biological sample by chloroform extraction. The aqueous solution was made strongly basic, and the sulfamide hydrolyzed to the corresponding secondary amine by heating at 100°. The amine produced was then determined by a methyl orange technique (6).

EXPERIMENTAL

Reagents

All organic solvents used were of analytical reagent grade. In addition, the following stock solutions are required: McIlvaine buffer, pH 5.0; 2:4-dinitrofluorobenzene (0.1%) solution in *n*-hexyl acetate (DNFB reagent) (the solution is stored in a brown bottle, and kept at 0–5°); methyl orange reagent (a solution of methyl orange (500 mg.) in water (100 ml.) is washed with chloroform. Before use, the aqueous solution is diluted 1:1 with saturated boric acid solution); and a solution of concentrated sulfuric acid (2%) in absolute ethanol.

Procedure

For Sulfamylureas Derived from Alkyl and Cycloalkyl Amines.—The biological sample (1 ml., either plasma or urine), in a glass-stoppered glass centrifuge tube is adjusted to pH 4–5 with 0.5 *N* hydrochloric acid and pH 5 buffer (1 ml.) added, followed by *n*-hexyl acetate (2 ml.). The mixture is mechanically shaken for 30 minutes, then centrifuged to separate the layers. A portion of the hexyl acetate layer (1 ml.) is transferred to a fresh glass-stoppered centrifuge tube containing the DNFB reagent (0.5 ml.). The tube is loosely stoppered and heated in an oil bath at 125–135° for 10 minutes. The solution is allowed to cool for 30 minutes, and the absorbance read at 398 mμ in a Beckman DU spectrophotometer against *n*-hexyl acetate as a blank.

For Sulfamylureas Derived from Pentafluoropropylamine.—The biological sample (1 ml.) is adjusted to *ca.* pH 4–5 with 0.5 *N* hydrochloric acid. The solution is buffered with pH 5 buffer (1 ml.), and extracted by shaking with ethyl acetate (2 ml.) for 30 minutes. A portion of the ethyl acetate layer (1 ml.) is carefully pipeted onto ashless filter paper, folded to contain a small ball of cotton (about 4 mg.), and the whole dried under an infrared lamp for 15 minutes. Potassium chlorate (about 1 mg.) is added, the paper carefully folded to contain this and the cotton ball, and placed in the platinum basket of a Schöniger flask (500 ml. capacity). The combustion is carried out in oxygen in the usual manner, and the gases absorbed in water (about 50 ml.). The flask and contents are allowed to stand for 20 minutes, and then the contents (together with washings) are transferred to a separator (250-ml. capacity), the final volume being about 150 ml. The fluoride ion concentration is then determined by the method of Johnson and Leonard (7).

For Sulfamides.—The biological sample (10 ml.) is made basic (pH 12) with sodium hydroxide solution and shaken with chloroform (10 ml.) for 15 minutes. The layers are separated by centrifugation, and a sample of the aqueous layer (5 ml.) transferred to a fresh tube containing 30% sodium hydroxide solution (1 ml.). The tube is stoppered, and the solution is heated on a steam bath for 20 minutes. After cooling, sodium chloride (*ca.* 3 Gm.) is added, and the solution shaken with benzene (20 ml.) for 30 minutes. The mixture is centrifuged, and a portion of the benzene layer (15 ml.) is washed with 0.1 *N* sodium hydroxide solution (5 ml.). Part of the benzene layer (8 ml.) is transferred to a tube containing isoamyl alcohol (0.4 ml.) and methyl orange reagent (0.6 ml.). The mixture is shaken for 10 minutes, centrifuged, and a portion of the benzene layer (6 ml.) transferred to a colorimeter tube containing 2% sulfuric acid in ethanol (1 ml.). The contents are mixed thoroughly and the absorbance read immediately in a Bausch & Lomb Spectronic 20 spectrophotometer at 540 mμ.

For Amines.—The biological sample (5 ml.) is alkalinized with 30% sodium hydroxide solution (1 ml.), sodium chloride (about 3 Gm.) is added, and the mixture shaken with benzene (20 ml.) for 30 minutes. A portion of the benzene layer (15 ml.) is separated and the sulfamide procedure followed from this point.

Calculations

All assays of unknown samples are related to standard curves prepared by submitting to the entire assay, pooled control urine or plasma samples, to which known amounts of the compound to be assayed had been added. The sulfamylurea assay obeys Beer's law over the range 10–200 mcg./ml. The sulfamylureas investigated varied widely in oil-water partition coefficients [0.03– <4 in the system cyclohexane/0.1 *N* hydrochloric acid (3)], and this affected the percentage recoveries from plasma and urine. However, in all cases, recovery was between 70–100%. The absorbance at 398 mμ of the 2,4-dinitrophenylamine derivative corresponding to an initial sulfamylurea concentration of 100 mcg./ml. fell in the range 0.410 to 0.560. The sulfamide assay follows Beer's law over the range 5–100 mcg./ml. and is subject to variations similar to those of the sulfamylurea assay. Sulfamylureas do not interfere in the sulfamide assay, but amines must be removed prior to hydrolysis. The amine assay has a range of 1–15 mcg./ml. Neither sulfamylurea nor sulfamide interfere in this assay.

Determination of Wavelength for Measurement of Dinitrophenyl Derivatives

The dinitrophenyl derivatives of all amines studied (cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, *n*-propyl, *n*-butyl) exhibit a plateau in the ultraviolet at 390–410 mμ, together with a peak at a lower wavelength (*ca.* 350 mμ), in agreement with previously reported observations (4, 8–11). At the higher wavelength blank values are considerably reduced, and errors arising from the absorbance of 2:4-dinitrophenol are also minimized (12). Accordingly, the higher wavelength is utilized in the assay procedure.

Stability Studies

Sulfamylureas (at concentrations *ca.* 100 mcg./ml.) could be recovered after heating at 100° for 20 minutes with 30% sodium hydroxide essentially quantitatively (92–100%). At concentrations up to 200 mcg./ml., sulfamylureas cause no interference in the sulfamide assay.

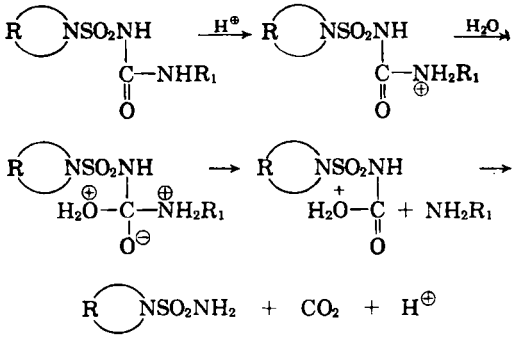
Sulfamides (at concentrations of *ca.* 60 mcg./ml.) could be recovered after 30 minutes at 100° in aqueous acid (pH 2) to the extent of 70–90%. Under assay conditions, concentrations of sulfamide in excess of 100 mcg./ml. cause no interference in the sulfamylurea assay.

Both sulfamylureas and sulfamides could be recovered essentially quantitatively (90–100%) after 48 hours at 37° from urine (pH 5–8).

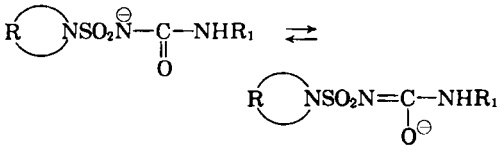
DISCUSSION

The determination of a substituted urea by the DNFB procedure was first reported by Spingler (4) who applied it to tolbutamide. Carmichael (8) extended the procedure to the determination of chlorpropamide. The present method is a modification of the original and incorporates an improvement since high blanks due to hemolyzed plasma samples have been eliminated. This has been achieved by buffering the plasma to be extracted to pH 5, at which pH the color from hemolyzed samples is not extracted by hexyl acetate. In addition, in selecting the higher absorption wavelength of the dinitrophenyl derivatives for assay use, the reliability of the assay has been further increased.

The development of assays capable of differentiating between two closely related compounds such as a sulfamylurea and its sulfamide metabolite is a reflection of the different stabilities of these two classes of compounds under hydrolytic conditions. By analogy with the proposed mechanism for the hydrolysis of amides (13), the hydrolysis of the sulfamylureas in acid solution may be envisioned as

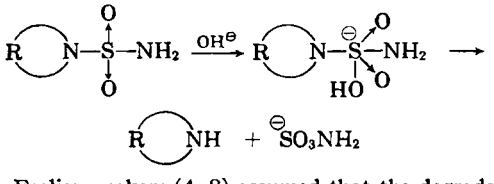


The initial (and most probably, rate determining) step will be the protonation of the sulfamylurea. If the group R_1 is of sufficient electronegativity, the availability of electrons at the nitrogen, and thus protonation, will be limited. The high electronegativity of the pentafluoropropyl group presumably restricts electron availability at the nitrogen to such an extent that the series of sulfamylureas containing that group was resistant to hydrolysis. For this reason, an alternative assay procedure for these compounds was developed. In alkaline solution, the sulfamylureas will exist in the salt form



The high electron density at the carbonyl group of this form which hinders attack by OH^-, accounts for the observed stability of the sulfamylureas in basic solution.

The hydrolytic behavior of the sulfamide metabolites can be explained on similar grounds. Much weaker acids than their sulfamylurea counterparts, they resist protonation and are therefore stable under acidic conditions. In basic solution however, the hydrolysis probably proceeds by



Earlier workers (4, 8) assumed that the degradation of the substituted urea occurred upon heating at 130°, the solvent being inert. We have found,

however, that both the sulfonyl and sulfamylureas are stable when heated alone in pure hexyl acetate but that prior washing of the hexyl acetate with dilute hydrochloric acid causes the reaction to proceed smoothly. The requirement of both acid and water becomes clear from the preceding discussion of the reactions involved.

The analytical method described for the fluorine containing sulfamylureas is novel because direct use is made of the fluorine substituent in the molecule. The electronegativity of the pentafluoropropyl group increases the acid strength of the series, evidenced by a decrease in pKa [from 7.5 to 8.3 in the cycloalkyl series to 6.5 in the pentafluoropropyl series (3)], enhancing the acid stability of these compounds to the point where they are resistant to hydrolysis under the assay conditions described for the sulfamylureas of the cycloalkyl series. The plasma and urine blank values in the combustion assay are low (*ca.* 1 mcg./ml.), being limited more by the cleanliness of the analytical apparatus than by biological contaminants.

The DNFB assay has been most extensively studied in the sulfamylurea series, but is also applicable to the sulfonylureas (tolbutamide, chlorpropamide,[1] acetohexamide, and cycloheptolamide). Plasma and urine blanks are low (about 5 mcg./ml.) and the assay has a wide range (10–200 mcg./ml.).

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Assays for sulfamylurea hypoglycemic agents and their metabolites in blood plasma and urine have been described. Sulfamylureas were determined by degradation to the corresponding primary amine which was determined spectrophotometrically after reaction with 2:4-dinitrofluorobenzene. The compounds in a series of fluorine-containing sulfamylureas were determined as fluoride ion after Schöniger combustion. One class of metabolites, the sulfamides, was hydrolyzed under basic conditions to give a secondary amine, which was then determined by the methyl-orange-dye–complex technique. The remaining amine metabolites were assayed directly by the methyl orange procedure. Although the assay has been primarily studied for the sulfamylureas, it is equally applicable to the determination of the hypoglycemic sulfonylureas. The high blanks caused by hemolyzed plasma samples in currently used sulfonylurea assays have been eliminated in the assay described.

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Relation of pH to Preservative Effectiveness II

Neutral and Basic Media

By BILLIE WICKLIFFE and DURWARD N. ENTREKIN

Liquid trypticase soy broth media was buffered with Prideaux and Ward's universal buffer to neutral and basic pH levels (pH 7 to 10 inclusive). Preservative solutions of various concentrations were added to tubes containing the media. After inoculation of the tubes with a slurry obtained from soil samples suspended in water, these preparations were examined for preservative effectiveness over a 6-month period. In most cases preservative effectiveness varied with pH alteration. The preservative activity ranged from negligible with cinnamic acid and some of its derivatives, the amides of bromal and dichloroacetaldehyde, sorbic acid and dehydroacetic acid-sodium, to fair in the case of parabens and salicylanilide, to good with cetrimide, chlorophenesin, vanillic acid esters, hexylresorcinol, hexachlorophene, and phenylethanol.

THE DEGREE of dissociation and the pH have been shown to influence the ability of preservatives to act in a chemical or physical way to prevent multiplication of organisms (1–3). The acidity or alkalinity of a product is just one of the many factors involved in the selection of effective preservatives for pharmaceutical preparations (1, 4–6). Antiseptics are said to be effective

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at certain pH ranges, and this pH range is different for each antiseptic or group of antiseptics (2, 3).

Some preservatives active in acid pH are not effective at neutral or basic pH levels (7). Studies concerning the parabens as preservatives showed greater concentrations were needed for alkaline than for acid or neutral syrups (8). Antifungal activities of many preservatives have been observed at acid and basic pH levels (9–12).

Bacterial growth has been shown to be inhibited at pH 2 without the use of additional preservative material (13); it has been stated that asepsis also occurs above pH 9 (14).

The presence of buffers in test preparations has been shown to have great influence on the activity of the preservatives employed (15–17). Preservatives incorporated in different buffer mixtures of approximately the same pH value will exhibit varying germicidal activity (17).

Previous work in this series gave results of preservative effectiveness in acid media (13). The work presented here expands this study to include an investigation of the effects of neutral and alkaline pH levels.

EXPERIMENTAL

The procedure as outlined in previous work (13) was followed in this test with the following exceptions:

(*a*) Prideaux and Ward's universal buffer was used in this experiment as it would yield pH values over the entire range to be tested, *i.e.*, pH 7, 8, 9, and 10 (18). This buffer alone has no bactericidal effect at any pH level used in this test (19).

(*b*) The soil sample used for the inoculum had been checked and contained Gram-positive and Gram-negative spore forming rods, cocci, yeast, and mold. To insure the inoculum containing some organism which would multiply at all pH levels included in this investigation, a suspension of *Alcaligenes* was added to the soil sample. The *Alcaligenes* was isolated from a milk of magnesia suspension with pH approximately 10. A pure culture of *Alcaligenes* was grown on an agar slant for several days and washed off with normal saline to prepare the fortifying suspension.

(*c*) Buffered media was autoclaved at 15 p.s.i. for 15 minutes to effect the sterilization. The media buffered to pH 8, 9, and 10 resulted in lower pH readings after this procedure. Therefore, to obtain media with the desired pH values, solutions were prepared at correspondingly higher pH levels which upon autoclaving fell into the desired pH range.

It was noted that the media darkened during the process of sterilization. Media at pH 7 turned yellow, and the color change increased with the increase in alkalinity to pH 10 which turned a light brown. A slight precipitate was formed in the pH 10 media.

Many preservatives included in the previous work (13) and several preservatives not included were used in this experiment (Table I). Solid preservatives were prepared by weight/volume per cent and liquid preservatives by volume/volume per cent. Results were recorded as: visible growth (+), static condition (⊕), and complete killing of organisms (. .).

RESULTS AND CONCLUSIONS

Cinnamic Acid.—In this study no preservative activity was observed with the aromatic materials tested. Cinnamyl acetate, cinnamic alcohol, and methyl, ethyl, and propyl cinnamate at concentrations of 0.0375, 0.075, and 0.15% were included in the test. These indicated no preservative activity

Table I.—Preservatives Selected for Use and Their Sources

Preservatives	Source
p-Hydroxybenzoates	
Methyl parasept	Heyden Newport Chemical Corp.
Ethyl parasept	Heyden Newport Chemical Corp.
Propyl parasept	Heyden Newport Chemical Corp.
Butyl parasept	Heyden Newport Chemical Corp.
Chemocides	
Cetremide (Chemocide CETB)	Chemo Puro Manufacturing Corp.
Chlorophenesin (Chemocide PCPD)	Chemo Puro Manufacturing Corp.
Salicylanilide (Chemocide SA)	Chemo Puro Manufacturing Corp.
Cinnamyl derivatives	
Cinnamyl acetate	Fritzsche Brothers, Inc.
Cinnamyl alcohol	Fritzsche Brothers, Inc.
Methyl cinnamate	University of Georgia Research Laboratories
Ethyl cinnamate	University of Georgia Research Laboratories
Propyl cinnamate	University of Georgia Research Laboratories
Vanillic acid derivatives	
Vanillic acid	E. R. Squibb & Sons
Vanillic acid, ethyl ester	E. R. Squibb & Sons
Vanillic acid, propyl ester	E. R. Squibb & Sons
Vanillic acid, isobutyl ester	E. R. Squibb & Sons
Amides of bromals and dichloroacetaldehydes	
Dichloroacetaldehyde chloroacetamide	W. D. Easterly, Jr., University of Arkansas
Bromal chloroacetamide	W. D. Easterly, Jr., University of Arkansas
Dichloroacetamide phenylacetamide	W. D. Easterly, Jr., University of Arkansas
Dichloroacetamide benzamide	W. D. Easterly, Jr., University of Arkansas
Miscellaneous	
Dehydroacetic acid-sodium	The Dow Chemical Co.
Sorbic acid	Union Carbide Chemical Co.
Hexachlorophene	K & K Laboratories, Inc.
Hexylresorcinol	Winthrop Laboratories
Phenylethanol	Mann Research Laboratories

over the test period, with the ethyl and propyl cinnamate showing no activity at the end of the second month. It should be noted that the solubility of these preservatives in the media was exceeded in most cases; therefore, the material available to exert the preservative action in these tubes was less than the amounts indicated. These materials maintained a static condition in many of the tubes during the earlier readings, particularly at the neutral or weakly basic levels. However, they lost their activity fairly rapidly and eventually exhibited no preservative action. Although these esters have been shown to be effective antifungals (20), they were ineffective as preservatives against mixed organisms at neutral and alkaline pH values.

Chemocides (Table II).—The chemocides as a group showed varying degrees of preservative activity. Chlorophenesin was effective only at the maximum concentration (0.5%) used. Similar results have been recorded at these concentrations in media buffered at pH levels 3 through 6 (13). Salicylanilide gave inconsistent results at the lower concentration and neutral pH. At pH 8 and 9 salicylanilide was effective at all concentrations used in this experiment when examined at the end of the test period. It should be noted, however, that conditions of stasis were observed in earlier readings, indicating that this material is not an immediate destroyer of all microorganisms. At pH 10, conditions of stasis developed in tubes at the 3-month reading which at earlier readings showed effective preservative activity. This static condition was observed because growth was evident in the subculture, but no growth was seen in the preserved tube. It is believed that the tubes were contaminated during addition of sterile water used to keep the volume of the test media constant. However at the 6-month reading, preservative effectiveness was observed at 0.1 and 0.14% concentrations. This indicates that at pH 10 salicylanilide is an effective preservative even after 6-months' storage at this extreme pH. It has been shown (13) that salicylanilide is more effective at pH 5 or below in tests run on media buffered to acid levels (pH 3 to 6).

Cetremide, a cationic quaternary detergent, was effective in all concentrations and all pH levels tested throughout the 6-month test period. Cetremide is therefore valuable as a preservative in these concentrations in both acid (13) and basic media, pH 3 and 10 inclusive.

p-Hydroxybenzoates (Table III).—The preservative activity of the parabens increased as the length of the side chain increased. These results agree with previous reports on their effectiveness as preservative or antifungal agents at the pH levels included in this test (6, 9, 13, 21). However, it has been reported that this relationship between chain length and activity does not exist at pH 6 since methyl and ethyl esters were more active at pH 6 than the propyl and butyl esters (13). Propyl and butyl parabens were effective at pH 9; however, all parabens studied, with one exception, were ineffective at pH 10, probably because of hydrolysis of the esters. The exception noted is the butyl ester at its maximum concentration. While the ethyl and propyl esters were not completely soluble in the concentrations used at pH 7, 8, and 9, these were soluble in media buffered to pH 10. This may be explained by the formation of a salt at the more alkaline level. At the lower concentration and higher pH levels, these esters became less active as time elapsed and many cultures changed from static conditions to those of visible growth. This, too, was probably due to hydrolysis of the chemical. At the lower pH levels, however, these static conditions improved to the point of showing effective preservative action.

Vanillic Acid (Table IV).—Vanillic acid gave negative results similar to that of the cinnamic acid derivatives. The esters of vanillic acid, however, showed preservative action; this activity increased as the length of the side chain increased. Vanillic acid was prepared at lower concentrations than its esters due to its decreased solubility. The solubility

TABLE II.—SIX-MONTH READINGS OF CHEMOCIDES

pH	Blank	Concentration, % w/v		
Chlorophenesin (Chemocide PCPD)		0.05	0.1	0.5
7	+	+	+	..
8	+	+	+	..
9	+	+	+	..
10	+	+	+	..
Salicylanilide (Chemocide SA)		0.06	0.1	0.14
7	+	.., +[a,b]	⊕[a]	.., +[a,b]
8	+	..[a]	..[a]	..[a]
9	+	..[a]	..[a]	..[a]
10	+	+	..[a]	..[a]
Cetremide (Chemocide CETB)		0.025	0.05	0.1
7	+	..	..	..
8	+	..	..	..
9	+	..	..	..
10	+	..	..	..

[a] Precipitate formed in tubes on the addition of the preservative solution; therefore, apparently a saturated solution at this temperature has been produced. [b] Duplicate tube did not produce identical readings.

TABLE III.—SIX-MONTH READING OF *p*-HYDROXYBENZOATES

pH	Blank	Concentration, % w/v		
Methyl Paraben		0.1	0.2	0.3
7	+	+	⊕, ..[b]	..
8	+	+	+	..
9	+	+	+	⊕
10	+	+	+	+
Ethyl Paraben		0.05	0.1	0.2
7	+	+	..	..[a]
8	+	+	⊕, +[b]	..[a]
9	+	+	+	..[a]
10	+	+	+	+
Propyl Paraben		0.075	0.1	0.125
7	+	..[a]	⊕, ..[a,b]	..[a]
8	+	..[a]	..[a]	..[a]
9	+	⊕[a]	..[a]	..[a]
10	+	+	⊕	⊕
Butyl Paraben		0.03	0.04	0.05
7	+	..	..	..
8	+	..	..	..
9	+	..	..	..
10	+	+	+	..

[a] Precipitate formed on the addition of the preservative solution. This precipitate floated on the media. Apparently a saturated solution at this temperature has been produced. [b] Duplicate test did not produce identical readings.

of the esters in the media was exceeded in many instances resulting in an "oil-like" substance floating on the test media and red-brown "oil-like" sediment precipitating in the tubes. Therefore, percentages given in the table were not always true values.

The propyl ester was the most effective of those studied; its activity at pH 10 was slightly decreased, possibly due to the formation of alkali salts (22) and/or hydrolysis. This loss of effectiveness at pH 10 agrees with previous work (22). The activity of the ethyl ester was similar to that of the propyl ester, except that increased concentration of the preservative was required. The isobutyl ester was intermediate in its action. These esters produced stasis in many of their earlier readings; however, on standing this condition of stasis changed to completion preservation. These results showed some variation from results obtained in acid media where the ethyl ester was most active (13).

TABLE IV.—SIX-MONTH READING OF VANILLIC ACID DERIVATIVES

pH	Blank	Concentration, % w/v		
Vanillic Acid		0.5	0.1	0.2
Growth was evident in all tubes before the end of the test period				
Vanillic acid, ethyl ester		0.1	0.4	0.8
7	+	..	..	..[b]
8	+	+	..	..[b]
9	+	+	..	..[b]
10	+	+	+	..[b]
Vanillic acid, propyl ester				
7	+	..	..[b]	..[b]
8	+	..	..[b]	..[b]
9	+	..	..[b]	..[b]
10	+	+	..[b]	..[b]
Vanillic acid, isobutyl ester				
7	+	+	..[b]	..[b]
8	+	..	..[b]	..[b]
9	+	..	..[b]	.., +[a,b]
10	+	⊕	..[b]	..[b]

[a] Duplicate test did not produce identical readings. [b] Oily precipitate formed on addition of preservative stock solution to media. Apparently a saturated solution at this temperature has been produced.

TABLE V.—SIX-MONTH READING OF AMIDES OF BROMAL AND DICHLOROACETALDEHYDE

pH	Blank	Concentration, % w/v		
Bromal chloroacetamide		0.1	0.2	0.3
7	+	+	.., ⊕[a]	⊕
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
Dichloroacetaldehyde chloroacetamide				
		0.1	0.2	0.4
7	+	+	+	⊕
8	+	+	+	+, ⊕,[a]
9	+	+	+	+
10	+	+	+	+
Dichloroacetaldehyde benzamide				
		0.125	0.25	0.5
7	+	+	+	⊕
8	+	+	+	⊕
9	+	+	+	+
10	+	+	+	+
Dichloroacetamide phenylacetamide				
7	+	+	+	⊕, ..[a]
8	+	+	+	+
9	+	+	+	+
10	+	⊕, +[a]	+	+

[a] Duplicate tubes did not produce identical readings.

TABLE VI.—SIX-MONTH READING OF MISCELLANEOUS PRESERVATIVES

pH	Blank	Concentration, % w/v		
Sorbic Acid		0.1	0.2	0.3
7	+	+	+	.., ⊕[b]
8	+	+	+	.., +[b]
9	+	+	+	+
10	+	+	+	+
Dehydroacetic Acid-Sodium		0.01	0.2	0.5
Growth was evident in all tubes by the end of the test period				
Hexylresorcinol		0.02	0.05	0.1
7	+	..[a]	..[a]	..[a]
8	+	..[a]	..[a]	..[a]
9	+	+[a,c,d,f]	⊕,[a,c,d,f]	..[a,c,d,f]
10	+	+[d,f]	+	+[a]
Hexachlorophene		0.005	0.015	0.025
7	+	..	..[a]	..[a]
8	+	..	..	..
9	+	..	..	..
10	+	..	..	..
Phenylethanol		1.0	2.0	3.0
7	+	..	..[e,f]	..[e,f]
8	+	.., +[b]	..[e,f]	..[e,f]
9	+	..	..[e,f]	..[e,f]
10	+	..	..[e,f]	..[e,f]

[a] Tubes appear cloudy on the addition of the preservative solutions. [b] Duplicate test did not produce identical readings. [c] Oily precipitate formed on addition of preservative stock solution to media. [d] Dark reddish precipitate formed on addition of preservative stock solution to media. [e] Liquid preservative floated on media. [f] Apparently a saturated solution at this temperature has been produced.

Amides of Bromals and Dichloroacetaldehyde (Table V).—The amides of bromals and dichloroacetaldehyde included in this test, although exhibiting antifungal activity in previous tests (23), showed negligible preservative action in these neutral and basic pH ranges. There was slight activity in the range of pH 7 and 8 at the highest concentrations used. As with many of the other materials used in this study, conditions of stasis in the earlier stages of the test reverted to that of visible growth at or before the 6-month reading. It is interesting to note that even though earlier workers (23) had encountered some difficulty with the solubility of the bromal amides, no difficulty was encountered concerning the solubility of any of these compounds in the test media and in the concentration used here.

Miscellaneous (Table VI).—The preservatives collected in a miscellaneous group gave varied results ranging from no action (sorbic acid and dehydroacetic acid-sodium) to complete action (hexachlorophene).

Sorbic acid, an unsaturated fatty acid, was not an effective preservative within the limitations of this test, showing slight activity only at the maximum concentration (0.3%) and the lower pH levels studied. Growth appeared within 2 weeks after inoculation in a majority of these tests. Earlier tests had shown this to be an effective preservative at pH 5 and below (13) but to exhibit no antifungal activity at pH 9 (11). As the pH increases, ionization increases, thus possibly explaining the loss of preservative activity in these tests.

Dehydroacetic acid-sodium gave negative results similar to those of sorbic acid. The highest concentration at pH 7 inhibited visible growth for only 2 weeks. The material was effective in earlier studies in the acid pH range (13).

Hexylresorcinol exhibited preservative action at the lower pH levels tested in all concentrations. A dark reddish-brown precipitate was formed on the addition of this preservative to the media. The quantity of this precipitate increased and darkened as the concentration of the preservative and the pH increased at all concentrations and at all pH levels. As alkalinity of the media increased, the effectiveness of the preservative decreased, showing slight activity at 0.05% at pH 9 and no activity at any concentration used at pH 10. This decline in preservative action developed late in the test period. At 2 weeks only one tube showed a condition of stasis, while all other tubes showed no microbes present. It is believed that these tubes, as those in the salicylanilide series, then became contaminated during the addition of the water used to restore the volume of the test media. These contaminants combined with the hexylresorcinol's loss of effectiveness at pH 10 resulted in the growth observed late during this test. Hexylresorcinol and hexachlorophene were both shown to be effective at acid pH levels in previous work (13).

Hexachlorophene was effective at all pH levels in all concentrations tested in this series. Therefore, hexachlorophene has been shown to be effective over a pH range from 3 to 10, while hexylresorcinol exhibited activity between pH 3 and 9 (13).

It should be noted that the solubility of phenylethanol was exceeded in all tests, except for the lowest concentration used. Since the solubility of phenylethanol in water is 2.0%, it may be assumed that the tubes containing higher percentages of this material will actually contain only this amount in solution, with the remainder of the preservative separating into a second phase. Phenylethanol showed preservative activity in all concentrations studied at all pH levels, except for 1.0% at pH 8. This was not consistent and must be due to experimental error.

SUMMARY

The effectiveness of several preservatives, both those in present day usage and a few experimental materials, at neutral and basic pH levels was observed over a 6-month test period. It was shown that bacterial growth occurs at all pH levels tested between 7 and 10.

As in the previous work done in this series of tests (13), a fortified soil sample was used as the inoculum in an effort to check the preservative effectiveness against a large number of organisms. Trypticase soy broth was the test media for this investigation with Prideaux and Ward's universal buffer used to maintain the desired pH levels.

Many of the materials tested, including cinnamic acid and some of its derivatives, the amides of bromals and dichloroacetaldehyde, sorbic acid, and dehydroacetic acid-sodium, were ineffective as preservatives. Others, the parabens and salicylanilide, showed some preservative activity; while cetremide, chlorophenesin, vanillic acid esters, hexylresorcinol, hexachlorophene, and phenylethanol exhibited very good preservative action. Those in the latter group therefore warrant further study.

It was seen that pH is a factor which must be considered if judicious selection of preservative agents is to be made.

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Toxicity of Plastics Used in Medical Practice II

Toxicity of Citric Acid Esters Used as Plasticizers

By D. B. MEYERS, J. AUTIAN, and W. L. GUESS

The acute and chronic toxicity of triethyl citrate, acetyl triethyl citrate, tributyl citrate, and acetyl tributyl citrate was studied in a series of small animals. The behavior response to parenteral administration of the esters was observed in rats, mice, frogs, and rabbits. Mice were used to determine the LD_{50} for each compound. All of the esters exhibited a marked effect on the CNS evidenced by increased respiration rate, convulsions, and cord depression. When applied locally to the nerve, the citrates rapidly blocked nerve conduction. A profound depressor action was observed in cats and rabbits which was attributed to a direct cardiac inhibitory effect. Chronic administration for a period of 2 weeks resulted in a depression of weight gain; in the animals that received acetyl tributyl citrate evidence of a change in the blood picture was observed. Histopathological examinations revealed no damage to the liver, kidney, lungs, and spinal cord in the chronic animals.

MUCH PROGRESS has been made in the use of plastics for various devices used in medicine and pharmacy in the last 5 years. Advancements in plastic technology have made possible many improvements in existing devices which would not have been possible with more conventional materials. However, certain problems have been noted which indicate that greater care should be taken in selecting the plastic device (1–3).

Tubings for the administration of drug and blood products or for the collection of body fluids may be composed of one or more polymeric materials. Usually the polymer is of the vinyl type, if softness, flexibility, and clarity are desired. Plasticizers and other additives must be added to this polymeric material to give the final device the desired physical and chemical properties. Thus, it is possible to have a host of plastic formulas, referred to as polyvinyl chloride, but each differing from the other in composition. In most instances the various ingredients used for polyvinyl chloride tubings which are to be used in medical practice have been approved as food additives by the FDA.

In 1960, Autian and Kapadia (4) reported that certain polyvinyl chloride tubings released a constituent to several hydroalcoholic solvent systems. They did not, however, investigate the possible biological effect of the leached constituent. Meyler, Willebrands, and Durrer (5) in the same year reported that certain polyvinyl chloride tubings released a constituent to blood which in turn caused cardiac arrest and ventricular fibrillation on the isolated perfused heart of rats. Keith, *et al.* (6), reported that one lot of plastic tubings (vinyl type) appeared to enhance the hemolytic effect on perfused blood. This observation was further substantiated with *in vitro* studies by Hirose and associates (7). Lawrence, *et al.* (8), noted that a number of commercially available polyvinyl chloride tubings used as part of administration devices released a constituent into tissue causing a toxic response when implanted for short periods of time in animals. In this particular study the offending agent in the plastic was not the plasticizer but one of the other additives.

Even though the work of Lawrence, *et al.*, revealed that the plasticizers were not causing adverse effects by the experimental method employed, there is no assurance that the same plasticizers or other plasticizers will not produce a biological effect when administered in a different manner to animals. Since present vinyl tubings contain a large proportion of one or more plasticizers and since these tubings will have contact with drug systems or body tissues for varying periods of time, it was thought prudent to investigate the pharmacological properties of one series of plasticizers (citric acid esters) which had previously been reported as quite safe by oral feeding (9).

EXPERIMENTAL

The compounds used in this study included triethyl citrate (TEC), acetyl triethyl citrate (ATEC), tributyl citrate (TBC), and acetyl tributyl citrate (ATBC). The esters were commercial samples containing less than 1% foreign material.[1] Because of the nearly insoluble nature of the butyl

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[1] Samples supplied by Charles Pfizer and Co., Inc., Brooklyn, N. Y.

derivatives, all the esters were administered suspended or dissolved in 3% acacia throughout the experimental work. Ester concentration was varied to be suitable for each particular study.

Behavior Studies.—The general overt effects of the esters were first observed following i.p. administration of graded doses into Swiss albino mice. Both of the ethyl citrates produced a very rapid loss of righting reflex without loss of consciousness in doses slightly exceeding 400 mg./Kg. Usually the animals regained their posture within 15 minutes. Respiration rate was markedly increased and frequently clonic convulsions were observed. The butyl derivatives failed to produce the rapid loss of righting reflex, but did evoke an increase in respiration which was frequently accompanied by clonic convulsions. Some writhing was observed during the first 10 minutes after injection of the butyl compounds. All of tne esters produced the same signs of stimulation in Wistar rats when given i.p. with both TEC and ATEC, again causing a rapid loss of righting reflex of short duration when the dosage exceeded 400 mg./Kg. The stimulating properties of the esters were confirmed by intravenous administration into rabbits. Marked increases in motor activity and respiration were observed following doses of 100 mg./Kg.

Doses of 1000 mg./Kg. placed in the ventral lymph sac of the frog resulted in a short period of clonic activity, followed by the complete abolishment of all reflex activity.

Acute Toxicity.—The i.p. LD_{50}'s for the esters were determined in Swiss albino mice weighing between 16 and 20 Gm. Doses were chosen from a logarithmic scale and the median lethal dose calculated by method of Bliss (10). All deaths occurred in the first hour following administration of TEC and ATEC, but in the case of the butyl compounds a 72-hour observation period was required. The LD_{50} with standard error for TEC was 1750 ± 68 mg./Kg.; for ATEC, 1150 ± 185 mg./Kg.; for TBC, 2900 ± 210 mg./Kg.; and for ATBC, >4000 mg./Kg. Death was attributed to circulatory collapse and postictal depression.

Chronic Toxicity Studies.—To study cumulative effects of the citrates, a 14-day chronic toxicity test was carried out in which mice weighing from 16–20 Gm. were given daily i.p. doses which approximated one-fifth of the acute median lethal dose. Five groups of 20 mice each were selected and the weight, RBC count, WBC count, clotting time, and hemoglobin level of each individual determined. Group *A* received daily injections of TEC 350 mg./Kg.; Group *B*, 230 mg./Kg. of ATEC; Group *C*, 580 mg./Kg. of TBC; and Group *D*, 900 mg./Kg. of ATBC. Group *E* received daily injections of the 3% acacia vehicle and served as the control. The animals were weighed daily and at the end of the 2-week period the RBC count, WBC count, clotting time, and hemoglobin level were redetermined.

Figure 1 shows that all of the compounds inhibited normal weight gain. At the 7-day period this inhibition was significant within 95% confidence limits. It was most striking in the case of ATBC which, although given in the largest doses, had exhibited the lowest acute toxicity.

There were no significant differences in the RBC counts, the WBC counts, the clotting times, and the hemoglobin levels in the mice that received TEC, ATEC, or TBC. However, significant (95% confidence) fall in the RBC count and hemoglobin levels in those animals administered ATBC occurred.

In a further study of the blood picture changes due to ATBC, two albino rabbits were administered 450 mg./Kg. of ATBC daily for 14 days. Two others were given 900 mg./Kg. daily for 7 days. At the end of the period, the RBC count, hemoglobin level, and a differential WBC count were made; red bone marrow smears from the femur bone were examined. In all animals there was a fall in the red blood cell count ranging from 0.5 to 2.5 million and a corresponding fall in hemoglobin levels. However, the basophil count and the bone marrow smears gave no indication of aplastic anemia.

At the end of the chronic toxicity study, two mice from each group were sacrificed; slides were made of liver, lung, and kidney tissues. Examination of these slides did not reveal pathological cellular changes in these organs.

On the day following withdrawal from the chronic test, hexobarbital sleeping time studies were conducted in the remaining mice. There was no significant difference in the sleeping time within the various groups. This confirmed that liver function was not impaired by chronic administration of the esters.

Effects of the Esters on Neural Muscular Transmission.—Behavior studies indicated that the citrate esters produced some neurological effects. Therefore, a series of studies was set up to determine the nature of the neural involvement. In the first experiment, white rats were anesthetized with pentobarbital, and right anterior tibialis muscle freed and arranged for recording contractions, and shielded electrodes placed central to a pledget of cotton around the sciatic nerve. Stimulus was supplied through the electrodes by a stimulator set to deliver a current which would produce a submaximal response every 10 seconds. After obtaining a normal response, 3 drops of the ester were placed on the cotton. All four esters produced a complete sciatic nerve block which was reversible when the cotton was removed and the nerve washed with saline. This activity was confirmed using the isolated gastrocnemius muscle-sciatic nerve preparation from the frog.

To determine central nervous system involvement, pentobarbital anesthetized white rats were set up so that the contralateral reflex could be recorded. After freeing the anterior tibialis muscle in the left leg and arranging it for recording contractions, shielded electrodes were placed around

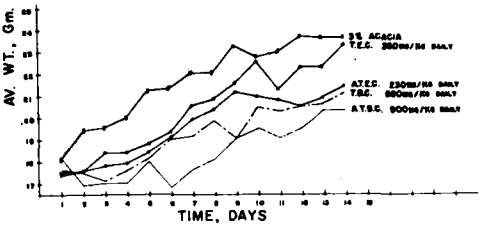


Fig. 1.—The effect of some citric acid esters administered i.p. on weight gain of mice.

the sciatic nerve in the right leg. The stimulator was set to deliver a series of five impulses 10 seconds apart when activated by a switch. After determining a normal submaximal contralateral reflex, each ester was administered through the jugular vein which had previously been cannulated. Figure 2 shows that all of the esters were capable of completely blocking this reflex. The duration of blockage varied according to the ester. Considerably larger doses of ATBC were required to affect the block, but the duration was much longer. In contrast, TBC block occurred in response to the lowest dose but lasted longer than that produced by either of the ethyl esters.

To verify that cord depression and not myoneural junction blocking was the cause of the inhibited contralateral reflex, rats were prepared so that the sciatic nerve stimulation and anterior tibialis muscle contraction occurred on the same side. Doses of the esters which were twice as great as those which abolished the contralateral reflex failed to impair myoneural junction transmission.

Local Anesthesia Action.—All four of the esters temporarily abolished the corneal reflex when 3 drops of a 5% suspension were placed in the conjunctival sac of the rabbit. As expected, the butyl esters were longer in duration than their ethyl counterparts. This local anesthetic effect was confirmed by the intradermal administration of

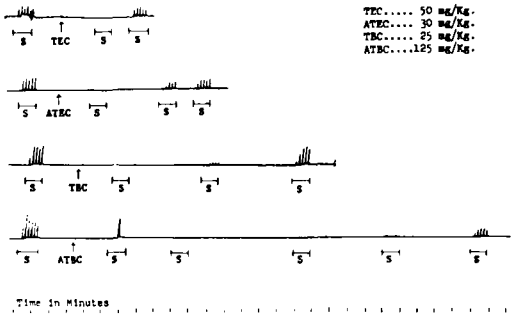


Fig. 2.—Effect of some citric acid esters administered i.v. on the contralateral reflex of the rat.

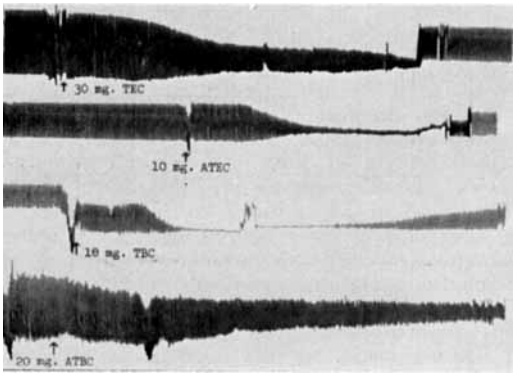


Fig. 4.—Effect of some citric acid esters on rate and amplitude of the isolated rabbit heart.

0.1 ml. of a 2% suspension of the esters into shaved areas on the back of guinea pigs. The duration of insensitivity to pricking of the surrounding area with a pin was 12 to 20 minutes for TEC. However, TBC produced a deadened area for a period of over 2 hours.

Cardiovascular Action of the Citrate Esters.—Acute toxicity studies indicated probable cardiovascular collapse, so a study of the effect of the citrate esters on blood pressure was indicated. Rabbits and cats were used in this study. Blood pressures were recorded from the cannulated carotid artery with a mercury manometer. A tambour connected to the tracheal cannula simultaneously recorded respiration. The esters were administered through the ear vein or through the cannulated jugular. Figure 3 is a tracing showing the typical responses obtained from varying doses of the esters in rabbits. There was an apparent dose-response relationship with all the compounds, and all of them produced complete loss of blood pressure when administered in toxic doses. Interestingly, the comparative doses required to produce a fall in blood pressure for the esters were quite well related to those which were required to produce death. ATBC with a high LD_{50} (greater than 4000 mg./Kg.) did not produce a significant fall in blood pressure in low

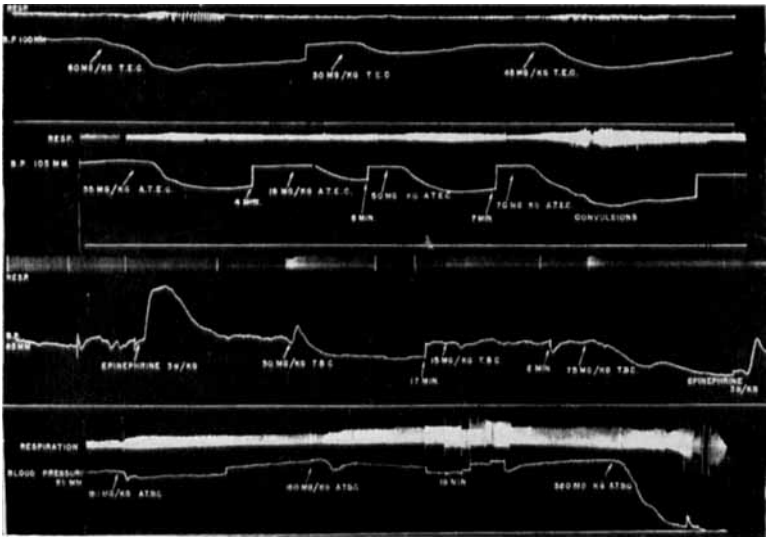


Fig. 3.—Influence of some citric acid esters on blood pressure and respiration of rabbits under pentobarbital anesthesia.

doses. The depressor effect of the esters was similar in cats and rabbits. It could not be blocked by atropine, and it could be reversed by epinephrine. This was indicative that the effect was due to direct smooth muscle relaxation or to the cardiac depression which was quite apparent.

To show that the depressor effect was primarily due to cardiac inhibition, the esters were tested on the isolated rabbit heart after the method of Langendorff (11). As can be seen in Fig. 4, all of the compounds brought about a sharp decline in both rate and amplitude of the heart beat. If given in carefully controlled doses, the heart returned to normal, but higher doses resulted in complete heart arrest. Coronary flow was also markedly diminished, precluding the possibility of significant direct smooth muscle relaxation.

SUMMARY AND CONCLUSIONS

The results of this study indicate that the four citric acid esters used as plasticizers have well defined and marked pharmacological activity when administered parenterally. All four have local anesthetic action and can block neural transmission when they come in direct contact with a nerve trunk. All of them diffuse from the blood stream into the cord in sufficient concentration to depress cord function. At the same time they apparently stimulate the higher centers. Although they vary widely in potency, both the ethyl esters and the butyl esters depress cardiac activity sufficiently to produce cardiovascular collapse. Chronic administration of three of them (TEC, ATEC, and TBC) does not appear to have an effect on the blood picture, but there are indications that ATBC may inhibit production or increase destruction of red blood cells. No species variation was observed throughout the investigation.

One must take care not to extrapolate the results reported in this paper to actual polyvinyl chloride devices which might contain plasticizers as reported here, since the biological activity will be elicited only when the plasticizer is released into the body fluids and, further, that the magnitude of the response shall have a direct relation to the concentration released. The biological responses reported here for a group of plasticizers should, however, alert those manufacturing plastic devices to learn more about the ingredients they employ in their plastic formulation if these items are to be introduced into medical practice.

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Rheology of Gelatin Films

By ROBERT A. CASTELLO* and JERE E. GOYAN‡

A procedure has been developed for the evaluation of the viscoelastic properties of gelatin films employed in soft gelatin capsule formulations. It involves the measurement of the tensile relaxation modulus of the film and the fitting of the resultant curve on an analog computer to obtain the appropriate constants. A theory was proposed for the mechanism of stress relaxation in gelatin films. Based upon this theory, an equation for the tensile relaxation modulus was derived. This equation was of the same form as the empirical equation which fit the stress relaxation curves of the gelatin films.

ALTHOUGH MUCH INFORMATION has been gathered concerning changes in the rheological properties of gelatin solutions upon thermal aging, little is known of the characteristics of gelatin films. Since the rheological behavior of gelatin films influences the properties of capsules molded from them, information concerning changes in this behavior might be helpful in evaluating technological problems encountered in the encapsulation process.

The viscoelastic properties of gelatin have been investigated by several authors. Among them, Ferry (1, 2) has studied the concentration effect on gel rigidities, and Tobolsky (3, 4) has studied stress relaxation as a tool in the investigation of gelation mechanisms. In this study the stress relaxation model has been

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adapted for the investigation of soft gelatin capsule formulations.

The field of viscoelasticity is intimately concerned with two basic physical laws. The first of these is Newton's law of viscosity which states that the stress on a perfectly viscous body is directly proportional to the rate of strain and is independent of the strain, giving

$$\sigma = \eta d\epsilon/dt \qquad \text{(Eq. 1)}$$

where σ = stress, $d\epsilon/dt$ = rate of strain, and η = coefficient of viscosity. The second is Hooke's law which states that the stress on a perfectly elastic body is proportional to the strain and is independent of the rate of strain, giving in the case of simple extension

$$\sigma = E\epsilon \qquad \text{(Eq. 2)}$$

where σ = stress, ϵ = strain, and E = Young's modulus of elasticity.

The basic viscoelastic equations may be derived from these two equations; Reiner (5) has done this for the stress relaxation modulus. Stress relaxation may be defined as the change in stress with time of a viscoelastic body under constant strain. Upon differentiating Eq. 2 with respect to time

$$d\sigma/dt = Ed\epsilon/dt \qquad \text{(Eq. 3)}$$

Then, combination of this with Newton's equation gives

$$2d\epsilon/dt = (1/E)\, d\sigma/dt + (1/\eta)\sigma \qquad \text{(Eq. 4)}$$

Under the conditions of stress relaxation, the strain is constant. Therefore, $d\epsilon/dt$ is equal to zero and the equation becomes

$$d\sigma/dt = (E/\eta)\sigma \qquad \text{(Eq. 5)}$$

By separating the variables and integrating

$$\sigma(t) = \sigma_o exp - (E/\eta)t \qquad \text{(Eq. 6)}$$

This then describes the change in stress with time of a viscoelastic system under constant strain.

A very wide class of substances exhibit mechanical behavior in which elastic and viscous behavior are superposed. Much interest has been generated in this complex mechanical behavior and several authors (6–8) have employed mechanical models depicting these phenomena.

Nearly a century ago, Maxwell first postulated the stress relaxation equation. This equation corresponds to a mechanical model consisting of a spring and dashpot in series (see Fig. 1) which is therefore called a Maxwell element. In this model, the spring obeys Hooke's law,

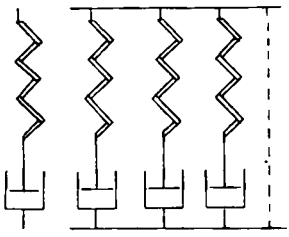


Fig. 1.—Maxwell and Wiechert mechanical models for stress relaxation.

and the resistance to flow in the dashpot is supplied by a Newtonian viscous oil.

The Maxwell equation is

$$\sigma(t) = \sigma_o exp - (t/\tau) \qquad \text{(Eq. 7)}$$

where τ = the Maxwell relaxation time (the time required for the stress to equal $1/e$ times its initial value). In terms of the Maxwell model, the spring would have a Young's modulus of E, and the oil a viscosity of η. Therefore

$$\tau = \eta/E \qquad \text{(Eq. 8)}$$

The Maxwell model describes adequately the stress relaxation of a single polymer chain, but polymeric systems such as gelatin solutions are described as being composed of n polymer chains. A theory that accounts for the presence of a number of chains was proposed by Wiechert. The mechanical model suggested by Wiechert is simply a large number of Maxwell elements placed in parallel as shown in Fig. 1. The equation for stress relaxation of the Wiechert model is

$$\sigma(t) = \sum_{i=1}^{n} \sigma_i\ exp(-t/\tau_i) \qquad \text{(Eq. 9)}$$

where σ_i = partial stress contributed by the ith element ($\sigma_o = \Sigma\sigma_i$) and τ_i = Mawell relaxation time of the ith element. A similar analogy can be made as with the Maxwell model so that

$$\tau_i = \eta_i/E_i \qquad \text{(Eq. 10)}$$

where η_i and E_i are the coefficient of viscosity and Young's modulus of the ith elements, respectively.

EXPERIMENTAL

Several different types of gelatin were employed in this study. Specifications for these samples appear in Table I. The formulation used consisted of:

Gelatin (on an anhydrous basis)...	43%
Glycerin U.S.P.....	18%
Distilled water.....	39%

In the preparation of the sample, the gelatin was weighed and placed in a 2-L. resin reaction flask equipped with a Gerald K. Heller model GT-21 motor and controller.[1] The stirrer shaft was inserted through the center part of the resin flask

[1] G. K. Heller Co., Las Vegas, Nev.

TABLE I.—SPECIFICATIONS OF GELATIN SAMPLES[a]

Sample	Source	Viscosity, Millipoise	Bloom	pH	Raw Material	Process	% Moisture
33-59A	Amer. Agr. Chem. Co.	44.5	250	5.7	Porkskin	Acid	9.8
33-59B	Amer. Agr. Chem. Co.	47.1	225	5.55	Ossien	Lime	9.4
33-59C	Amer. Agr. Chem. Co.	51.3	281	5.8	Porkskin	Acid	9.3
33-59D	Amer. Agr. Chem. Co.	50.5	290	5.55	Porkskin	Acid	9.5
33-59E	Amer. Agr. Chem. Co.	44.7	203	5.45	Calfskin	Lime	13.1
33-59F	Amer. Agr. Chem. Co.	47.1	150	5.6	Calfskin	Lime	10.3
33-59G	Amer. Agr. Chem. Co.	29.5	165	4.95	Porkskin	Acid	10.6
33-59H	Amer. Agr. Chem. Co.	25.6	177	5.65	Porkskin	Acid	8.9
33-59I	Amer. Agr. Chem. Co.	29.8	177	5.15	Porkskin	Acid	10.1
33-59J	Amer. Agr. Chem. Co.	35.8	161	5.5	Ossien	Lime	8.6
33-59K	Swift	37 to 43	195 to 205	4.4 to 4.8	Porkskin	...	10.0
33-59L	Vyse	37 to 43	120 to 130	5.5 to 7.0	Calfskin	...	10.4
33-59N	Riches Nelson	30	200	5.0	Ossien	Acid	12.9

[a] The authors thank the Lederle Division of American Cyanamid Corp. for supplying gelatins 33-59K and 33-59L.

with a ball and socket joint. The flask was immersed in a water bath at 90°. The glycerin and water were mixed and heated to 90°, then added to the gelatin. The age of the melt was calculated from the time of this addition. The gelatin was allowed to melt, and the sample was then stirred for 30 minutes to insure homogeneity. After stirring, the sample was transferred to a 1-L. round bottomed flask which was immersed in a water bath at 60° ± 0.1°. Transfer was accomplished by a vacuum supplied by a water aspirator. This transfer also served to deaerate the gelatin melt.

Film strips were cast on the apparatus shown in Fig. 2. The brass hopper was equipped with a semicircular valve to control the flow of gelatin. It was inserted through a port in the top of the drum assembly and was adjusted with set screws at the four corners of the hopper assembly to a position 0.010 in. above the drum surface. The casting thickness of the film was set at 0.025 in. with an adjustable gate in front of the hopper. The temperature of the hopper was controlled by a Glas-col heating mantle which was made to hopper dimensions. It was adjusted to maintain a 60° temperature in the sample. The films were cast on an 8-in. diameter chrome plated drum which was driven by a 1/6 h.p. Boston gear ratio motor[2] having a gear reduction of 1600 to 1. At the time of the casting, the valve was opened and the motor turned on. The film was cast at a rate of 1 r.p.m. The films were stripped from the casting drum and placed on stainless steel sheets to be cut into test strips. Test strips were cut with a razor blade using a template. The dimensions of the test portion were 1 × 5 cm. The actual film thickness was measured with a micrometer at the time of tensile measurements.

Film strips were aged in a blue M vapor-temp humidity chamber model VP-100 which had been modified.[3] The solenoid valve was bypassed, and a water-alcohol mixture was circulated through the cooling coils by means of a centrifugal pump. The cooling mixture was contained in a refrigerated water bath and was kept at a temperature of −2°. The centrifugal pump was connected through an electrical relay which was controlled by a resistor whose resistance varied with humidity. The dry bulb temperature was adjusted as normally in the humidity cabinet. The relay was then adjusted

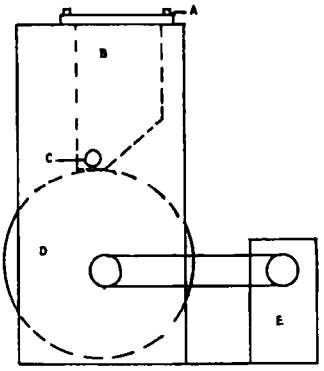


Fig. 2.—Film casting apparatus.
A, Set screw
B, Hopper
C, Valve
D, Drum
E, Motor

so that the pump would shut off when the proper wet bulb depression was obtained on a wet bulb thermometer mounted inside the cabinet. The cover for the humidity cabinet which was originally an inverted bell jar was changed to a Plexiglas cabinet which would accommodate the tensile strength apparatus. Access to the inside of the cabinet was gained through an 8 × 12-in. door which was held in place by wing nuts at each of the corners. The edges of the door and the bottom of the Plexiglas cover were sealed with sponge rubber to prevent transfer of moisture from the atmosphere to the controlled humidity air inside the cabinet. A circulating fan was placed in the cabinet to insure that variations in the humidity at various levels in the cabinet would be negligible.

Tensile measurements of the films were made on a Gardner Laboratories tensile strength apparatus[4] mounted inside the humidity cabinet. This apparatus was modified in the following manner. The spring for recording the force was replaced by a Statham Laboratories model G-1 transducer,[5] and the output voltage was recorded on a model G-10 Varian Associates recorder.[6] A Starret dial indicator[7] was mounted on the apparatus to measure film elongation. The indicator had a range of 0.25 in. and was graduated in 0.001 in. At the time of measurement, the sample was instantaneously placed under a small strain by turning the handle on the worm gear at the top of the apparatus. The stress was followed with time and was recorded on

[2] Marwedel, San Francisco, Calif.
[3] Fisher Scientific Co., Pittsburgh, Pa.
[4] Gardner Laboratories, Bethesda, Md.
[5] Statham Instruments, Hato Rey, Puerto Rico.
[6] Varian Associates, Palo Alto, Calif.
[7] Marwedel, San Francisco, Calif.

TABLE II.—COMPUTER CONSTANTS OF GELATIN FILM 33-59B DURING THERMAL AGING OF MELT AT 60°C.

Melt Age, Hr.	Pre-exponentials × 10^{-8} (dynes cm.$^{-2}$) A	B	C	D	Exponentials (sec.$^{-1}$) α	β	γ
7	4.30	4.68	4.38	3.26	0.922	0.261	0.046
24	4.04	4.66	3.80	2.72	0.922	0.261	0.046
48	5.58	4.82	3.24	2.74	0.922	0.261	0.046
72	5.28	3.80	3.78	2.66	0.922	0.261	0.046

the Varian recorder at a chart speed of 16 in. per minute. The elongation was measured to the nearest 1/1000 in. on the dial indicator, and the film thickness was measured with a micrometer for the calculation of the cross-sectional area.

Effect of Thermal Aging of the Melt.—A sample was prepared and hydrolyzed at 60° for 96 hours measured from the time of addition of the glycerin-water mixture to the dry gelatin. Film strips were cast at 24-hour intervals. These strips were aged in a humidity cabinet at 30° and 49% relative humidity for 3 hours. It had previously been determined that the film moisture content equilibrated during this aging period. Stress-time plots were recorded and the tensile relaxation moduli were calculated. Duplicate test strips were run for all measurements and agreed within 5% error. The stress relaxation curves were fitted on an analog computer and the constants determined. All of the curves fit

$$E(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} + D \quad \text{(Eq. 11)}$$

A typical set of constants is shown in Table II.

Film Storage at Constant Relative Humidity.—A gelatin melt was prepared and cast at 24, 48, and 72 hours. Several test strips were cut from each casting and aged in a humidity cabinet at 30° and 49% relative humidity. Tensile relaxation measurements were made at 48-hour intervals for 336 hours after casting. The stress relaxation curves were again fitted on the computer. Sample constants are shown in Table III.

Refrigerator Storage of Melt.—A sample was prepared and maintained at 60° for 30 hours. At 28 hours, a sample was cast and aged in the humidity cabinet at 30° and 43% relative humidity for 3 hours. The tensile relaxation modulus was determined. At the end of the 30-hour period, the sample was placed in a refrigerator at 3° for 15 days. The sample was removed from the refrigerator and heated an additional 5 hours at 60° to melt it. At this time, additional test strips were prepared in the same manner and the tensile relaxation moduli determined.

RESULTS AND DISCUSSION

All of the stress relaxation curves for a given gelatin melt were fitted using the same set of decay constants regardless of the time of thermal aging. This was true not only for samples cast at various times from the same melt but also for samples cast from subsequent melts containing the same gelatin. These exponential decay constants could then be used as parameters to identify a specific gelatin.

As the gelatin was aged, the values of the initial modulus, E_o, and the equilibrium modulus, E_e, decreased indicating that the number of cross links in the network was decreasing. These values did not decay in a regular fashion as did the viscosity values of the diluted gelatin melt (see Fig. 3). In most cases there appeared to be an induction period with little change in the tensile properties of the film and then a marked decrease usually appearing in the 48-hour sample. Therefore, the decay in the modulus could not be adequately explained by a scission of the polymer chain and subsequent loss of cross-linking sites from the molecule. If this were so, the decay would be expected to parallel the loss in viscosity.

Another cause for the tensile modulus decay may be proposed based on the concept of crystallinity in polymer systems. As the time of thermal aging increased, the polymer chains would contract and become more amorphous. When the gelatin temperature was lowered to the gel point during film casting, there would be fewer bonding sites oriented for cross-linking and the tensile modulus of the film would be lower. If this were the case, it would be expected that after extended periods at low temperatures the gelatin molecules could reorient and the modulus would increase.

This type of behavior was demonstrated during humidity cabinet storage of the gelatin films. The moduli increased with time of storage in the humidity cabinet indicating an increase in the number of cross-links and an increase in the crystallinity of the films. Since the increase of the tensile properties was a rate process, a treatment similar to Bisschop's for crystalline polymers was employed (9, 10). The equilibrium modulus of the film was plotted *versus* time of storage in the humidity cabinet. These plots for the 48-hour casting and 72-hour casting are shown in Fig. 4. As reported in the literature, these plots yielded straight lines whose slopes could be used as an estimation of the gel rate. It was interesting to note that the rate

TABLE III.—COMPUTER CONSTANTS OF GELATIN FILM 33-59J DURING HUMIDITY CABINET AGING 48-HOUR CASTING

Film Age, Hr.	Pre-exponentials × 10^{-8} (dynes cm.$^{-2}$) A	B	C	D	Exponentials (sec.$^{-1}$) α	β	γ
48	4.22	1.54	1.54	0.85	0.874	0.252	0.043
96	4.50	1.28	1.28	0.79	0.874	0.252	0.043
144	4.14	1.45	1.94	1.25	0.874	0.252	0.043
192	3.70	2.16	2.36	1.55	0.874	0.252	0.043
240	3.63	1.81	1.65	1.24	0.874	0.252	0.043
288	3.61	2.60	2.46	2.02	0.874	0.252	0.043
336	3.15	3.38	2.83	2.00	0.874	0.252	0.043

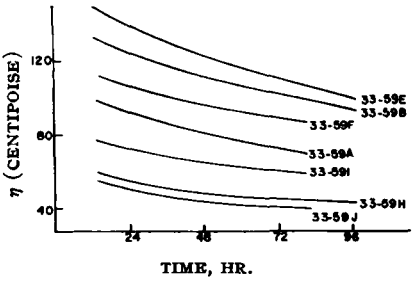


Fig. 3.—Viscosity of gelatin melts *vs.* time of thermal aging.

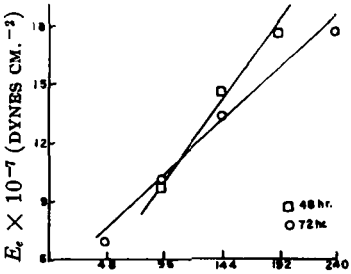


Fig. 4.—Equilibrium modulus gelatin film 33-59J *vs.* time of humidity cabinet aging.

for the 48-hour casting was greater than that for the 72-hour casting. Thus, it appears that one of the effects of thermal aging of the melt is a decrease in the rate of gelation after casting.

Samples stored in the refrigerator for extended periods showed increased tensile properties over the thermally degraded melts which had previously been cast. This held true even though the samples had to be reheated at 60° for an additional 5 hours to reach casting consistency. These results again support the proposition that an orientation mechanism may account for the decay of tensile properties in gelatin films. At the refrigerator temperature the sample could reorient and increase its crystallinity allowing for a greater number of cross-links to be formed upon remelting and casting.

Experimental evidence favors the concept of crystalline-amorphous transitions in gelatin films. Assuming this concept to be true, the films would be expected to become more amorphous during thermal aging. This should result in a shift of the cross-link distribution to increase the number of short relaxation time cross-links. The phenomenon of stress relaxation could then be explained on the basis of an entropy change in the system which had been induced by the elongation of the polymer chains forcing them into a more oriented and more highly crystalline form. This concept may be investigated from a theoretical viewpoint to determine what type of equation for stress relaxation would be predicted. The film, as cast, may be considered to consist of polymer chains of varying degrees of crystallinity. During stress relaxation there is crystal growth, and these chains are transformed to the crystalline state. A series of reactions would be occurring during this process corresponding to the model

$$A \xrightarrow{\alpha} B \xrightarrow{\beta} C \xrightarrow{\gamma} D \qquad \text{(Eq. 12)}$$

where A corresponds to the amorphous chains, B and C to intermediate degrees of crystallinity, and D to the crystalline chains. The rate constants for the transformations of these chains to more crystalline forms are α, β, and γ.

Assuming that these are all first-order transformations, a series of differential equations for the change in concentration of these cross-links with time may be

$$\frac{d[A]}{dt} = -\alpha[A] \qquad \text{(Eq. 13)}$$

$$\frac{d[B]}{dt} = \alpha[A] - \beta[B] \qquad \text{(Eq. 14)}$$

$$\frac{d[C]}{dt} = \beta[B] - \gamma[C] \qquad \text{(Eq. 15)}$$

$$\frac{d[D]}{dt} = \gamma[C] \qquad \text{(Eq. 16)}$$

These equations may then be solved for the concentration of the cross-links.

The tensile modulus of the film should be proportional to the concentration of the cross-links and may be

$$E(t) = k_1[A] + k_2[B] + k_3[C] + k_4[D] \qquad \text{(Eq. 17)}$$

By substitution of the values for the concentration of the cross-links into the above equation one may obtain the equation for the tensile relaxation modulus, which on rearrangement yields

$$E(t) = k_4(A_o + B_o + C_o + D_o) + \left[\frac{k_1(\alpha-\beta)(\alpha-\gamma) + k_2(\alpha-\gamma) + k_3\alpha\beta - k_4\beta\gamma}{(\alpha-\beta)(\alpha-\gamma)}\right] A_o e^{-\alpha t} + \left[\frac{k_2\alpha\beta - k_2\alpha(\beta-\gamma) + k_4\alpha\gamma}{(\alpha-\beta)(\beta-\gamma)}\right] A_o e^{-\beta t} + \left[\frac{k_2(\beta-\gamma) + k_3\beta + k_4\gamma}{(\beta-\gamma)}\right] B_o e^{-\beta t} + \left[\frac{k_4\alpha\beta - k_3\alpha\beta}{(\gamma-\alpha)(\gamma-\beta)}\right] A_o e^{-\gamma t} + \left[\frac{k_3\beta + k_4\beta}{(\gamma-\beta)}\right] B_o e^{-\gamma t} + (k_3 - k_4)\, C_o e^{-\gamma t} \qquad \text{(Eq. 18)}$$

This predicts for the tensile relaxation modulus an equation which would fit the Wiechert mechanical model with the following boundary conditions

at $t = 0$

$$E_o = k_1A_o + k_2B_o + k_3C_o + k_4D_o \qquad \text{(Eq. 19)}$$

at $t = \infty$

$$E_\infty = k_4(A_o + B_o + C_o + D_o) \qquad \text{(Eq. 20)}$$

Since the experimental curves also fit this model, it may be proposed that the stress decay can be attributed to a change in the crystalline-amorphous ratio in gelatin films.

SUMMARY

Information gathered to this point from stress relaxation measurements seems to support the theory of crystallite formation in gelatin films. Results of humidity cabinet aging studies indicate that the increase in the tensile properties of the films is a kinetic process whose rate is influenced

by the degree of thermal aging of the melt. The decrease in the rate of gelation may be explained on the basis of dissolution of the smaller crystallites at elevated temperatures. At the time of casting, these crystallites are not present to serve as nuclei, and the rate of crystallization would be decreased. The crystallites reappear after an induction period at low temperatures. This may be demonstrated by storage of the thermally aged melt in a refrigerator for a period of time. Subsequent measurement of the tensile relaxation modulus of films cast from this melt show an increase in its value. This reversal of the thermal degradation of the melt lends support to the crystalline-amorphous concept.

Viscosity studies of the melt proved ineffectual in describing changes occurring in the tensile properties of films cast from the melt. Results indicated an orderly decrease in molecular weight with thermal aging. Changes in the tensile properties could not be reconciled with changes in molecular weight. Not only were these changes not parallel with the decrease in viscosity but also there was no way to explain the reversal of thermal changes during low temperature storage of the melt. Although some changes in the tensile properties may result from a scission of the polymer chain during hydrolysis, it does not appear to be the *major* contributing factor.

To obtain a complete picture of the changes occurring in the rheological properties of gelatin films, it seems that, in addition to viscometric studies, one must measure some viscoelastic property whereby one may obtain information about the cross-linking processes occurring during gelation. It is felt that these changes may be responsible for some of the technological problems arising in the use of gelatin systems in the pharmaceutical industry.

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Powdered Particle Interactions: Suspension Flocculation and Caking III

By ROBERT G. WILSON* and BERNARD ECANOW

The chemistry of bismuth subnitrate in aqueous suspension has been investigated and explained in terms of coordination theory. Particular attention has been given to the reaction that occurs between phosphate ions and the surface of the bismuth subnitrate particles. Flocculation and caking phenomena are reviewed and interpreted in the light of these new findings.

IN A PREVIOUS paper (1) flocculation phenomena for bismuth subnitrate suspension systems were reported in terms of microscopic data and the relative suspension heights obtained from a series of controlled flocculation experiments. Strong bonds were seen to form between the particles while in the suspended state. The particles were held in fixed relative positions as they settled and were unable to shift and slide past one another to squeeze out the suspension medium and form a dense, compact sediment. Instead, the flocculated particles settled into the framework of a bulky sediment.

In this prior work (1) some effort was made to suggest the probable nature of the reacting groups, but only tentative conclusions could be drawn. The work has continued with a closer inspection of the reactions of bismuth subnitrate in aqueous suspension in the hope that such information could provide a better insight into the mechanics of flocculation.

The literature (2–7) contains little detailed information on bismuth subnitrate or its reactions. A number of empirical formulas have been assigned to the compound. It is evident that the composition and properties of the crystalline material will vary, depending upon the method of preparation and the nature of changes that may occur when the material is subjected to further processing. The structure of the commercial product cannot be reduced to lattice formulas that describe an exact crystal geometry.

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* Fellow of the National Science Foundation.

MATERIALS AND METHODS

Materials.—Bismuth subnitrate N.F. from the same lot, supplied by Mallinckrodt Chemical Works,

by the degree of thermal aging of the melt. The decrease in the rate of gelation may be explained on the basis of dissolution of the smaller crystallites at elevated temperatures. At the time of casting, these crystallites are not present to serve as nuclei, and the rate of crystallization would be decreased. The crystallites reappear after an induction period at low temperatures. This may be demonstrated by storage of the thermally aged melt in a refrigerator for a period of time. Subsequent measurement of the tensile relaxation modulus of films cast from this melt show an increase in its value. This reversal of the thermal degradation of the melt lends support to the crystalline-amorphous concept.

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IN A PREVIOUS paper (1) flocculation phenomena for bismuth subnitrate suspension systems were reported in terms of microscopic data and the relative suspension heights obtained from a series of controlled flocculation experiments. Strong bonds were seen to form between the particles while in the suspended state. The particles were held in fixed relative positions as they settled and were unable to shift and slide past one another to squeeze out the suspension medium and form a dense, compact sediment. Instead, the flocculated particles settled into the framework of a bulky sediment.

In this prior work (1) some effort was made to suggest the probable nature of the reacting groups, but only tentative conclusions could be drawn. The work has continued with a closer inspection of the reactions of bismuth subnitrate in aqueous suspension in the hope that such information could provide a better insight into the mechanics of flocculation.

The literature (2–7) contains little detailed information on bismuth subnitrate or its reactions. A number of empirical formulas have been assigned to the compound. It is evident that the composition and properties of the crystalline material will vary, depending upon the method of preparation and the nature of changes that may occur when the material is subjected to further processing. The structure of the commercial product cannot be reduced to lattice formulas that describe an exact crystal geometry.

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* Fellow of the National Science Foundation.

MATERIALS AND METHODS

Materials.—Bismuth subnitrate N.F. from the same lot, supplied by Mallinckrodt Chemical Works,

St. Louis, Mo., was used throughout the experiments. Monobasic potassium phosphate, reagent grade, supplied by Merck & Co., Rahway, N. J., was used in preparing the phosphate solutions. All other materials used were of reagent grade.

Equipment.—A Birrell wrist-action shaker, model DD, was used to agitate the bismuth subnitrate suspensions undergoing time reactions. Spectrophotometric determinations were made on a Coleman, Jr., spectrophotometer, model 6-A. A Beckman extended range pH meter was used to obtain the pH data.

Methods.—PHOSPHATE DETERMINATIONS.—The concentration of phosphate ion was determined through use of the molybdovanadophosphoric acid method, described by Boltz (8).

NITRATE DETERMINATIONS.—The concentration of nitrate ion was followed by the phenoldisulfonic acid method, described by Boltz (9).

EXPERIMENTAL WORK

Attention was first directed toward the reaction that occurs between phosphate ions and bismuth subnitrate in aqueous suspension. A series of solutions of known phosphate ion concentrations was prepared and used to obtain the upper curve of Fig. 1. The same solutions of phosphate ion were then used as the suspending media for a series of 2% bismuth subnitrate suspensions, and the lower curve was obtained. The downward shift in phosphate ion concentration was the result of its reaction with the bismuth subnitrate. If no reaction had taken place, the two curves would have been identical.

It was soon found that the amount of phosphate ion removed from the suspension medium varied with time. Three sets of a series of 100 ml. 2% bismuth subnitrate suspensions were prepared, with each set at a different level of initial phosphate ion concentration. The results are shown in Fig. 2, which plots the concentration of phosphate ion remaining in the suspension medium *versus* time. The results indicate that the uptake of phosphate

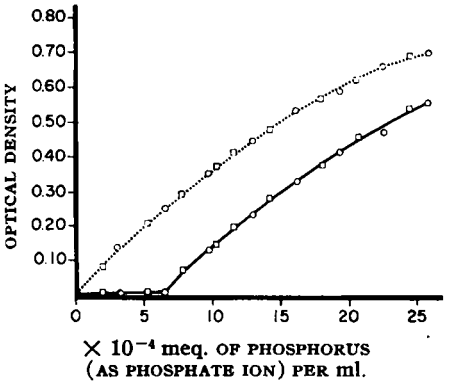


Fig. 1.—A plot of optical density *vs.* phosphate ion concentration. The dotted line presents the data obtained from a series of solutions of known phosphate ion content. The solid line plots data on the concentration of phosphate ion remaining in the suspension media, after bismuth subnitrate 2% concentration was added to each of these known solutions. The experiment was duplicated, with one set of data shown as O and the other as □.

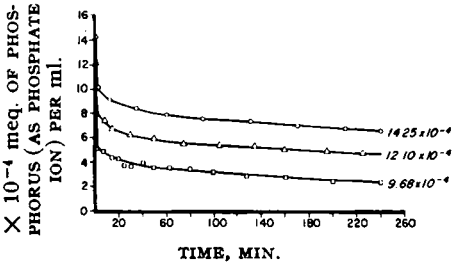


Fig. 2.—A plot of the concentrations of phosphate ion remaining in 2% bismuth subnitrate suspensions *vs.* time from the moment of preparing the suspensions. The three curves present data from suspensions prepared at three different initial phosphate ion concentrations. The curves have been marked with these initial concentrations.

ion is independent of its initial concentration in the suspension medium, and that the rate at which reactive sites appear on the surfaces of the particles is the controlling factor. It should be noted that all three of the initial concentrations used were sufficient to cause the flocculation of the suspended particles. The amount of phosphate removed from the suspension medium has been calculated and plotted as one of the curves of Fig. 5.

It was found that the amount of phosphate ion required to cause flocculation increases as an aqueous bismuth subnitrate suspension is aged. For a 2% suspension, the requirement rose from 1.0×10^{-4} to 1.4×10^{-4} meq. phosphorus (as phosphate ion) per milliliter of suspension, during the first 15 minutes. No aging effect could be shown beyond this initial period of time by this technique.

Next, studies were conducted on the changes in pH that result from the suspension of bismuth subnitrate powder in water. The data could be converted to hydrogen ion concentration where this was of assistance in interpreting the phenomenon. The suspension medium under test was first adjusted to pH 7.00, and the bismuth subnitrate then added all at once in an amount calculated to give a 2% suspension. The data for bismuth subnitrate suspended in water have been plotted as one of the curves of Figs. 3 and 4. The same data were converted to milliequivalents of hydrogen ion released with time and plotted as one of the curves of Fig. 5.

Comparable pH *versus* time plots were obtained for 2% bismuth subnitrate suspensions containing different amounts of phosphate ion. The results are shown in Fig. 3. Buffering by the phosphate ion tended to shift the initial portion of the curves upward and to the right so that it was not clear whether the release of hydrogen ion was the same in each medium. The experiment was repeated using chloride ion as the flocculating agent. It showed an ability to flocculate comparable to that of phosphate ion, but no buffering effect was expected to cloud an analysis of the results. The data have been plotted in Fig. 4. As can be seen, the presence of chloride ion enhanced the initial release of hydrogen ions. However, the effect was short lived, for after the first 40 seconds the pH gradually rose, indicating the removal of hydrogen ions from the suspension medium. This response to the presence of chloride ion was particularly

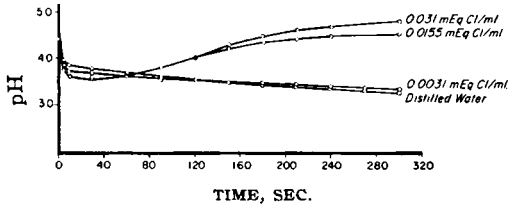


Fig. 3.—A plot of pH *vs.* time for freshly prepared bismuth subnitrate suspension systems. The bismuth subnitrate in dry powder form was added to solutions containing different concentrations of chloride ion to form a series of 2% suspensions. The chloride solutions were adjusted to pH 7.00 prior to the addition of the solid material. The curves have been marked to show the initial chloride ion concentrations, and are compared against the results obtained with distilled water.

marked when its concentration in the suspending medium was increased to over ten times the amount required to cause flocculation.

The concentration of nitrate ion in the bismuth subnitrate suspension medium also varied with time. The data have been plotted as one of the curves of Fig. 5. It can be seen that hydrogen ion is released in an amount almost double that of the nitrate ion, and thus the interaction of bismuth subnitrate with water is not one of simple hydrolysis to form nitric acid.

The final phase of the work was concerned with the disappearance of phosphate ion with time as a bismuth subnitrate suspension is aged. Reference to Fig. 5 will show that no 24-hour value has been given for the disappearance of phosphorus (as phosphate ion) from the suspension medium. This is because, at low levels of phosphate ion concentration, all of the ion disappeared from the suspension medium within the first 24 hours. After several trials, a 48-hour reading was obtained by raising the initial phosphate ion concentration to 200×10^{-4} meq. phosphorus (as phosphate ion) per ml. After 48 hours on the shaking machine, the pH was buffered at 4.05 by the phosphate remaining in solution, and 104×10^{-4} meq. of phosphorus (as phosphate ion) had been removed from each milliliter of the suspension medium. Nitric acid was then added to bring the pH down to 3.0 and the suspension shaken for another 48 hours. There was no further uptake of phosphate ion. The pH was reduced to 1.5, and all of the remaining phosphate ion was removed from the suspension medium during an additional 48 hours of shaking.

DISCUSSION OF RESULTS

The empirical formulas that have been assigned to bismuth subnitrate may be representative of the bulk substance of the crystal or powder, but do not necessarily provide an adequate description of the crystal surface. As a crystal forms, its surface is an amorphous, hydrated, electrostatically charged region of transition between the mother liquor and the fully formed crystalline material. Dean (10) suggests this for a colloid such as α-Fe_2O_3, in which the iron and oxygen atoms in the surface region may either be thought of as adsorbed ions, or as atoms belonging to the crystal structure but having unsatisfied valence and coordination bonds which are in interaction with the molecules of the solution.

In commercial processes, it seems the pH generally rises as the bismuth subnitrate is formed. Thus the surface composition of the crystal would be strongly influenced by the final pH of the precipitation medium and this effect would be further accentuated by the loss of nitric acid that occurs during the washing step. The drying step also acts upon the surface, rather than the bulk substance of the crystal. Adsorbed water molecules are driven off

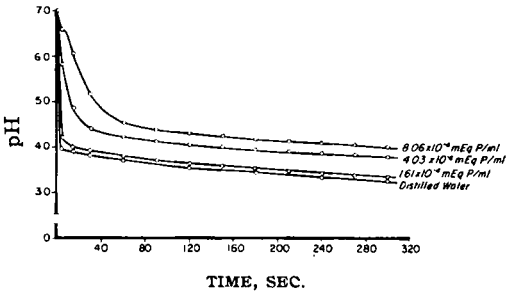


Fig. 4.—A plot of pH *vs.* time for freshly prepared bismuth subnitrate suspension systems. The bismuth subnitrate in dry powder form was added to solutions containing different concentrations of phosphate ion to form a series of 2% suspensions. The phosphate solutions were adjusted to pH 7.00 prior to the addition of the solid material. The curves have been marked to show the initial phosphate ion concentrations and are compared against the results obtained with distilled water.

and hydroxy groups at the surface are converted to oxy bridges.

The reactions of bismuth subnitrate can be understood within the framework of Werner's coordination theory (11). Dean (10) has discussed coordination theory pictorially in terms of the ferric ion. The water molecules of a coordination shell are held so strongly that the bonds between the iron atom and the oxygen atoms of the water molecules resemble covalent bonds. Two electrons from the oxygen atom serve to hold the water to the charged metallic atom and are drawn toward the metal atom so that the bonds between the hydrogen atoms and the oxygen are weakened. The hydrated ion becomes acidic and yields hydrogen ions to the solution.

These concepts can be extended to the bismuth subnitrate suspension system. When bismuth subnitrate forms a 2% suspension in water, nitrate ions appear in the suspension medium, and the pH falls from 7.00 to 3.95. These facts have been summarized in Fig. 5. Utilizing coordination theory, the reaction sequence shown in Fig. 6 (reactions *a* through *e*) is proposed.

In these reaction steps, the bismuth atom has been assigned a coordination number of 8. This finds justification in the crystal structure of the compound, bismuth oxychloride. Wells (12) and Hückel (13) have both discussed the layered structure of the BiOCl crystal, in which the bismuth atom has been shown to be 8-coordinated. Very similar formulas could be drawn if it should later be shown that the bismuth atom in bismuth subnitrate is 6-coordinated.

The data plotted in Fig. 3 show the change in

pH that takes place when bismuth subnitrate is added to a neutral solution of sodium chloride. There is a rapid release of hydrogen ion, just as is seen when bismuth subnitrate is added to water, but the release is enhanced by the presence of the chloride ion. We believe this may be a case in which the solubility of a very slightly soluble substance is increased by the presence of other ions (14). The nitrate concentration is in a range where the Debye-Hückel effect would be of significance.

As nitrate ions leave the crystal surface, unsatisfied bismuth sites would become available for covalent-type bonding with water of coordination. As the covalent-type bonds are formed, hydrogen ions are released to the suspension medium, and the pH falls. Figure 3 also shows a pH reversal effect, due to the presence of chloride ion. This seems due to the insolubility of bismuth oxychloride or its precursors and to the high electronegativity of the chloride atom. By forming a Bi-Cl bond having more of an ionic than covalent character,

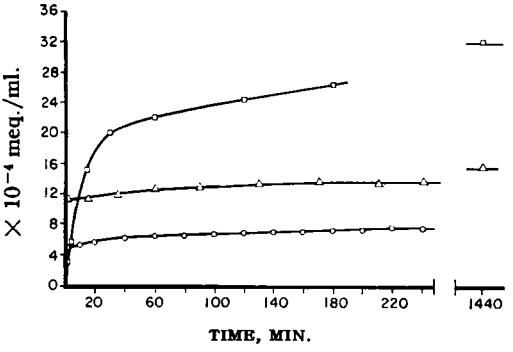


Fig. 5.—A summary plot showing ion concentrations *vs.* time. The data was obtained from several different suspension systems. Key: □, the curve on hydrogen ion concentration *vs.* time when bismuth subnitrate is added in dry powder form to give an aqueous 2% bismuth subnitrate suspension; △, the curve on nitrate ion concentration *vs.* time when bismuth subnitrate is added in dry powder form to give an aqueous 2% bismuth subnitrate suspension; ○, the curve on loss of phosphate ion concentration *vs.* time, following the addition of bismuth subnitrate in dry powder form to a solution containing monobasic potassium phosphate.

the strength of the covalent-type bonds between the bismuth atom and its water of coordination is diminished. With a weakening of these bonds, the oxygen atoms of the water of coordination become correspondingly more negative, and there is some recall of the hydrogen ions previously released. The net effect is a rise in the pH of the solution. The change in pH with time, when bismuth subnitrate is added to water containing the phosphate ion, has been shown in Fig. 4. Buffering is one reason for the difference in response to phosphate or chloride ion, but another factor is that the phosphate group is not so electronegative as is the chlorine atom. The significant pH reversal seen with chloride ion would thus not be expected from the medium containing the phosphate ion.

The aging of a bismuth subnitrate suspension can be partly understood in terms of reactions *a* through *e* in Fig. 6. Figure 5 shows that the nitrate and hydrogen ion concentrations of the suspension medium increase rapidly following the addition of the bismuth subnitrate, and the concentrations of both ions asymptotically approach equilibrium levels. As aging proceeds, respective sites appear due to the ionization of the nitrate group. However, this ionization accounts for but a part of the reactive sites that ultimately appear on the crystal surface. The particle surface reactivity increases to such an extent that the phosphate ion concentration will drop from 200×10^{-4} to 96×10^{-4} meq. of phosphorus (as phosphate ion) per milliliter, over a 48-hour period. The exact reason for this increase in the number of reactive sites is unknown. It is assumed that it is the end result of an opening of oxy bridges as the crystal surface becomes increasingly hydrated.

As evidence of the change in surface charge and reactivity with time, it is of interest to consider the deflocculation of a phosphate ion flocculated bismuth subnitrate suspension. If such a suspension is freshly formed and continuously shaken while it ages, it will revert to the deflocculated state. The shaking action tends to separate the flocculated particles while their surface is being altered by the hydration mechanism. A positive particle surface

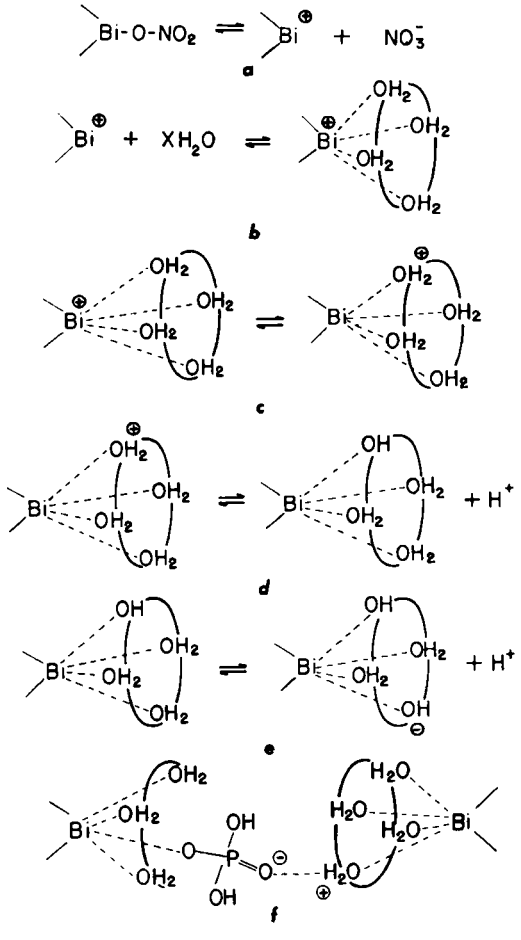


Fig. 6.—Proposed reaction sequence utilizing the coordination theory.

charge would result, and there may be a further reduction in the negativity of the surface as reacted phosphate groups form second bonds with the crystal surface when adjacent bismuth sites become available. This type of reaction at the crystal surface may be inferred from the crystal structure of a salt such as aluminum phosphate (15).

Thus, aging processes combined with vigorous agitation will cause the flocculated system to revert to a deflocculated state in which the individual particles have a net positive surface charge. In the absence of shaking, there is no apparent tendency to deflocculate on aging for a period of several months. However, if a suspension flocculated by a small amount of phosphate ion is allowed to age for several months and then shaken, it will promptly deflocculate. It should be noted that the hydration phenomenon does not cause an increase in the amount of phosphate ion required to cause flocculation of the system. Also, the deflocculated system may be reflocculated by the addition of a relatively small amount of phosphate ion. It is not necessary to add phosphate ion in an amount sufficient to satisfy all of the reactive sites on the aged crystal surfaces. This serves to reinforce the thought that only a few phosphate bridges are required to bond the bismuth subnitrate particles into a floc.

Where an excess of phosphate ion is present and the pH is held at a relatively high level, as was the case when the suspension medium initially contained 200×10^{-4} meq. of phosphorus (as phosphate ion) per milliliter, the suspension remains in the flocculated state throughout the aging process. Shaking is instrumental in breaking the larger flocs into smaller aggregates, but there is no reversion to the completely deflocculated suspension that might be expected. The reversal of charge (irregular series) for iron oxide particles suspended in water has been discussed by Van Wazer (16). The particles initially carry a positive charge and will be flocculated by the addition of polyphosphate ions. However, if more polyphosphate is introduced into the system, there is a reversal of particle charge, and the system reverts to the deflocculated state. In the case of bismuth subnitrate, this type of deflocculation was not seen despite the vigorous shaking action to which the suspension was subjected.

From the above, it appears that the surface charge on the bismuth subnitrate macroparticles was not so effective in preventing flocculation or in causing deflocculation as the surface charge in a typical colloidal system. This finding emphasizes the difference between a colloidal and noncolloidal suspension system. For the noncolloidal bismuth subnitrate, flocculation phenomena must be explained in terms of the number of bonds formed between the particles and in terms of the strengths of such bonds. The chemistry of the ferric ion and of the bismuth subnitrate crystal surface point to at least two types of bonds. These are illustrated in reaction *f* of Fig. 6. This bonding mechanism would imply that the nitrate ion could be responsible for the flocculation of a bismuth subnitrate suspension. Indeed this is the case, for a degree of flocculation is seen when a concentrated solution of potassium nitrate is added to a bismuth subnitrate suspension.

One other phenomenon remains to be considered. When a bismuth subnitrate suspension was flocculated with a large excess of phosphate ion, a little more than half of the phosphate was removed from the suspension medium as the suspension was aged with shaking for a 48-hour period. The balance of the phosphate could be removed by adjusting the pH to 1.5 and continuing the shaking process. This seems due to the action of the acid upon those groups in the crystal surface which were formed later in the manufacturing process. Reactive sites appear in large number as the crystal surface is etched by the acid.

SUMMARY

The surface chemistry of bismuth subnitrate particles in aqueous suspension has been discussed in terms of Werner's coordination theory. This new approach to the chemistry of bismuth subnitrate has been of value in explaining the phenomena associated with bismuth subnitrate suspensions. Data are reported which support the application of the theory to this special case involving a suspended solid. Aging phenomena have also been reported for the bismuth subnitrate suspension system.

Coordination theory has been used in a further discussion of the nature of the bonds that exist between the particles of a phosphate ion flocculated bismuth subnitrate suspension. An important difference between colloidal and noncolloidal systems is based on this point. To bond noncolloidal particles, relatively strong bonds must be present in sufficient number over those areas of particle surface that come in contact with each other. As particle size decreases, fewer bonds or bonds of a weaker nature, can serve to bind the particles together into a floc.

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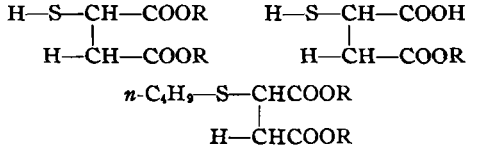
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Synthesis and *In Vitro* Evaluation of the Antifungal Activity of a Series of Thiomalic Acid Derivatives

By WOODROW R. BYRUM, JOHN E. WINTTER, and JACK T. BRYAN*

Mono and diesters of thiomalic acid with various alcohols were prepared, and the *S-n*-butyl derivatives of the diesters were prepared. These compounds were analyzed, various physical constants were determined, and the fungicidal and fungistatic activities were measured, using undecylenic acid as the standard for comparison against *Trichophyton mentagrophytes*. Several of the compounds under the conditions of the tests exhibited fungicidal and/or fungistatic activity.

THIOMALIC ACID (mercaptosuccinic acid, 2-mercapto-1,4-butanedioic acid) possesses two structural features which are present in the structures of some commercially available antifungal compounds—namely, the $-\overset{|}{\underset{|}{C}}-S-$ group and the carboxylic acid group. Barry, *et al.* (1), found that *S*-alkylthiomalic acids and their half esters strongly inhibited the growth of various bacteria. This paper reports the preparation of a series of esters of thiomalic acid and *S-n*-butylthiomalic acid; the general formulas for these compounds are



where R is n-C_3H_7—, n-C_4H_9—, and n-C_5H_{11}—. The fungistatic and fungicidal activities of these compounds are also reported.

EXPERIMENTAL

The esters listed in Table I were prepared by standard procedures. The following examples illustrate the methods and conditions used.

Diesters.—The dipropyl, dibutyl, and diamyl thiomalates were prepared as follows. A mixture of 15 Gm. (0.1 mole) of thiomalic acid, 45 ml. of the appropriate alcohol, and 4 ml. of concentrated sulfuric acid was refluxed for 3 hours. The excess alcohol was removed by distillation under reduced pressure, and the residual liquid was poured with stirring into 150 ml. of a saturated aqueous solution of sodium bicarbonate. When neutralization of the acid was complete, the crude esters were recovered by ether extraction.

Monoesters.—The monopropyl, monobutyl, and monoamyl thiomalates were prepared as follows. A mixture of 15 Gm. (0.1 mole) of thiomalic acid, 0.1 mole of the appropriate alcohol, 4 ml. of concentrated sulfuric acid, and 35 ml. of dioxane was refluxed for 12 hours. The volume of the reaction mixture was then reduced by distillation under reduced pressure. The residual liquid was poured with stirring into 150 ml. of a saturated aqueous solution of sodium bicarbonate. The alkaline mixture, which had assumed a wine color, was extracted with several portions of ether to remove any diester produced by the reaction. Concentrated hydrochloric acid was added until the aqueous mixture was acid to litmus. This discharged the color and caused the monoester to separate as an oil. Ether extraction of the acidified mixture resulted in the isolation of the crude monoesters.

S-n-Butyldiesters.—The dipropyl, dibutyl, and diamyl *S-n*-butyl thiomalates were prepared according to the method of Barry, *et al.* (1). Clean, finely divided sodium metal, 0.5 Gm. (22 mmoles), was dissolved in 22 ml. of dry ethanol. The appropriate thiomalic acid diester (22 mmoles) and *n*-butyl bromide (22 mmoles) were added to the sodium ethylate solution and refluxed for 3 hours. The mixture was cooled, and the liquid layer was filtered from the solid sodium bromide. After removing the excess ethanol by distillation under reduced pressure, the residual liquid was poured into 20 ml. of water, the aqueous mixture was extracted with ether to remove the ester. The crude dialkyl *S-n*-butyl thiomalates were obtained upon evaporating the ether.

The crude esters were all light yellow viscous liquids with the monoesters being most viscous and darkest in color. Distillation at pressures of 1–3 mm. of mercury produced distillates that were colorless. If prolonged exposure to high bath temperature occurred during the distillation of the esters, the distillate assumed a yellow color. This decomposition was prevented by flushing the apparatus with nitrogen before distillation and by furnishing nitrogen to the aspirator tube during the reduced pressure distillation and distilling as rapidly as possible. Distillation reduced the yield of ester materially except for dialkyl *S-n*-butyl thiomalates. The monoesters were particularly subject to decomposition during distillation. The pure esters were characterized (see Table I) and used for the antifungal tests.

Relative Solubilities of the Mono and Diesters.—The relative solubilities of the mono and diesters were determined in some common solvents. These data are recorded in Table II.

FUNGICIDAL AND FUNGISTATIC STUDIES

Fungicidal Test.—The method of testing the esters of thiomalic acid for fungicidal activity was, with

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TABLE I.—DERIVATIVES OF THIOMALIC ACID

$$R_2\text{—}S\text{—}CH(COOR_1)\ (\alpha)$$
$$H\text{—}CH(COOR)\ (\beta)$$

R	R_1	R_2	Formula	Yield, %	Boiling Range, °C./mm. Hg	Sulfur, %[a] Calcd.	Sulfur, %[a] Found	$d_4^{20°}$	$n_D^{20°}$	$(R_L)D$ Calcd.	$(R_L)D$ Found
n-C_3H_7—	*n*-C_3H_7—	H—	$C_{10}H_{18}O_4S$	60	135–139/1	13.69	13.59	1.0700	1.4579	59.37	59.72
n-C_4H_9—	*n*-C_4H_9—	H—	$C_{12}H_{22}O_4S$	58	150–155/1	12.23	12.28	1.0332	1.4573	68.61	69.18
n-C_5H_{11}—	*n*-C_5H_{11}—	H—	$C_{14}H_{26}O_4S$	57	165–175/1	11.04	10.95	1.0074	1.4564	77.93	78.40
n-C_3H_7—	H—	H—	$C_7H_{12}O_4S$	46	125–132/2	16.69	16.60	1.2298	1.4858	45.43	44.84
n-C_4H_9—	H—	H—	$C_8H_{14}O_4S$	44	145–152/2	15.55	14.89	1.1857	1.4811	50.05	49.49
n-C_5H_{11}—	H—	H—	$C_9H_{16}O_4S$	41	164–170/3	14.56	14.67	1.1500	1.4799	54.71	54.38
n-C_3H_7—	*n*-C_3H_7—	*n*-C_4H_9—	$C_{14}H_{26}O_4S$	55	124–127/1	11.04	11.13	1.0285	1.4602	78.13	77.34
n-C_4H_9—	*n*-C_4H_9—	*n*-C_4H_9—	$C_{16}H_{30}O_4S$	52	140–144/1	10.07	10.01	1.0011	1.4590	87.37	86.95
n-C_5H_{11}—	*n*-C_5H_{11}—	*n*-C_4H_9—	$C_{18}H_{34}O_4S$	50	153–158/1	9.26	9.40	0.9811	1.4585	96.69	96.45

[a] Analytical method (2).

TABLE II.—RELATIVE SOLUBILITY OF THE MONO AND DIESTERS[a]

Thiomalate Ester	Water	Benzene	Acetone	Pet. Ether	Methyl Alcohol	Chloroform	Tetrahydrofuran
Monopropyl	−	+++	+++	−	++	+++	+
Dipropyl	−	+++	+++	+	++	+++	+
Monobutyl	−	+++	+++	−	++	+++	+++
Dibutyl	−	+++	+++	+	+++	+++	+++
Monoamyl	−	+++	+++	−	+++	+++	+++
Diamyl	−	+++	+++	+++	+++	+++	+++

[a] Insoluble in ten parts (volume) of solvent, −; soluble in three to four parts, +; soluble in equal part, ++; soluble in less than equal part, +++.

minor modifications, the procedure described by Burlingame and Reddish (3) and as adapted and modified by Golden and Oster (4) for use in testing alcohol soluble compounds.

The culture medium used was Sabouraud's dextrose agar containing 10% horse serum and maintained at a pH of 5.6 to prevent bacterial contamination. Petri dishes of this medium were streaked with a culture of *T. mentagrophytes* (No. 640 Emmons) and incubated at 28 ± 1° for 10 to 15 days. Cultures of this age were cut into disks of 1-cm. diameter with a sterile cork borer and transferred aseptically to seeding tubes containing 10 ml. of 0.5%, 1.0%, and 2.0% acetone solutions of each compound listed in Table III. After a 2-minute contact with the test compound, each disk was placed in 10 ml. of sterile broth and shaken gently for 3 minutes. This was done to free the culture of water soluble material. The disk was then removed from the broth and washed with 10 ml. of acetone in the same manner for 5 minutes, thereby removing the test compound from the culture. Once again each disk was immersed separately in sterile broth for 3 minutes to remove the acetone and then was spread culture side down over the surface of a sterile slant of Sabouraud's agar. These slants were incubated at 28 ± 1° for 14 days and then observed for growth. The results are outlined in Table III.

Undecylenic acid was used as a control in the same concentrations as the test compounds and exhibited 100% fungicidal activity at each concentration. Acetone which was used as a solvent for the test compounds was also used as a control and, as with other investigators (4, 5), had no effect upon the test fungus. All tests were performed in triplicate.

Fungistatic Test.—The method of testing for the presence of fungistatic activity was a modification of the procedure described by Kligman and Rosenweig (6) and Collins and Wiese (7). Sabouraud's dextrose agar was the culture medium used and was maintained at a pH of 5.6 to prevent bacterial contamination. Ten compounds, each in concentrations of 0.5%, 1%, and 2.0%, were tested in triplicate.

Petri dishes of Sabouraud's medium were streaked with a culture of *T. mentagrophytes* (No. 640 Emmons). A sterile filter paper disk, 13 mm. in diameter, was placed in each acetone solution of the test compounds and the controls. Each disk was picked up with sterile forceps, the excess liquid drained off by touching the disk to the wall of the container, and then immediately dropped onto the agar surface of a Petri dish which had been streaked with the test organism. These Petri dishes were then incubated at 37° for 7 days, at which time the zones of inhibition were measured. These data are recorded in Table IV and represent the results from two separate determinations.

DISCUSSION

Analysis of the diesters by standard methods for the mercapto group (8) showed them to be between 98–100% pure. The monoesters were submitted to the same analytical procedures, but the free carboxyl group interfered in these assay methods. Since the *S*-alkyl thiomalates lacked a free mercapto group, the sulfur contents of all of the derivatives of thiomalic acid prepared for this study were determined by the Parr sodium peroxide bomb method described by Smith and Shriner (2).

The structure indicated for the monoesters in Table I assumes that the ester linkage involves the β-carboxyl group, the group farthest removed from the carbon bearing the mercapto group. No experimental evidence for this structure verifies this assumption; however, further study of this problem is underway in this laboratory. Mention may be made that Heilbron (9) indicates β-carboxyl involvement in the structure for thiomalic acid monamide.

TABLE III.—NUMBER OF CULTURES UPON WHICH THIOMALIC ACID ESTERS EXERTED FUNGICIDAL ACTION[a]

	Concn., %		
Thiomalate	0.5	1.0	2.0
Free acid	0	0	0
Dipropyl	0	3	4
Dibutyl	0	0	4
Diamyl	0	5	6
Monopropyl	2	6	6
Monobutyl	0	1	3
Monoamyl	6	6	6
Dipropyl *S*-butyl	0	6	6
Dibutyl *S*-butyl	0	0	3
Diamyl *S*-butyl	6	6	6

[a] Each figure represents the results from a total of six determinations.

TABLE IV.—FUNGISTATIC ACTIVITIES OF THIOMALIC ACID ESTERS[a]

	Concn., %		
Thiomalate	0.5	1.0	2.0
Free acid	0	0	0
Dipropyl	0	2	5
Dibutyl	0	0	0
Diamyl	0	0	3
Monopropyl	5	7	15
Monobutyl	6	10	18
Monoamyl	8	15	29
Dipropyl *S*-butyl	3	7	40
Dibutyl *S*-butyl	0	0	0
Diamyl *S*-butyl	6	10	28

[a] Zones of inhibition measured in millimeters.

The method by which the monoesters were prepared yielded diesters as a side product to the extent of 10–15%. An attempt to increase the yield of monoester and to reduce the amount of discoloration during preparation by using polyphosphoric acid as the catalytic agent resulted in comparable yields but less discoloration during the reflux period.

An attempt has been made to prepare salts of the monoesters with various metal compounds. While some precipitates were obtained, these have not been properly characterized and were not tested for antifungal activity. The product produced with cupric ion was dark violet and probably represented a mixture of compounds in which the copper exists in both valence states as has been suggested for free thiomalic acid (10). Since metallic ions can form mercaptides and some can cause the oxidation of the mercapto group, additional study of the monoester salts will be undertaken.

Oster and Golden (11) found that the fungistatic values obtained by using several dermatophytes, *T. mentagrophytes*, *E. inguinale*, and *T. purpureum*, ran closely parallel. We decided (as they did) to use one organism, *T. mentagrophytes*, as the representative test organism to save time and reduce the number of tests. Because of its known antifungal activity, undecylenic acid was used as the standard in these comparative tests. Complete inhibition of growth was produced by undecylenic acid at all concentrations. Triplicate tests of the acetone solvent showed no antifungal effect.

The inhibition of growth around the filter paper disks was measured in millimeters and represents an average of three measurements.

SUMMARY

Mono and diesters of thiomalic acid with several alcohols and diesters of *S-n*-butyl thiomalic acid with the same alcohols were prepared. These compounds were analyzed for their sulfur content, various physical constants were determined, and the fungicidal and fungistatic activities were determined using *T. mentagrophytes* as the test organism. Undecylenic acid was used as the standard for comparisons.

In the fungicidal tests, disks of an agar culture of the test organism were exposed to various concentrations of the thiomalic acid derivatives. After washing in broth and in acetone to remove the test compounds, the disks were placed culture side down on the surface of a sterile slant of Sabouraud's agar. The slants were observed for growth after incubation. The absence of growth indicated that the compounds exerted a fungicidal action. Both monoamyl thiomalate and diamyl *S*-butyl thiomalates were fungicidal at each concentration in all tests. Diamyl, monopropyl, and dipropyl *S*-butyl thiomalates exhibited fungicidal activity at 1 and 2% concentrations. Thiomalic acid exhibited no fungicidal activity.

In the fungistatic test method sterile filter paper disks impregnated with acetone solutions of the test compounds were placed on Petri dishes of Sabouraud's agar streaked with the test organism. Inhibition of growth after incubation was indicative of fungistatic activity. Thiomalic acid was inactive, dipropyl and diamyl thiomalate showed slight activity at the highest concentration used, and monopropyl, monobutyl, monoamyl, dipropyl, and diamyl *S*-butyl thiomalates showed activity at all concentrations.

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Evaluation of Polymeric Materials II

Screening of Selected Vinyls and Acrylates as Prolonged-Action Coatings

By ROBERT JAY NESSEL*, H. GEORGE DEKAY, and GILBERT S. BANKER

Acrylic and vinyl polymers, copolymers, and derivatives were evaluated as potential sustained-action coatings. Based on a screening of films of the polymeric materials for solubility characteristics, free film formation, and drug permeability, the *n*-butyl half ester of poly(methyl vinyl ether)/maleic anhydride offered the most *in vitro* promise of controlling drug release. The *n*-butyl half ester had no cumulative toxic effects based on a short-term feeding study.

IN THE SELECTION of polymeric films for application as protective, enteric, or sustained-release coatings to solid pharmaceutical dosage forms, the solubility, film formation, drug permeability, and toxicity of the coating materials must be considered.

In the past the usual method of evaluation entailed the application of the new coating agent directly to tablets, pills, etc., and the testing of disintegration rates. However this approach was very time consuming, especially when many substances were to be tested, and the method provided only limited information with which to interpret and analyze results. In 1953, Antonides (1) developed a test procedure for screening film-forming materials using microscope slides as a coating surface. Kanig and Goodman (2) and Munden, *et al.* (3), have evaluated several chemical and physical properties of polymers using the free polymer films as the test samples. These studies of film evaluation were not directed toward sustained-release products.

EXPERIMENTAL

Preparation of Polymer Derivatives.—In addition to the commercially available polymers studied in this project, several derivatives were prepared to produce polymeric materials with solubility properties more nearly approaching that desired in a prolonged-action coating (insoluble or slowly soluble in gastric media and slowly soluble in intestinal media). The polymers screened in this study are listed in Table I and were selected for investigation based on their reported low toxicity and solubility characteristics.

Of the polymers studied, poly(methyl vinyl ether)/maleic anhydride or PVM/MA was unique in the number of derivatives which were available or could be prepared from it. The derivatives commercially available or available as experimental chemicals were the methyl, ethyl, and 2-ethylhexyl half or partial esters of PVM/MA. In addition, the *n*-butyl and isobutyl half esters of PVM/MA were synthesized, which provided a series of derivatives that decreased in solubility with an increase in the degree of esterification or with an increase in the length of the substituted aliphatic group.

The *n*-butyl half ester was prepared by slurrying 100 Gm. of Gantrez AN119 in 500 ml. of *n*-butyl alcohol in a three-neck standard taper round-bottom flask, fitted with an air mixer, thermometer, and a reflux column. The mixture was heated between 107 and 115° with the aid of a heating mantle. After 23 hours of refluxing, the polymer was precipitated by pouring the alcoholic solution of the half ester into water, and allowing it to stand until the alcohol was substantially removed by diffusion into the water. The half ester was then filtered off, air-dried, cut into pieces, additionally air-dried, recut, and finally vacuum dried at 60° and 65 mm. Hg.

The isobutyl half ester was prepared by placing 200 Gm. of Gantrez AN139 in a three-neck flask, as previously described, with 860 Gm. of isobutyl alcohol. The resulting mixture was heated between 85 and 90° for 26 hours. Precipitation, filtration, and drying of the half ester were accomplished in the same manner described for the *n*-butyl ester.

The degree of esterification of the poly(methyl vinyl ether)/maleic acid esters was calculated by the modified acid value procedure of the manufacturer of the anhydride. The acid value determinations agreed within 5% of the theoretical values supplied by the manufacturer for the *n*-butyl and isobutyl half ester products. The two grades of anhydride (Gantrez AN119 and 139) used in the half ester syntheses differ only in molecular weight. The choice of the polymer anhydride grade used in the synthesis of the half esters is dependent on the viscosity of the formed half ester in its alcoholic solution. The highest molecular weight grade of PVM/MA did not lend itself to the synthesis method for the half esters defined above.

Film Preparation and Evaluation.—Film samples of the polymers listed in Table I were made by preparing solutions of the polymers in appropriate solvent systems (Table II), with the films then being cast from these solutions by the mercury substrate technique (3). According to this method, the polymer solution was poured on a layer of mercury contained in a Petri dish, after which the film was allowed to air-dry. Film thickness was controlled by diluting the stock polymer solutions (Table II) with acetone, as necessary, according to solution viscosity, to produce dry films having a thickness of 0.003 to 0.006 in.

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TABLE I.—TABULATION OF POLYMERS SCREENED

Commercial Name	Chemical Designation	Physical Form
Gantrez AN119 & 139[a,b]	Poly(methyl vinyl ether)/maleic anhydride	Powder
PVM/MA methyl half[b] ester	Poly(methyl vinyl ether)/maleic acid methyl half ester	Powder
PVM/MA ethyl half[b] ester	Poly(methyl vinyl ether)/maleic acid ethyl half ester	Powder
PVM/MA 2-ethylhexyl[b] half ester	Poly(methyl vinyl ether)/maleic acid 2-ethylhexyl partial ester	Powder
PVM/MA isobutyl half ester	Poly(methyl vinyl ether)/maleic acid isobutyl half ester	Powder
PVM/MA *n*-butyl half ester	Poly(methyl vinyl ether)/maleic acid *n*-butyl half ester	Powder
Butvar B-90[c]	Polyvinyl butyral	Powder
Lemac 541-20[d]	Vinyl acetate copolymer—1	Beads
Neovac V27N[e]	Vinyl acetate copolymer—2	Emulsion
Polectron 130[b]	Vinyl pyrrolidone/ethyl acrylate copolymer	Emulsion
Neocryl BT-4[e]	Acrylic copolymer	Emulsion
Acrysol ASE-75[f]	Acrylic/methacrylic copolymer	Emulsion
Carboset 511[g]	Acrylic resin	Solution
Pentalyn 255[h]	Polymeric resin	Solid

[a] Hereafter referred to as PVM/MA. [b] General Aniline and Film Corp., New York, N. Y. [c] Shawinigan Resins Corp., Springfield, Mass. [d] Borden Chemical Co., New York, N. Y. [e] Polyvinyl Chemicals Inc., Peabody, Mass. [f] Rohm and Haas Co., Philadelphia, Pa. [g] B. F. Goodrich Chemical Co., Cleveland, Ohio. [h] Hercules Powder Co., Wilmington, Del.

TABLE II.—POLYMER SOLUTIONS EMPLOYED IN PRELIMINARY SCREENING TESTS

Polymer, Chemical Designation	Polymer Wt., Gm.	Acetone, ml.	Ethyl Alcohol, ml.	Butyl Acetate, ml.	Butyl Alcohol, ml.
PVM/MA	10	50	50	..	..
PVM/MA esters	10	50	50	..	..
Polyvinyl butyral	10	50	..	25	25
Vinyl acetate copolymer 1	10	50	..	25	25
Vinyl acetate copolymer 2[a]	..	..	..	..	..
Vinyl pyrrolidone/ethyl acrylate copolymer[a]	..	..	..		
Acrylic copolymer[b]	10	50	50	..	..
Acrylic/methacrylic copolymer[b]	10	50	50	..	..
Acrylic resin[a]	..	..	..	..	..

[a] Films were cast from the polymer solution or emulsion as commercially supplied. [b] Polymer solids were recovered by dilution of the polymer emulsion with water, followed by the addition of 1*N* sodium hydroxide until solution of the polymer occurred. Precipitation of the polymer from solution was then accomplished by the addition of 1*N* sulfuric acid.

The removal of the unplasticized polymeric films as a complete sheet was the criterion for free film formation. Film strips were cut from the film disks using a microscope slide as a template Film solubility was evaluated by visual observation using two such film strips of each polymer. One sample was placed in 50 ml. of simulated gastric fluid at 38° and was agitated at low speed with a magnetic stirrer for a maximum of 2 hours or until dissolution of the film occurred. This procedure was repeated in intestinal fluid using the other film strip. The simulated gastric and intestinal fluids were prepared according to the following formulas (4):

GASTRIC FLUID	
Sodium chloride	2.0
Pepsin	3.2
Hydrochloric acid	2.5
Distilled water, *q.s.*	1000.0

INTESTINAL FLUID	
Pancreatin	10.0
Ox bile extract	4.0
Potassium biphosphate, 0.2*M*	250.0
Sodium hydroxide, 0.2*M*	205.0
Distilled water, *q.s.*	1000.0

Film Permeability and Drug Binding Studies.—To determine the permeability of the films to drug, a small dialysis cell (5) was employed with 26-mm. diameter samples of the polymer films being used as the dialyzing membrane. Four milliliters of a stock solution containing 20 mg./ml. of *d*-amphetamine sulfate was pipeted into one side of the cell through the side arm. Buffer solutions of pH 2.4 and 6.8 were prepared according to the U.S.P. XVI, and were used in addition to the simulated gastric and intestinal fluids previously described. Into the other side of the dialysis cell 4 ml. of gastric fluid was pipeted through the side arm. The side arms were then sealed, the dialysis cell was placed in a rocker device in a water bath at 37 ± 2° for 0.5 hour, and the amount of drug which had permeated the membrane at that time was determined. The cell was then refilled on the test solution side (opposite the drug solution side) with another 4 ml. of gastric fluid, dialyzed as previously described for 1.5 hours, and the dialyzate determined. Following this determination, pH 2.4 buffer, pH 6.8 buffer, and artificial intestinal fluid were placed in turn in the test solution side of the cell, and the dialyzate determined at the time intervals indicated in Table IV.

The same dialysis cell was used in the study of drug binding to the polymeric materials, but in this study the polymer film sample used in the earlier study was replaced with Visking Nojax casing[1] as the dialysis membrane. Four milliliters of water was placed on the fluid side or test solution side of the cell and 4.0 ml. of a solution of 20 mg./ml. of *d*-amphetamine sulfate plus polymer solution or dispersion resulting from the dissolution or dispersion of a 26-mm. film disk was placed on the drug side of the cell. The dialysis cell was rotated for 24 hours in the water bath, the amount of drug in solu-

[1] Visking Co., Chicago, Ill.

tion on both sides of the cell was determined, and the amount of polymer drug binding was calculated.

Short-Term Feeding Study of PVM/MA n-Butyl Half Ester.—In an effort to obtain the LD_{50} for the *n*-butyl half ester of PVM/MA, the most promising polymer studied based on preliminary screening, dosages of 5, 10, 15, and 20 Gm./Kg. of animal weight were selected for administration based on the reported LD_{50} of the parent polymer in rats of 8 Gm./Kg. (6). However, due to the low density of the polymer material and the large volume to be fed, these doses could not be successfully administered in any form. For this reason a short-term feeding study was undertaken.

Two groups of six female Holtzman rats were used in this study for preliminary determination of the short-term toxic feeding effects, if any, of the polymer half ester based on gross changes in animal well being and on liver weights. One group of six animals was observed as the test group and the other as the control group. The test animals received daily doses of 5 Gm. of polymer per kilogram of body weight mixed with 15 Gm. of ground food. The polymer was milled to 40-mesh to insure a uniform mixture. The control animals received a corresponding quantity of lactose mixed with their food. All animals were housed in individual cages and received water *ad libitum*. Prior to daily dosing, each animal was weighed and any change in general animal appearance was noted. This procedure was followed until the tenth day when the animals were sacrificed and the livers removed. The liver weights were determined on a wet and dry basis and were converted to grams of liver per 100 Gm. of rat weight. The average dry liver weight for each group of six animals was calculated and compared to ascertain whether any of the polymer had accumulated in the liver.

RESULTS AND DISCUSSION

Free Film Formation and Film Solubility.—Utilizing the 10% solutions described in Table II, unplasticized films were cast as previously described, and on drying were evaluated for free film formation and solubility. These results are shown in Table III. The polymeric resin and the acrylic resin were immediately eliminated from further study since free films could not be obtained. The vinyl pyrrolidone/ethyl acrylate, acrylic copolymer, and polyvinyl butyral did not appear to demonstrate adequate solubility and were eliminated. The vinyl acetate copolymers, PVM/MA, and the PVM/MA methyl and ethyl half esters showed good promise as enteric coatings, but doubtful direct application as prolonged-action coatings. On the basis of solubility in simulated gastric and intestinal fluids, the acrylic/methacrylic copolymer film and the PVM/MA butyl and ethylhexyl films demonstrated the most promise and were further investigated.

The solubilities of the PVM/MA half ester series varied considerably and ranged from the methyl and ethyl half esters, which were quite soluble at low pH values, to the ethyl-hexyl and butyl half esters, which were insoluble to slightly soluble, until the dissolution media became slightly alkaline. The heptyl half ester of PVM/MA is reported to be soluble only under very alkaline conditions, and half esters synthesized with alcohols containing more than seven straight chain carbon atoms are reported insoluble (7). Lappas and McKeehan (8) in a preliminary communication also report that variations in ester chain length affect the dissolution pH of PVM/MA esters. Film dissolution in gastric and intestinal media is one indicator of the potential of a polymer film as a sustained-release coating, but it is not the absolute or even necessarily an accurate measure of the polymer's potential in this regard should the polymer film prove to be permeable to drug molecules.

Film Plasticization.—The ultimate objective of this project was to produce a highly plasticized flexible film which would remain intact about a coated granule after compression in a tablet matrix. A high degree of film plasticization was therefore important. The four polymers showing the most promise as a result of the unplasticized film solubility study (the two butyl and the ethylhexyl esters of PVM/MA and the acrylic/methacrylic copolymer) were plasticized with a number of agents and were reappraised for solubility characteristics. The water insoluble plasticizers studied included ricinoleic and linoleic acids, castor oil, dibutyl and diethylphthalate, and sorbitan monooleate. Diethylphthalate and sorbitan monooleate were the most effective plasticizers of the PVM/MA ester films. The most flexible films, of the films which retained their integrity, resulted from the addition of 60% w/w of

TABLE III.—SOLUBILITY AND FILM FORMATION OF SELECTED POLYMERS

Polymer, Chemical Designation	Free Film Formation	Solubility	
		Gastric Fluid	Intestinal Fluid
PVM/MA 119 and 139	Yes	P. Sol.[a]	Sol.[b] (5 min.)
PVM/MA methyl half ester	Yes	P. Sol.	Sol. (15 min.)
PVM/MA ethyl half ester	Yes	P. Sol.	Sol. (30 min.)
PVM/MA 2-ethylhexyl half ester	Yes	Insol.	P. Sol.
PVM/MA isobutyl half ester	Yes	Sl. Sol.[c]	P. Sol.
PVM/MA *n*-butyl half ester	Yes	Sl. Sol.	P. Sol.
Polyvinyl butyral	Yes	Insol.	Insol.
Vinyl acetate copolymer 1	Yes	Insol.	Sol. (15 min.)
Vinyl acetate copolymer 2	Yes	Insol.	[d]
Vinyl pyrrolidone/ethyl acrylate copolymer	Yes	Insol.	Insol.
Acrylic copolymer	Yes	Insol.	[d]
Acrylic/methacrylic copolymer	Yes	Sl. Sol.	Sol. (1 hr.)
Acrylic resin	No	...	...
Polymeric resin	No	...	...

[a] Partially soluble—according to weight loss on a dry basis, after 2 hours in gastric or intestinal media, 10 to 50% of the polymer dissolved. [b] Soluble—50% or more of the polymer dissolved (dry weight basis). [c] Slightly soluble—less than 10% of the polymer dissolved (dry weight basis) after 2 hours. [d] No visible solubility, but the film fell apart on handling.

TABLE IV.—FILM PERMEABILITY AND DRUG BINDING AT DECREASING HYDROGEN ION CONCENTRATIONS

Time, hr.	Permeable, %	Bound, %	pH
	PVM/MA ISOBUTYL HALF ESTER		
0.5	0.00	0.00	1.2
1.5	0.00	0.00	1.2
2.5	0.00	0.00	2.4
4.5	0.00	0.00	6.8
6.0	Dissolved	10.8[a]	7.6
		20.5[b]	
	PVM/MA *n*-BUTYL HALF ESTER		
0.5	0.00	0.00	1.2
1.5	0.00	0.00	1.2
2.5	0.00	0.00	2.4
4.5	0.00	0.00	6.8
6.0	Dissolved	0.00[a]	7.6
		6.6[b]	
	ACRYLATE/METHACRYLATE COPOLYMER		
0.5	0.00	0.00	1.2
1.5	0.00	0.00	1.2
2.5	0.00	0.00	2.4
4.5	0.00	0.00	6.8
6.0	0.00	0.00	7.6

[a] Per cent of drug binding as indicated by equilibrium dialysis. [b] Per cent of drug binding based on filterable drug in solution after dissolution of the polymer film in the permeability study.

sorbitan monooleate and 30% w/w of diethylphthalate, based on polymer weight. This system necessitated a change in the solvent system, from the alcohol-acetone solvent previously used for the polymers alone, to a system of equal parts of acetone and ethyl acetate. The alcoholic solvent system did not adequately carry the phthalate plasticizer, which came out of solution before the polymer to form an oily or greasy film. The acetone-ethyl acetate solvent system containing 5% PVM/MA butyl or ethylhexyl ester, 3% sorbitan monooleate and 1.5% diethylphthalate, produced a series of films which were completely clear, very flexible, and only slightly greasy to nongreasy. The general solubility characteristics of the plasticized films were identical to the unplasticized films (Table III); insoluble to slightly soluble in gastric fluid and partially soluble in intestinal fluid.

The acrylic/methacrylic copolymer was plasticized with 40% w/w of sorbitan monooleate and was dissolved in a solvent system of 3 parts of acetone to 1 part of alcohol U.S.P.

Film Permeability to d-Amphetamine Sulfate.—Films ranging in dry thickness from 0.003 to 0.006 in. were cast from 5% solutions of the *n*-butyl and isobutyl half esters of PVM/MA and the acrylic/methacrylic copolymer in an acetone-ethanol (3:1) solvent system. One per cent by volume of sorbitan monooleate plasticizer was added to each solution. The results of this study (Table IV) indicated that all three films were impermeable to drug at all pH values studied.

Polymer-Drug Binding.—Drug binding to the polymers was determined by an equilibrium dialysis method which involved the dialysis of colloidal solutions or dispersions of polymer and drug across a semipermeable membrane that permitted the drug free passage while restricting the polymers. The concentration of drug which had permeated the membrane after 24 hours was compared to the theoretical concentration of drug which should have been present based on dialysis of the drug alone (about 8% of the *d*-amphetamine sulfate in the concentration studied was bound to the Nojax membrane). The per cent of bound drug was then calculated as

$$\% \text{ Bound} = \frac{\text{Theoretical drug concn. at equilibrium} - \text{drug concn. found}}{\text{Theoretical drug concn.}}.$$

According to this method only the isobutyl half ester bound the drug, and then at only one pH level and to an extent of only 10.8% (Table IV).

Drug binding was also calculated from analysis of the permeability data, by assaying total drug in solution in the cell after "dissolution" of the polymer film "membranes." According to this method 20.5% of drug was bound to the isobutyl half ester and 6.6% of drug to the *n*-butyl half ester colloidal polymer dispersion (Table IV).

At this point in the study, of the three polymers remaining, the *n*-butyl half ester of PVM/MA appeared to be the most promising. Its solubility characteristics were superior to the acrylic/methacrylic copolymer and were equal to the isobutyl half ester, while the *n*-butyl ester produced less drug binding than the isobutyl derivative. The acrylic/methacrylic copolymer, due to its extremely high molecular weight, produced solutions which were too viscous for spray application in concentrations above about 1.5%.

Short-Term Feeding Study of PVM/MA n-Butyl Half Ester.—The results of the feeding study are shown in Table V. From a comparison of the mean dry liver weight for each group of animals (variation in average group weights was less than 10 mg.), it can be concluded that the polymer did not accumulate in the liver. No gross physical changes were observed in the animals which were fed polymer.

SUMMARY

Fourteen acrylic and vinyl polymers and derivatives were systematically studied for their potential as sustained-action coatings based on their film forming characteristics, film solubility, flexibility, permeability, and drug binding properties. With-

TABLE V.—RAT LIVER WEIGHTS AFTER SUB-ACUTE DOSING WITH *n*-BUTYL HALF ESTER OF PVM/MA

Final Rat Wt., Gm.	Wet Liver, Gm.	Gm. Wet Wt./100 Gm. Rat	Dry Liver, Gm.	Gm. Dry Wt./100 Gm. Rat
	CONTROLS[a]			
212	7.095	3.347	2.105	0.993
218	7.715	3.535	2.300	1.055
204	7.460	3.657	2.300	1.128
226	8.615	3.812	2.565	1.135
236	8.295	3.515	2.419	1.025
$\bar{X}$	7.836	3.573	2.338	1.067
	POLYMER FED ANIMALS			
232	9.115	3.929	2.666	1.149
224	8.175	3.650	2.487	1.110
209	7.100	3.397	2.078	0.994
223	7.285	3.267	2.106	0.944
210	8.150	3.889	2.415	1.150
210	7.765	3.697	2.315	1.102
$\bar{X}$	7.931	3.637	2.345	1.075

[a] One animal died.

out exception, the carboxylic acid polymers (acrylic and maleic acids), demonstrated the most potential as prolonged-action coatings based on solubility in simulated gastric and intestinal fluids. Solubility of a maleic acid polymer varied in aqueous media at a range of pH levels with the degree of esterification of the polymer and with the ester chain length. Butyl half esters of the maleic acid polymer demonstrated good potential solubility properties for a prolonged-action coating. The intact films studied were impermeable to drug in solution. Polymer-drug binding was significant with only one of the three final polymers studied. Poly(methyl vinyl ether)/maleic anhydride *n*-butyl half ester demonstrated the best *in vitro* potential as a sustained-action coating. The polymer showed no toxic effects in a preliminary short-term feeding study.

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One-Phase Solvent Systems for Paper Chromatography

Mixtures of *n*-Butanol, Acetic Acid, and Water

By T. J. BETTS

Whenever possible, one-phase solvent systems, made up of the minimum number of constituents, should be used for paper chromatography. Two-phase systems should be replaced by their equivalent one-phase mixtures giving stability, accuracy, and simplicity. A "phase diagram" of the type shown is required for all commonly used three-component paper chromatographic solvent mixtures to facilitate the design of one-phase solvents for particular problems. Mixtures of *n*-butanol, glacial acetic acid, and water were studied with this concept in mind.

SOLVENT MIXTURES used for paper chromatography may consist of one or two phases. The two-phase mixtures have been traditionally used for paper chromatography, and may themselves be divided into *direct phase* mixtures (1) in which the phase flowing over the paper is an organic substance such as *n*-butanol or phenol which has been saturated with a more polar liquid such as water, and *reversed phase* mixtures (2) in which the mobile phase is the more polar of the two. *Zaffaroni type* paper chromatography is an example of the direct phase method in which the paper is impregnated with a relatively nonvolatile stationary phase such as formamide, the mobile phase being a less polar liquid such as benzene (3). The paper may also be impregnated for reversed-phase chromatography, being made hydrophobic with a grease.

With the Zaffaroni method, the results obtained obviously depend greatly on partition effects between the two phases. The term "partition" has been almost automatically associated with paper chromatography since the original paper of Consden *et al.* in 1944 (4), but some workers consider that in some cases other phenomena are involved such as adsorption (5,6) or ion exchange (7,8). Considering, for example, that tannins can be resolved with pure water as solvent (9), or hydroxyanthraquinones with pure toluene (10), the mechanism of partition is unlikely to be an explanation for all separations obtained by paper chromatography, even allowing for the formation of cellulose-water complexes (11).

The preoccupation with partition, however, has resulted in the use of many two-phase solvent systems; the conception apparently was that this would insure that partition takes place. The use of a two-phase system means that the composition of the mobile phase is not known, so that it cannot be varied in a controlled manner. In 1960, Smith (12) advocated the use of "monophasic" solvents in place of two-phase mixtures because "emulsions sometimes formed (during their preparation) and . . . a drop in

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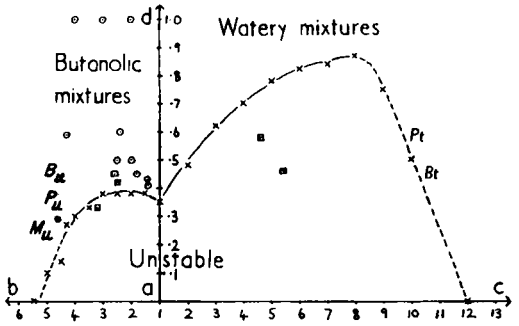


Fig. 1.—Quantities of glacial acetic acid required to just form one phase with various mixtures of *n*-butanol and water. Key: *a–b*, volume of *n*-butanol mixed with unit volume of water; *a–c*, volume of water mixed with unit volume of *n*-butanol; *a–d*, volume of glacial acetic acid required to form one phase of the above mixtures, in terms of the unit volume, *n*-butanol or water (whichever least). Temperatures of mixtures, 19–23°. Points marked x were calculated from experimental results. Points marked ⊙ represent one-phase mixtures in the literature. Points marked ⊡ were obtained from analyses, and represent the composition of the upper (P_u) and lower (P_l) phases of the Partridge mixture, the upper (B_u) and lower (B_l) phases of the Baker mixture, and the upper phase of the Munier mixture (M_u).

temperature during the overnight run sometimes led to the separation of water on the paper and resulted in unsatisfactory separations." This paper offers further support to his plea for simplicity. Although one-phase systems have been used since 1948 (13), the literature abounds with two-phase systems, those consisting of *n*-butanol, glacial acetic acid, and water being probably the most frequently used of all. Examination of the literature revealed 15 apparently different two-phase mixtures of this type, and 14 one-phase mixtures. The composition of some of the one-phase mixtures is shown in Fig. 1. This multiplicity led to the choice of mixtures of butanol, acetic acid, and water for study in relation to the concept of one-phase solvent systems.

EXPERIMENTAL

First, the minimum quantity of glacial acetic acid required to form one phase of various mixtures at approximately 21° of *n*-butanol and water was determined. From these results, the values used in plotting Fig. 1 were calculated. (Similarly shaped curves are obtained if ethanol or acetone is used instead of acetic acid.) Figure 1 indicates whether a particular mixture will form one phase. It might be anticipated that unstable mixtures which break down into two phases would form layers each of just stable composition. This hypothesis was tested by the analysis of the phases of typical two-phase mixtures. These were analyzed for their content of acetic acid and water, the butanol present being calculated. The acetic acid content of a phase was determined by titration of 5 or 10-ml. portions with normal aqueous alkali to phenolphthalein. The water content was determined by titration of 0.05 to 0.25-ml. portions (measured with a micrometer syringe) with Karl Fischer reagent.

Analysis of Partridge's Solvent Mixture

This well-known solvent mixture was first used by Partridge in 1948 to examine sugars (14) and has been used for many other substances since then. It is prepared by shaking together 40 ml. of *n*-butanol, 10 ml. of glacial acetic acid, and 50 ml. of water. The upper phase (of approximately 51 ml.) which separates on standing is usually used as the mobile phase. Analytical results:

Upper Phase.—63.5% butanol, 10.7% acetic acid, 25.8% water are contained. [Smith (12) analyzed this upper phase as butanol–acid–water = 62:12:26 at 20°.]

Lower Phase.—16.1% butanol, 9.4% acetic acid, 74.5% water are contained. Converting these analyses to proportions corresponding to Fig. 1 gives:

Upper Phase.—2.5 butanol:0.42 acetic acid:1 water.

Lower Phase.—1 butanol:0.58 acetic acid:4.6 water.

From Fig. 1 it is apparent that the upper phase should be a stable mixture as it contains acetic acid in slight excess of the amount required to form one phase from the proportions of butanol to water present. Surprisingly, the lower phase appears to be an unstable mixture containing insufficient acetic acid for stability.

Analysis of Baker's Solvent Mixture

This solvent mixture was first used by Baker *et al.* (15) to examine amines and has since been used for hydroxyanthraquinone aglycones (16) and glycosides (17). If it is prepared by shaking together 50 ml. of *n*-butanol, 10 ml. of glacial acetic acid, and 40 ml. of water, an upper phase of approximately 72 ml. separates on standing and is the mobile phase. Analysis gives:

Upper Phase.—63.8% butanol, 11.2% acetic acid, 25.0% water, or a ratio of 2.6 butanol:0.45 acetic acid:1 water. This stable mixture is very similar to the upper phase of the Partridge mixture, although it is prepared from different proportions of butanol and water. From the results for the upper phase, the following was calculated:

Lower Phase.—1 butanol:0.46 acetic acid:5.4 water. Like the lower phase of the Partridge mixture, a mixture of this composition should be unstable.

Analysis of Munier's Solvent Mixture

This represents solvent mixtures even more rich in butanol. It was used by Munier and Macheboeuf (18) to examine alkaloids, but is imprecisely defined. *n*-Butanol–glacial acetic acid (100:10) saturated with water may be prepared with the minimum quantity of water to give two phases, or with—for example—a quantity of water equal to that of the organic solvent mixture. For this work, 50 ml. of *n*-butanol with 5 ml. of glacial acetic acid required 17 ml. of water to form two phases. An upper phase of approximately 69 ml. separated on standing. Analysis results:

Upper Phase.—70.7% butanol, 7.3% acetic acid, 22.0% water, or a ratio of 3.2 butanol:0.33

TABLE I.—AVERAGE R_f VALUES (×100) OBTAINED WITH VARIOUS MIXTURES OF *n*-BUTANOL AND WATER MADE INTO ONE PHASE[a]

Solvent Mixture	Morphine	Thebaine	Quercetin[b]	Quercitrin	Aloe-emodin	Barbaloin	Leucine	Rhamnose
n-Butanol–glacial acetic acid–water								
(*1*) 1.5:0.4:1	59	78	70	79	90	80	66	45
(*2*) 2.25:0.4:1	53	74	68	77	89	76	61	36
(*3*) 2.5:0.4:1[c]	46	71	69	78	89	74	61	33
(*4*) 2.8:0.4:1	43	70	73	79	90	75	59	31
(*5*) 2.8:0.8:1	54	75	65	72	87	73	67	38
(*6*) 2.8:1.6:1	64	80	56	66	84	71	72	45
(*7*) 3.0:0.4:1	42	70	72	78	90	73	57	32
(*8*) 4.5:0.2:1	33	60	st[d]	st[d]	87	71	41	27
(*9*) 5.5:nil:1	st[d]	st[d]	st[d]	st[d]	85	67	28	22
n-Butanol–ethanol–water								
(*10*) 3.0:0.6:1	45	63	67	75	86	72	47	35
n-Butanol–acetone–water								
(*11*) 3.0:0.6:1	46	65	81	80	91	76	42	32

[a] These results were obtained at 16–20°, on Whatman No. 1 paper (unwashed), using the ascending technique overnight. The spots of morphine and thebaine were revealed by spraying with Dragendorff's reagent (Munier's modification). The rhamnose spots were revealed by aniline phthalate solution and the leucine spots by ninhydrin solution, with the subsequent application of hot air. Only solvent mixtures *5* and *6* contain acetic acid in excess of the amount required to form one phase. The other mixtures contain practically the minimum of acetic acid, or ethanol, or acetone to form one phase. [b] With *n*-butanol–water (3:1), 0.6 part of ethanol or acetone are required to form one phase, but only 0.4 part acetic acid. R_f values of quercetin are difficult to estimate, as the spots are prone to tailing. [c] This one-phase mixture represents the upper phase of the Partridge mixture. Using the two-phase Partridge mixture (with both phases present in the tank) the following R_f values were obtained: 45, 71, 68, 75, 89, 76, 61, 32 (substances in the same order as in Table). [d] Streaks obtained instead of spots.

acetic acid:1 water. Figure 1 suggests that this is hardly a stable mixture, and if kept apart from the lower phase, it does deposit globules.

Paper Chromatography with One-Phase Solvent Mixtures

The changes in R_f values of a selection of naturally occurring compounds (representative of those normally subjected to paper chromatography in these laboratories) in response to change in composition of solvent mixtures of *n*-butanol, glacial acetic acid, and water was studied. This could be done readily with one-phase mixtures. Results are given in Table I.

DISCUSSION

In addition to the points against the use of two-phase solvent mixtures for paper chromatography made by Smith (see above), the following emerge from the above results. The preparation of two-phase mixtures may result in the use of a mobile phase readily inclined to become unstable. The formation of phase apparently unstable on separation may be due to the separated phase providing a vapor phase for its new container, or just due to partial evaporation or slight temperature change. The visible breakdown of separated phases may be just due to the formation of butyl acetate. But whatever the cause, this potential instability, noticed under the conditions in which chromatography is performed, is something to be avoided. Also, change in the formula of a two-phase three-component mixture may cause no (or very little) change in the composition of the mobile phase. In addition, the preparation of the solvent is always easier and quicker than even nonemulsifying two-phase mixtures, possible variation in the composition of the mobile phase of a two-phase mixture due to temperature differences when it is prepared is prevented, and wastage of nonmobile phase is eliminated. The vapor of a one-phase mixture can fill the atmosphere of the chromatographic tank as well as the nonmobile phase of a two-phase mixture if it is placed in the tank for a suitable period before running the chromatogram.

Figure 1 represents the type of curve likely to be obtained with most three-component mixtures where two components are relatively immiscible, and the third is miscible with both the others. Such curves should be available for all commonly used three-component paper chromatographic solvents to facilitate selection and modification of solvent mixtures. Figure 1 reveals that the upper phase of the Partridge solvent mixture contains *n*-butanol and water in proportions representing one of their least miscible combinations, so that a relatively large amount of acetic acid is needed to form a stable mixture. In fact, this upper phase contains a slight excess of acetic acid for this purpose. These observations may be of value in designing other paper chromatographic solvent mixtures, a procedure which is rarely recorded by workers.

Table I indicates that the upper phase of the Partridge mixture can be replaced with complete success by a one-phase mixture of *n*-butanol–glacial acetic acid–water (63:11:26), or roughly (25:4:10), or still more approximately but satisfactorily (5:1:2). The lower phases of the Partridge and Baker mixtures present a problem not resolved here. The analysis results indicate that they have a composition which is thermodynamically impossible in that it is unstable, yet the lower phase of the Partridge mixture, separated from the upper phase, does not break down on chilling to minus 5° (unlike the upper phase). Yet it is impossible to prepare a one-phase mixture of the composition indicated from the analysis by mixing the con-

stituents in any manner. The tendency to form actually or theoretically unstable phases is a point against the use of two-phase mixtures, for they might break down during chromatography. Another point against their use is that superficially dissimilar two-phase systems (Partridge and Baker) use a mobile phase of practically identical composition. A change in formula may be meaningless.

The function of the acetic acid in mixtures with butanol and water has been implied to be the regulation of the amount of water that will dissolve in the butanolic phase and vice versa. But substances with basic or acidic character may be affected by the acetic acid. An acidic response could be the suppression of ionization so that ion exchange does not take place with the paper. A basic response could be the formation of the acetate, this salt being the substance actually chromatographed. From Table I (mixtures 7, 10, 11), as acetic acid can be replaced by a suitable quantity of ethanol or acetone without greatly affecting the R_f values of aloe-emodin, barbaloin, morphine, quercitrin, and rhamnose, these substances would appear to be unaffected by the acid. However, there are signs of some response by morphine and rhamnose and also leucine when the acetic acid is reduced or eliminated, as is possible with butanol-rich mixtures. In some cases this may be due to the high butanol content of the solvent mixture. But whatever the cause of these responses, the correct amount of ethanol or acetone may be substituted for acetic acid in some cases, thus avoiding the problem of esterification which otherwise occurs. Table I should be helpful in designing solvents for particular problems.

The results in Table I agree with the observation of Harasawa and Danjo in 1957 (19) that "generally effects of variation of composition (of solvent mixtures) are relatively small." With the mixtures examined, increase in the proportion of butanol to the other constituents tends to decrease R_f values. But increasing the proportion of acetic acid at the same time tends to offset this effect in some cases.

The ideal one-phase paper chromatographic solvent is a pure liquid such as water or toluene. Two-component one-phase mixtures may cover the many cases where this ideal is unrealizable. Only if these fail should three-component mixtures be used. It is an admission of unsuccessful application to invoke the use of mixtures with four or more components. The proportions of solvent mixtures should also be expressed as simply as possible. It is unlikely that the mixture of butanol–acetic acid–water "40:12:29" (20) will produce any result that 3:1:2 will not do. Chromatographers should remember that there is no virtue in complexity for its own sake.

SUMMARY

1. A "phase diagram" in modified form was obtained for mixtures of *n*-butanol and water, made into one phase by the addition of the minimum quantity of glacial acetic acid when necessary.

2. The phases of some well-known two-phase mixtures of *n*-butanol, glacial acetic acid, and water used as paper chromatographic solvents were separated and analyzed, and found to be apparently unstable in some cases, by reference to the "phase diagram."

3. Some naturally occurring compounds were paper chromatographed with various one-phase mixtures of *n*-butanol, glacial acetic acid, and water designed with reference to the "phase diagram," and changes in R_f values produced by changes in the solvent mixture studied.

4. The use of one-phase mixtures as solvents for paper chromatography whenever possible is recommended and comments are made on their design.

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Factors Influencing the Release of a Drug from a Prolonged-Action Matrix

By JACK LAZARUS, MIRIAM PAGLIERY, and LEON LACHMAN

The influence of drug particle size, drug concentration, and granule size on the *in vitro* release rate of the water-soluble drug tripelennamine from a compressed waxy granular matrix was studied. The rate of release is affected to a greater degree by drug concentration and crystal size than by the size of the matrix granules. At a given drug concentration, a faster release rate was observed as the crystal size increased.

THE DESIRED duration of continuous and uniform release from an oral sustained-action dosage form is generally about 8 to 12 hours. The various methods and processes which are employed to accomplish such prolonged release, and the *in vitro* test procedures used, have been the subject of many reports, patents, and reviews (1–8).

In the early development stages of these dosage forms, their *in vitro* release rates of the active substances serve as a guide for further formulation work. Although many of the studies reported on in the literature mention the factors which influence the release of the drug from the dosage form, there are few published investigations describing the effect of the different variables on the release rate, both *in vivo* and *in vitro*.

Sjogren and Fryklof (9) studied the effect of water solubility and concentration of a drug on its release rate from a tablet which was prepared by compressing a granulate containing the active substance and an insoluble plastic material. The plastic formed a coherent and porous matrix in which the active substance was dispersed. They found that the rate of release was directly dependent upon the solubility, as well as the concentration, of the drug. The release of sparingly soluble substances from the matrix was low, and was found to be increased by the addition of physiologically inert but readily soluble material such as sodium chloride, mannitol, urea, and polyethylene glycol 6000. A recent United States patent also recommends the use of water soluble substances to increase the release of a drug dispersed in a compressed plastic tablet (10). Simoons (11) also employed a soluble substance to aid the release of a small quantity of a sparingly soluble drug from a tablet matrix.

From the aforesaid references and information obtained in our laboratory, it seems necessary to have substances present in the matrix which are soluble in the test fluid in order to facilitate the release of the drug. However, in certain instances when the drug itself is soluble and is present in sufficient concentration, soluble additives are generally not required.

The present investigation was designed to determine the influence of the following three major factors on the *in vitro* release of a freely soluble drug: (*a*) particle size of the drug, (*b*) granule size prior to compression, and (*c*) concentration of active substances.

EXPERIMENTAL

Sizing of Material.—The drug substance employed was tripelennamine HCl U.S.P. having a solubility of more than 1 Gm. in 1 ml. of water.

The crystals were sized on 8-inch diameter U. S. sieve series screens set on a Rotap testing sieve shaker and shaken until two successive weighings did not show a variation of more than 0.2% between weighings.

The crystals taken for study were designated as 40-, 80-, and 120-mesh size since they were retained on these screens. The 40-mesh material passed through a 30-mesh screen, the 80-mesh through a 70-mesh screen, and the 120 mesh through a 100-mesh screen.



Fig. 1.—Various fractions of tripelennamine HCl. The linear distance between each white scale line is 100 microns.

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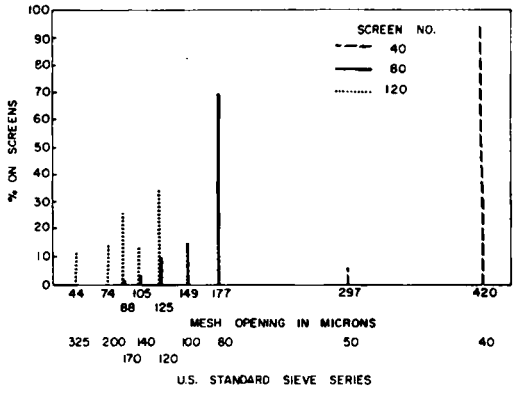


Fig. 2.—Particle size distribution of screened tripelennamine HCl.

A small sample of the sieved crystals was further analyzed on 3-inch diam. stainless steel screens on the vibratory shaker. Photomicrographs of the different crystal fractions were taken to demonstrate the magnitude of the range of particle sizes (Fig. 1). The linear distance between each white scale line is 100 microns. The differences in size should be of value in establishing a mental picture of the pore sizes created in the matrix when the crystals dissolve. Examination of the crystals obtained from each fraction revealed that many fine fragments of crystalline matter clung to the larger particles and further shaking of the screens on the Rotap failed to completely remove these crystals. The data presented in Fig. 2 shows the size distribution of the above crystal fractions.

Core Material.—Equal parts of hydrogenated castor oil (castorwax) and stearyl alcohol were used as the matrix material, since it was considered best in terms of future studies to have a core that was essentially unaffected by pH changes. When stearic acid was used in place of stearyl alcohol, dissolution of the matrix material occurred in simulated intestinal fluid as illustrated in the photograph shown in Fig. 3. The term core is used instead of tablet because, in the commercial tripelennamine HCl prolonged action tablets,[1] it is the core of the compressed coated tablet which provides the prolonged release, while the initial dose is contained in the coating. The coating does not contain any waxy material. The same batch of raw materials was used throughout the tests performed in this study.

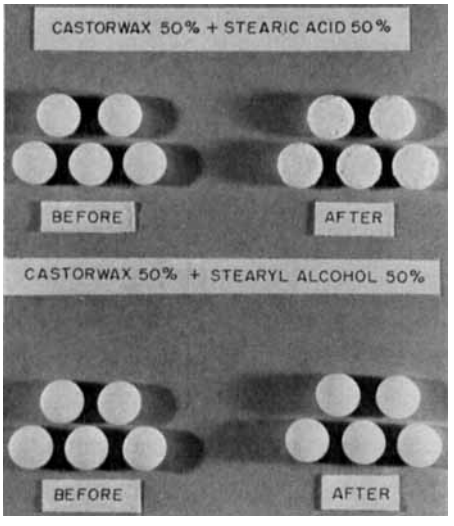


Fig. 3.—Effect on wax cores of 7-hour immersion in simulated intestinal fluid U.S.P. XVI.

The stearyl alcohol and castorwax were melted at 80–85°, and the screened tripelennamine HCl was dispersed in the melt. The liquid dispersion was solidified into flake form by congealing on a chilled stainless steel drum. The flakes were stored at 6° for 16 hours and then passed through a No. 12 mesh screen of the Fitzpatrick comminutor mill. One-third of the granules was mixed with 0.5% magnesium stearate and compressed. The other $^{2}/_{3}$ of the granulation was divided in half and passed through a No. 20 and No. 30-mesh screen on the Fitzpatrick Homoloid mill, respectively. After lubrication of these fractions with magnesium stearate, the granules were compressed. The 12-, 20-, and 30-mesh granules were each prepared to contain 15, 25, and 35% drug.



Fig. 4.—The Stoll-Gershberg apparatus modified to decrease moisture loss by evaporation.

The cores were compressed on a Stokes F machine, using flat-faced $^{12}/_{32}$-inch punches to a thickness of 4.5 to 4.6 mm. and a hardness of 4 to 5 as measured on the Strong-Cobb hardness tester.

For this investigation, the core thickness and hardness were maintained constant while the weight varied somewhat. The surface area was the same for all the tablets.

In Vitro Release Rate.—The release rate of the tablets was determined in a modified U.S.P. tablet disintegration apparatus consisting of baskets fitted with a 30-mesh screen, a glass immersion tube with a coarse fritted glass filter for removing samples of the liquid and a snug-fitting plastic cover to prevent moisture loss as illustrated by Fig. 4.

Purified water (600 ml.) was used as the eluting liquid, since preliminary studies in our laboratories indicated that the release of tripelennamine from a similar core material was the same whether the eluant was water or simulated gastrointestinal fluids. Six cores were used in each test. The disintegration apparatus was maintained at 37 ± 1° in a constant temperature water bath. The test

[1] Marketed as Lontabs by Ciba Pharmaceutical Co.

fluid was sampled at prescribed time intervals over an 8-hour period. Four milliliters of solution was removed at each sampling time and replaced with 4 ml. of purified water. At the end of the tests, the amount of water remaining in the beaker was measured and was found, generally, to exhibit a 5% loss of water over the 8-hour period. Without the plastic covering, a 20–25% loss in water was experienced.

The amount of water lost by evaporation from the beaker was prorated on an hourly basis, and from the amount of eluant in the beaker at sampling time, the total amount of drug present in the solution was calculated. The tripelennamine content of the sample was determined by diluting 1 ml. of solution with methanol and measuring its absorbance in a Beckman model DU spectrophotometer at 246 mμ.

THEORETICAL CONSIDERATIONS

Factors which control the amount of drug released from the matrix are as follows: (*a*) solubility and dissolution rate of drug; (*b*) porosity of tablet, (*c*) hardness of tablet, (*d*) geometry of matrix, (*e*) granule size, (*f*) drug concentration, (*g*) particle size of drug, and (*h*) matrix substances.

Higuchi (12) has indicated that the Eq. 1 relationship holds when the release mechanism from a granular matrix occurs by diffusional movement through porous or intergranular openings.

$$Q = \sqrt{\frac{Dt}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 1)}$$

where Q = the amount of drug released per unit exposed area after time t, D = the diffusivity of the drug in the permeating fluid, τ = the tortuosity factor of the capillary system $\cong 3$, A = the amount of drug present in the matrix per unit volume, C_s = the solubility of the drug in the permeating fluid, and ϵ = the porosity of the matrix.

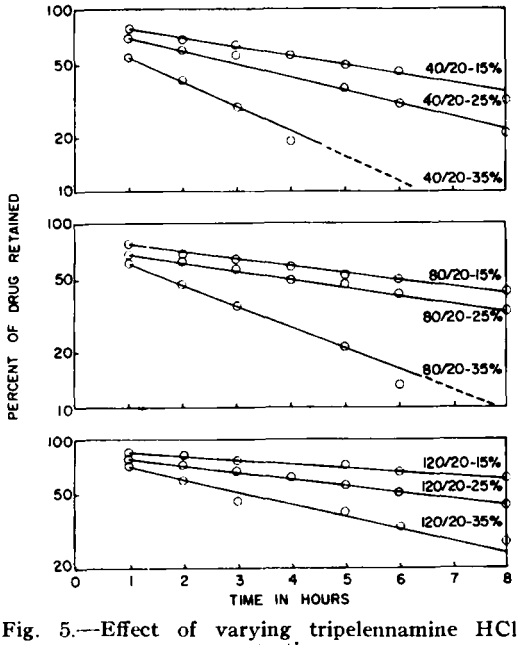


Fig. 5.—Effect of varying tripelennamine HCl concentration.

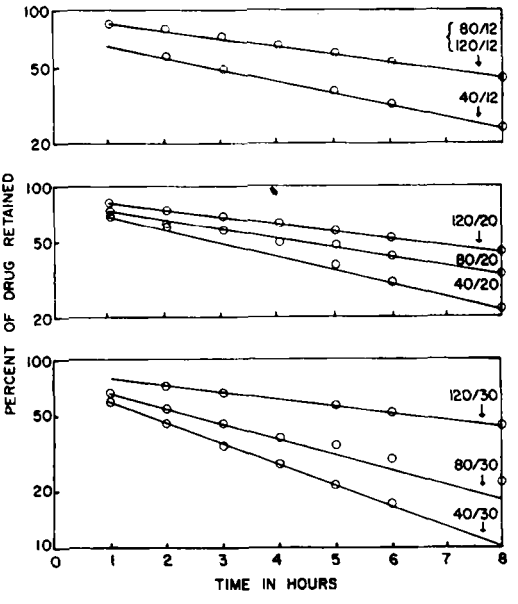


Fig. 6.—*In vitro* release rates of 25% tripelennamine HCl cores.

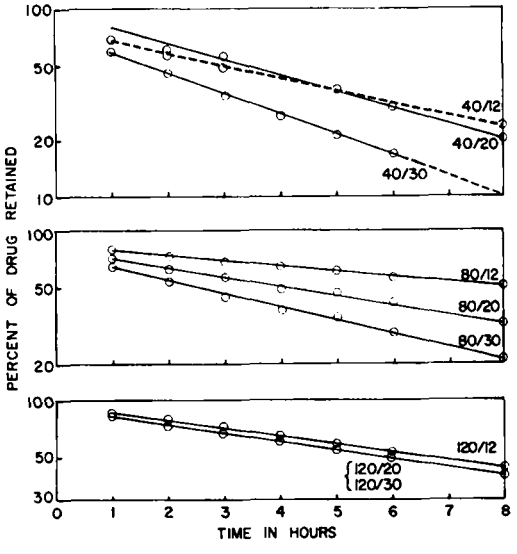


Fig. 7.—*In vitro* release rate of 25% tripelennamine HCl cores.

According to Eq. 1, a reduction of the release rate of a drug from a granular matrix can best be achieved by decreasing those factors which are proportional to the amount released, such as: (*a*) decreasing the solubility and the dissolution rate of the drug results in a decrease of C_s; (*b*) reducing the value of A will tend to decrease the release rate; (*c*) although hardness and porosity of a compressed tablet are related, in the present study, it was necessary to arrive at a predetermined hardness for the tablets since the granules containing the smaller size drug crystals yielded a softer tablet under the application of maximum pressure than the granules with the larger size crystals; and (*d*) the geometry of the tablets will affect the tortuosity factor which has an inverse influence on the release rate.

RESULTS AND DISCUSSION

The logarithm of the per cent drug retained in the cores was plotted against time in evaluating the influence of drug size, granule size, and drug concentration on the release of tripelennamine HCl. The straight lines obtained indicate that the *in vitro* release, for the system under consideration, follows an apparent first-order rate pattern. The *in vitro* release rates were based on the average release from six tablets.

The effect of varying the drug concentration while the granule and the crystal size were maintained constant can be seen from the representative plots in Fig. 5. In each case the release rate increased as the concentration increased. It can be seen from the graph that there was a greater rate increase as the concentration changed from 25 to 35% than for the corresponding increasing from 15 to 25%.

The graphs in Fig. 6 indicate that release obtained when the crystal size was varied while the granule size and concentration were maintained constant. Increasing the crystal size yielded faster release rates for each particular granule size.

When the drug concentration and crystal size are maintained constant, the release rate increases as the granules of the drug dispersion in the wax become smaller as illustrated in Fig. 7. This effect is expected since the grinding action to which the granules are subjected fractures the wax covering exposing more crystal surfaces. No significant effect on the release rate was noted when the size of the granules containing the 120-mesh crystals was varied.

The plots in Figs. 5–7 can be represented by $\log C = -kt/2.303 + \log C_o$, where the release rate is equal to the slope k and C the per cent residual drug concentration at time t.

Table I summarizes the release rate constants in hours^{-1} for the core formulations containing different crystal size, granule size, and drug concentration. The relationships between the three variables can be seen from the table. For the system studied, the drug size and concentration in the core are approximately equivalent in their influence on the rate of release and the granule size showing a lesser over-all effect.

The effect that a droplet of water has on the surface of a placebo wax core and a core containing the drug substance can be seen in Figs. 8–11. The wax cores are hydrophobic in nature, and a droplet of water on them assumes the characteristic shape. The core containing the tripelennamine HCl is readily wetted as seen by the difference in the contact angle of the water droplet to the core surface as illustrated in Fig. 8. The photomicrographs in Fig. 9 show the surface of a placebo core before and after treatment with one drop of water for 1 minute. The droplet was removed from the core by absorption onto filter paper and the core was then air dried. No surface changes were observed and the intergranular spaces or cracks do not appear to be altered.

TABLE I.—*In vitro* RELEASE RATES IN HOURS^{-1} OF TRIPELENNAMINE HCl CORES

Crystal Granule Size	Drug Concn. in Cores 15%	25%	35%
40/12	0.062	0.153	0.290
40/20	0.104	0.167	0.311
40/30	0.157	0.252	0.289
80/12	0.062	0.094	0.110
80/20	0.085	0.107	0.269
80/30	0.106	0.184	0.260
120/12	0.049	0.094	0.083
120/20	0.051	0.101	0.156
120/30	0.057	0.101	0.152

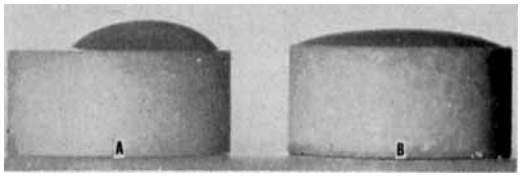


Fig. 8.—Wetting effect of a drop of water on (*A*) placebo compressed granular matrix and (*B*) a compressed granular matrix containing tripelennamine HCl.

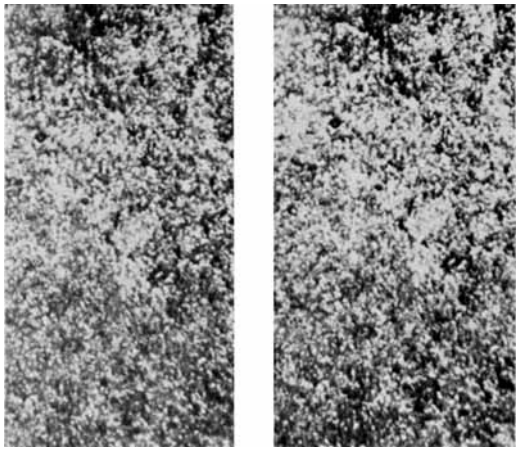


Fig. 9.—Left, surface of a placebo core before treatment with water; and right, same surface after treatment with one drop of water.

The surface appearance of the drug core is approximately the same as the placebo as evidenced by the photomicrograph shown in Fig. 10. Oblique lighting was used to illuminate the tablet and to emphasize the surface defects. When the tripelennamine HCl core is treated with water, porous areas and pitting develop on the surface due to the dissolution of the drug crystals as demonstrated in Fig. 10 (right side). It is obvious from the photomicrographs in Fig. 10 that the test fluid will more readily penetrate the core containing the drug than the placebo. Faster penetration would be due to dissolution of the drug at the exposed surface of the tablet causing pitting and permitting further penetration of fluid more readily. Penetration into the placebo core is by the more limited pathway of capillarity.

The observation of more rapid penetration of fluid into the core containing the drug than into the placebo was substantiated by the use of an indicator, an aqueous solution of sodium fluorescein. The cores were immersed into the solution and were removed periodically, then broken in half for examination under visual and ultraviolet light. With longer immersion the drug cores showed an increasing degree of penetration of the solution

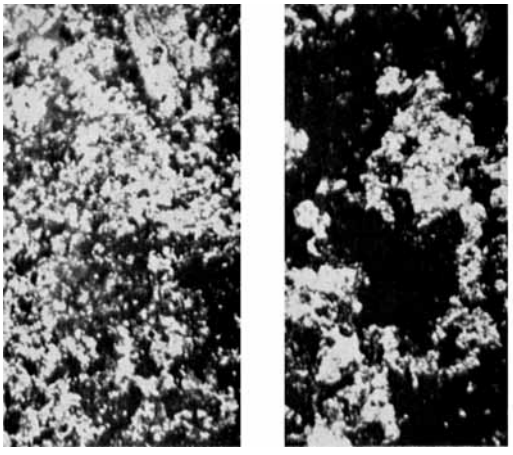


Fig. 10.—Left, surface of core containing tripelennamine HCl before treatment with water; and right, same surface after treatment with one drop of water.

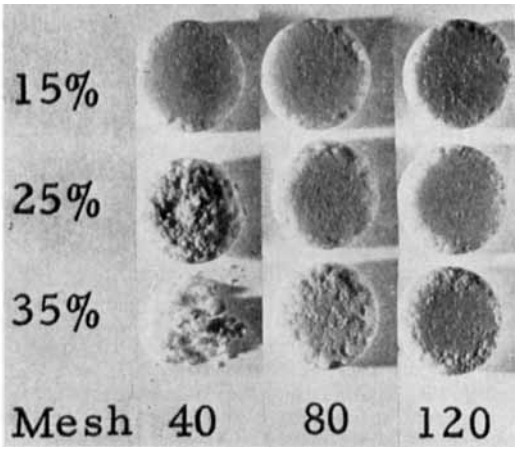


Fig. 11.—Tripelennamine HCl cores after immersion in water.

into the cores together with a gradual increase in the depth of color on the surface of the cores. The placebos, on the other hand, only showed a slight penetration of the solution into the matrix and an insignificant increase in surface color intensity with time.

A possible explanation for the release of a freely soluble drug from the compressed granular wax matrix may now be suggested. As the surface crystals dissolve on contact with the fluid, cavities develop which permit further attack by the fluid. The bonding strength of the granules of the matrix weakens as the pores increase in size and eventually the outer layer of granules slough off and exhibit an eroded appearance.

The effect of an 8-hour immersion in the bathing fluid on the cores is shown in Fig. 11. The matrix with the larger crystals (40 mesh) shows more disintegration than the smaller sized crystals. The small crystals, which are quantitatively more numerous at equivalent concentrations, create small cavities upon dissolution, which appear to be structurally less deleterious to the matrix. As the drug concentration is increased, the proportion of wax to drug decreases, resulting in less protection of the crystals against attack by the fluid and greater erosion occurs.

The data presented in this report represent our initial findings from the investigation that is presently under way to determine the critical physical chemical parameters needed for formulating prolonged action dosage forms with predictable release rates. From this information, it appears that the critical factors which affect the release rate of a drug in a compressed granular matrix of uniform physical properties are drug concentration, drug particle size, and granular size of the matrix.

SUMMARY

In the case studied, the crystal size and drug concentration exerted the greatest influence on the release rate, while the size of the granule as measured prior to compression plays a lesser role.

At the same concentration, the larger crystals gave a more rapid release.

Penetration of the fluid into the matrix appears to be primarily due to the dissolution on the surface of the matrix of water soluble material.

A modification of the *in vitro* U.S.P. tablet disintegration apparatus is described which has resulted in a reduction of the amount of fluid lost from the beaker by evaporation during the test run.

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Absorption and Retention of Fluoride from Ingested Stannous Fluoride Dentifrice

By SHARAD S. DESHPANDE and JOHN F. BESTER

Absorption, excretion, and skeletal retention of fluoride were studied in rats fed daily 1.0 mg. of fluoride from stannous fluoride and from a dentifrice containing stannous fluoride. Inhibiting effect of calcium ion accompanying ingested dentifrice upon absorption of fluoride from the intestinal tract was observed. Approximately 50.0 per cent of the ingested fluoride from stannous fluoride and 30.0 per cent of that from the dentifrice were absorbed. There was a steady increase in urinary excretion of fluoride as time progressed. The major portion of absorbed fluoride appeared to be retained in the skeleton. Femurs of rats receiving fluoride from stannous fluoride stored more fluoride than those receiving fluoride from dentifrice. The rats showed a diminishing ability to accumulate fluoride in their skeletons with increase in age.

THE DISCOVERY of the role of fluoride in the prevention of dental caries has initiated considerable research on this element and its metabolism. Stannous fluoride received its first attention when Muhler and Day (1) observed the superiority of this substance over sodium fluoride in protecting rats fed a cariogenic diet. Radike and Muhler (2) showed that a concentration of 10 p.p.m. of stannous fluoride fed to hamsters was roughly twice as effective in preventing caries as the same concentration of sodium fluoride.

Since these early observations there has accumulated incontrovertible evidence that fluoridation of drinking water decreases the incidence of caries. There has also been the introduction of a number of fluoride-containing dentifrices. Neither development has been without controversy; among the areas of dispute have been concern over the quantity of fluoride most desirable and its method of administration. As early as 1956, Hillenbrand (3), speaking for the American Dental Association, pointed to the evidence supporting fluoridation of drinking water as the most effective procedure. A summary of additional evidence was provided in a monograph by Elwell and Easlick (4).

The use of fluoride as an anticariogenic agent is primarily a long term procedure; hence, its possible deleterious effects upon particular patients must be considered. Hillenbrand (3), in referring to a fluoride-containing dentifrice, stated, "It is expected that nearly all of this fluoride will be discharged by rinsing the residual dentifrice from the mouth. Small children, however, might swallow an appreciable portion of the dentifrice, thus contributing to the possibility of dental fluorosis, especially where adequate or excessive levels of fluoride are present in the drinking water." It has been recognized for years that impairment of growth, mottled tooth enamel, and defective bone structure are the chief toxic effects of fluoride in greatly excessive quantities (5).

Very few studies have been made on the retention of fluoride following ingestion of stannous fluoride at high levels. In most of the feeding experiments in rats, sodium fluoride has been used (6–9). The data in all these studies confirm that growth of the skeleton has great influence upon the storage of fluoride in the bones, as young rats retain more fluoride than do old. A retarding effect on growth was observed by Muhler and Day (1) when fluoride was administered to rats at a concentration of 159 p.p.m. Weddle and Muhler (10) gave 2.0 mg. fluoride (as stannous fluoride) to rats by stomach tube daily, for 14 days, and found that 46.0% of the total ingested fluoride was stored in the skeleton. Mitchell (11) reported that from 95.6 to 96.2% of ingested fluoride could be stored in bone, and, in a later report (12) found that continuous feeding of fluoride resulted in a greater retention of the element in bone than did intermittent feeding of the same amount of fluoride. Zipkin (13) found that the concentration of fluoride in bone increased in an essentially linear fashion with increasing concentration of fluoride in drinking water up to 4.0 p.p.m. Largent (14) expressed the opinion that an approximate balance between the intake and output of fluoride is achieved following the establishment of an approximate equilibrium in which the tissues—primarily the skeleton—reach and maintain a certain level of concentration.

Along with skeletal storage, absorbability of fluoride from the gastrointestinal tract must be considered. Among various ions influencing the absorption of fluoride from the tract, calcium would appear to be the most important. An inhibiting effect upon absorption by calcium was observed by Muhler and Weddle (15), by Wagner

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and Muhler (16), and by Krylova and Gnoevaya (17). The form of inorganic salt accompanying ingested fluoride, its concentration, and its solubility, exert an effect upon absorption of fluoride and upon its storage in the skeleton (18).

All the previous studies of absorption and retention of fluoride from stannous fluoride were conducted on animals receiving the chemical in relatively low concentrations. Because of the possibility of ingestion of dentifrice indicated by Hillenbrand (3) and the apparent lack of knowledge of absorption and retention of fluoride from this source, this study was undertaken. Uptake, excretion, and skeletal retention of fluoride from dentifrice were compared to that of fluoride from stannous fluoride and comparisons made with control rats on a normal diet.

EXPERIMENTAL

A total of 90 rats, Wistar strain, aged 8 weeks was used. The deviation in weight was not more than 5.0 Gm. from the mean. The animals were divided into three groups. Each group contained an equal number of males and females. They were housed in steel cages in groups of five. Feeding cones were used, permitting the animals free access to food. A dentifrice containing stannous fluoride was chosen for the experimental study. As the dentifrice was insoluble in water, it was decided to administer it in food instead of in drinking water. The special food was prepared as follows: 15 Gm. of dentifrice, containing 1.0 mg. fluoride per Gm. made into a thin suspension with distilled water, was evenly mixed with 225.0 Gm. of rat pellet ration. The feed was dried and reweighed to determine the exact amount of dentifrice incorporated. This served as a 3-day supply for each five rats, each rat receiving approximately 1.0 mg. fluoride per 15.0 Gm. of diet. After each 3-day period, any food remaining in the feeding cones was weighed and discarded. In order that the fluoride intake for each five rats remain constant, the amount of fluoride in fresh ration was then increased to compensate for the quantity that was not consumed from the preceding ration. This procedure allowed each rat to receive 1.0 mg. fluoride per day. For the group of rats receiving fluoride supplement in the form of stannous fluoride,[1] 61.9 mg. stannous fluoride (15.0 mg. fluoride) was accurately weighed and dissolved in 25.0 ml. distilled water. The solution was evenly sprayed over 225.0 Gm. of ration and dried. This also was a 3-day supply for five animals. The same procedure for correcting the fluoride intake after each 3-day period was employed. Both of the food supplies were freshly prepared each 3 days because of the instability of stannous fluoride in water. Drinking water for the animals was not restricted. Thus one group of animals, consisting of 15 males and 15 females, each received 1.0 mg. fluoride daily from dentifrice, and a second group of the same number of animals of each sex received a like amount of fluoride daily from stannous fluoride. A third similar group was fed the same diet without fluoride addition.

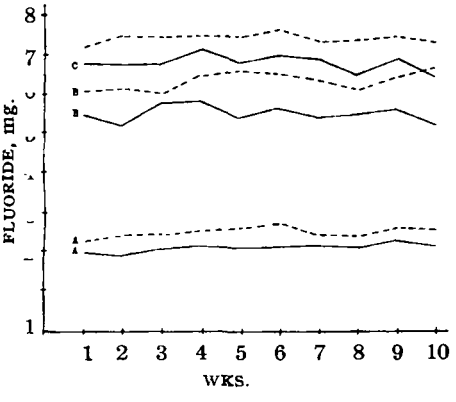


Fig. 1.—Twenty-four-hour fecal excretion of fluoride. (Control animals, *A;* animals receiving stannous fluoride, *B;* animals receiving dentifrice, *C;* solid line, males; broken line, females.)

TABLE I.—PER CENT ABSORPTION OF INGESTED FLUORIDE[a]

Wk.	Sex	Absorbed From—Dentifrice	Absorbed From—Stannous Fluoride
1	M	32.9	51.5
	F	30.0	44.5
2	M	31.5	47.2
	F	28.6	45.8
3	M	33.0	48.6
	F	25.8	45.6
4	M	29.0	48.6
	F	28.6	42.9
5	M	34.3	51.5
	F	30.0	42.8
6	M	30.0	47.3
	F	29.0	44.6
7	M	31.5	51.5
	F	31.5	42.9
8	M	33.0	48.6
	F	27.5	44.6
9	M	30.0	48.6
	F	27.5	41.6
10	M	33.0	51.5
	F	30.0	40.0
Mean	M	31.6 ± 0.55	49.5 ± 0.57
	F	28.9 ± 0.52	43.5 ± 0.58

[a] Total administered less that excreted in feces, expressed as percentage.

Analyses of the diets showed that untreated ration had an average fluoride content of 25.0 p.p.m.; that treated with dentifrice had 91.0 p.p.m.; that treated with stannous fluoride had 93.0 p.p.m.

A metabolism study, similar to that of Weddle and Muhler (19), was carried out on the animals weekly for 10 weeks. Two rats of each sex from each group were taken for each weekly study. Urine and feces, collected over 24 hours, were analyzed for fluoride content.

Skeletal deposition of fluoride was studied by determining fluoride content of the femurs of the rats. The femurs were chosen because they have been shown to have an adequate fluoride content (18). Two rats of each sex from each group were sacrificed every 2 weeks by a blow on the head. The femurs were removed from both legs, cleaned

[1] Obtained from City Chemical Corp., New York, N. Y.

TABLE II.—PER CENT RETENTION OF ABSORBED FLUORIDE[a]

Wk.	Sex	Absorbed from Dentifrice	Absorbed from Stannous Fluoride
1	M	82.9	81.0
	F	84.8	79.4
2	M	81.4	82.0
	F	83.5	79.7
3	M	82.0	79.7
	F	81.9	79.5
4	M	79.0	79.4
	F	82.5	78.0
5	M	82.0	80.5
	F	73.4	74.9
6	M	79.6	88.5
	F	83.4	78.8
7	M	80.0	80.2
	F	83.7	77.4
8	M	80.9	78.9
	F	80.6	78.1
9	M	77.7	78.9
	F	80.6	76.5
10	M	79.6	80.0
	F	81.1	75.4
Mean	M	80.5 ± 0.51	79.9 ± 0.97
	F	81.6 ± 1.00	77.8 ± 0.54

[a] Total absorbed less that excreted in urine, expressed as percentage.

of all extraneous tissue, weighed, and analyzed for fluoride content. The method used for distillation of the fluoride from the samples was that of Willard and Winter (20), with the acceptable modification (19) that calcium oxide was used as fluoride fixative in the preliminary ashing procedure instead of calcium hydroxide. Fluoride in the distillate was determined colorimetrically using a Bausch & Lomb spectronic 20 colorimeter and the method of Revinson and Harley (21). The method depends upon quantitative decolorization of thorium-chrome Azurol S dye complex by fluorine.

RESULTS AND DISCUSSION

The absorption of fluoride from ingested stannous fluoride or from dentifrice was found by comparing the urinary and fecal excretion of fluoride in the appropriate groups of rats. The total excretion of fluoride in feces by the two experimental groups and the control group is shown in Fig. 1. The fecal excretion of fluoride from each of the sources was relatively constant over the 10-week study period. However, the fact that fecal excretion of fluoride between the groups differed indicates a difference in amount and degree of absorption of fluoride from the two sources.

On the basis of knowledge of weekly excretion of fluoride in urine and feces of the two experimental groups, the percentage absorption of ingested fluoride (Table I) and the percentage retention of absorbed fluoride (Table II) were calculated. Table I shows that an average of 68.4% of the ingested fluoride from dentifrice was excreted in the feces of the male rats, while the females excreted 71.1%. This amount of fluoride in the feces may be considered as the unabsorbed part of that ingested. Statistical evaluation of the data in Table I shows that there is a significant difference in the amount of fluoride absorbed by male and female rats (t = 3.66). The same significant difference obtains in capacity to retain fluoride (Table II). In the present studies, the dentifrice fed to the rats contained 42.0% calcium orthophosphate. Each milligram, therefore, of ingested fluoride from the dentifrice was accompanied by 0.42 Gm. calcium orthophosphate, yielding approximately 0.1 Gm. of calcium ion. This level of calcium was probably responsible for the interference with the absorption of fluoride from the intestinal tract (15–17).

It is interesting to note from Table II that approximately 81.0% of the absorbed fluoride from the dentifrice and 78.0 to 80.0% of the absorbed fluoride from the solution of stannous fluoride were retained by the animals. Hence, in spite of lower absorption rates of fluoride from the dentifrice, a major portion of that which was absorbed must have been retained.

Numerous reports (14, 22, 23) on urinary fluoride excretion show that, at a given level of fluoride ingestion over a sufficient period of time, a steady state of equilibrium is established, so that urinary excretion of fluoride essentially equals absorption. The data in Table II show that such a state of equilibrium had not been reached after 10 weeks as fluoride excreted was still only a relatively small percentage of that absorbed. Figure 2 indicates a small, but steady, increase in urinary excretion of fluoride over the study period, indicating that growing rats have a diminishing ability to retain fluoride. This is in agreement with earlier reports (7, 9).

Fluoride balances in the two experimental groups at each weekly interval appear in Table III. High fluoride balance from ingestion of fluoride from solution of stannous fluoride, compared to that from dentifrice, is clearly evident. These positive

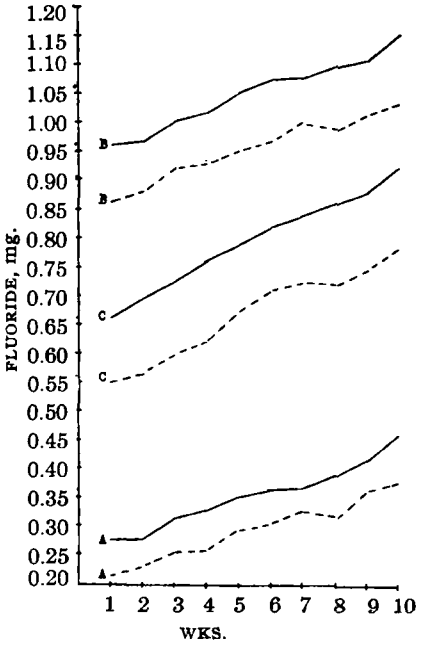


Fig. 2.—Twenty-four-hour urinary excretion of fluoride. (Control animals, *A;* animals receiving stannous fluoride, *B;* animals receiving dentifrice, *C;* solid line, males; broken line, females.)

TABLE III.—FLUORIDE BALANCE IN RATS[a]

Interval, Wk.	Fluoride from Stannous Fluoride Male	Fluoride from Stannous Fluoride Female	Fluoride from Dentifrice Male	Fluoride from Dentifrice Female
1	41.7	35.1	27.3	25.4
2	43.1	36.4	25.5	23.8
3	38.7	36.3	27.5	21.5
4	38.5	35.0	22.6	23.7
5	41.4	32.0	28.1	25.1
6	37.0	35.0	24.0	23.5
7	41.2	33.2	25.1	24.8
8	38.3	34.7	26.4	23.2
9	41.1	31.1	23.4	23.2
10	38.3	30.1	26.1	24.5
Mean	39.9 ± 0.64	34.0 ± 0.70	24.6 ± 0.67	23.8 ± 0.37

[a] All percentages obtained by the following formula:

$$\frac{\text{Amount of fluoride ingested per week} - \text{Amount of fluoride excreted in feces and urine}}{\text{Amount of fluoride ingested per week}} \times 100$$

fluoride balances in both groups of rats confirm previous reports (24, 25).

It is known that retention of absorbed fluoride occurs primarily in the skeleton (10, 11). The retention of this fluoride, represented by accumulation of the element in the femurs, is shown in Table IV and Fig. 3. The amount of fluoride retained in the femurs of the rats receiving a solution of fluoride was definitely at a higher level than that found in rats given fluoride in dentifrice. Although females from both experimental groups retained less fluoride than did males, when these absolute amounts of femur fluoride were converted to parts per million of ashed weight, it became apparent that females retain a higher proportionate amount of fluoride. This is in accord with the findings of Weddle and Muhler (19). It is also evident that the skeleton was still able to accumulate more fluoride, as there is no indication of the femurs having reached saturation levels at the end of 10 weeks

In conclusion, it would appear that the fluoride in stannous fluoride-containing dentifrices is appreciably absorbed from the gastrointestinal tract when the dentifrice is ingested. Accumulation of the element in bone can and does occur and that accumulation continues for weeks if the quantity ingested is sufficient.

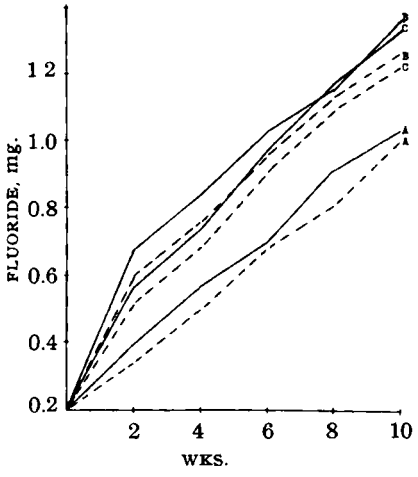


Fig. 3.—Amount of fluoride in both femurs. (Control animals, *A;* animals receiving stannous fluoride, *B;* animals receiving dentifrice, *C;* solid line, males; broken line, females.)

TABLE IV.—DEPOSITION OF FLUORIDE IN FEMURS OF RATS

Wk.	Group	Sex	Dry Wt. of Femurs, Gm.	Ashed Wt. of Femurs, Gm.	Fluoride Content, mg.	Fluoride, mg./Gm., Ashed Wt.
2	Control	M	0.482	0.243	0.190	0.79
		F	0.369	0.185	0.150	0.83
	Stannous fluoride	M	0.476	0.247	0.475	1.90
		F	0.354	0.184	0.400	2.20
	Dentifrice	M	0.481	0.243	0.375	1.54
		F	0.383	0.202	0.320	1.60
4	Control	M	0.719	0.368	0.370	1.02
		F	0.523	0.269	0.310	1.10
	Stannous fluoride	M	0.726	0.362	0.640	1.80
		F	0.510	0.250	0.560	2.20
	Dentifrice	M	0.792	0.363	0.550	1.50
		F	0.512	0.262	0.500	1.90
6	Control	M	0.849	0.437	0.500	1.10
		F	0.603	0.317	0.490	1.50
	Stannous fluoride	M	0.863	0.447	0.830	1.80
		F	0.634	0.326	0.760	2.30
	Dentifrice	M	0.852	0.430	0.770	1.70
		F	0.623	0.310	0.720	2.30
8	Control	M	0.977	0.494	0.710	1.40
		F	0.765	0.397	0.620	1.60
	Stannous fluoride	M	1.023	0.513	0.960	1.80
		F	0.776	0.397	0.940	2.40
	Dentifrice	M	0.952	0.482	0.970	2.02
		F	0.732	0.357	0.900	2.50
10	Control	M	1.438	0.720	0.920	1.29
		F	0.978	0.491	0.800	2.20
	Stannous fluoride	M	1.369	0.713	1.160	1.60
		F	0.935	0.480	1.060	2.20
	Dentifrice	M	1.423	0.708	1.140	1.50
		F	0.912	0.454	1.020	2.20

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EXPERIMENTAL

Antitussive Assays in Guinea Pigs

Method.—The method employed is a modification of that described by Winters and Flataker (2). Test animals (350 to 450 Gm. body weight) were individually placed for 1 minute in a 1-L. exposure chamber—a plastic cylinder—into which ammonia vapors were introduced. Vapors were delivered at 10 p.s.i. from an Ohio nebulizer, which contained 200 ml. of 0.5% ammonia water (Merck 28% stock) in the reservoir. The concentration of ammonia in the exposure chamber was relatively constant under the experimental conditions used.

Ammonia-induced coughs were recorded on a paper oscillograph (Grass model 5) *via* a pressure transducer (PT 5-A) which was connected to the exposure chamber and the oscillograph. The transducer was capable of recording respiratory rate as well as body movements and by proper manipulation of the sensitivity gain of the preamplifier, the gross body movement measurements could be made less apparent. Prior to the antitussive evaluation, the animals were screened to determine their susceptibility to ammonia vapors. Animals that coughed at a frequency of between 10–40 coughs per minute were selected for the drug study which was generally performed at least 2 hours afterwards. In any particular group, the test animals were selected in a manner such that their cough frequency response did not vary more than 10 per minute. This arbitrary selection helped to diminish wide variability within any test group. For the most part, the variations in mean cough frequency response among the test groups were within the range of 10 coughs per minute.

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TABLE I.—CONSTITUENTS OF AEROSOLIZED TERPENES (CONGESTAID)–FREON FORMULATION

Menthol	Dipropylene glycol
Thymol	Triethylene glycol
Camphor	Alcohol SDA-40
Eucalyptol	Propellant 11
Medicated odor	Propellant 12

Control animal data have therefore been accumulated for over 1000 animals. From this collection, it was established on the basis of a distribution pattern that the mean is skewed to the left and that there are two peaks for the frequency responses. In this study, the peak with the range of 10–40 coughs per minute was selected. The determination of statistical significance of the antitussive data is based upon the assumption that the cough frequency response of each group of animals may be treated as a binomial effect and as such analyzed by the method of Mainland and Murray (4).

Effects of Freon Propelled Formulation of Mixtures of Terpenes, Glycols, and Alcohol.—The antitussive effects of a freon-propelled formulation of terpenes, glycols, and alcohol, as shown in Table I, were evaluated in guinea pigs. The formulations were prepared to be delivered by a metered valve system. A 10-L. rectangular plastic exposure chamber was first sprayed either for 1 second or 10 seconds with the test formulation and then the animal was placed within for 3 minutes and then removed to be placed in the challenging chamber. The results are illustrated by the example in Fig. 1. The aerosolized terpenes formulation significantly reduced the induced ammonia coughs by 33 and 41% at the 60 to 120 minute intervals, respectively. Not only was there a reduction in cough paroxysmals but the general character of the respiratory pattern of the animals was improved (Fig. 1). Respiration was more even, deeper, and unrestrained.

Concentration in Exposure Chambers.—The effects of chamber concentration of aerosolized terpenes was determined by placing guinea pigs for 3 minutes in chambers of different volumes (see Table II) in which a 1-second spray (2 Gm. of total spray) was dispersed and evaluating the antitussive effects over a period of 4 hours. In the case of the maximum chamber volume, a 10-second spray (27 Gm. of total spray) was used.

The results as shown in Table II illustrate that the antitussive activity occurs immediately and generally persists for about 1 to 2 hours. The concentration effect of the different chamber volumes was noted in the duration of antitussive activity. In the chamber volume of 1 liter, the treated animals retained their protection for as much as 4 hours whereas those treated in the large volume chambers had a correspondingly shorter duration of protection.

Bronchospasms

Fifty guinea pigs were employed in this study and the bronchospasms were induced by either exposing the animals to aerosolized 0.5% histamine dihydrochloride or 1.0% acetylcholine hydrochloride in a 10-L. chamber. The respiratory responses of the animals were recorded as described for the antitussive study and in addition an observer with a stop watch also noted the onset time for bronchospasm to occur. The bronchospasm was witnessed by the observer as a spasm of the diaphragm which correlated with the oscillographic recording (Fig. 2).

In these studies it was technically impossible to use the same animal as its own control or for more than one test level evaluation. Therefore, separate groups of control nontreated animals were employed for each study. It was determined, that aerosolized histamine dihydrochloride produced bronchospasms in 45.2 seconds and 60% of the animals died and for the survivors their respirations were severely embarrassed. Animals exposed to a 10-second spray of aerosolized terpenes for 3 minutes in a 10-L. chamber were able to tolerate the histamine challenge; and the bronchospasm did not occur until 57 seconds of exposure (Fig. 2) and only 20% of the animals died with the survivors manifesting only mild to moderate respiratory distress.

Acetylcholine-induced bronchospasm occurred in control animals after 48 seconds and the animals were severely affected. Exposure to a 10-second spray of aerosolized terpenes did not alter or influence the acetylcholine-induced bronchospasm.

Antitussive Studies in Humans

Method.—The method used is an adaptation of that described by Bickerman (3). Essentially, it involves for each subject the determination of the supraminimal exposure time to citric acid aerosol

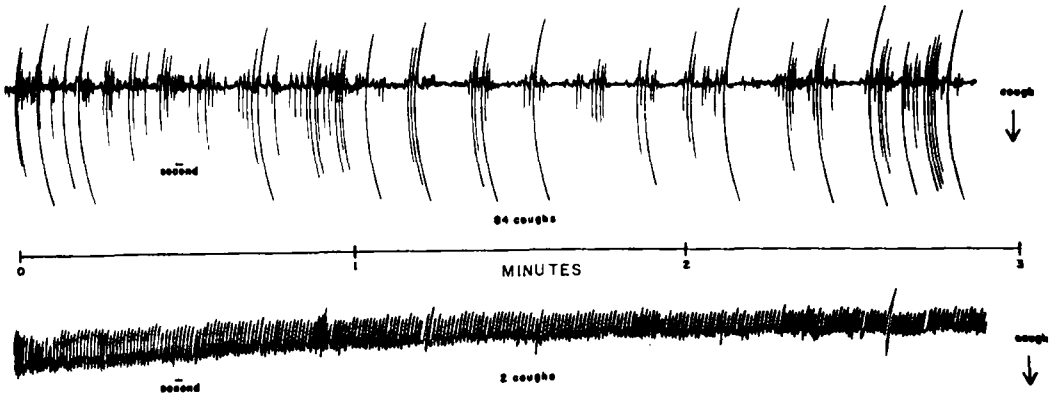


Fig. 1.—Oscillographic recording of respiratory rate and depth of guinea pigs exposed to ammonia vapors before treatment (top) and effects of aerosolized terpenes treatment on respiration of ammonia vapor-treated animals (bottom). The respiration is irregular and the depth shallow during ammonia vapor exposure and the animal exhibited cough paroxysmals. Following aerosolized terpenes treatment, this animal only exhibited one cough and the respiration was regular and deep as compared with 84 coughs prior to treatment.

TABLE II.—INFLUENCE OF EXPOSURE CHAMBER VOLUME ON THE ANTITUSSIVE EFFECTS OF AEROSOLIZED TERPENES (CONGESTAID) IN GUINEA PIGS TREATED WITH NEBULIZED AMMONIA VAPORS

Exposure Chamber Volume (liter)	N[a]	Per Cent Inhibition of Cough — Minutes after Treatment				
		0	30	60	120	240
1[b]	40	...	51	88	53	48
10[b]	50	47	76	73	22[d]	17[d]
235[b]	70	95	62	55	39	42
25,000[c]	20	44	28[d]	...	...	...

[a] N = Number of animals evaluated. [b] 1-second spray of aerosolized terpenes. [c] 10-second spray of aerosolized terpenes. [d] Nonsignificant effect; all other effects were significant at the 95% confidence level.

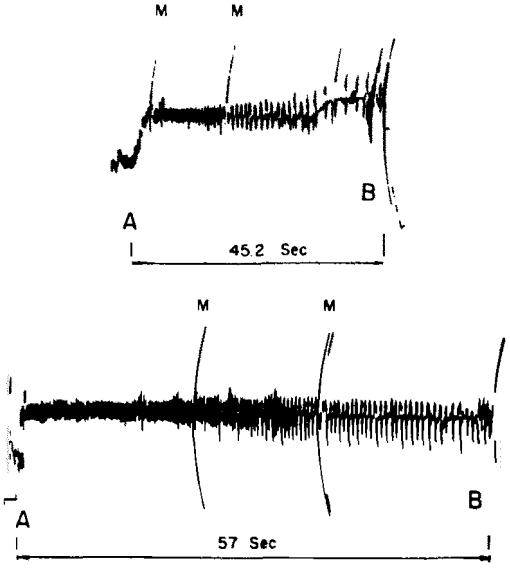


Fig. 2.—Oscillographic recording of histamine-induced bronchospasms in guinea pigs and the delaying effects of aerosolized terpenes toward the bronchospasms. The control record (top) shows at A the initiation of histamine exposure and at B the occurrence of bronchospasm after 45.2 seconds. Following aerosolized terpenes treatment (bottom), the respiration was more regular and the occurrence of bronchospasm after 57 seconds was delayed. Bronchospasm was always characterized by a reduction in the inspiratory volume followed by an explosive expiration. This feature contrasts bronchospasm activity (B) from gross body movements (M).

(25% aqueous solution) for the production of a cough response. The exposure time is regulated by a solenoid valve (Hoke valve) which can be manually activated through a timer (Eagle signal, 1–5 seconds) so that the volume of irritant delivered is fixed and reaches the subject at the onset of inspiration. The amount of aerosolized citric acid delivered for each exposure time employed has been determined and this relationship is curvilinear as illustrated in Fig. 3.

In this study, a panel of 12 male college students (21–25 years) was selected. They represented a group of trained subjects who had been used for at least 15 other determinations and were capable of reliably distinguishing between the antitussive effects of placebo and codeine phosphate (15 and 30 mg. *per os*). The subjects were seated in a comfortable position and a face mask (Willson mono mask) was securely fastened over the nose and mouth. During the pretreatment test periods, each subject was exposed to various amounts of citric acid until the supraminimal threshold amount was determined. Once the supraminimal threshold, as expressed as exposure time, was established, the subject was then given five challenges at intervals of 30 seconds per period of testing. The cough responses were recorded oscillographically by means of a pressure transducer (PT5) and a Grass model 5 recorder.

After each period of citric acid challenging, the subject was asked to rinse his mouth with the tap water. A 10-second spray of aerosolized terpenes was applied to a 4 × 4-in. gauze pad and the subject inhaled deeply the vapors for 5 minutes. The antitussive effects of the aerosolized terpenes were then determined 30 and 60 minutes afterwards. The assay was repeated twice within 24 hours and the results are shown in Tables IIIa and IIIb.

It was observed that the best effects occurred 60 minutes after treatment although at 30 minutes about half of the subjects exhibited antitussive benefits. The most obvious changes were in the cough frequency and cough numbers. Cough frequency refers to the subject's response to a challenge of citric acid. For example, each subject was given five exposures of aerosolized citric acid per test intervals. If, after treatment with aerosolized terpenes, the subject responded with a coughing episode to only three challenges, his cough frequency response was reduced by 40 per cent. Cough numbers refers to the total coughs recorded for all five citric acid challenges. Thus if a subject responded twice to each challenge of citric acid he would exhibit 10 coughs per test; if after aerosolized terpenes the

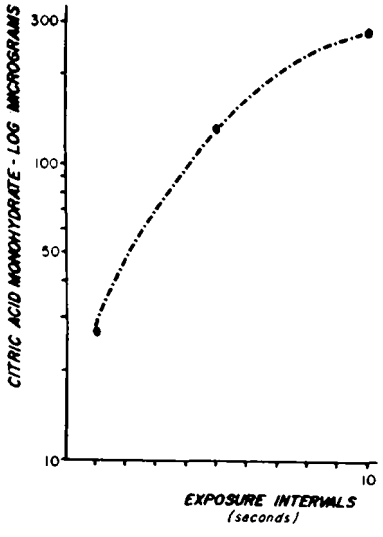


Fig. 3.—Relationship of delivered aerosolized citric acid and exposure intervals used in inducing coughs in humans.

TABLE IIIa.—FIRST TEST—MEAN PER CENT CHANGE FROM CONTROL[a]

Subject	No.	30 Minutes				60 Minutes			
		Frequency	Coughs	Velocity	Onset	Frequency	Coughs	Velocity	Onset
N. P.	1	0	10	0	0	0	−10	−8	8
J. H.	2	0	0	0	0	−20	0	0	0
W. A.	3	0	−30	−34	11	0	−20	−34	33
E. W.	4	−20	−58	0	+33	−20	−50	0	27
R. C.	5	0	17	0	−13	0	−17	0	−13
R. M.	6	0	0	0	0	0	0	0	0
S. S.	7	−20	−50	−23	−7	−40	−50	6	23
M. W.	8	−40	−75	13	23	−80	−90	13	62
J. K.	9	0	25	0	0	−20	−38	0	0
A. H.	10	−80	−33	−33	−27	−60	−67	−36	−13
B. K.	11	−20	−6	−6	9	−40	−13	5	14
P. E.	12	−80	−29	−29	−18	−80	−82	−29	−18

[a] A positive result indicates per cent increase over the control reading; a negative result indicates per cent decrease from the control reading. The above data were determined by taking the average of five challenges, one every 30 seconds, at each time interval.

TABLE IIIb.—SECOND TEST—PER CENT CHANGE FROM THE CONTROL

Subject	No.	30 Minutes				60 Minutes			
		Frequency	Coughs	Velocity	Onset	Frequency	Coughs	Velocity	Onset
N. P.	1	0	0	0	11	0	9	0	0
J. H.	2	0	17	0	0	20	−17	80	7
W. A.	3	0	−33	−20	0	0	−33	−20	0
E. W.	4	−20	−56	9	−25	−60	−67	0	−25
R. C.	5	20	0	67	0	0	−13	67	0
R. M.	6	0	0	0	0	0	25	0	7
S. S.	7	20	−34	−39	0	−40	−70	−22	0
M. W.	8	0	−39	0	0	0	−28	0	0
J. K.	9	0	25	0	0	−20	−37	0	0
A. H.	10	−20	−25	24	−13	−50	−50	−16	−6
B. K.	11	−20	−11	56	46	0	0	82	15
P. E.	12	−47	−56	−22	−41	−60	−63	−22	−41

total cough number was only five his cough was reduced by 50%. As shown in the Tables IIIa and IIIb, at the 60-minute period, the cough frequency was reduced in 8 out of 12 and 5 out of 12 subjects, not affected in 4 out of 12 and 6 out of 12 subjects, and in one instance increased. Similarly, it was found that the cough number was reduced in 10 out of 12 and in 9 out of 12 subjects. Therefore, aerosolized terpenes were capable of suppressing both the subject's cough response in terms of cough frequency and cough number.

The effects of aerosolized terpenes on cough velocity and onset time were not clearly established. In almost 50% of the subjects, the cough velocity was decreased whereas in several instances it was appreciably elevated in spite of obvious reductions in cough frequency and in cough numbers. The onset time to cough was not appreciably altered by aerosolized terpenes, although there was some delay.

DISCUSSION

The results of this investigation have demonstrated that the introduction of aerosolized terpenes, alcohol, and glycols in an atmosphere into which animals are exposed will afford a significant antitussive action capable of suppressing ammonia-induced coughs. Moreover, the dispersement of a combination of these materials in a freon-propelled system is equally antitussive and antihistaminic in animals exhibiting bronchospasm following exposure to histamine dihydrochloride aerosols. Similarly, it was found that inhalation of aerosolized terpenes vapors by human volunteers also reduced the frequency and severity of citric acid-induced coughs. These findings agree with those observed by Sadove (5) and Calesnick (6). By way of comparison, we have observed that the effects of aerosolized terpenes were greater than those obtained with 15 and 30 mg. *per os* of codeine phosphate.

It has been taught that the volatile oils were capable of stimulating the production of mucin secretion by the goblet cells in the epithelium of the bronchi and that this action produced a protective layer over the sensitive bronchial surfaces. However, in addition to this it may be deduced from our animal data that this is not the only action of the volatile oils. Since it was demonstrated (Fig. 1), that following an exposure to aerosolized terpenes, the respiratory rate and depth and ease of breathing were facilitated, it must be concluded that some other pharmacological effects are also elicited. Among the possibilities is that of producing a relaxation of the smooth muscle of the bronchi and bronchioles. Justification for this may be inferred by the demonstration of an antihistaminic action following the use of aerosolized terpenes. The data do not permit us to make any strong claim for assuming that there is generalized smooth muscle relaxation since aerosolized terpenes did not antagonize the effects of aerosolized acetylcholine hydrochloride. Nonetheless, it can be stated that following the aerosolized terpenes treatment, the flow of air in the respiratory passages is enhanced as is evident in the animal data and in the human data obtained by us and Sadove (5).

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Quantitative Photochemical Decomposition of Drugs I

Design and Calibration of an Instrument

By JAMES C. PRICE and GEORGE E. OSBORNE

An instrument for facilitating photochemical decomposition studies has been designed, constructed, and calibrated. The instrument supplies light of narrow bandwidth, which can be varied with respect to wavelength and to some extent with respect to intensity; the instrument provides for monitoring, recording, and integration of the incident and exit beams to and from the reaction mixture; it provides for keeping the reaction mixture at a constant and predetermined temperature and for stirring the mixture. Stoppered reaction cells exclude air when desirable. Short and long term stability of the instrument appear to be good, and response to light of any one wavelength with respect to intensity is practically linear.

THE PROBLEM of the decomposition of drugs as a result of the absorption of radiant energy in the form of light has become more acute as the number and complexity of pharmaceuticals have increased. The quantitative study of the influence of light upon drug stability is unfortunately difficult. Not only are many of the drug entities very complex, but also the presence of adjuncts in a finished, marketable, and acceptable preparation may tend to confound the study of such systems. Add to these problems the complicated task of measuring and controlling the light energy absorbed by the system and of separating the effects produced by the light from other unknown and uncontrollable factors, and it becomes little wonder that investigations in this area in the discipline of pharmacy have, until recently, been more or less qualitative. It is to the problem of the measurement and control of the quality and quantity of light that the efforts of this work have been primarily directed.

INSTRUMENT DESIGN

The design of an instrument for photochemical studies must include the following considerations.

The instrument should supply radiant energy of proper wavelength range, of suitably narrow bandwidth, and of sufficient intensity to make decomposition studies practicable.

The instrument should measure the quantity of energy absorbed by the drug sample by accounting for (*a*) the intensity and quantity of the light energy entering the drug system and (*b*) for the intensity and quantity of energy leaving the system.

Ideally, the instrument should provide for concurrent and constant analysis of the effects of the radiant energy on the drug system.

The components of such an instrument then should include: (*a*) a light source, (*b*) a monochromator or other means of isolating a narrow portion of spectrum, (*c*) radiant energy detection devices and associated equipment for the measurement of light entering and leaving the sample, and (*d*) a means of keeping the reaction system in a constant and controlled environment.

When the components of this system are to be selected, it is helpful in some instances to postulate properties of ideal components, considering both the behavior of radiant energy and the requirements for the study of photochemical decomposition reactions. Available components of design which most closely approximate those of the ideal can then be selected within budgetary limits.

Design and Selection of Components

Light Source.—The ideal light source for photochemical studies should produce line spectra, the intensity of which could be varied at will by changing the input energy. The source should be stable, have a long life, be economical of space and energy requirements, be readily obtainable and inexpensive. The lamps which most nearly approach these requirements are the mercury vapor medium pressure arcs.

The lamp selected for the subject instrument was 250-watt mercury vapor Uviarc, manufactured by the General Electric Co. The lamp was rated for 1000 hours useful life, and it supplied useful mercury spectral lines at 254, 265, 281, 297, 303, 313, 365, 405, 436, 546, and 578 mμ wavelengths. The lamp was readily obtainable and had a low first cost. In

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addition, it could be operated from the regular alternating current lines through a current-limiting transformer. The addition of a variable transformer ahead of the current-limiting transformer made it possible to vary the output intensity of the lamp through a limited range.

The Monochromator.—Quantitative photochemical studies almost always require that the light used to irradiate a chemical substance or a drug be of narrow bandwidth, *i.e.*, as nearly monochromatic as possible. Ideally, one should be able to select any bandwidth for a given experiment, and the device used for limiting the bandwidth should not cause any light loss in the process.

Although filters can be used to isolate a narrow portion of spectrum, they do not offer the versatility and ease of preselection of wavelength that is possible with a prism or diffraction grating monochromator. For these reasons, a diffraction grating monochromator was designed as a part of the instrument. A diffraction grating was chosen as the dispersing element in the monochromator, rather than a prism, primarily because its linear spectral dispersion facilitated wavelength calibration; in addition, good quality replica gratings were available at a reasonable cost.

The grating selected was a Bausch & Lomb Certified-Precision replica, plane reflection type, with 52 × 52-mm. ruled area, and 1200 lines per millimeter. The grating was blazed for 3000 Å. wavelength.

The optical layout for the monochromator was based on the Czerny-Turner modification of the system described by Ebert, as reported by Fastie (1, 2). The Czerny-Turner modification differs from the Ebert system only in that two small, off-axis, spherical mirrors are used instead of one large one.

The optical arrangement of the parts of the monochromator are shown in Fig. 1, which shows also the reaction cell, the optical blank, the detectors, and the light path taken in the instrument. Light from the source is collimated by a large, first surface, spherical, concave mirror and is focused at the entrance slit with the aid of a small plane mirror. Light from the entrance slit is deflected by a second plane mirror to a spherical concave mirror which renders the beam parallel and sends it to the diffraction grating. The diffracted light is then focused by a second spherical concave mirror on the reaction cell exit slit. However, a portion of the light is focused on the optical blank exit slit with the quartz plate beam splitter. Light from the exit slits goes through the reaction cell and the optical blank, respectively; the photocell detectors monitor the light exiting from the reaction cell and the optical blank cell. The rectangular objects on either side of the silica cells are water jackets and holders for the

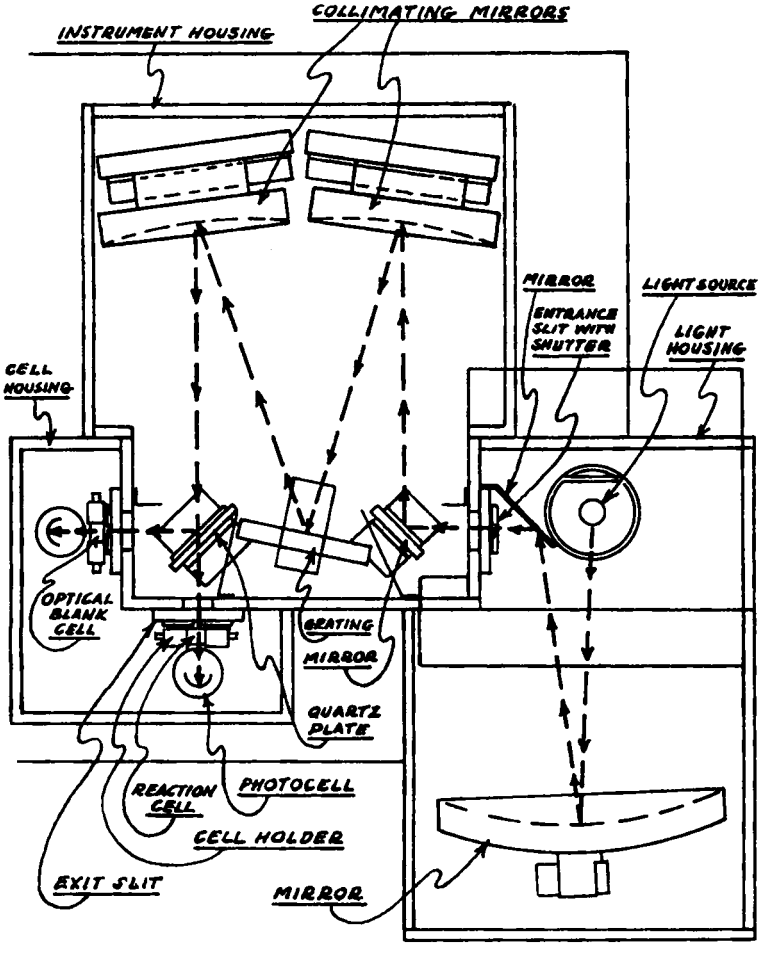


Fig. 1.—Light path diagram of the instrument showing the light source, the monochromator, the cells and cell holders, and the photocell light detectors.

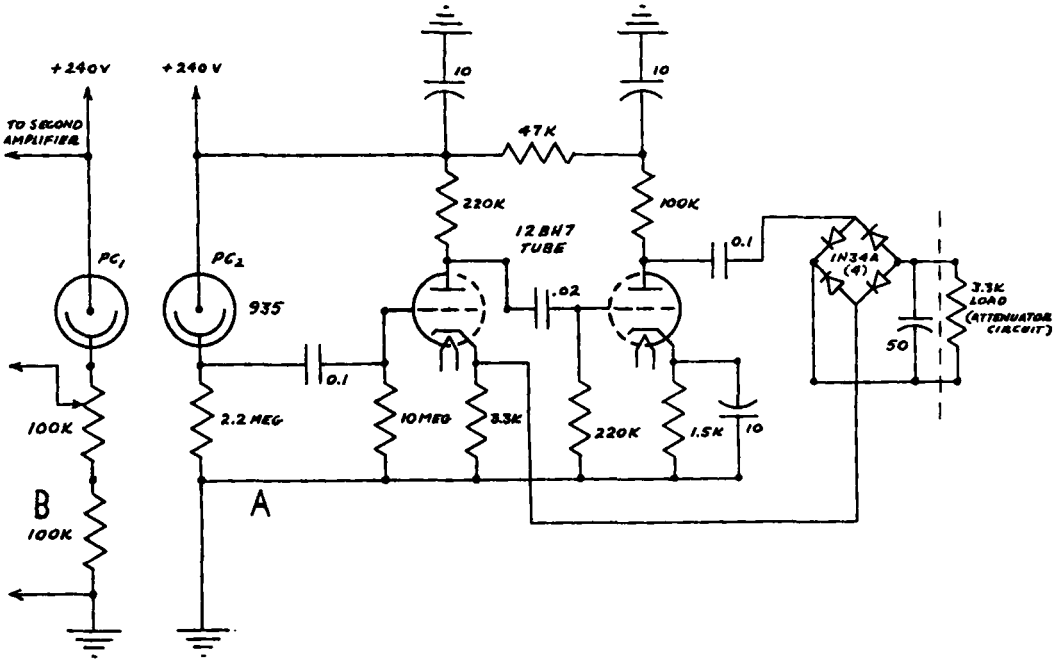


Fig. 2.—*A*, Photocell and photocell amplifier circuit for the optical blank channel. *B*, Photocell circuit for the reaction cell channel (amplifier circuit is identical to *A*). All resistance values are in ohms: k = 1000, meg = 1,000,000. Capacitance values are in microfarads.

cells. Not shown on the diagram is a small electric motor with a magnet attached to its shaft, which serves to stir the reaction mixture (*via* a small magnetic stirrer).

The collimating mirror for the light source is a concave mirror, 6.5 in. in diameter, 3.8-in. focal length; the two concave mirrors in the monochromator proper are identical 4.25-in. diameter, 9-in. focal length, aluminized mirrors. The quartz plate beam splitter is a 1-in. by 2-in. silica microscope slide. The slits are fixed at 2.1-mm. width and 18-mm. length. The diffraction grating, described previously, is rotated with a gearbox from a surplus type A-10 sextant. The degree indicator on the sextant gearbox serves as a means of selecting wavelength.

The reaction cells are ordinary, 1-cm. light path, silica spectrophotometer cells with Teflon stoppers. They are held in place against the slits by the water jacket assembly.

Electronic Components

Light Detectors.—The ideal detector should give a linear response with respect to intensity, should respond only to the spectral range of interest, and should show the same response for the same intensity for any wavelength within the range of interest. Thermopiles are classically the detectors of choice for photochemical studies (chiefly because of their linear response which does not change with wavelength); they have the disadvantages of low output, slow response, and sensitivity to temperature changes and heat radiation. In addition, a display of their output requires either very sensitive galvanometers or elaborate amplifiers. Vacuum type photocells, though having a varying response with wavelength, are insensitive to heat radiation and temperature changes; when a modulated signal is used, their output is easily amplified. Their response time is rapid, and for a given wavelength, their output is linear with respect to intensity. For these reasons, photocells were selected as detectors in this instrument. They were type 935 vacuum phototubes having an S-5 spectral response.

The output signals from the photocells were approximately electrically balanced by using different values of cathode resistors in each phototube. A voltage divider potentiometer in the cathode resistance of the photocell monitoring the light from the reaction cell permitted balancing the two signals exactly (Fig. 2).

Photocell Amplifiers.—The primary requirement of the amplifier is to give stable, linear amplification. Alternating current amplifiers with negative feedback to compensate for nonlinearity fulfill these requirements. Since the signal from the photocells was modulated at a 120 cycle rate (the light output from the mercury vapor lamp was modulated at this rate because of the alternating current operation), an alternating current amplifier could be used, and the difficulties of direct current amplification were avoided. The amplifier constructed for the photocells consisted of a dual-triode tube and a full-wave bridge rectifier to provide a rectified output signal. Negative feedback through the rectifier to the cathode of the first stage helped to correct for nonlinearity in the amplifier and the rectifiers. In the circuit shown in Fig. 2, a type 12BH7 tube gave very nearly linear response over the full range of output from the phototubes. A type 12AX7 tube gave slightly more linear response over a shorter range.

The rectified output from each photocell amplifier goes to an attenuator circuit, which allows the selection of one-half or full output to go to the recorder.

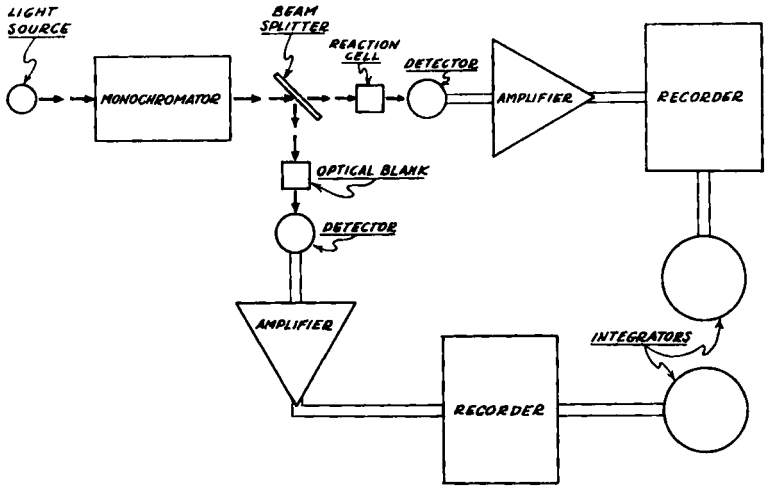


Fig. 3.—Block diagram showing the relationship of the components of the instrument.

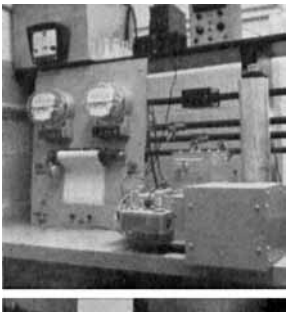
Recorder and Integrator Systems.—The recorder and integrator systems were based on a light integrator described by Miller (3). Two servo-motor-amplifier systems were used to record and integrate the signal from each photocell amplifier. A complete description of the recorder and integrator systems is included in the thesis (4) from which this report is derived.

Summary of Instrument Design

The relationship of the individual components in the instrument is shown in Fig. 3. Light from the Uviarc source is rendered monochromatic by the monochromator; the monochromatic beam is split; the main portion of the beam is directed to the reaction cell, and the other portion of the beam is directed to the optical blank. Output from each photocell detector is amplified, and the signal intensity is recorded. The signal is then integrated or summed over the time period of exposure of the reaction cell to light. Figure 4 (*A* and *B*) shows the instrument.

CALIBRATION OF THE INSTRUMENT

Monochromator Calibration.—The monochromator was calibrated with the mercury arc source, using known lines of the mercury spectrum as references. A plot of wavelength *versus* dial indication was linear.



A



B

Fig. 4.—*A*, Overall view. The recorder and integrators and their associated controls are on the left. The housing on the right contains the light source, monochromator, reaction and optical blank cells, and the photocell detectors. *B*, Top view of light source, monochromator, and cell housing assembly. Cell compartment is shown open.

Recorder Calibration.—The recorder was calibrated to determine its linearity by using known fractions of the voltage required to drive the recorder to full scale indication. The response was linear within the readability of the chart indication. This result was expected since the recorder linearity is primarily dependent upon the linearity of the balancing potentiometer, and this unit is specified by the manufacturer to be linear within 0.5%.

Integrator Linearity Calibration.—The integrator assemblies were calibrated for linearity in terms of recorder chart indication *versus* revolutions per minute of the integrator rotors. The results confirmed those of Miller (3) that the linearity and accuracy of the integrators were better than 1% of the full registration rate.

Calibration for Linearity of Over-All Response.—The over-all response linearity (excluding the integrators) was determined by utilizing Beer's relationship. Dilutions of dyes were made up, and the transmittance of several dilutions were determined for each channel of the instrument. This procedure was repeated for the most useful spectral lines of the mercury lamp from 254 mμ wavelength to 436 mμ wavelength. The results showed that the instrument was practically linear down to about 15% transmittance for all wavelengths tested. The responses of both channels were very closely parallel.

Calibration of Integrators in Terms of Quanta Per Revolution of Integrator Rotor.—This calibration was performed using the uranyl oxalate actinometer system first studied in detail by Leighton and Forbes (5). The quantum yields for this reaction have been carefully worked out by these and other workers (6, 7) for several wavelengths of the mercury spectrum.

The actinometer solution was exposed to a given wavelength of light for a sufficient length of time to give a titratable difference between the exposed and unexposed solutions; the number of molecules of oxalate decomposed was calculated; then from the known quantum yield of the reaction, the number of quanta absorbed by the solution was calculated. The number of integrator rotor revolutions of the channel monitoring the reaction cell exit was sub-

tracted from the number of rotations of the optical blank integrator, and the result was divided by the number of quanta absorbed by the solution to obtain the number of quanta per revolution of the integrator rotor.

Because of the difference in phototube response at different wavelengths, the calibration procedure was carried out for the most useful wavelengths of the mercury spectrum, *i.e.*, 254, 265, 281, 303, 313, 365, and 405 mμ.

STABILITY OF THE INSTRUMENT

Short term stability of the instrument has been observed to be excellent with no noticeable drift after a 30-minute warm-up period. Long term stability has been checked at one wavelength (254 mμ) using the potassium ferrioxalate chemical actinometer system described by Parker (8) and later tested by Baxendale (9); the calculated values for quanta per revolution of the integrator rotor were within 1% of the original calculated values.

SUMMARY AND CONCLUSIONS

An instrument for facilitating photochemical decomposition studies has been designed, constructed, and calibrated. The instrument supplies light of narrow bandwidth, which can be varied with respect to wavelength and to some extent with respect to intensity; the instrument provides for monitoring, recording, and integration of the incident and exit beams to and from the reaction mixture; it provides for keeping the reaction mixture at a constant and predetermined temperature and for stirring the mixture. Stoppered reaction cells exclude air when desirable. Short and long term stability of the instrument appear to be good, and response to light of any one wavelength with respect to intensity is practically linear.

Considering that the cost of the parts and materials to build the instrument was minimal, this instrument or a modification of it would be a useful tool for pharmacy laboratories doing photochemical decomposition studies.

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(3) Miller, O. E., *ibid.*, p. 661.
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Use of Models in Determining Chemical Pharmaceutical Stability

By LLOYD KENNON

The area of chemical kinetics as it has been applied to the prediction of the stability of drugs in pharmaceutical formulations has been quite extensively discussed and reviewed during the past few years. In spite of this, a method has been developed which will, it is hoped, be especially useful in pharmaceutical product development work as distinguished from academic research studies in which the goals are to determine reaction rates or orders or to elucidate reaction mechanisms. The present method is based upon the construction of reference reaction paths which result logically when one considers the usual values of pertinent heats of reaction and considers normal shelf-life goals. Two results which have emerged from consideration of these paths are rules of thumb having a sound theoretical basis and a naturally arising logical assay schedule. The schedule arises because of the temperatures chosen for sample storage (RT, 37°, 45°, 60°, and 85°) and would change if another set of storage conditions was employed.

MORE FREQUENT USE of chemical kinetics is now being made in the course of product development work in the pharmaceutical industry. The intrinsic value of such approaches is now considered to be significant—as the many academic and industrial publications in the scientific literature bear witness.

The pharmaceutical literature is replete with examples of stability data which are presented much in the same manner as product development laboratory reports. Many papers present tables which show stability assay results obtained after formulations were stored under the usual accelerated conditions or tables which indicate composition changes induced or prevented when modifications are made in the formulation under study. Although useful, such information must be considered qualitative or semiquantitative when compared to the results of more rigorous studies made since 1950 wherein parameters such as reaction rate constants and heats of activation were determined. It may be noted, however, that concepts such as kinetic studies and

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tracted from the number of rotations of the optical blank integrator, and the result was divided by the number of quanta absorbed by the solution to obtain the number of quanta per revolution of the integrator rotor.

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MORE FREQUENT USE of chemical kinetics is now being made in the course of product development work in the pharmaceutical industry. The intrinsic value of such approaches is now considered to be significant—as the many academic and industrial publications in the scientific literature bear witness.

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half-lives, as they relate to consumer products, were known and used before 1950 in other industries, especially in the food industry; as an example we may cite the two initial papers of Oswin (1), who studied the kinetics of package life. Also noteworthy is the fine, well-referenced review article concerning the prediction of drug and pharmaceutical formulation stability published by Garrett (2).

The use of kinetics as described in recent works is, however, not as straightforward as it might be; from the standpoint of the formulating pharmacist. The reason appears to be that most of the emphasis and effort have been placed on determining the exact kinetic paths followed by degrading drug compounds, rather than on correlating data to discover where the many roads led. However, as will be described, it is possible to correlate the data and construct certain kinetic paths which can be used for purposes of comparison during formulation development work. Such illustrative paths might be termed "reference decompositions" and serve then as models useful for making product stability evaluations.

It should be stressed that the method to be described here is aimed toward being especially useful in pharmaceutical development work as distinguished from academic research studies in which the goal is to determine precise reaction rates and reaction orders or to elucidate reaction mechanisms. Such studies require somewhat modified and more extensive experimental design. Studies of the latter type are still necessary and should be employed in cases where application of this shorter method indicates borderline or subminimal stability. The present approach, we also realize, may not be avidly embraced or endorsed by the purists; however, there is no intention to deprecate fundamental studies, but rather the intent is to use them to evaluate old empirical conclusions and methods and revise the latter so that intuition and empirically based relationships will be no longer necessary.

RATIONALE AND CONSTRUCTION OF THE REFERENCE REACTION PATH

Initially it may be appropriate to make some comments on heats of activation and on the role they play in the present method. Heats of activation are related to reaction rate constants (through the well-known Arrhenius equation) such that a rate constant's rate of change with temperature is affected by the heat of activation. Thus the course of degradation, that is, the amount of breakdown as a function of time and temperature, followed by a compound depends only on the value of the heat of activation—not on the order of the reaction or on any other factors. To make the rate of degradation of a reference decomposition proceed as *unfavorably* as possible, that is, to allow the formulation to be stable at the higher temperatures for the maximum possible periods of time, one need only assign a very low value to the heat of activation. It may be useful to amplify the concept of the unfavorable reaction just mentioned. By "unfavorable" it is meant that the reaction course (*i.e.*, the stability assay results) would tend to be misleading because the assay results obtained on samples stored at the *higher* temperatures would stay high for *longer* periods of time. Normally, a formulator wants weak formulations to manifest their weaknesses after *short* periods of storage at elevated temperatures.

To construct the unfavorable reference reaction path under discussion, a suitably low value for the heat of activation, such as 10 Kcal./mole, must be chosen. Almost no measurable reactions (and in pharmaceutics we deal with reactions that are much slower than the instantaneous and hence barely measurable) have activation energies this low. Most activation energies are in the 15–60 Kcal./mole range and are virtually sure to be found within

TABLE I.—HEATS OF ACTIVATION OF DEGRADATIVE REACTIONS OF SELECTED DRUG COMPOUNDS[a]

Compound	ΔHa	Reference
Actinospectacin	20	3
APAP	18	4
Ascorbic acid	16, 23, 23	5
Aspirin	13, 15, 18	6, 7
Atropine	13, 14, 25	8, 9
BAL	16, 24	10, 11
Barbital	11, 15, 16	12
Benzocaine	19	13
N-Butylformamide	20	14
Chloramphenicol	20, 20, 21, 21, 23, 24, 24, 35	15–18
Chlorobutanol	19	19
Dye—FD&C Blue No. 2	22, 23	20
Dye—FD&C Red No. 1	22, 47	20
Dyes—Mixture of FD&C Yellow No. 6 and D&C Red No. 33	20	21
Epinephrine	23	22
Filipin	9	23
Folic acid	17, 27	5
Fumagillin	9, 17	24
Glucose	31, 31, 32	25, 26
Homatropine	11, 12, 13	9, 27
Hydrocortisone	7, 17, 20	28, 29
Isoamyl nitrite	20	30
Methylphenidate	15, 25	31
Methylprednisolone	14	32
Morphine	23	33
Naphazoline	5, 16	34
2-PAM	17, 17, 29	35
Pantothenyl alcohol	20, 21	5, 36
Phenethicillin	18	37
Prednisolone	11	32
Procainamide	13, 13	13, 38
Procaine	12, 17	13, 39
Riboflavin	20	40
Salicyl alcohol	24	41
Streptozotocin	21, 21, 27	42
Thiamine	13, 21, 24, 25, 26, 26, 29	5, 36, 43
Vitamin A	15, 23	5
Vitamin B_{12}	23, 26	5, 36

[a] Note: The listing of two or more values for the heat of activation indicates that different degradative pathways may be possible, that the reaction is composed of multiple steps, that more than one determination was made, or that different salts or reaction conditions were employed in the kinetic study.

the 10–100 Kcal./mole range. Table I, in addition to serving as a set of references to many quantitative kinetic studies, lists the heats of activation of degradative reactions of a number of drug compounds reported since 1950; the average value found was 19.8 Kcal./mole. Of course, it might be noted that these heats of activation necessarily fall in a range which made study of the reactions convenient. Many drug compounds' degradation reactions must have heats of activation higher than those listed in the table.

In addition to what has been said about the 10 Kcal./mole value, it should be pointed out that, of course, values lower than this exist (as shown in Table I), but reactions with such low heats of activation take place so readily that generally stable, salable products cannot be formulated, at least not without heroic measures being taken. This point is illustrated by the work of Tingstad and Garrett (23). In a study of filipin degradation a heat of activation of 9 Kcal./mole was found. The compound's stability picture was this: crystalline filipin had a half-life of 43 hours in air. The authors hasten to add that their study may have been too severe, because filipin stored at 25° exhibited "only" a loss of 25% after one year in a closed amber bottle.

One last concept that should be discussed concerns the fact that a specious relationship is not being drawn between thermodynamics and kinetics. Situations are possible wherein thermodynamic characteristics mirror kinetic behavior. Meloche and Laidler (44) studied substituent effects on the acid and base hydrolyses of various benzamides (unsubstituted, nitro, chloro, two different methyls). These workers found that changes in the hydrolysis rates were influenced mainly by changes in activation energy such that the rates of both alkaline and acid hydrolysis decreased as the activation energies increased. Values of 22.0 to 27.1 Kcal./mole were found for the acid hydrolysis reactions, 16.1 to 23.1 Kcal./mole for the alkaline. Furthermore, it is possible in a sense to change the heat of activation of a degradative reaction by changing the vehicle so that a different mechanism of decomposition is brought about. The work of Stern *et al.* (34) illustrates this concept effectively. These authors point out that naphazoline in acidic or neutral solution is "relatively stable," in basic solution it is "readily prone to hydrolysis"; the heat of activation of the hydrolytic reaction in acid is 16.4 Kcal./mole, in base 4.9 Kcal./mole.

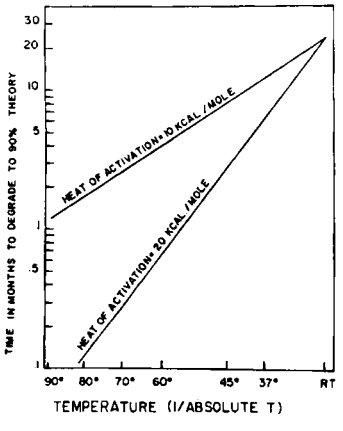


Fig. 1.—Two-year shelf-life goal reference decomposition.

TABLE II.—MAXIMUM AND MINIMUM TIMES AT WHICH POTENCY MUST BE AT LEAST 90% OF LABEL CLAIM WHEN STORED AT INDICATED TEMPERATURE TO OBTAIN A SHELF-LIFE OF TWO YEARS AT ROOM TEMPERATURE

	Maximum	Minimum
37°	12 months	6.4 months
45°	8.3 months	2.9 months
60°	4.1 months	3 weeks
85°	6 weeks	2.5 days

TABLE III.—ASSAY SCHEDULE

	Times at which samples are to be assayed, months							
Temp., ° C.	1	3	4	6	8	12	18	24
60	X	X	X					
45		X	X	X	X			
37				X	X	X		
RT					X	X	X	X

The remaining parameter which must be specified before the reference reaction path can be constructed is the degree of stability desired. General consideration of pharmaceutical industrial practice indicates that a reasonable standard is that potency should fall no more than 10% from its original or its labeled concentration (if an overage is used) in two years when the product is stored at room temperature.

Thus, standard kinetic equations were used to calculate the paths which reactions would follow if a 10% potency loss (or a drop to 90% of label claim) in two years at room temperature is permitted. By choosing activation energies of 10 and 20 Kcal./mole, both of which are conservatively low, and plotting the time in months which a formulation would take to drop to 90% potency *versus* $1/T$, the graph (Fig. 1) illustrated results. Table II presents essentially the same data as the graph.

USE OF THE GRAPH OR TABLE

From what has been stated so far and upon consideration of the picture presented by the graph and Table II, it can be seen that a logical course of action is presenting itself.

It is necessary only to see if the potency of a formulation is above 90% of its original or of its labeled concentration after storage at various temperatures for certain periods of time as compared to the model. If it is, the formulation is meeting the requirement of a two-year shelf-life, as far as kinetics will indicate, and apparently, formulation efforts were successful. Thus the formulation in question is assayed according to the schedule shown in Table III. If the assays are over 90% of label claim at the minimum times shown (indicated by the 20 Kcal./mole line on the graph) at the respective temperatures, the assays will be over 90% after two years at RT, in all probability; if the assays are still over 90% at the maximum times shown (indicated by the 10 Kcal./mole line on the graph), it is a certainty (kinetically speaking) that a potency of over 90% will be maintained after two years at RT. Naturally, values greater than 90% or values of 90% after longer times than those shown by the model indicate a shelf-life of more than two years. Studies at 85° are optional; this temperature may be too high for general use in stability prediction studies. Also, with regard to storage condi-

tions, the times and temperatures suggested in Tables II and III may be changed at will by choosing different points on the lines of the graph; *e.g.*, taking 70° as a storage temperature, the maximum and minimum times would be, respectively, 2.6 months and 9 days.

As implied, overages make no difference in the use of this method of evaluating stability characteristics. Overages are merely one means of assuring that potency will not be less than 90% of label claim after two years when greater stability cannot be built into the formulation in other ways. When using data from assays on formulas which contain overages, it is the per cent of *label claim* that should be used just as in the case of formulas in which there are no overages.

NOTE ON POSSIBLE MISUSE OF THE GRAPH

As stated previously, this method is not intended to help in the determination of exact kinetic paths. Thus if, *e.g.*, the following two points were obtained: 80°, 1 month, and 45°, 4 months (to drop to 90%, etc.), it would appear that a line drawn through these points would cross the 25° line at about 10 months to indicate an unsuitable product. *This is not a true picture.* No line *should* be drawn through these points because the only aim in using this method is to see if the two points are above the 20 Kcal./mole line, which they are. Incidentally, in this example, two such points (if valid) would indicate an activation energy of about 8.8 Kcal./mole—something which is ruled out for reasons previously discussed as being an unreasonable possibility. The seeming paradox would have to be considered as due to experimental errors either in assay, timing, or temperature or due to the presence of additional degradative reactions. Naturally, two or more points which form a line with a *greater* slope than either of the two lines shown on the graph would tend to indicate a higher activation energy and longer than two-year shelf-life and would cause no concern.

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By WILLIAM L. SCHALKER and MURIEL C. VINCENT

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The numerous disadvantages of the usual sugar coating have lead to recent investigations for newer materials and methods of coating. In

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the search for a substitute for the sucrose in tablet coating formulas, many natural and synthetic materials have been studied, and a number of them have been found to be satisfactory as film coating agents—polyethylene glycol 6000 in ethanol (1), polyethylene glycol 6000 and carboxymethylcellulose in hydroisopropanol (2), sodium carboxymethylcellulose and hydroxyethylcellulose applied over a shellac prime coat (3), zein in isopropanol (4), polyvinylpyrrolidone and acetylated monoglycerides (5), and polyethylene oxide water soluble resins (6).

EXPERIMENTAL

This investigation was concerned with the evaluation of dioctyl sodium sulfosuccinate (DSS)[1] as a film coating for sugar coated and noncoated compressed tablets with regard to methods of film application and the testing of several environmental effects. The tablets which were used in the experimental work are listed in Table I.

Coating.—The method of application of DSS coating solutions was similar to that for conventional sugar coating solutions; a model 29 Stokes polishing and coating machine equipped with a 10-in. copper coating pan was used.

Solutions containing between 15 and 25% DSS in alcohol produced relatively satisfactory results, although the film coat was somewhat soft and slightly tacky regardless of the thickness applied, drying time, or drying temperature. The surface tackiness became more pronounced with time, probably due to the slight hygroscopic nature of DSS.

This condition of coating softness was remedied by the addition of sodium benzoate to produce a comparatively hard surface. Propylene glycol was included in the formula to improve the glossy appearance and enhance the plasticity of the film coat.

A typical coating formula had the following percentage composition: DSS, 20; sodium benzoate, 2–15; propylene glycol, 0.5; alcohol 70% w/w, to make 100.

The best results were obtained by applying three to five coats at 15-minute intervals with sufficient of the coating solution to moisten the tablets completely. Cooled air for drying gave more desirable results than hot air since the tablet coating had a tendency to become uneven and flake off if dried too rapidly. Uneven coatings could be easily smoothed by the application of 70% w/w alcohol.

Application of Color.—The addition of varying concentrations of certified dyes (FD&C Blue No. 1, FD&C Red No. 3, FD&C Yellow No. 5) to the DSS coating solution failed to give uniformity of color to the tablets. However, if a dye concentration of 0.5% in diluted alcohol was included in the tablet granulation and the tablets coated in the usual manner with 0.25% dye added to the coating solution, satisfactorily colored tablets resulted.

Uniform coloring of tablets was also accomplished by the addition of 0.25 to 0.5% dye solution in diluted alcohol to the uncoated tablets just prior to applying a shellac prime coat or DSS coat. The normal coating process would follow, using 0.25% dye in the coating solution.

[1] Marketed as Aerosol OT by the American Cyanamid Co., Pearl River, N. Y.

TABLE I.—TABLETS AND COATINGS USED IN THE TESTS

Code	Tablet	Size and Type	Maker
1	Sodium chloride	10 gr., standard punch 3/8 in.	A
2	Placebo	250 mg., oval	B
3	Ferrous sulfate	5 gr., coated	C
4	Ferrous sulfate	5 gr., coated	D
5	Effervescent aspirin and sodium phosphate	7 1/2 gr., standard punch 3/8 in.	A
6	Methenamine	5 gr.	E
7	Lactose-starch	4 gr., standard punch 11/16 in.	A
8	Lactose-starch	7 gr., standard punch 3/8 in., colored	A
OT	...	DSS coated	..
OT-B	...	DSS 15% sodium benzoate	..
OT-W	...	DSS coated and polished	..
P-OT	...	Shellac prime coated and coated with DSS	..

All prime coats were composed of freshly prepared solutions of 20 Gm. white arsenic-free shellac to 40 ml. alcohol (7).

Taste.—A bitter aftertaste was noted with the DSS coatings. The addition of 2% saccharin sodium to the coating solution would almost completely mask this taste, although some aftertaste was noted from the saccharin itself. Sodium cyclamate in a concentration of 5% proved more desirable, and a mixture of 2.5% sodium cyclamate and 0.25% saccharin was equally successful. A polish coat would also eliminate most of the bitter taste.

Weight of Coating.—The DSS coating increased the weight of commercial sugar coated tablets approximately 5 to 12 mg. per tablet and of noncoated compressed tablets about 20 to 45 mg. per tablet. The surface texture and the difference of tablet size were the main factors influencing the variance in weight.

Polish Coat.—The polishing procedure was accomplished in a 20-in. galvanized iron, canvas-lined polish pan. The tablets were sprayed with a warm solution of a 2% wax mixture (eight parts white beeswax, five parts carnauba wax) in chloroform (8). This polish coat slightly improved the glossy appearance of the film coat, lessened the effect of humidity, and partially masked the taste of DSS.

Durability Test.—This test was designed to compare the effect of physical trauma on coated and noncoated tablets. Samples of 100 tablets of each type were placed in clear 4-oz. dry prescription squares, filling the containers from one-half to one-third their capacity. The bottles were shaken lengthwise through a total distance of about 3 in. 200 times per minute in a size 2 bottle shaker (International Equipment Co., Boston, Mass.).

The tablets were inspected at 15-minute intervals during a 2-hour shaking period and were screened after each time interval to remove any powder that resulted from tablet deterioration. At each inspection there was a progressively greater wear on the

TABLE II.—TABLET DURABILITY TEST

Tablet Formula	Time, Min. 15	30	45	60	75	90	105	120
1 S[g]	CB[d]	CB	D	D	D	D	D	D
OT	A[b]	BB[c]	BB	CB	CB	CB	CB	CB
P-OT	A	BB	BB	BB	CB	CB	CB	CB
OT-W	A	A	BB	BB	BB	BB	CB	CB
2 H[h]	O[a]	A	A	BB	BB	CB	CB	CB
OT	O	O	O	O	A	A	A	A
P-OT	O	O	O	O	O	O	A	A
OT-W	O	O	O	O	O	O	A	A
3 M[f]	A	BW	BW	BW	BW	CW	CW	CW
OT	O	O	A	A	A	A	A	BW
OT-W	O	O	A	A	A	A	A	A
4 M	A	A	BW	BW	CW	CW	CW	CW
OT	O	O	A	A	A	A	A	A
OT-W	O	O	O	O	O	O	A	A
5 S	CB	CB	D	D	D	D	D	D
OT	O	A	A	A	BW	CW	CW	D
P-OT	O	O	A	A	A	BW	BW	CW
OT-W	O	O	A	A	A	BW	CW	CW
6 S	CB	D[e]	D	D	D	D	D	D
OT	BW[c]	BW	CB	CB	CB	CB	CB	D
P-OT	BW	BW	BW	BW	BW	BW	CW	CB
OT-W	A	BW	CW	CW	CB	CB	CB	CB
7 H	BW	BW	CW[d]	CW	D	D	D	D
OT	O	A	A	A	A	A	A	BW
P-OT	O	O	A	A	A	A	A	A
OT-W	O	O	A	A	A	A	A	A

[a] O, No effect. [b] A, Very slight powdering or chipping of coat. [c] BW, BB, Wearing of edges or breaking of tablet. [d] CW, CB, Coating completely worn off or tablets in small chips and powdering. [e] D, Tablet completely disintegrated. [f] M, Manufacturer's coating. [g] S, Soft punched, as indicated by Monsanto hardness tester. [h] H, Hard punched, as indicated by Monsanto hardness tester.

nontreated tablets compared to that of the DSS coated tablets. The coated soft punched tablets were particularly susceptible to disintegration, which might be attributed to the fact that the coating did not provide enough protection against the prolonged shaking period.

Table II gives the complete results of these tests.

Shelf-Life Studies.—Samples of film coated tablets were stored in clear tightly stoppered 2-ounce dry prescription squares for 6 months at temperatures fluctuating between 15 and 37°. The tablets were exposed to indirect sunlight over this period, and the bottles were handled at intervals that would simulate drugstore treatment. Upon examination there were no visual changes to the tablet coatings. There was some apparent, though slight, adhesion of the tablets due to moisture entering the bottles at times of relatively high humidity. This condition was

TABLE III.—TABLET HUMIDITY TEST

Tablet Formula	Time, Hr. 1	3	6	9	24	36	48	60
1 S[g]	A[b]	A	A	A	B	B	C	C
OT	A	A	A	B	B	B	C	C
P-OT	A	A	A	B	B	B	B	B
OT-W	O[a]	A	A	A	B	B	B	B
2 H	O	A	A	A	B	B	B	B
OT	A	A	A	A	B	B	B	B
P-OT	A	A	A	A	A	B	B	B
OT-W	O	O	A	A	B	A	A	A
3 M[f]	O	A	A	B	B	B	C	D
OT	A	A	A	B	B	B	C	D
OT-W	O	A	A	A	B	B	B	C
4 M	A	A	A	B	B	B	C	D
OT	A	A	A	B	B	B	C	D
OT-W	O	A	A	A	B	B	C	C
5 S	A	B[c]	B	B	C[d]	C	D[e]	D
OT	O	A	B	B	C	C	D	D
P-OT	O	A	A	A	B	C	C	D
OT-W	O	O	A	A	A	B	C	C
6 S	A	A	A	B	B	C	D	D
OT	A	A	A	B	B	C	D	D
P-OT	A	A	A	B	B	C	C	D
OT-W	O	A	A	A	B	B	C	C
8 H[h]	O	O	A	A	A	A	B	B
OT	O	A	A	A	A	A	B	B
P-OT	O	A	A	A	A	A	B	B
OT-W	O	O	A	A	A	A	B	B

[a] O, No effect. [b] A, Slight softening of tablet surface. [c] B, Surface coat or surface deteriorated. [d] C, Tablet breaking. [e] D, Complete deterioration of tablet. [f] M, Manufacturer's coating. [g] S, Soft punched. [h] H, Hard punched.

TABLE IV.—TABLET DISINTEGRATION TEST

Tablet Formula	Control Min.:Sec.	Time Variation for Coated Tablets: OT Min.:Sec.	P-OT Min.:Sec.	OT-W Min.:Sec.
1	5:51	−0:18	+2:16	+1:18
2	83:20	−25:06	−2:40	−23:05
3	81:45	−21:08	...	−18:31
4	96:56	−20:09	...	−15:48
5	3:30	−0:04	+1:00	+0:17
6	2:59	−0:05	+1:03	+0:47
7	18:40	−1:07	−0:56	−1:05

corrected by the addition of a desiccant packet to each container.

Humidity Test.—A glass desiccator equipped with a porcelain crucible holder and crucibles and filled with approximately 2 in. of water acted as a humidity control chamber. The sealed desiccator was allowed to stand 48 hours at 25° before it was used. One tablet was placed in each of the seven crucibles and examined frequently over a period of 60 hours. Duplicate series were performed with each tablet variety.

Table III shows these humidity effects. The tablets coated with DSS and wax polish proved to be most durable in this respect, with the prime coated-DSS coated tablets and the DSS-coated tablets following in that order.

U.S.P. Disintegration Tests.—These tests were performed using procedures and equipment prescribed in the U.S.P. XV (9). The temperature of the inner bath of the disintegration unit (Scientific Glass Apparatus Co., Bloomfield, N. J.) was maintained at 37 ± 2°. Distilled water was used as the tablet solvent in the inner bath and was changed with each batch of tablets.

Table IV gives the average results obtained from duplicate tests on six tablets of each type. This table shows that the disintegration rates were not impeded by the DSS coatings. The shortened disintegration time was particularly noticeable with tablets coated only with DSS. This was to be expected as a result of Cooper and Brecht's (10) work with 21 surface-active agents which indicated that DSS was an effective additive to tablet granulations to reduce disintegration time.

SUMMARY AND CONCLUSIONS

Upon examination of the shortcomings of sugar coatings and the many tests required of these coatings, it can quickly be surmised what constitutes the desirable features of an ideal tablet coating. Once a list of these factors is relatively complete, it can be used as a means of measuring the coating results. An ideal coating must be physically stable, therapeutically compatible, chemically inert, economically feasible and, finally, have a patient product acceptability.

The DSS coating has shown exceptional resistance to heat, light, and trauma; on the other hand, an inherent weakness toward environmental moisture is prevalent. This hygroscopic nature of the compound is a condition that can be counteracted, but still remains a disadvantage as far as this coating is concerned. DSS can serve as a final protective coating for either sugar or uncoated tablets.

The materials in the basic coating formula are quite safe in regard to toxicity. DSS exhibits a very low order of toxicity. The acute oral LD_{50} for mice has been determined to be approximately 1 Gm./Kg. of body weight (11).

The coating is almost completely noncaloric in composition, even when sweetened and flavored. The coating will not hinder disintegration of the tablets; thus, it will not slow therapeutic absorption of the coated drugs.

No incompatibility has been encountered while applying the coating to the various tablets. DSS is compatible with all medications normally dispensed in tablet form.

The chief advantage of film coating is the reduction of operational costs. DSS coatings are easily applied to tablets using conventional coating equipment; therefore, no conversion of equipment now in use is necessary. There is a drastic reduction in the application time of this film coating, with about 1 to 3 hours required to coat a batch of tablets completely. This is far less time than is necessary for the sugar coating operation and, in turn, results in a lowered labor cost. The thinner film coating and resultant small change in tablet weight or size provide a substantial reduction in bottle size, shipping, and storage space needed. Less skill and experience are necessary to employ this coating procedure successfully compared to those required for the sugar coating process.

Probably the most important single factor in the use of medicinal agents today is patient acceptance. The thin coating provides tablets of smaller size so that swallowing is facilitated. Although the DSS coating is odorless, it produces an undesirable bitter aftertaste which can be masked with flavoring agents or limited by a wax polish coating. Since the DSS basic coating is colorless, a wide variety of colors may be employed. This formula gives a uniform, elegant, glossy appearance necessary for favorable acceptance.

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Solubility of Carbon Dioxide, Krypton, and Xenon in Aqueous Solution

By SHU-YUAN YEH and RICHARD E. PETERSON

The solubility of carbon dioxide, krypton, and xenon has been measured at 1 Atm. total pressure and at temperatures of 25, 30, 37, and 45° in distilled water, 0.9 per cent sodium chloride, and .066 *M* phosphate buffer solution at pH 7.0. The solubility of the gases studied in aqueous solution decreased as the temperature increased. Heats and entropies of solution have been calculated according to Eley's equation (16). The relation between $\Delta S°$ and $\Delta H°$ at various temperatures is exactly linear in every case, and the slope is approximately equal to $1/T$.

THE SOLUBILITY of krypton and xenon in water has been measured by many investigators (1–5,9). However, the results reported in the literature are not in agreement. The present study was undertaken to obtain more precise solubility coefficient data needed for calculation of body fat in a living body from the equilibration of these gases in the body. The solubility of carbon dioxide in water and normal saline has also been measured in order to test the apparatus and technique employed.

EXPERIMENTAL

Materials.—Research grade carbon dioxide, krypton, and xenon were purchased from the Matheson Co. According to their specifications, carbon dioxide contained maximum possible impurities of 0.3% of nitrogen or carbon monoxide and oxygen; krypton contained maximum possible impurities of 0.04% nitrogen and oxygen; xenon contained maximum possible impurities of 0.02% nitrogen and 0.05% krypton. No further analysis for the purity of the gases was made. No correction was applied to the experimental data obtained for the impurity of the gases.

The water used was redistilled from glass apparatus.

Sodium chloride solution (0.9%) and .066 *M* sodium phosphate buffer solution at pH 7.0 were made by weighing a proper amount of analytical grade salt and dissolving it in distilled water. The pH of the phosphate buffer solution was adjusted by using a Beckman model GS pH meter.

Apparatus and Manipulation.—The solubility apparatus used was essentially the one previously described (6), except that the absorption flask (125 ml.) did not have a mercury sealing cup, and a few drops of water were added to the gas buret to saturate the gas with water vapor before an experiment was conducted.

Degassing of Solution.—The absorption flask was charged with about 100 ml. of freshly boiled solution assembled on the solubility apparatus, frozen in a low temperature bath, evacuated, then boiled in a warm water bath. This process was repeated three times. The saturation of the solution with the gas was done essentially by the procedure previously described (6). The weight of the solution was determined at the conclusion of the experiment.

The densities and vapor pressures of distilled water were found in the literature (7). The densities of sodium chloride and phosphate buffer solution were determined in the usual way, their vapor pressure data being taken from the literature (8) and extrapolated between the concentrations.

RESULTS AND DISCUSSION

The solubility of carbon dioxide, krypton, and xenon in distilled water, 0.9% sodium chloride solution, and .066 *M* phosphate buffer solution at pH 7.0 were measured at 1 Atm. total pressure and temperatures of 25, 30, 37, and 45°. Each solution was measured three or four times. The standard deviation of each measurement closely approximated ±1.0% from the values reported in Tables I, II, and III. Possible systematic errors of those measurements would be predominantly due to the barometric pressure reading, which was assumed to have ±0.2 mm. error, and the temperature, which was assumed to have ±0.05° error. Therefore, the volume of the absorption flask calibrated by filling with a known volume of air might have ±0.034-ml. difference. Also, if the densities of the solution had an error of 1×10^{-4}, there would be ±0.01-ml. difference in volume for a 100-ml. solution. In terms of Ostwald coefficient, it would make a difference of about ±0.0005. Therefore, for the large solubility of carbon dioxide and xenon in water, it would cause approximately an 0.2% deviation, and for the smaller solubility of krypton, it would cause a 1% deviation from other investigators' values.

The results obtained from those measurements, in terms of Ostwald solubility coefficient, are presented in Tables I, II, and III. The literature values taken were reduced in terms of Ostwald solubility coefficient for uniform comparison. The data obtained from present studies agree well with the results of Morrison and Johnstone (4) and are considerably lower than those of Valentiner (2) and Antropoff (1).

The solubility of carbon dioxide, krypton, and xenon in normal saline and in phosphate buffer solution at pH 7 was less than in pure water. The de-

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The authors acknowledge valuable discussions with Professor Henry S. Frank, Department of Chemistry, University of Pittsburgh, Pittsburgh, Pa.

TABLE I.—SOLUBILITY OF CARBON DIOXIDE IN AQUEOUS SOLUTIONS

Soln.	Temp., °C.	Ostwald Solubility Coefficient	Lit. Value	$-\Delta H$ cal./mole	$-\Delta S$ cal./deg./mole
Distilled water	45	0.5453	0.558[a]	3912	20.0
	37	0.6282	0.638[a]	4310	21.3
	30	0.7264	0.738[a]	4657	22.4
	25	0.8129	0.828[a]	4906	23.2
0.9% NaCl	45	0.5100	...	3923	20.2
	37	0.5876	...	4322	21.2
	30	0.6800	...	4670	22.6
	25	0.7576	...	4919	23.4

[a] See *Reference 7*.

TABLE II.—SOLUBILITY OF KRYPTON IN AQUEOUS SOLUTION

Soln.	Temp., °C.	Ostwald Solubility Coefficient	Lit. Value	$-\Delta H$ cal./mole	$-\Delta S$ cal./deg./mole
Distilled water	45	0.0441	0.0441[b]	2337	20.0
	37	0.0481	0.0511[c]	3250	22.9
	30	0.0539	0.0540[b]	4037	25.5
			0.0566[a]		
	25	0.0581	0.0611[a]		
			0.0582[b]	4603	27.4
0.9% NaCl	45	0.0411	...	1957	19.0
	37	0.0444	...	3181	22.9
	30	0.0499	...	4252	26.4
	25	0.0542	...	5017	28.9
Phosphate	45	0.0417	...	2941	22.0
	37	0.0462	...	3369	23.4
	30	0.0516	...	3744	24.6
	25	0.0558	...	4012	25.5

[a] See *Reference 2*. [b] See *Reference 3*. [c] See *Reference 5*.

TABLE III.—SOLUBILITY OF XENON IN AQUEOUS SOLUTION

Soln.	Temp., °C.	Ostwald Solubility Coefficient	Lit. Value	$-\Delta H$ cal./mole	$-\Delta S$ cal./deg./mole
Distilled water	45	0.0737	...	2946	20.9
	37	0.0827	...	4045	24.4
	30	0.0958	0.0108[a]	5006	27.6
			0.0934[b]		
	25	0.1068	0.1195[a]	5692	29.9
0.9% NaCl	45	0.0680	...	3729	26.6
	37	0.0778	...	4158	24.9
	30	0.0895	...	4533	26.1
	25	0.0976	...	4801	27.0
Phosphate	45	0.0688	...	3585	23.1
	37	0.0782	...	3977	24.3
	30	0.0893	...	4320	25.4
	25	0.0990	...	4566	26.3

[a] See *Reference 2*. [b] See *Reference 4*.

creased solubility of the gas in the sodium chloride and sodium phosphate solutions was essentially the same (8%). The decrease in the solubility of the gas in aqueous solution is due to the "salting out" effect of the electrolytes in the solution as reported by previous investigators (19).

Effect of Temperature on Solubility.—The solubility of carbon dioxide, krypton, and xenon in aqueous solution decreased as temperature was raised (Tables I, II, and III). The relation between the solubility and temperature fitted the equations of Valentiner (2), Horiuti (10), and Kobatake and Hildebrand (11) very well, but did not fit the Clapeyron equation, *i.e.*, linear relationship between logarithm β and $1/T$. Lanning (12), who measured the solubility of rare gases in water and organic solvents, found that the solubility of gas in organic solvents fitted the Clapeyron equation very well, but not that of gas in water. Clever, *et al.* (18), also reported that the relation between temperature and the solubility of rare gases in organic solvents fitted the Clapeyron equation very well, and that their solubility increased as temperature increased. The decrease in gas solubility in an aqueous system as temperature is raised is due to modification of water structure as the gas atoms dissolve. The change is in the direction of greater crystallinity of water structure, as pointed out by Frank and Evans (13)—the water builds a microscopic "iceberg" around the gas atoms. As the temperature is raised, these "icebergs" melt, and the amount of gas trapped in the water is decreased. Similarly, Pauling stated that the nonhydrophilic molecules—krypton, xenon, etc., are trapped in clathrate structure, which he called krypton hydrate, xenon hydrate, etc. (14). The decrease of gas solu-

bility in normal saline and phosphate buffer is probably due to the effect of electrolytes which decrease in "icebergs" forming or clathrate tendencies.

Free Energy, Heats, and Entropies of Solution.—Lange and Watzel (15), Eley (16), and Bulter (17), using the gas-solubility data of Valentiner, have calculated the heats and entropies for the solution of rare gases in water. Eley plotted both the values of $\Delta H°$ and $T\Delta S°$ against T giving straight lines of slope $R(B-1)$. Lange and Watzel have derived values of $\Delta H°$ and $\Delta S°$ similar to Eley's and observed their strong dependence on temperature. Bulter calculated the values of $\Delta S°$ and $\Delta H°$ at 25° and found that there is a linear relation between the heats and entropies of solution. The $\Delta H°$ and $\Delta S°$ values presented in Tables I, II, and III were calculated according to the equations derived by Eley (16). Plotting the values of $\Delta H°$ and $T\Delta S°$ against T gave straight lines of slope $R(B-1)$. Plotting $\Delta S°$ against $\Delta H°$ also gave straight lines of slope approximately equal to $1/T$ (3.3×10^{-3}). Those plots showed that the data fitted the following equation well:

$$T\left(\frac{\partial \Delta S}{\partial T}\right)_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p = \Delta C_p$$

Although ΔC_p can be calculated from the straight lines plot of either $\Delta H°$ or $T\Delta S°$ against T, this method is a very insensitive one.

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Synthesis of *N*-Methyl-1,3-dimethylbutylamine

By M. T. WU

A procedure has been described for the preparation of *N*-methyl-1,3-dimethylbutylamine from methyl isobutyl ketone and methylamine. The method involves low pressure hydrogenation of ketimine in the presence of platinum catalyst.

RELATIVELY low molecular weight organic compounds with one or more electronegative atoms—such as nitrogen or oxygen—can dissolve salt-free water without losing their identity to a separate phase. The solvent then releases the purified water during a slight temperature increase. In view of the interesting physical properties of *N*-ethyl-*n*-butylamine and *N*-methyl-*n*-amylamine (1, 2), similar secondary amines seem worthy of study. This paper describes the synthesis of *N*-methyl-1,3-dimethylbutylamine.

EXPERIMENTAL

N-Methyl-1,3-dimethylbutylamine.—Methylamine (31 Gm., 1 mole) was dissolved in 385 Gm. absolute ethanol; the solution was chilled in ice. Methyl isobutyl ketone (300 Gm., 3 moles) was added dropwise, and the resulting solution slowly generated heat which was removed by additional cooling. This was divided into approximately four equal quantities. These were reduced catalytically over 8 Gm. of 10% platinum-on-charcoal at a maximum pressure of 60 p.s.i. The total amount of hydrogen absorbed corresponded to 99.5% of the theoretical quantity. After removing the catalyst by filtration, the filtrate was added to an excess of dilute hydrochloric acid at a temperature below 20°. The acidic solution was then concentrated to a small volume to remove ethanol, methyl isobutyl ketone, and any other volatile product. After making the acidic solution basic with an excess of aqueous sodium hydroxide, the water insoluble layer was removed, and the water layer was extracted four times with ether. The combined water-insoluble layers were dried over potassium hydroxide pellets and finally over calcium hydride. The ether solution was distilled carefully through a 28 × 1-in. metal helices-packed column from a small quantity of calcium hydride to yield 78.9 Gm. (69% on the basis of the methyl amine employed) of *N*-methyl-1,3-dimethylbutylamine, b.p. 122 to 122.5°, n_D^{20} 1.4131, d_4^{20} 0.7458, MR_D (calcd.) 38.53, MR_D (obs.) 38.13.

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N-Methyl-1,3-dimethylbutylamine.—Methylamine (31 Gm., 1 mole) was dissolved in 385 Gm. absolute ethanol; the solution was chilled in ice. Methyl isobutyl ketone (300 Gm., 3 moles) was added dropwise, and the resulting solution slowly generated heat which was removed by additional cooling. This was divided into approximately four equal quantities. These were reduced catalytically over 8 Gm. of 10% platinum-on-charcoal at a maximum pressure of 60 p.s.i. The total amount of hydrogen absorbed corresponded to 99.5% of the theoretical quantity. After removing the catalyst by filtration, the filtrate was added to an excess of dilute hydrochloric acid at a temperature below 20°. The acidic solution was then concentrated to a small volume to remove ethanol, methyl isobutyl ketone, and any other volatile product. After making the acidic solution basic with an excess of aqueous sodium hydroxide, the water insoluble layer was removed, and the water layer was extracted four times with ether. The combined water-insoluble layers were dried over potassium hydroxide pellets and finally over calcium hydride. The ether solution was distilled carefully through a 28 × 1-in. metal helices-packed column from a small quantity of calcium hydride to yield 78.9 Gm. (69% on the basis of the methyl amine employed) of *N*-methyl-1,3-dimethylbutylamine, b.p. 122 to 122.5°, n_D^{20} 1.4131, d_4^{20} 0.7458, MR_D (calcd.) 38.53, MR_D (obs.) 38.13.

Anal.—Calcd. for $C_7H_{17}N$: C, 72.97; H, 14.87; N, 12.16. Found: C, 72.95; H, 15.01: N, 12.19.

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Adaptation of Commercial Viscometers for Special Applications in Pharmaceutical Rheology III. The Tackmeter

By JOHN H. WOOD and EDWARD A. LAPHAM

A variant of the tackmeter originally developed by Green has been constructed. This instrument is suited to the precise measurement of tack instead of the usual subjective evaluation of lotions, creams, and ointments. It may be used in laboratory screening to compare relative tack of different formulations as a function of drying time or conditions and as a control procedure for formulations. It has been usefully applied as a measure of the tack left by materials rubbed into the human skin.

IN THE APPLICATION of lotions, ointments, or creams to the skin for therapeutical and/or cosmetic purposes, the resultant tackiness or stickiness of the area is often of concern—because of skin to skin adhesion for certain areas of the body or because of adhesion to a protective dressing.

The evaluation of the relative merits of different formulations has primarily been subjective. It was felt desirable to quantitate this, preferably by a direct measure which could compare similar formulations and also determine any aging changes as they might occur.

The problem of tack has been a continuous one in the printing industry. Green published a description of a tackmeter (1) which was, "a mechanical finger, paralleling in execution the finger tap-out test in the evaluation of pull-resistance, but capable of placing a numerical value on its measurements."

The instrument described here is a simplification of the Green tackmeter which permits evaluation of tack of lotions, creams, and ointments, both on air drying on plates and after application—with or without rub-in on the skin.

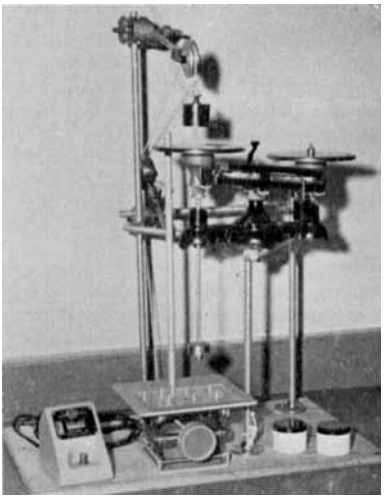


Fig. 1.—The tackmeter assembly with outer cup rotated upward to display the finger.

EXPERIMENTAL

The tackmeter assembly is shown in Fig. 1. The balance is a double beam Ohaus, Harvard Trip model. The assembly is connected to the beam collar underneath the frame casting by a connector and pin. The pin and hole in the vertical shaft is already present in this model. The collar must leave sufficient clearance for the bottom arm of the parallelogram of the balance to move freely. The connecting rod ($^1/_4$ in.) may be of any convenient length. The rod is connected to the collar by a set screen to permit easy removal for cleaning. The finger assembly is as shown. The finger diameter is $^5/_8$ in.; the inner diameter of the cup is $1^1/_4$ in. The thread for the rotation of the cup is 28 per inch. Two $^3/_4$-in. slots were cut in the cup to permit viewing the finger in operation. The whole assembly was of 316 stainless steel construction.

In use, the procedure may vary somewhat with the type of material being studied. For the following of the tack on air drying of a lotion, rings of 25-mm. glass tubing in $^1/_4$-in. lengths were cemented with Eastman 910 adhesive to a glass plate. A measured volume, 0.2 ml., was transferred to this cup and allowed to dry under prescribed suitable conditions. The finger was recessed to give the desired thickness of test lotion. To do this, the finger was contacted by a planar surface; then the cup was rotated downward until it, too, was in contact. Since the outer wall of the cup was scribed at every 10°, angular rotation of the cup could be readily determined using the pointer attached to the rod (and visible in the figure). The cup was rotated until the finger was recessed to the desired distance. Each 10° of rotation represented was 0.000992 in. of relative motion. The balance was counterweighted for the weight of the tackmeter. A 500-Gm. weight was placed on the left-hand pan and the plate carrying the dried lotions raised sufficiently by a jack, so that the finger could be located in the center of the cup. The jack was then further raised so that the cup bore on the plate. The desired drawing weight was placed on the right-hand pan. A stop clock was started as the 500-Gm. weight was removed; the time for the finger to break clear was noted. For the comparison of notably different lotions, the weight required to break clear in 6–10 seconds was determined, while for very similar lotions the differences in time for the same load were determined. Typical data are reported in Table I.

For ointments and creams, several modifications of this procedure were satisfactory. A film could be drawn by a doctor plate technique if the material

TABLE I.—COMPARISON OF TACKINESS OF SEVERAL LOTIONS ON DRYING

Sample	Withdrawal Wt. and Time of Break — 3 Hr. Drying	6 Hr. Drying
1	2 Gm. for 65 sec.	20 Gm. for 30 sec.
2	2 Gm. for 1.1 sec.	2 Gm. for 6 sec.
3	2 Gm. for 10 sec.	15 Gm. for 15 sec.
4	2 Gm. for 2.7 sec.	3 Gm. for 19 sec.

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TABLE II.—COMPARISON OF TACKINESS OF SEVERAL CREAM PREPARATIONS RUBBED IN THE SKIN FOR 20 SECONDS

Sample	Withdrawal Wt., Gm.	Time for Withdrawal, sec.
1	4	129, 165, 234, 212
2	4	101, 65, 71
3	2	6, 2, 2

was not too shear sensitive. Alternately, a weighed geometric spot was applied to the glass. In either case, the finger was brought to bear in a similar manner.

For actual measurement on human skin, the sample was applied in the appropriate manner and the finger applied directly with the 500-Gm. load. The cup was rotated back and clear for this. In general, the finger was contacted for 20 seconds before withdrawal; voluntary motion or quivers often induce the break-away after longer periods of contact. Typical data are shown in Table II.

DISCUSSION

The fundamental equations covering the use of the tackmeter were derived by Green (1), and the rheology of tack has been discussed by Bikerman (2).

The time required for break is inversely proportional to the viscosity function of the sample, the weight used for drawing, and the square of the sample film thickness, but is proportional to the fourth power of the radius of the finger. Thus, it is possible to derive a viscosity term from the tackmeter to define the sample film. In this work, it has not been necessary, or even desirable, to do this, since all desired comparisons can be readily made in units of withdrawal time and pulling weight. If, however, some knowledge of absolute viscosities of dried films is desirable, then the instrument may be calibrated by standards and used as a rheometer.

Obviously, the choice of finger dimensions and film thickness permit an extremely wide range of study. In the study on human skin, the bearing weight and duration of contact could conceivably be of different magnitudes in some applications.

Since the primary value of this instrument is for empirical testing, the finger surface may readily be modified to permit the attachment of dressings if it is felt to be important.

SUMMARY

The tackmeter described permits the measure of tack of lotions, creams, and ointments. It may be utilized on human skin and as a laboratory test.

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(1) Green, H., *Ind. Eng. Chem. Anal. Ed.*, **13**, 632(1941).
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Assay Methods for Total Neomycins B and C

By W. T. SOKOLSKI, C. G. CHIDESTER, O. S. CARPENTER, and W. M. KANESHIRO

Data from assays of neomycin C sulfate and mixtures of neomycin C sulfate and neomycin B sulfate were obtained using six cylinder-plate methods. Statistical analyses show that with three commonly used methods, neomycin C responses are only 35 to 50 per cent of neomycin B responses. With three modified methods, the response of neomycins B and C are approximately equal.

NEOMYCIN FERMENTATIONS produce two major antibacterial components—neomycin B and neomycin C. Commercial preparations are composed mostly of B, but may contain as much as 50% C (1). Kaiser (2) found that the U.S.P. neomycin standard contained about 15% C. The present assay systems do not give equivalent responses for B and C and with significant differences in C content between the test and standard preparations, the assay results are not in terms of total neomycin relative to the standard.

Some problems in the assay of neomycin were discussed in a previous paper (3). The purpose of this study was to compare responses of neomycin B and neomycin C obtained by three assay methods commonly used and by three methods which were designed to and did reduce differences between the responses of neomycins B and C.

EXPERIMENTAL

Neomycin Preparations.—The physical and chemical properties of the neomycin B preparation and the neomycin C preparation were described by Ford, *et al.* (4). With a radioisotopic assay procedure (2), the neomycin B preparation was found to contain 11% C, while the neomycin C preparation was found to be free (<1%) of neomycin B. Both preparations were sulfate salts, and potency values for Tables I–III were recorded as neomycin sulfate. The potency values for Table IV are recorded in terms of neomycin base equivalent.

The Upjohn Co. control standard, potency 742 mcg./mg., was used to determine the potencies of commercial neomycin sulfate lots (Table IV).

Preparation of Neomycin Solutions.—The neomycin powders were weighed after drying in a vacuum oven at 60° for 3 hours. One milligram per milliliter stock solutions were made with distilled water. Stock mixtures in ratios of 1:1 and 9:1 (9 neomycin B + 1 neomycin C) were prepared from these solutions. Working solutions were prepared by diluting the stock solutions with the buffer appropriate to the method.

Assay Methodology.—The cylinder-plate method was used for all assays. Twenty by 100-mm. plastic Petri dishes (Plastomatic Corp., Malvern, Pa., cat. No. 94), were used with glazed porcelain tops. Six stainless steel cylinders (inside dimensions 6 × 10 mm.), were placed on each plate with a Shaw

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TABLE I.—AVERAGE POTENCIES OF NEOMYCIN C AND NEOMYCIN C:B MIXTURES

Assay Method	C: No. Assays	C: Av.[b] Potency	C: 95% C.L.	1C:1B[a]: No. Assays	1C:1B[a]: Av.[b] Potency	1C:1B[a]: 95% C.L.	1C:9B[a]: No. Assays	1C:9B[a]: Av.[b] Potency	1C:9B[a]: 95% C.L.
1	10	452	409–499	5	666	588–756	11	865	789–948
2	9	379	354–406	11	678	626–734	11	898	848–950
3	10	373	347–402	6	660	620–702	6	877	854–900
4	8	1077	982–1132	13	1054	982–1132	13	975	927–1028
5	10	1153	1064–1249	7	1009	926–1100	5	967	778–1200
6	12	1108	1047–1171	11	961	894–1033	9	927	872–985

[a] Ratios of the neomycins C and B preparations; the neomycin B preparation contained 11% C. [b] Micrograms of neomycin B sulfate per milligram.

TABLE II.—STANDARD ERRORS ESTIMATED FROM AMONG ASSAY VARIATION

Assay Method	S.E. Of Log Potency	S.E. As % of Potency
1	0.0506	11.7
2	0.0430	9.9
3	0.0337	7.8
4	0.0441	10.2
5	0.0534	12.3
6	0.0405	9.3
Av.	0.0445	10.2

dispenser. Using a 3 × 3 assay design (5), three concentrations of neomycin B were applied to each plate alternately with three corresponding concentrations of (*a*) neomycin C, (*b*) 1:1 mixture, or (*c*) 9:1 mixture. There were nine replicate plates in each assay. The assays were run by two technicians over a period of 6 months. Individual assay results and weighted mean averages were calculated on the IBM 1620 computer.

Media.—All media ingredients were purchased from Baltimore Biological Laboratories (BBL) or Difco Laboratories (Bacto).

Preparation of Inoculum.—The inoculum for the assay plates in all but *Method 4* was a suspension of *Staphylococcus aureus* FDA 209P prepared as follows. Two BBL trypticase soy seed agar (TSA) slants, cultured overnight, were washed with BBL antibiotic assay broth (AAB) into a Roux bottle containing 300 ml. TSA. The Roux bottle was incubated for 18 hours at 32°, after which the growth was suspended in 40 ml. AAB. The suspension was adjusted so that a 1:10 (1 + 9) dilution with distilled water gave a 65% light transmission at 650 mμ in an 18 × 150-mm. test tube with a Lumetron model 401 spectrophotometer. The undiluted suspension was stored at 4° for as long as 2 weeks. The inoculum rate in *Methods 1, 2, 5,* and *6* was 0.3 ml. suspension per 100 ml. agar at 48°. The inoculum rate in *Method 3* was 0.05%. The inoculum in *Method 4* was 0.3% of a *Bacillus subtilis* UC 564 aqueous spore suspension (6) containing 2 × 10^9 spores/ml.

Procedures Currently Used for Neomycin Determinations.—Dilutions of 10, 20, and 40 mcg./ml. were made from the neomycin B stock solution and the 1:1 and 9:1 stock mixtures with 0.1 *M* pH 7.9 potassium phosphate buffer. However, because neomycin C was expected to have a smaller response than neomycin B, the neomycin C stock solution was diluted to 20, 40, and 80 mcg./ml.

Method 1.—Assay plates were prepared with 10 ml. base layers and 5 ml. *S. aureus* seed layers of BBL neomycin assay agar.

Method 2.—Assay plates were the same as in *Method 1*, except that 0.5% sodium chloride was added to the neomycin assay agar before autoclaving.

Method 3.—Assay plates were prepared with 15 ml. trypticase soy base layers and 4 ml. TSA seed layers inoculated with 0.05% *S. aureus*.

Methods for Total Neomycins B and C.—Neomycin C working solutions were equal in concentration to working solutions of neomycin B and B:C mixtures. The concentrations used in each assay are given in Table III.

The medium designated yeast beef agar (YBA)

TABLE IV.—POTENCIES OF COMMERCIAL NEOMYCIN SULFATE AS BASE EQUIVALENT

Neomycin Sulfate Lots	Neomycin C Sulfate[a], %	Potency, mcg./mg.[b] *Method 3*	Potency, mcg./mg.[b] *Method 6*	Corrected for C Bias from *Method 3*[c]
B	11	731	...	791
C	100	241	...	791 (assumed)
1	21	590	691	685
2	19	588	738	669
3	27	549	651	663
4	12	639	688	693
5	10	642	647	687

[a] Determined by radioisotopic assays. [b] Micrograms of neomycin standard per milligram. [c] Estimated by using the 241 value for 100% C and the 791 value for total neomycin.

TABLE III.—DOSES AND AVERAGE ZONES FOR NEOMYCIN C *vs.* NEOMYCIN B

Assay Methods	No. Assays	Neomycin B[a] d	Neomycin B[a] z	Neomycin B[a] d	Neomycin B[a] z	Neomycin B[a] d	Neomycin B[a] z	Neomycin C[a] d	Neomycin C[a] z	Neomycin C[a] d	Neomycin C[a] z	Neomycin C[a] d	Neomycin C[a] z
1	10	10	16.2	20	17.6	40	18.4	20	16.6	40	17.6[b]	80	18.6
2	9	10	15.9	20	17.6	40	19.2	20	15.2	40	17.2[b]	80	18.5
3	10	10	12.8	20	14.4	40	15.9	20	12.2	40	13.8	80	15.2
4	4	5/7[c]	13.5	10/14	15.0	20/28	16.1	5/7	13.9	10/14	15.1	20/28	16.3
	4	10	14.6	20	15.6	40	16.7	10	14.5	20	15.8	40	16.8
5	5	20	16.4	40	17.9	80	19.3	20	16.7	40	18.4[b]	80	19.7
	3	7	14.4	14	16.2	28	17.3	7	14.9	14	16.4[b]	28	17.4
6	12	10	16.6	20	18.0	40	19.1	10	16.8	20	18.2[b]	40	19.3

[a] d = Dose, mcg./ml.; z = average zone diameter, mm. [b] Significant curvature on C. [c] Two assays 5, 10, 20; two were 7, 14, 28.

was prepared as follows. Bacto peptone (0.6%), 0.3% Bacto yeast extract, and 0.15% Bacto beef extract were dissolved in distilled water, and the solution was adjusted to pH 7.9 with 1 *N* sodium hydroxide. One and one-half per cent Bacto Noble agar was added, and the medium was autoclaved.

Method 4.—Plates were prepared with 20 ml. base layers of BBL streptomycin assay agar with yeast extract and 4 ml. YBA layers inoculated with 0.3% *B. subtilis*. Working solutions were prepared by diluting the stock solutions with 0.1 *M* pH 7.9 tris-hydroxymethyl aminomethane (tris) buffer.

Method 5.—The medium was YBA with 0.2% sodium chloride added before autoclaving. Assay plates were prepared with 10 ml. base layers and 5 ml. *S. aureus* seed layers. Neomycin stock solutions were diluted with 0.1 *M* pH 7.9 tris buffer as in *Method 4*.

Method 6.—Assay plates were the same as in *Method 5*, except that 0.2% glucose was added to the seed agar before autoclaving. Working solutions were prepared by diluting neomycin stock solutions with 0.25 *M* pH 7.9 tris buffer.

RESULTS AND DISCUSSION

Methods 1, 2, and *3* (with some variation in the preparation of inoculum and in volume of agar medium) are commonly used for neomycin determinations. Table I indicates that these methods have a 50 to 65% negative bias in the response of the neomycin C preparation relative to the neomycin B preparation. This bias is also evident in the assays of B:C mixtures.

With *Methods 4, 5,* and *6*, the neomycin C responses were 98 to 125% of the responses of the neomycin B preparation. It is not certain, however, that the high responses reflect a positive neomycin C bias because the actual potencies of these preparations are unknown. In fact, potency values of neomycin sulfate preparations as free based equivalent in general are relative to standards of uncertain purity. The present U.S.P. standard with an assigned value of 700 mcg. neomycin base equivalent per milligram was reported to contain 13.7 to 18.6% neomycin C (2). Potency estimates as now obtained are dependent on the extent of neomycin C bias in assay method and the B-C content in the test and standard preparations.

The standard errors of assay potency (Table II) did not differ significantly among the six methods; the average standard error of potency was 10%. A considerable number of individual assays failed the usual validity tests specified in U.S.P. XVI (7). However, all assays were included in the analyses for Tables I, II, and III.

The average zone diameters for neomycin C compared to neomycin B in all six methods are given in Table III. The neomycin C responses showed significant curvature with all but *Methods 3* and *4* and *Method 5* at high doses. Among assays that did not pass validity tests, curvature was the main cause of failure. This may reflect a basic difficulty in comparing preparations of high neomycin C content with preparations of high neomycin B content.

Some commercial lots of neomycin sulfate varying from 10 to 27% neomycin C were assayed by *Methods 3* and *6* against The Upjohn Co. (TUC) control neomycin standard which contained only 4% neomycin C and had an assumed value of 742 mcg. base equivalent per milligram. These two assays were chosen for comparison because both use *S. aureus* with salt-free media. As expected, the assay results with *Method 3* were consistently lower than with *Method 6* due to the neomycin C bias. An approximation of the extent of bias was made by using the *Method 3* potency results on the B and C preparations which were 731 and 241 mcg. base equivalent per milligram, respectively. Knowing the response of C alone (no B present in the C preparation), the following determinations were made: 0.11C + 0.89B = 731 (*Method 3* value, Table IV) 1.0C = 241, 0.11 (241) + 0.89B = 731 = total neomycin response, and total neomycin base = 791.

Using the same method for calculation, corrections were made for C bias on *Method 3* results for commercial neomycin sulfate lots (Table IV). The corrected values are rough estimates and should not be regarded as absolute. Exact measurements would consider the 4% C in the TUC standard and the sulfate content of each preparation as well as the neomycin base content for which a method is not available. It is obvious that the corrected values are relatively close to the values obtained with total neomycin *Method 6*.

Of the three total neomycin methods, the *B. subtilis* method (*Method 4*) is the most applicable in routine operation for three reasons: (*a*) the media are commercially available; (*b*) the test organism as a spore suspension is stable for long periods; (*c*) the inhibition zone edges are sharp and easily read.

In the development of total neomycin assay methods, one of the major considerations was the effect of the ionic strength of the assay medium on the response of neomycin. Salt increases the slope of the dose-response curve (6), [as does Noble agar (3)], but salt also adversely affects the activities of the neomycins (3, 8, 9). Neomycin C is affected more than neomycin B so that by decreasing the ionic strength of the assay medium, the response of neomycin C relative to neomycin B is increased. In a medium such as the YBA medium used in this study, the neomycin C response can be made to approximate the neomycin B response by adjustment of ionic strength.

SUMMARY

The commonly used assay procedures for neomycin have a negative bias for neomycin C. The response of neomycin C in agar-diffusion assays is affected by the ionic concentration of the medium, more so than is B. A total of six assay methods is described which include three presently used procedures with a neomycin C bias and three procedures which additively assay the B and C components in neomycin.

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Coniferyl Aldehyde as a Constituent of Oils Containing Eugenol

By SHOUKRY KHALIL WAHBA* and JOSEPH E. SINSHEIMER

The presence of coniferyl aldehyde in eugenol, clove oil, myrica oil, pimenta oil, and sassafras oil has been demonstrated. The characteristic cherry red developed by coniferyl aldehyde with phloroglucinol and hydrochloric acid can be used for a colorimetric assay of coniferyl aldehyde and contributes a significant portion of the color developed by eugenol-containing oils with these reagents.

A CHARACTERISTIC cherry red is produced when a phloroglucinol-hydrochloric acid reagent is allowed to react with clove oil (1). Eugenol has been considered responsible for this test (2, 3), and since many allyl compounds give a positive test, it has been suggested as a functional group test for this moiety (2).

Adler (4), however, has explained the positive phloroglucinol reaction of lignin and its prototype compounds upon the formation of coniferyl aldehyde or related compounds. He was able to isolate the 2,4-dinitrophenylhydrazone of methylconiferyl aldehyde from methyleugenol and to demonstrate that a purified sample of eugenol does not give a positive phloroglucinol test.

Although coniferyl aldehyde has not been listed as a constituent of *Eugenia caryophyllata* (5–7), it appears that it may be present in this plant and other eugenol-containing species as well as oils obtained from such plants. This investigation was undertaken to detect the presence of coniferyl aldehyde in eugenol-containing oils and to isolate the compound from eugenol U.S.P.

EXPERIMENTAL

Apparatus and Materials.—Spectrophotometric determinations were made with Beckman model DB, Zeiss PMQ II, and Perkin-Elmer model 137 spectrophotometers. All melting points were made on a Köfler hot stage and are corrected. Samples of eugenol, clove oil, myrica oil, pimenta oil, and sassafras oil met the present or former requirements for official purity. All chemicals were of reagent grade and were used without further purification.

Chromatographic Procedures.—One-tenth of a milliliter of alcoholic solutions containing 2% clove oil, myrica oil, pimenta oil, or eugenol; 0.01% coniferyl aldehyde, or 0.2% vanillin were chromatographed as suggested by Higuchi (9) on Whatman No. 1 filter paper, using the aqueous phase of a mixture of water:petroleum ether (90–100°):benzene:methanol (50:50:50:1) or butanol saturated with 3% aqueous ammonia.

Presence of vanillin and coniferyl aldehyde was detected by spraying with a 0.5% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid or with a 1% solution of phloroglucinol in a mixture of 10 ml. of hydrochloric acid and 90 ml. of alcohol.

Since sassafras oil has a low concentration of eugenol, a 0.5-ml. alcoholic solution containing 50% of this oil was chromatographed as a band on a 23 × 57-cm. sheet of Whatman No. 1 paper using the butanol-ammonia system. The zone corresponding in R_f to coniferyl aldehyde was extracted with alcohol and the concentrated extract rechromatographed as a single spot with the butanol-ammonia system. Coniferyl aldehyde was then detected with the phloroglucinol spray.

Isolation of Coniferyl Aldehyde.—The 2-Gm. brown residue remaining after distillation up to 250° of the major portion of 200 ml. of eugenol was dissolved in 5 ml. of ethanol. One-milliliter portions were chromatographed as bands on 23 × 57-cm. sheets of Whatman No. 1 filter paper using the aqueous system described above in a descending technique.

The coniferyl aldehyde zone was located with the aid of the phloroglucinol spray on a portion of this paper. This zone was extracted with alcohol, the solution concentrated to 1 ml. under reduced pressure, and rechromatographed in the same manner. The coniferyl aldehyde zone was then extracted with boiling benzene and the extract concentrated to 1 ml. Ten milligrams of yellow needles were isolated after cooling the solution overnight.

Colorimetric Determination of Coniferyl Aldehyde.—To a 2-ml. solution of from 1 to 6 mcg. of coniferyl aldehyde in acetone was added 1 ml. of a 0.2% solution of phloroglucinol in a mixture of 5 ml. of hydrochloric acid and 95 ml. of acetone. The cherry red which develops must be measured at 540 mμ within 10 to 15 minutes after the start of the reaction.

Rate of Formation of Coniferyl Aldehyde.—A mixture of 200 ml. of eugenol and 20 ml. of a 1% alcoholic solution of 2,4-dinitrophenylhydrazine was distilled under nitrogen. The fraction collected at 248° was tested at a concentration of 20 mg. in 2 ml. of acetone as outlined under the *Colorimetric Determination of Coniferyl Aldehyde.* Determinations were made immediately following distillation and at intervals up to 48 hours after the distillation.

RESULTS AND DISCUSSION

It was not only possible to indicate the presence of coniferyl aldehyde by paper chromatography but also to isolate this aldehyde from eugenol U.S.P. The residue after distillation of the major portion of a sample of eugenol was chromatographed on paper using the aqueous phase of the system suggested by Higuchi (9) for the separation of coniferyl aldehyde in his lignin studies. While the presence of two carbonyl compounds was indicated through the use of a 2,4-dinitrophenylhydrazine spray, only one of the compounds gave a cherry red test with a phloroglucinol-hydrochloric acid spray. The red produced by this latter spray was used to locate the coniferyl aldehyde zone. Extractions of this zone yield crystals melting at 78° which did not show a depression when mixed with an authentic sample of coniferyl aldehyde.[1] A 2,4-dinitrophenylhydrazone was also prepared from the isolated material and melted at 246–248°. A mixture of this hydra-

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[1] The authors are indebted to Dr. John C. Pew, Forest Products Laboratory, Madison, Wis., for generously furnishing us with a reference sample of coniferyl aldehyde.

TABLE I.—CHROMATOGRAPHIC CHARACTERISTICS OF CONIFERYL ALDEHYDE AND VANILLIN

Compd.	R_f Values[a] System I[b]	System II[c]	Phloroglucinol Color Reaction
Coniferyl aldehyde			
Reference	0.61	0.58	Cherry red
Isolated	0.60	0.58	Cherry red
Vanillin			
Reference	0.68	0.46	Pale yellow
Isolated	0.67	0.46	Pale yellow

[a] These values are for Whatman No. 1 paper at 21° and are averages with a standard deviation of less than .015. [b] The aqueous phase of a mixture of water: petroleum ether (90–100°): benzene: methanol (50:50:50:1). [c] Butanol saturated with 3% aqueous ammonia.

zone and one prepared from the authentic sample showed no depression in melting point. The infrared spectra of both the aldehyde and its hydrazone were identical with the reference materials.

The chromatographic characteristics of the isolated and reference coniferyl aldehyde are summarized in Table I. The second aldehyde present in the sample of eugenol is consistent in its chromatographic characteristics with those of vanillin as also summarized in Table I. For both coniferyl aldehyde and vanillin, a mixture of the isolated and reference aldehyde chromatographed as a single spot with characteristic R_f values. It is evident that coniferyl aldehyde can be separated from any vanillin present.

In addition to the isolation of coniferyl aldehyde from eugenol, the presence of this aldehyde in eugenol-containing oils was demonstrated by paper chromatography. When clove, myrica, and pimenta oils were chromatographed on paper using the butanol-ammonia system, a characteristic red developed with the phloroglucinol-hydrochloric acid spray and had the same R_f value as the reference coniferyl aldehyde.

The presence of coniferyl aldehyde can even be demonstrated for sassafras oil which contains eugenol as a minor constituent in the order of 0.5% (8). Thus, sassafras oil yields a chromatogram with the characteristic color and R_f value for coniferyl aldehyde but requires a preliminary concentration of the coniferyl aldehyde.

Coniferyl aldehyde can be determined colorimetrically with phloroglucinol under a variety of conditions. In this investigation, maximum stability was obtained with acetone as a solvent and by limiting the amount of hydrochloric acid used. For a concentration of 6 mcg. in 3 ml. of the reaction mixture, a maximum absorbance of 0.485 was obtained after 10 minutes and was stable for another 5 minutes. The color developed during this period was proportional to concentration up to 6 mcg. in 3 ml. of the reaction mixture. However, it should be noted that although coniferyl aldehyde contributes significantly to the color developed by eugenol-containing oils upon treatment with phloroglucinol and hydrochloric acid, it is not advisable to use this reaction for the direct determination of coniferyl aldehyde in these oils. Other constituents produce color under the same conditions. For example, vanillin produces a pale yellow, while safrol yields an intense red.

A positive cherry red phloroglucinol test on freshly pressed oil and clove buds demonstrated that the formation of coniferyl aldehyde also occurs at this point and is not an artifact due to processing of the oil or to the method of isolation used in this investigation. It becomes evident that coniferyl aldehyde must be widely present in eugenol preparations when the rapid rate of formation of the compound is noted. Eugenol was distilled under nitrogen and a colorless oil obtained which turns pale yellow after a 1-hour exposure to the atmosphere. A 20-mg. sample of the colorless distilled eugenol failed to give a positive test with phloroglucinol when examined immediately. However, when the distilled eugenol was exposed to atmospheric oxygen, a concentration of 20 mg. in 3 ml. of reaction mixture gave an absorbance of 0.010 after 5 minutes and at the same concentration reached a maximum absorbance of 0.325 after 22 hours of exposure to the atmosphere.

SUMMARY

Coniferyl aldehyde has been isolated from eugenol and shown to be present in eugenol-containing volatile oils.

Coniferyl aldehyde forms rapidly from eugenol and is essentially responsible for the characteristic cherry red color developed by eugenol-containing oils with phloroglucinol and hydrochloric acid.

A colorimetric procedure has been developed for the estimation of coniferyl aldehyde.

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Simplified Turbidimetric Assay of Lipase in Dosage Forms

By FREDERICK P. SIEGEL and BERNARD ECANOW

A simplified turbidimetric procedure for the standardization of lipase activity in dosage forms is described. The procedure compares in accuracy to the best titrimetric methods and simplifies the handling of a large number of samples to be assayed.

THE ASSAY OF lipase has practical consideration in the evaluation of enzyme activity in dosage forms and in serum as a diagnostic for acute pancreatitis.

The principle lipase procedures employed today are titrimetric, based on the hydrolysis of a glyceride substrate. The subsequent neutralization of the free fatty acids by a standard sodium hydroxide solution is usually expressed in lipase units. A lipase unit represents the enzyme activity resulting in the hydrolysis of glyceride equivalent to the release of 50 microequivalents of fatty acid.

Most techniques require from several to 24 hours of incubation to permit a titratable amount of fatty acid to be liberated and are further complicated by a difficulty in assessing a visual end point in opaque emulsion substrates. Lazo-Wasem (1) recently applied a nonaqueous titration procedure and circumvented the incubation time and end point problem. However, the required benzene extractions minimize its use with large numbers of samples and cut down its practicability in the laboratory.

The turbidimetric assay is a simplified procedure which can be conducted in the spectrophotometer and generally consumes not more than 30 minutes for the total routine. The application of this assay to dosage forms is discussed.

EXPERIMENTAL

Reagents.—The stock emulsion of 50% olive oil in water is prepared by the continental method employing powdered acacia, olive oil, and distilled water. The emulsion is homogenized in a colloid mill, stored in a cool place, and allowed to age for 3 weeks prior to use.

A working emulsion is prepared by placing 50 ml. of the stock emulsion with 300 ml. of a 0.2 *M* tris buffer, pH 8.0, in a separator. The diluted emulsion is shaken over a period of several hours; it is then allowed to sit for a period of about 12 hours to permit maximum creaming. The stable emulsion is carefully removed from the cream.

The stable emulsion is then carefully diluted in increments, with 0.2 *M* tris buffer, and read in the Coleman Jr. spectrophotometer to assess the ultimate final dilution required to produce an absorbance of approximately 0.65. The measurements are made in a 19-mm. cell at a wavelength of 600 mμ with a water blank.

The remainder of the stable emulsion is then diluted according to calculations and allowed to sit overnight to permit possible further creaming as the result of the dilution.

The diluted emulsion should yield an O.D. value of not less than 0.60.

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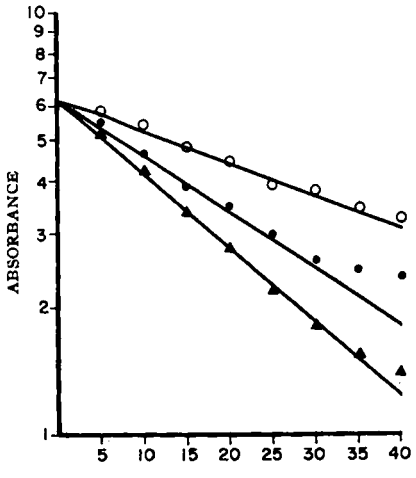


Fig. 1.—Semilogarithmic plot of absorbance of the reaction substrate *vs.* time. Key: ○, 0.6 u. lipase/ml.; ●, 1.0 u. lipase/ml.; and ▲, 1.4 u. lipase/ml.

Standard Curve.—Place 5 ml. of the working emulsion in 19-mm. Coleman cells. Add 1 ml. of a suspension containing 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 units of pancreatic lipase. Wilson's pancreatic lipase 3000 (3 u./mg.) was diluted in distilled water. The suspension was homogenized in a blender for about 1 minute.

A control is represented by 5 ml. of working emulsion plus 1 ml. of buffer. The point of addition of 1 ml. of enzyme suspension is designated zero time. The cell is mixed by inversion using a parafilm cover and read immediately as zero time. Absorbance readings are taken in intervals of several minutes at 600 mμ with a water blank. All operations were performed at 25°.

The logarithm of the absorbance will decrease linearly with time for approximately one half-time. This straight line region is employed to evaluate a half-time for each enzyme concentration. The half-time observed is inversely proportional to the enzyme concentration (Fig. 1 and Table I).

Methods.—In the analysis of an unknown, the lipase procedure entails the preparation of a suspension of the dosage form by homogenizing in the blender for about 1 minute, so as to yield a concentration of lipase in the approximate range of 0.5 to 1.0 u./ml.

A 1-ml. aliquot of the suspension is added to 5 ml. of the substrate. The tube is inverted several times and read as described under the *Standard Curve*. The half-time for the reaction of the un-

TABLE I.—STANDARD CURVE

u./1 ml. Aliquot	$T_{1/2}$, Min.
1.4	17
1.2	20
1.0	24
0.8	30
0.6	40
0.4	65

TABLE II.—ASSAY OF LIPASE-CONTAINING DOSAGE FORMS

Dosage Form	U. Lipase per Dosage Form	U. Lipase[a] Titrimetric Assay (1)	U.[a] Turbidimetric
Lipase 3000 powder	3400 u./Gm.	3400	3400
Lipase 3000 powder	3000 u./Gm.	3000	3000
Enteric coated tablet A	120 u./tablet	110	108
Enteric coated tablet B	130 u./tablet	110	120
Capsules A	100 u./capsule	105	105
Capsules B	200 u./capsule	190	200
Capsules C	250 u./capsule	230	240
Powder paper A	100 u./paper	100	105
Powder paper B	200 u./paper	195	205
Powder paper C	250 u./paper	240	240

[a] Mean values ten assays.

known is inversely related to the half-time of the standards: ($T^1/_2$ unknown/$T^1/_2$ standard) = (concn. of standard/concn. of unknown). The following data are used to illustrate the assay.

A sample labeled to contain 200 u. of lipase was diluted to 200 ml. A 1-ml. sample of the above suspension produced a half-time of 20 minutes. The half-time for 1 u./ml. of standard enzyme is 24 minutes. Then (20/24) = (1/X) and X = 1.2 u./ml. of lipase.

RESULTS AND DISCUSSION

Several commercial dosage forms were evaluated as well as several lipase concentrates employed in the manufacture of commercial dosage forms. Capsules and powder papers were also prepared from the concentrates in this laboratory and checked by this assay procedure (see Table II).

The rate of disappearance of the substrate turbidity is proportional to the release of fatty acids when compared to the method of Lazo-Wasem. Good recoveries of enzyme have been found with errors generally under 5%, but never exceeding 10%.

The substrate remains stable for a period of 2–3 weeks at 25°. It is advised that each new substrate batch be standardized with a standard lipase preparation, although duplications between batches have been good.

In assaying tablets of lipase the complete tablet is crushed and triturated to a fine powder. The material is then washed into a volumetric flask where it is brought to volume. The preparation is then homogenized in a blender.

One should be cautioned about the assay of lipase in a digestant tablet containing pepsin. The pepsin coat must be washed off prior to assay since the pepsin enzyme will inactivate lipase.

Caution in washing glassware is essential since it has been observed that certain surfactants will affect the rate of enzyme activity.

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Sensitivity of Color Tests for Nitrites, Nitrates, and Glyceryl Trinitrate I. Solutions in Distilled Water

By JAMES C. MUNCH, MERCEDES FRANCO, and BERNARD FRIEDLAND

Threshold concentrations detected (limens) for nitrites, nitrates, and glyceryl trinitrate have been determined for solutions in distilled water with 16 reagents. Generally the most sensitive tests detected 10 to 100 parts per million of these products; except chromotropic acid detected 2 parts per million of nitrite.

NITROGLYCERIN (glyceryl trinitrate) investigations require delicate methods for the detection and determination of nitrites, nitrates, and glyceryl trinitrate in tissue fluids, viscera, and pharmaceutical products. Some hundred reagents have been suggested in the literature (1–22) but our investigations showed that only a few considered specificity and threshold limits for determination. Results with these reagents indicated that sixteen showed promise, although close attention to details are required for successful use with them. Based on findings in testing solutions in distilled water reported in Table I, additional tests are under way for determinations in various pharmaceuticals, as well as tissue fluids and viscera.

Preparation of Test Solutions.—A concentrated solution was prepared by dissolving: (*a*) 1.50 Gm. of reagent grade sodium nitrite; (*b*) 1.37 Gm. of reagent grade sodium nitrate; and (*c*) 10 Gm. of an analyzed mixture of 1 part glyceryl trinitrate and 9 parts of beta lactose, in distilled water and making up each volume to 100 ml. Just before use each stock solution was diluted from 1% (10,000 parts per million) to 5,000, 2,000, 1,000, 100, and 10 parts per million for test. The sodium nitrite solution was further diluted to 2 p.p.m. for the test with chromotropic acid. If a negative test was obtained within 5 minutes using a concentration of 10,000 p.p.m., additional tests were not undertaken. Such negative results are indicated by (10,000) in Table I. Otherwise, the figures represent the limen (threshold concentration detected) for each product with the chosen reagent. The colors recorded are the final colors observed at the end of 5 to 10 minutes.

Preparation of Reagent Solutions.—Reagent chemicals were obtained from Eastman Kodak Co., Rochester, N. Y. Solutions were prepared at the

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TABLE I.—LIMITS OF SENSITIVITY OF COLOR REACTIONS, P.P.M.

Reagent, No.	Nitrate	Nitrite	Glyceryl Trinitrate
1. Antazoline	5,000 red-yellow	100 yellow	5,000 red-yellow
2. Benzidine	(10,000)[b]	10 yellow-brown	(10,000)
3. Chromotropic acid[a]	100 yellow[a]	2 yellow[a]	1,000
4. Diphenylamine	1,000 blue-purple	1,000 violet-blue-green	5,000 blue
5. Guaiacol	(10,000)	10,000 red-orange	(10,000)
6. Indole	10,000 pink	5,000 orange	5,000 pink
7. Michler ketone	10,000 yellow	5,000 yellow	10,000 yellow
8. Chicago acid	10,000 brown-orange	(10,000)	(10,000)
9. Amino G acid[a]	(10,000)	10 violet-blue[a]	5,000 violet-blue
10. Neutral red	(10,000)	5,000 red-orange	(10,000)
11. Phenol	(10,000)	1,000 yellow-brown	(10,000)
12. *m*-Phenylaminediamine	(10,000)	100 orange-brown	(10,000)
13. Resorcinol	(10,000)	100 brown	(10,000)
14. Thioglycolic acid	(10,000)	5,000 orange	(10,000)
15. Thiourea	(10,000)	10,000 red-brown	(10,000)
16. 2,4-Xylenol	1,000 yellow	(10,000)	100 yellow[a]

[a] Reagent most characteristic in distinguishing among nitrites, nitrates, and glyceryl trinitrate; also most sensitive. [b] (10,000) indicates negative reaction.

time of use, thus avoiding possible changes with aging. In general one drop of the test solution was added to one drop of the reagent solution on a spot plate and changes observed over a period up to 10 minutes. The reagent solutions were prepared as follows:

1. Antazoline; 2 - (N - Benzoylanilinemethyl)-imidazoline HCl.—Mix one drop of 1% aqueous solution with one drop of concentrated H_2SO_4.

2. Benzidine.—Prepare 1% solution in glacial acetic acid.

3. Chromotropic Acid: 4,5-Dihydroxy-2,7-naphthalene-disulfonic Acid.—Dissolve 0.5 Gm. in 100 ml. of concentrated H_2SO_4.

4. Diphenylamine.—Dissolve 1 Gm. in 100 ml. of concentrated H_2SO_4.

5. Guaiacol.—Dissolve 1 Gm. in 100 ml. of dilute H_2SO_4.

6. Indole.—Dissolve 15 mg. in 100 ml. of water. Just before use, add 1 ml. of this solution to 0.75 ml. of concentrated H_2SO_4.

7. Michlers Ketone; Tetramethyldiaminobenzophenone.—Dissolve 1 mg. in three drops of concentrated H_2SO_4, add about 1 mg. of test product; after standing 5 minutes add 1 ml. of distilled water.

8. Chicago Acid; S-Amino-1-naphthol-5,7-disulfonic Acid.—Dissolve 1 Gm. in 100 ml. of water.

9. Amino G Acid; 2-Naphthylamine-6,8-disulfonic Acid.—Dissolve 200 mg. in 100 ml. of water; mix one drop of reagent solution with one drop of test solution, then add one drop of 1% α-naphthylamine in dilute hydrochloric acid.

10. Neutral Red: Toluylene Red CI 825; Aminodimethylaminotoluaminozine HCl.—Dissolve 100 mg. in 100 ml. of alcohol.

11. Phenol.—Dissolve 10 Gm. in 100 ml. of water.

12. m-Phenylaminediamine.—Dissolve 100 mg. in 100 ml. of 10% acetic acid.

13. Resorcinol.—Dissolve 1 Gm. in 100 ml. of water, and add three drops of 10% aqueous solution of $FeCl_3$.

14. Thioglycolic Acid.—Dissolve 4 Gm. in 100 ml. of water.

15. Thiourea.—Dissolve 1 Gm. in 100 ml. of water. Mix one drop of test solution with one drop of reagent solution, then add one drop of 10% aqueous solution of $FeCl_3$.

16. 2,4-Xylenol.—Dissolve 100 mg. in alcohol; mix 0.1 ml. of this solution with 10 ml. of concentrated H_2SO_4.

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In Vitro Studies of Larvicidal Agents Against the Larvae of *Anaylostoma caninum*

By MARTIN E. HAMNER, LOUIS D. HAUSER, and THOMAS J. HOWARD

An investigation was conducted on larvicidal agents using a modification of the Baermann procedure for culture of the test larvae. *In vitro* tests against the larvae of *Ancylostoma caninum* indicated that *p*-chloro-*m*-xylenol is an effective larvicidal agent. The drug's activity is reduced by blood, plasma, and certain solubilizers.

THE LARVAL STAGES of helminths produce several diseases in man and animals. The most prominent such disease of man is larva migrans, a condition characterized by prolonged migration of a larval parasite in the skin or internal organs.

The term "cutaneous larva migrans," commonly known as "creeping eruption," refers to a migration of the larvae in the skin and is commonly caused by the larvae of the dog and cat hookworms, *Ancylostoma braziliense* and *A. caninum*.

Visceral larva migrans, the invasion of the internal organs, is primarily caused by the larvae of the cat and dog ascarid, *Toxocara canis*.

Many agents have been used in the treatment of larva migrans clinically, but an effective, safe, and highly satisfactory larvicidal agent is not at present available (1, 2).

METHOD

The larvae of *A. caninum* were isolated by a modified Baermann technique from fecal samples taken from infested dogs.

The Baermann procedure depends on the principle that nematode larvae will migrate out of fecal material into water maintained at a higher temperature than the fecal sample (3). A disadvantage of the method is that water when in contact with fecal material becomes soiled and turbid making examination difficult and shortening the life of the larvae unless a complete change of water can be effected. A high proportion of sluggish or inactive larvae may be produced. To overcome the difficulty, the fecal sample was suspended above the water level and kept moist with cloth wicks extending from the sample into the water. The larvae dropped into the water as they hatched, and contact of the larvae-bearing solution with the fecal sample was avoided.

The drugs tested were dissolved in water whenever feasible. If the agent was not water soluble, propylene glycol and/or Triton X-100[1] were used as solubilizing agents. The blanks used as controls consisted of water or an appropriate solution of the solubilizing agents in water. The drug concentrations referred to are the final concentrations after dilution with the larvae-bearing solution.

In vitro tests were conducted by placing 1 drop of the larvae-bearing solution in the well of the microscope slide and adding 1 drop of the solution to be tested. The slides were observed under the microscope at routine intervals.

Although normally lack of motility indicated death of the larvae, this was not true in all instances. Viable larvae which had become immotile would react visibly in a dilute solution of ethanol which appeared to act as a stimulant to them. One drop of ethanol-water (1:1) was added at the time of last observation. Larvae remaining immotile thereafter were considered dead.

EXPERIMENTAL

Initial studies tested the larvicidal properties of iodine in both free and combined forms.

In these tests only free iodine, as contained in Lugol's solution, was highly effective against the test larvae. Free iodine in Lugol's solution, diluted with water to a concentration of 0.55%, produced a 100% kill in 10 minutes. A concentration of 0.025% produced an 80% kill in 24 hours. These results were accepted as the standard for subsequent studies.

Screening tests were carried out on a series of compounds, and inactive agents were discarded. Results obtained with nicotine (alkaloidal), nicotine salicylate, phenol, creosote, and glaucarubin, all of which were less active than iodine, are recorded in Table I.

The chemical agent *p*-chloro-*m*-xylenol (PCMX) had a high level of larvicidal activity and was effective at much lower concentrations than was the standard. A concentration of 0.012% of PCMX produced 96% kill in 1 hour; 0.006% produced 100% kill in 24 hours (Table II).

PCMX has been reported to be an effective phenolic type germicidal and fungicidal agent. The compound is soluble in water only to the extent of 1 part in 3000, but is very soluble in acetone, ether, and alcohol. PCMX is reported to have an MLD in mice of 120 Gm./Kg. and an LD_{50} of 115 Gm./Kg. when injected as an aqueous suspension intraperitoneally (4).

It has been previously reported that the germicidal effectiveness of PCMX is partially destroyed by certain wetting or solubilizing agents (5). Further tests were conducted to determine the effects of solubilizing agents on the larvicidal properties of the drug. The most efficient solvent was a 5% aqueous solution of propylene glycol. Concentrations of propylene glycol of 10% or above were toxic to larvae, thus interfering with the tests. Other solubilizing agents used increased the survival time of the larvae, apparently through an inhibitory effect on the active agent. The results obtained with solubilizing agents are summarized in Table III.

PCMX, combined with agents listed in Table III in the concentrations indicated, was only one-half to one-fourth as effective as the same concentration of drug in a 5% aqueous solution of propylene glycol.

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[1] Marketed by Rohm and Haas Co., Philadelphia, Pa.

TABLE I.—RESULTS OF TESTS OF LARVICIDAL ACTIVITY OF VARIOUS AGENTS

Drug	Concn., %	Percentages of Larvae Dead or Immotile 10 Min.	30 Min.	1 Hr.	2 Hr.	24 Hr.
Iodine (as free iodine in Lugol's solution)	0.055	100	100	100	100	100
	0.05	82	94	98	[b]	88
	0.025	53	65	75	75	80
	Control[a]	0	0	0	0	0
Nicotine	0.25	0	0	0	0	100
	0.1	75	75	63	75	92
	Control	0	0	0	0	0
Nicotine salicylate	0.16	44	57	75	82	[b]
	Control	0	0	0	0	[b]
Phenol	0.05	13	0	0	63	100
	0.025	0	0	0	50	0
	Control	0	0	0	0	0
Creosote	0.05	0	38	[b]	[b]	50
	Control	0	0	[b]	[b]	10
Glaucarubin	0.05	0	7	7	43	0
	Control	0	0	0	10	0

[a] Larvae in aqueous suspension. [b] Readings not taken.

Additional tests were performed to determine the effects of blood constituents on the activity of PCMX. Results obtained with *in vitro* studies reveal that the agent's effectiveness is reduced by these substances.

TABLE II.—RESULTS OF TESTS OF LARVICIDAL ACTIVITY OF *p*-CHLORO-*m*-XYLENOL USING PROPYLENE GLYCOL 5% AS SOLUBILIZING AGENT[a]

Concn. of PCMX, %	10 Min.	30 Min.	1 Hr.	2 Hr.	24 Hr.
0.05	83	100	100	100	100
0.025	80	90	90	[b]	[b]
0.012	29	67	96	98	100
0.006	0	0	0	10	100
Control	0	0	0	0	10

[a] Results expressed as percentages of larvae dead or immotile. [b] Readings not taken.

TABLE III.—EFFECTS OF SOLUBILIZING AGENTS ON THE ACTIVITY OF PCMX

Solubilizing Agent	Concn. of Solubilizing Agent, %	Effect on Percentage Kill
Polysorbate 80[a]	0.5	Reduced
Dioctyl sodium sulfosuccinate	0.125	Reduced
Sodium lauryl sulfate	0.25	Reduced
Castile soap	0.25	Reduced
Triton X-100	0.50	Reduced
Polyethylene glycols 400, 1540, 4000, 6000	10.0	Reduced
Propylene glycol	10.0	Increased
Propylene glycol	5.0	No effect

[a] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

The external use of PCMX as a germicidal agent has been without reported ill effects to man or animals. Blood and tissue concentrations, up to 4 mg. per cent, have been recorded by Zondek and Finkelstein following administration to humans by intramuscular and percutaneous routes (6). In view of the reported low toxicity of the drug, it appears desirable to obtain data on blood levels required for larvicidal activity in the body. In our preliminary *in vivo* studies in dogs, doses of 200 to 400 mg./Kg., by oral and intramuscular administration, were nephrotoxic. Further work on systemic use of PCMX in dogs and guinea pigs is in progress.

In view of the drug's high rate of absorption by percutaneous administration, the use of PCMX in treatment of cutaneous larva migrans is a possibility. Also of interest is application of the compound as an area spray where man and animals may be exposed to infectious larvae of parasites.

REFERENCES

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Fluorometric Study of Antihistamines

By RICHARD E. JENSEN and RONALD T. PFLAUM

The reactions of 16 antihistamines with hydrogen peroxide and the fluorescent characteristics of the products are reported.

ANTIHISTAMINES containing an alkylamino substituent in the 2-position on a pyridine nucleus react with cyanogen bromide to yield fluorescent products (1). This reaction is the basis of a fluorometric method for the detection and determination of some of these antihistamines.

In the present work, the reactions of 16 antihistamines with a series of oxidants were studied. The fluorescence characteristics of the products formed upon treatment with hydrogen peroxide form the basis of this report. The corresponding studies on the cyanogen bromide systems were carried out for comparative purposes.

Excitation and fluorescence wavelengths and

Received September 11, 1963, from the Department of Chemistry, State University of Iowa, Iowa City.
Accepted for publication October 22, 1963.

TABLE I.—RESULTS OF TESTS OF LARVICIDAL ACTIVITY OF VARIOUS AGENTS

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In the present work, the reactions of 16 antihistamines with a series of oxidants were studied. The fluorescence characteristics of the products formed upon treatment with hydrogen peroxide form the basis of this report. The corresponding studies on the cyanogen bromide systems were carried out for comparative purposes.

Excitation and fluorescence wavelengths and

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relative intensities are reported which tend to show a correlation between fluorescence and chemical structure.

EXPERIMENTAL

All fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer equipped with an Osram xenon lamp source and a photomultiplier detector. The slits in the monochromator were arranged as follows: excitation beam, 1/8, 1/16, and 1/8 in.; fluorescent beam, 1/8, 1/16, 1/8, and 1/16 in. Quartz cells, 10 mm. square, were used.

The antihistamines were obtained from the sources listed in Table I. Stock solutions were prepared by dissolving accurately weighed samples of the antihistamine salts of approximately 50 mg. in 50 ml. of distilled deionized water.

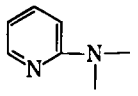
For the study of the effect of cyanogen bromide on antihistamines, 1 ml. of a freshly prepared saturated solution of cyanogen bromide was added to 1-ml. aliquots of the antihistamine stock solution. The mixtures were diluted to 10-ml. volume, allowed to stand for 30 minutes at room temperature, and then measured spectrofluorometrically.

In the study of the effect of hydrogen peroxide, 1 ml. of 3% hydrogen peroxide solution was added to 1-ml. aliquots of the antihistamine stock solution. The mixtures were diluted to 10 ml., heated in a 90° water bath for 30 minutes, and measured spectrofluorometrically.

RESULTS

The data obtained upon treatment of antihistamines with cyanogen bromide and hydrogen peroxide are summarized in Table I. Compounds are grouped according to structural formulation.

The first five antihistamines containing the alkylaminopyridyl grouping,



have an excitation wavelength of 340–345 mμ and a fluorescence maximum at 410 ± 3 mμ when treated with hydrogen peroxide. The situation is very similar for the cyanogen bromide system; excitation is 350 ± 5 mμ and a fluorescence at 415 ± 4 mμ. In every case, except for pyrilamine, the intensity of the fluorescence is much greater in the cyanogen bromide system. Thonzylamine, a 2-substituted pyrimidine derivative, reacts quite analogously to pyrilamine.

Compounds VII–X, containing a 2-picoline nucleus, in general developed a very weak fluorescence after treatment with cyanogen bromide. An excitation wavelength of 275–280 mμ resulted in fluorescence maxima between 388–467 mμ. The very weak intensity of the fluorescence may account for the fact that it had not been observed previously (1). Carbinoxamine (VII) exhibited only one set of fluorescence peaks, while the other compounds of this group revealed two sets of peaks upon treatment with hydrogen peroxide. This may be due to the ethereal linkage found only in carbinoxamine. Antazoline, a 2-substituted imidazole, reacts similarly to thonzylamine and to the compounds in the picoline group.

TABLE I.—FLUORESCENCE DATA ON ANTIHISTAMINES

	Antihistamine[g]	H_2O_2 System			CNBr System		
		Ex. mμ	Fl. mμ	Int.	Ex. mμ	Fl. mμ	Int.
I	Chlorothen[e] (Lederle Laboratories)	340	407	44	345	414	160
II	Methapyrilene[b] (Irwin, Neisler Co.)	345	409	86	350	412	361+[a]
III	Pyrilamine[c] (Dorsey Laboratories)	340	408	43	350	419	35
IV	Thenyldiamine[b] (Sterling Winthrop Laboratories)	345	412	96	355	414	369+[a]
V	Tripelennamine[b] (Ciba Pharmaceutical)	345	408	41	355	419	300+[a]
VI	Thonzylamine[b] (Warner-Lambert Laboratories)	330	407	5.4	345	419	0.6
VII	Carbinoxamine[c] (McNeil Laboratories)	335	394	3.6	275	467	0.3
VIII	Chlorpheniramine[c] (Schering Research)	350 360	436 446	4.0 3.7	280	447	3.0
IX	Doxylamine[d] (Wm. S. Merrell Co.)	370 345	449 436	2.8 2.2	280	388	0.1
X	Pheniramine[c] (Dorsey Laboratories)	330 370	352 457	7.2 5.8	275	434	0.4
XI	Antazoline[b] (Ciba Pharmaceutical)	330	415	2.8	350	410	0.6
XII	Chlorcyclizine[b] (Burroughs Wellcome)	345	451	1.5	...	...	...
XIII	Cyclizine[b] (Burroughs Wellcome)	305 335	417 449	1.3 1.0	...	...	...
XIV	Meclizine[f] (Chas. Pfizer & Co.)	310 345	420 444	1.4 1.4	...	...	...
XV	Diphenhydrazine[b] (Parke, Davis and Co.)	305 345	412 454	0.8 0.5	...	...	...
XVI	Promethazine[b] (Wyeth Laboratories)	345	384	6.6	320	449	2.4

[a] +, indicates that the intensity was greater than the instrument could detect using the described procedure and a 6 mμ band pass. [b] Weighed as the hydrochloride salt. [c] Weighed as the maleate salt. [d] Weighed as the succinate salt. [e] Weighed as the citrate salt. [f] Weighed as the dihydrochloride salt. [g] All concentrations are 100 mcg./ml.

The remaining antihistamines all give fluorescent products with hydrogen peroxide. The cyclizines, *N*-substituted pyrazines, are similar fluorometrically to the substituted picolines. Promethazine, an *N*-substituted phenthiazine, appears to fluoresce upon treatment with both hydrogen peroxide and cyanogen bromide. Promethazine hydrochloride undergoes a rapid decomposition in water resulting in a highly colored solution and a definite increase in acidity. It is possible that the observed fluorescence was due to a decomposition product in the reaction mixture.

Four oxidizing agents, potassium bromate, potassium bi-iodate, potassium periodate, and potassium persulfate were investigated in addition to hydrogen peroxide. Hydrogen peroxide provided the highest intensity of fluorescence when thenyldiamine was treated with equivalent amounts of each oxidant. The fluorescence intensity decreased in the order: $KIO_4 > KH(IO_3)_2 > KBrO_3 > K_2S_2O_8$.

Investigations are being continued on the antihistamines with special attention given to the quenching of the cyanogen bromide fluorescence and regeneration using hydrogen peroxide. The quantitative dependence of antihistamine concentration on fluorescence intensity and the effect of hydrogen ion concentration will be studied. Attempts will be made to determine the mechanisms of the reactions.

REFERENCE

(1) Pearlman, E. J., *J. Pharmacol. Exptl. Therap.*, **95**, 465 (1949).

Synthesis of Some Alkyl and Aminoalkyl Esters of Azobenzenedisulfonic Acid

By WILBERT G. WALTER* and TONY E. JONES

Two homologous series of dialkyl esters ranging from dimethyl to dibutyl of *p*- and *m*-azobenzenedisulfonic acid were synthesized for the first time. In addition, the hydrochloride salts of bis-diethylaminoethyl ester of *p*-azobenzene-4:4′-disulfonic acid and bis-2-di-*n*-butylaminoethyl ester of *m*-azobenzene-3:3′-disulfonic acid are reported.

EXCEPT FOR esters, some derivatives of azobenzenedisulfonic acid have been reported. In 1930, some isomeric azobenzenedisulfonchloramides (1) were made for consideration as possible antibacterial agents similar to the chloramine-T type compounds. Later in 1937, azobenzenedisulfonamide (2), with substituted amide groups such as 2-pyridyl and 2-thiazolyl which are known to be associated with sulfonamide drugs, was studied. Also, iodo derivatives of *p*-azobenzenedisulfonamide were prepared for study as radiopaque materials (3).

The purpose of the work reported here was to synthesize and investigate chemically some alkyl and aminoalkyl esters of *p*- and *m*-azobenzenedisulfonic acid. The objective was of interest because compounds having physiological activity might be produced. Azobenzenedisulfonic acid is conceived to undergo reduction to give aminobenzenesulfonic acid, since compounds such as prontosil (2′,4′-diaminoazobenzene-4-sulfonamide) (4) and azobenzene (5) are known to be reduced to give sulfanilamide and aniline, respectively. Aminobenzenesulfonic acid is a chemical isostere of aminobenzoic acid whose esters constitute an important class of local anesthetic compounds; it is also structurally related to the sulfonamide type drugs. By esterifying with alcohols that are found attached to active local anesthetic compounds of aminobenzoic acid—*e.g.*, diethylaminoethanol (procaine), ethanol (benzocaine), propanol (propaesin), *n*-butanol (butesin), compounds with activity of a similar nature might be produced. While these structure-activity relationships can be drawn about the derivatives, the products have not been subjected to bioassay to substantiate these inferences.

A similar method which Stern (1) used to prepare *m*-azobenzene-3:3′-disulfonic acid is also used here to prepare the *para* and *meta* isomers. In 1939, he reported a procedure satisfactory for preparing potassium *m*-azobenzene-3:3′-disulfonate, whereas the *para* isomer was prepared with more difficulty and by a different method—the reaction of fuming sulfuric acid on azobenzene. Although other methods (6) were tried during the course of this work, they gave poor yields or a mixture of azo products. The methods tried included the reduction of sodium nitrobenzenesulfonate with sodium amalgam and the dissolution of azobenzene in fuming sulfuric acid. The reaction of chlorosulfonic acid on azobenzene also proved unsatisfactory for preparing azobenzenedisulfonyl chloride (7).

The procedure employed to prepare the ester derivatives is as follows. Reduction and coupling of sodium nitrobenzenesulfonate (III) using zinc dust and potassium hydroxide to give sodium azobenzenedisulfonate (IV) (8), and conversion of this salt with phosphorus pentachloride to the acid dichloride (II), followed by esterification with the respective alcohol in anhydrous benzene.

Because an individual discussion on the preparation of each ester or isomeric compound would involve duplication, it was decided to describe in detail the preparation of four compounds representative of the group: sodium *p*-azobenzene-4:4′-disulfonate, *p*-azobenzene-4:4′-disulfonyl chloride, bis-(ethyl)-*p*-azobenzene-4:4′-disulfonate, and bis(diethylaminoethyl)-*p*-azobenzene-4:4′-disulfonate. References leading to compounds related to this work are given in Table I.

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Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

* Present address: School of Pharmacy, University of Mississippi, University.

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The purpose of the work reported here was to synthesize and investigate chemically some alkyl and aminoalkyl esters of *p*- and *m*-azobenzenedisulfonic acid. The objective was of interest because compounds having physiological activity might be produced. Azobenzenedisulfonic acid is conceived to undergo reduction to give aminobenzenesulfonic acid, since compounds such as prontosil (2′,4′-diaminoazobenzene-4-sulfonamide) (4) and azobenzene (5) are known to be reduced to give sulfanilamide and aniline, respectively. Aminobenzenesulfonic acid is a chemical isostere of aminobenzoic acid whose esters constitute an important class of local anesthetic compounds; it is also structurally related to the sulfonamide type drugs. By esterifying with alcohols that are found attached to active local anesthetic compounds of aminobenzoic acid—*e.g.*, diethylaminoethanol (procaine), ethanol (benzocaine), propanol (propaesin), *n*-butanol (butesin), compounds with activity of a similar nature might be produced. While these structure-activity relationships can be drawn about the derivatives, the products have not been subjected to bioassay to substantiate these inferences.

A similar method which Stern (1) used to prepare *m*-azobenzene-3:3′-disulfonic acid is also used here to prepare the *para* and *meta* isomers. In 1939, he reported a procedure satisfactory for preparing potassium *m*-azobenzene-3:3′-disulfonate, whereas the *para* isomer was prepared with more difficulty and by a different method—the reaction of fuming sulfuric acid on azobenzene. Although other methods (6) were tried during the course of this work, they gave poor yields or a mixture of azo products. The methods tried included the reduction of sodium nitrobenzenesulfonate with sodium amalgam and the dissolution of azobenzene in fuming sulfuric acid. The reaction of chlorosulfonic acid on azobenzene also proved unsatisfactory for preparing azobenzenedisulfonyl chloride (7).

The procedure employed to prepare the ester derivatives is as follows. Reduction and coupling of sodium nitrobenzenesulfonate (III) using zinc dust and potassium hydroxide to give sodium azobenzenedisulfonate (IV) (8), and conversion of this salt with phosphorus pentachloride to the acid dichloride (II), followed by esterification with the respective alcohol in anhydrous benzene.

Because an individual discussion on the preparation of each ester or isomeric compound would involve duplication, it was decided to describe in detail the preparation of four compounds representative of the group: sodium *p*-azobenzene-4:4′-disulfonate, *p*-azobenzene-4:4′-disulfonyl chloride, bis-(ethyl)-*p*-azobenzene-4:4′-disulfonate, and bis(diethylaminoethyl)-*p*-azobenzene-4:4′-disulfonate. References leading to compounds related to this work are given in Table I.

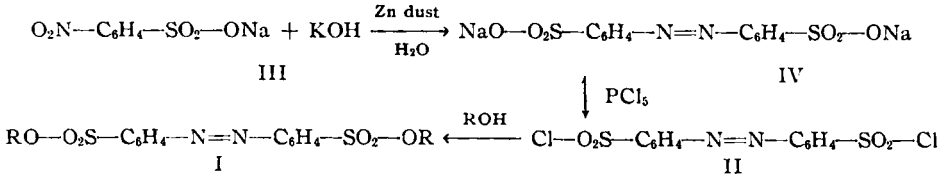
Received May 6, 1963, from the School of Pharmacy, University of Colorado, Boulder.

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Abstracted in part from a thesis submitted by Wilbert G. Walter to the Graduate School, University of Colorado, Boulder, in partial fulfillment of Master of Science degree requirements.

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R = alkyl or aminoalkyl group.

EXPERIMENTAL

Sodium p-Azobenzene-4:4′-disulfonate.—Sodium *p*-nitrobenzenesulfonate (100 Gm.) and potassium hydroxide (140 Gm.) were dissolved in distilled water (1000 ml.) and the solution filtered. On the addition of potassium hydroxide, the temperature increased to 97°, and the solution turned a dark orange. While the mixture was being stirred mechanically and maintained at 103–104°, six portions (15 Gm. each) of zinc dust were added in 15-minute intervals until the solution became colorless. Before each new portion of zinc dust was added, the progress of the reduction was followed by "spotting" the reaction mixture on filter paper. A regular grading of the color, from a faint yellow (after the first addition of zinc dust) to orange (azoxy condition) until colorless (hydrazo condition), was observed.

The zinc residues were removed by filtration, washed with two 50-ml. portions of hot distilled water, and the washings added to the filtrate. The hydrazo compound oxidized rapidly to the azo compound on exposure to air as observed when the solution became deeply colored. The reaction was aided by raising the temperature to 80–90° and mechanically stirring for 3 hours or until the color had become constant (dark reddish orange). The completeness of the color change could be followed by colorimetric comparison.

The temperature of the solution was raised to 85° and carbon dioxide passed through the mixture for 2 hours. A white precipitate was formed, which was removed by filtration; the filtrate was concentrated and chilled. Small orange crystals (32 Gm.) of sodium *p*-azobenzene-4:4′-disulfonate were collected. Further concentration of the solution yielded more of the product.

Purification was accomplished by dissolving the crystals in a solution consisting of 25 ml. of water and 100 ml. of ethanol and recrystallizing. Yield, 78 Gm. (91.2%). (See *References* in Table I.)

p-Azobenzene-4:4′-disulfonyl Dichloride.—Anhydrous sodium *p*-azobenzene-4:4′-disulfonate (10 Gm.) was mixed with phosphorus pentachloride (20 Gm.) and heated to a temperature of 190° for 35 minutes. Partial softening, then liquefaction of the mass occurred after 10 minutes. The mass was stirred at intervals with a glass rod to insure a complete reaction. After the mass developed a constant dark red, it was slowly poured over ice while several small quantities of ice water were added to the reaction flask. Crude *p*-azobenzene-4:4′-disulfonyl dichloride that formed was broken up, isolated, and washed with ice cold distilled water until free of soluble materials, then dried, and melted at 217–218° uncorrected. The dichloride compound could be recrystallized from most organic solvents; benzene, ligroin, and ether were used. Crystallization from benzene yielded a product melting at 219–220° uncorrected. Yield, 8.9 Gm. (90.8%). (See *References* in Table I.)

Bis-(ethyl)-p-azobenzene-4:4′-disulfonate.—*p*-Azobenzene-4:4′-disulfonyl dichloride (1 Gm.) was dissolved in anhydrous benzene (140 ml.) and absolute ethanol (25 ml.). The solution was refluxed for 30 minutes, then evaporated under reduced pressure to one-half of its original volume and chilled. The orange crystalline flakes (0.53 Gm.) obtained by filtration were air dried. Further evaporation of solvent yielded more product (0.20 Gm.) which melted at 155–156° uncorrected. Drying under reduced pressure in the presence of a drying agent did not alter the melting point of the ester product. Yield, 0.73 Gm. (69.5%).

Anal.—Calcd. for $C_{16}H_{18}N_2O_6S_2$: C, 48.24; H, 4.51. Found: C, 47.84; H, 4.47.

Bis-(diethylaminoethyl)-p-azobenzene-4:4′-disulfonate.—*p*-Azobenzene-4:4′-disulfonyl dichloride (2 Gm.) was dissolved in anhydrous benzene (350

TABLE I.—DERIVATIVES OF AZOBENZENEDISULFONIC ACID R—O_2S—C_6H_4—N=N—C_6H_4—SO_2—R

R Group	Isomer	M.p., °C. Uncorrected	Formula	Calcd., % C	Calcd., % H	Found, % C	Found, % H	Ref.
NaO—	*para*	...	$C_{12}H_8N_2Na_2O_6S_2$	...	...	...	...	(1, 3, 8–11, 15–17)
Cl—	*para*	219–220°	$C_{12}H_8Cl_2N_2O_4S_2$	...	...	...	...	(1, 7, 15, 17)
CH_3O—	*para*	201.5 to 202°	$C_{14}H_{14}N_2O_6S_2$	45.47	3.78	45.10	3.89	...
CH_3CH_2O—	*para*	155–156°	$C_{16}H_{18}N_2O_6S_2$	48.24	4.51	47.84	4.47	...
$CH_3CH_2CH_2O$—	*para*	161–162°	$C_{18}H_{22}N_2O_6S_2$	50.70	5.16	50.78	5.27	...
$CH_3(CH_2)_3O$—	*para*	156–157°	$C_{20}H_{26}N_2O_6S_2$	52.84	5.76	52.20	5.66	...
$(CH_3CH_2)_2$-NCH$_2$CH$_2$O— HCl	*para*	183.5 to 184°	$C_{24}H_{38}Cl_2N_2O_6S_2$	46.99	5.89	47.43	6.51	...
NaO—	*meta*	...	$C_{12}H_8N_2Na_2O_6S_2$	...	...	...	...	(1, 8, 12–14)
Cl—	*meta*	164–166°	$C_{12}H_8Cl_2N_2O_4S_2$	...	...	...	...	(1, 8, 12)
CH_3O—	*meta*	136–137°	$C_{14}H_{14}N_2O_6S_2$	45.47	3.78	45.09	3.77	...
CH_3CH_2O—	*meta*	102–103°	$C_{16}H_{18}N_2O_6S_2$	48.24	4.51	47.82	4.63	(8) ...
$CH_3CH_2CH_2O$—	*meta*	79–80°	$C_{18}H_{22}N_2O_6S_2$	50.70	5.16	50.61	5.20	...
$CH_3(CH_2)_3O$—	*meta*	75–76°	$C_{20}H_{26}N_2O_6S_2$	52.84	5.76	52.70	5.68	...
$[CH_3(CH_2)_3]_2$-NCH$_2$CH$_2$O— HCl	*meta*	154–155°	$C_{32}H_{56}Cl_2N_2O_6S_2$	52.98	7.44	53.37	7.60	...

ml.). The solution was warmed to 65°, and diethylaminoethanol (1.24 Gm.) in anhydrous benzene (50 ml.) was slowly added dropwise; no visible reaction was observed. The reaction mixture was refluxed for 4 hours at 65°. After this time, the hydrochloride salt which had precipitated was removed by filtration, washed with two 50-ml. portions of anhydrous benzene, and dissolved in absolute methanol (100 ml.). Anhydrous ether was added until the solution became slightly cloudy; the solution was then stored in a refrigerator (6°) for 24 hours. The product was recrystallized twice using the above procedure. The reddish orange crystals which formed (1.03 Gm.) melted at 184–185° uncorrected. The product was not subjected to further purification. After drying under reduced pressure in the presence of a drying agent, the product melted at 184–185° uncorrected. Yield, 1.02 Gm. (25%).

Anal.—Calcd. for $C_{24}H_{36}N_2O_6S_2$: C, 46.99; H, 5.89. Found: C, 47.43; H, 6.51.

CONCLUSION

The synthesis and chemical investigation of some alkyl and aminoalkyl esters of *p*- and *m*-azobenzenedisulfonic acid have been reported. Two homologous series of dialkyl esters ranging from dimethyl to dibutyl and the hydrochloride salts of bis-(2-di - *n* - butylaminoethyl) - *m* - azobenzene - 3:3′-disulfonate and bis-(diethylaminoethyl)-*p*-azobenzene-4:4′-disulfonate are reported. Discussion and references leading to the preparation of *p*- and *m*-azobenzenedisulfonic acids and their chlorides, metallic salt, and amide derivatives are also presented. The compounds were not subjected to pharmacological evaluation.

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By J. THURØ CARSTENSEN

Data presented show that the logarithm of the pseudo first-order rate constant for the degradation of vitamin A when moisture is abundantly present is linearly related to the water vapor pressure.

IT HAS BEEN established by us through numerous experiments that vitamin A in oil solution, when stored under an inert atmosphere, follows (*a*) a pseudo first-order degradation scheme and (*b*) lends itself to conventional Arrhenius plotting according to Garrett (1). This is exemplified in Figs. 1 and 2.

It has also been established that vitamin A acetate and palmitate encased in gelatin, acacia, and like substances, upon tableting into conventional uncoated tablets and/or chewable tablets, follow a similar pattern, provided that extrusion is allowed for. Examples of this are shown in Fig. 3.

While these facts are easily explained on a thermodynamical basis, *we have encountered the somewhat surprising phenomenon that in the case where moisture is abundantly present, the logarithm of the pseudo first-order rate constant* (k) *is linearly related to the water vapor pressure* (p). This holds true for water-micelle systems of vitamin A palmitate and also vitamin A acetate and palmitate beadlets in sugar coated tablets (see Figs. 4 and 5). To verify the better fit of a straight line (*e.g.*, the data in Fig. 4), the least square fit lines were drawn (*a*) with respect to $y = \log k = f(1/T)$ (T being absolute temperature) and (*b*) $y = \log k = f(p)$. The sum $\Sigma(y_i - \alpha x_i - \beta)^2 = S^2$ is not, in this case, of good comparative measurement because of the different abscissa; however, the relative standard deviation of the slopes (α) calculated in cases (*a*) and (*b*) give relative comparisons of which abscissa gives the best linear fit.

The values $S_\alpha/\alpha\,(1/T) = 0.485/3.463 = 0.14$ and $S_\alpha/\alpha\,(P) = 0.000479/0.0132 = 0.036$ demonstrate numerically what is obvious to visual inspection. The important qualitative fact is that in the case of $f\,(1/T)$ the intermediate points lie below the terminal line segment, whereas in case of $f\,(p)$ the points scatter, implying curvature for $f\,(1/T)$ but not for $f\,(p)$. There is no ready thermodynamic explana-

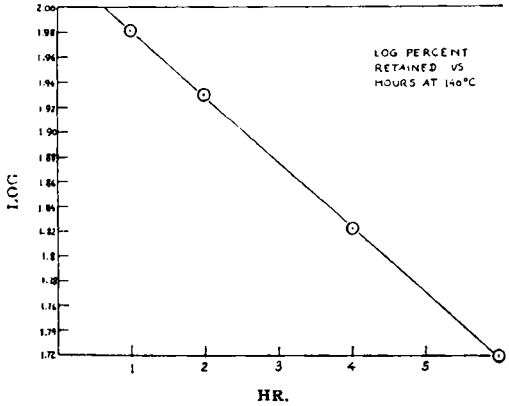


Fig. 1.—Vitamin A alcohol in Tween-Drew oil base, 500,000 units/ml., stored under nitrogen in glass bottles. Plot shows per cent potency retained as a function of hours at 140° C.

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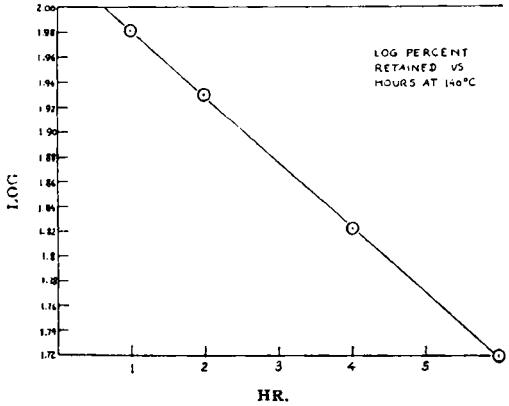


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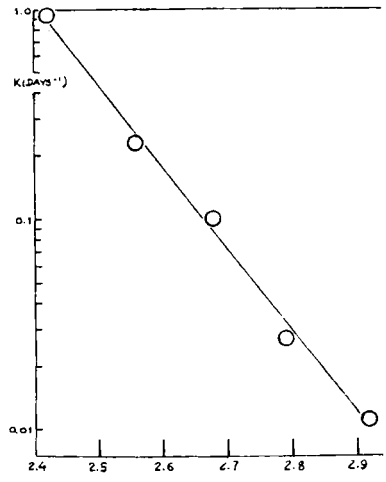


Fig. 2.—Vitamin A alcohol in Tween-Drew oil base. Plot shows pseudo first-order rate constants as a function of reciprocal absolute temperature (reciprocal °Kelvin).

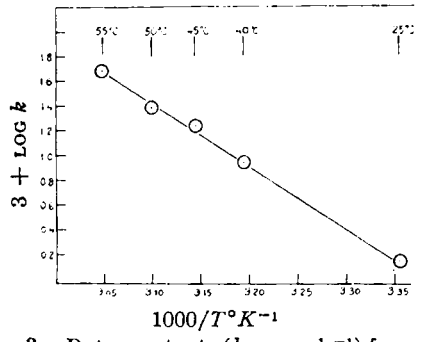


Fig. 3.—Rate constants (k = week^{-1}) for pseudo first-order rate constants of vitamin A palmitate beadlets in dry-slugged, mannitol-base, multivitamin chewable tablet. Log k is plotted against reciprocal absolute temperature.

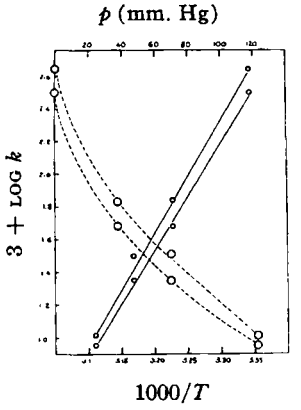


Fig. 4.—Plots of the pseudo first-order rate constant (k = month^{-1}) for vitamin A palmitate beadlets in sugar - coated tablets. Dotted lines are regular Arrhenius plots ($1/T$ scale); full lines are log k *vs.* water vapor pressure (p-scale).

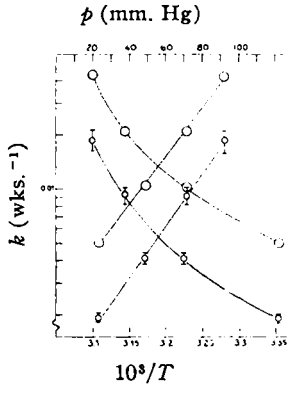


Fig. 5.—Plots of the pseudo first-order rate constants (k) for vitamin A palmitate oil in a multivitamin drop (Tween, micelle, glycerin, water). Dotted lines are regular Arrhenius plots ($1/T$-scale); full lines are log k *vs.* water vapor pressure (p-scale). Two different formulations (same vitamin A raw material) are shown.

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The more deep rooted explanations to this will be the subject of subsequent publications.

REFERENCE

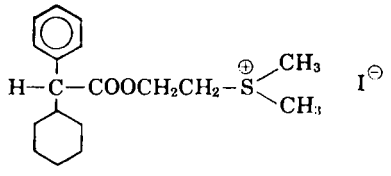
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β-(α-Phenyl-α-3-thenylacetoxy) Ethyldimethylsulfonium Bromide as a Potential Antispasmodic

By HEINO A. LUTS*, W. A. ZUCCARELLO‡, J. F. GRATTAN‡, and W. LEWIS NOBLES

A comparative structure-activity evaluation was made to determine the potentiality of 3-methylthiophene as a possible active isoster of an agent which has demonstrated marked antispasmodic activity.

In 1953, Protiva and Exner (1) reported on the spasmolytic activity of the sulfonium compound, thiospasmin.



Since that time, several structural modifications have been made. Neesby and his associates (2) in this country, utilizing the same sulfonium moiety in several model compounds, found more favorable therapeutic ratios for these derivatives. It has been suggested that a structure-activity study involving thiophene analogs should include the 3-substituted

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* Present address: Structure-Activity Research, Inc., Oxford, Miss.
‡ Pharmacological studies were made by these authors at Smith, Miller and Patch, Inc., New York, N. Y.

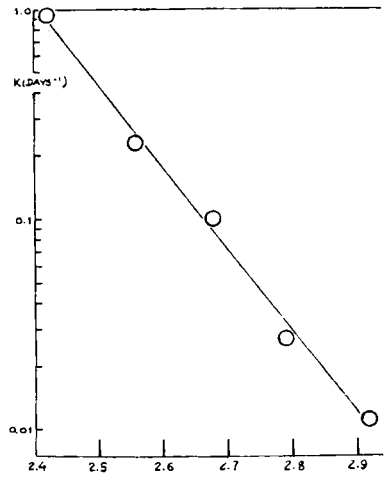


Fig. 2.—Vitamin A alcohol in Tween-Drew oil base. Plot shows pseudo first-order rate constants as a function of reciprocal absolute temperature (reciprocal °Kelvin).

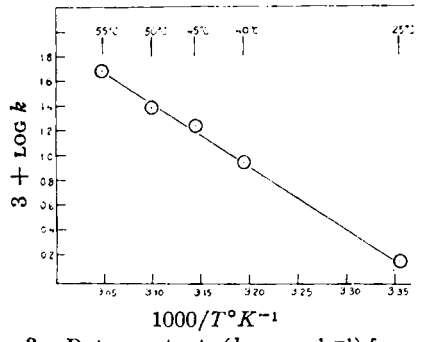


Fig. 3.—Rate constants (k = week^{-1}) for pseudo first-order rate constants of vitamin A palmitate beadlets in dry-slugged, mannitol-base, multivitamin chewable tablet. Log k is plotted against reciprocal absolute temperature.

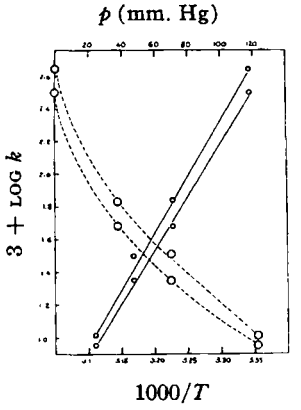


Fig. 4.—Plots of the pseudo first-order rate constant (k = month^{-1}) for vitamin A palmitate beadlets in sugar - coated tablets. Dotted lines are regular Arrhenius plots ($1/T$ scale); full lines are log k *vs.* water vapor pressure (p-scale).

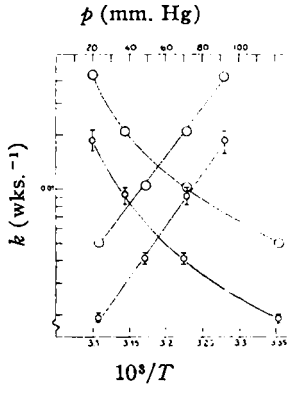


Fig. 5.—Plots of the pseudo first-order rate constants (k) for vitamin A palmitate oil in a multivitamin drop (Tween, micelle, glycerin, water). Dotted lines are regular Arrhenius plots ($1/T$-scale); full lines are log k *vs.* water vapor pressure (p-scale). Two different formulations (same vitamin A raw material) are shown.

tion for this. Attempts to relate (a) the absolute temperature to the water vapor pressure in the Arrhenius equation or (b) relate the water activity to the water vapor pressure lead to equations proportionalizing log k to log p not p.

The more deep rooted explanations to this will be the subject of subsequent publications.

REFERENCE

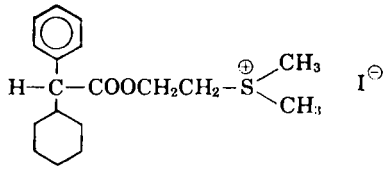
(1) Garrett, E. R., This Journal, 51, 811(1962).

β-(α-Phenyl-α-3-thenylacetoxy) Ethyldimethylsulfonium Bromide as a Potential Antispasmodic

By HEINO A. LUTS*, W. A. ZUCCARELLO‡, J. F. GRATTAN‡, and W. LEWIS NOBLES

A comparative structure-activity evaluation was made to determine the potentiality of 3-methylthiophene as a possible active isoster of an agent which has demonstrated marked antispasmodic activity.

In 1953, Protiva and Exner (1) reported on the spasmolytic activity of the sulfonium compound, thiospasmin.



Since that time, several structural modifications have been made. Neesby and his associates (2) in this country, utilizing the same sulfonium moiety in several model compounds, found more favorable therapeutic ratios for these derivatives. It has been suggested that a structure-activity study involving thiophene analogs should include the 3-substituted

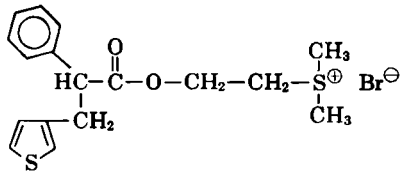
Received April 12, 1963, from the School of Pharmacy, University of Mississippi, University.
Accepted for publication September 6, 1963.
Abstracted from a thesis submitted by Heino A. Luts to the Graduate School, University of Mississippi, in partial fulfillment of Doctor of Philosophy degree requirements.
* Present address: Structure-Activity Research, Inc., Oxford, Miss.
‡ Pharmacological studies were made by these authors at Smith, Miller and Patch, Inc., New York, N. Y.

thiophene analog (3, 4). It is the purpose of this paper to present data on the synthesis of the latter compound and an evaluation of its biological activity.

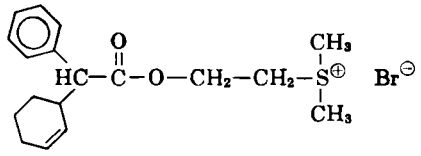
The sulfonium salt which serves as the basis for this paper was prepared by reacting phenyldiethylmalonate (I) with 3-thenylbromide (II) in ethanol in the presence of sodium. The compound thus formed (III) was then decarboxylated to yield a substance (IV); this was subsequently reacted with β-chloroethylmethylsulfide (5) (V), yielding the compound (VI). The quaternary salt of this agent was formed with methyl bromide to yield the compound (VII).

PHARMACOLOGY

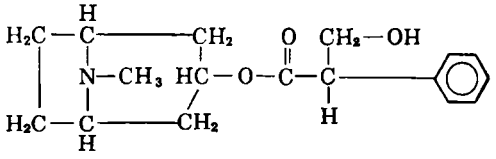
The antispasmodic activity of the 3-methylthiophene analog was compared with that of the cyclohexenyl derivative and of atropine. The findings, together with the formulas of each, are presented below.



Compound VII.—β-(α-Phenyl-α-3-thenylacetoxy) Ethyldimethyl sulfonium Bromide



CDS-216.—Dimethylsulfonium Ethyl-α-phenyl-α-(2,3-cyclohexenyl) Acetic Acid Bromide (2)

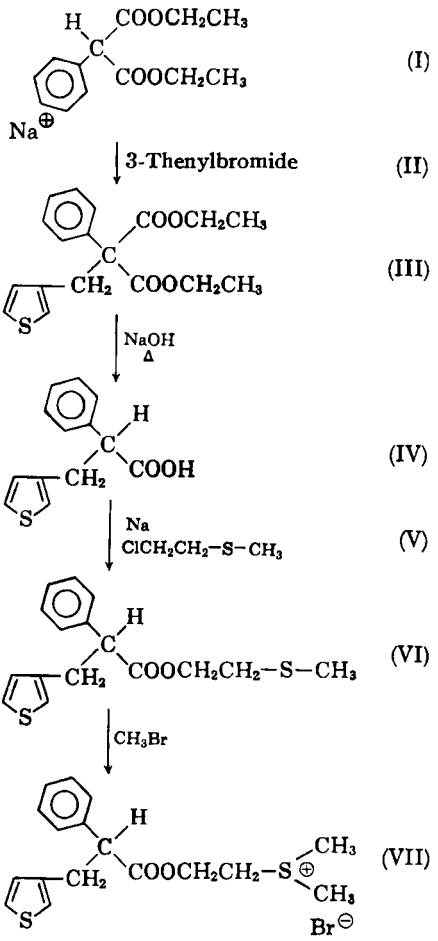


Atropine

PHARMACOLOGIC STUDIES

Acute Toxicity.—The median lethal dose (LD_{50}) of compound VII, when administered intraperitoneally in mice, was estimated to be 93 mg./Kg. The LD_{50} of dimethylsulfonium ethyl-α-phenyl-α-(2,3-cyclohexenyl) acetic acid bromide (hereafter designated CDS-216) was 72 mg./Kg. Mice receiving toxic doses of compound VII exhibited tremors, mydriasis, increased respiratory rate, and clonic convulsions. Death appeared to result from respiratory arrest.

In Vitro Spasmolytic Action—Isolated Rabbit Ileal Segment.—The ability of compound VII to antagonize the spasmogenic effect of acetylcholine was only one-twentieth that of CDS-216 or atropine. It would appear that substitution of the cyclohexenyl group of CDS-216 with methylthiophene was followed by a marked reduction in antispasmodic activity. Like atropine, compound VII reduced both the tonus and amplitude of contraction of normal smooth muscle.



General Scheme of Synthesis

Antagonism of the Effects of Carbaminoylcholine (Carbachol) or Faradization of the Peripheral End of the Sectioned Right Vagus Nerve.—Compound VII, when administered intravenously in pentobarbitalized rabbits at dose levels of 0.5 to 1.0 mg./Kg., affected a slight, transient reduction in the hypermotility of the ileum, *in situ*, induced by carbachol (6). These dose levels of compound VII prevented the cardiac slowing following stimulation of the vagus nerve. Again, the effect was evanescent in nature. In the main, compound VII possessed only one-tenth to one-twentieth the activity of either CDS-216 or atropine in these tests.

Tremorine Antagonism.—Since the antitremorine activity of many compounds parallels their cholinolytic action, the ability of compound VII to prevent the tremors and/or lacrimation following tremorine administration was examined (7). Whereas atropine and CDS-216 prevented both the tremors and the lacrimation at dose levels below 1 mg./Kg., compound VII did not have effect on the tremors when given at dose levels as high as 40 mg./Kg., although lacrimation could be prevented at dose levels of 10 mg./Kg. and above. Substitution of the methylthiophene with the cyclohexenyl moiety was fol-

lowed by increased ability to antagonize tremors and lacrimation induced by tremorine.

Inhibition of the Chromodacryorrhea Induced by Acetyl-β-methylcholine (Mecholyl).—Compound VII antagonized the action of Mecholyl on the parasympathetic effector cells of the accessory lacrimal glands in the rat. The median protective dose (PD_{50}) of compound VII was 1.4 mg./Kg., while that for atropine was shown to be 0.3 mg./Kg. In this test, compound VII was approximately one-fifth as active as atropine, but only slightly less active than CDS-216 (PD_{50} of 0.9 mg./Kg.).

Mydriatic Action.—Compound VII, when applied topically to the cornea of the rabbit, did not have effect on pupillary diameter at concentrations to 1.0%. CDS-216, at a concentration of 1.0%, caused only slight mydriasis; atropine, at a concentration of 0.5%, produced maximal pupillary dilation.

Effect on Hexobarbital Hypnosis.—When administered at several dose levels, compound VII did not alter either the time of induction or the duration of sleep produced by hexobarbital.

Other Pharmacologic Actions.—Compound VII was devoid of significant analgesic activity and possessed no local anesthetic action. It exerted neither an adrenolytic effect nor a potentiating action on the pressor response to *l*-epinephrine, and did not antagonize the vasodepressor action of histamine. At the dose levels employed, compound VII appeared to potentiate the action of serotonin on the isolated rat uterus.

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α-Phenyl-α-3-thenylacetic Acid.—This agent was prepared by standard malonic ester synthesis, namely by refluxing 24.1 Gm. (0.1 mole) of the sodium salt of phenyldiethylmalonate with 17.6 (0.1 mole) of 3-thenylbromide (8) in ethanol. After refluxing for 12 hours, the reaction mixture was filtered and the solvent evaporated. The residue was then saponified with alcoholic KOH and the alcohol evaporated and the residue acidified with hydrochloric acid. The resulting mixture was then extracted twice with 100 ml. of benzene and dried over potassium carbonate. After filtering and evaporating the benzene, the residue was heated in high-vacuum to yield a semisolid. Recrystallization of this material from benzene-petroleum ether gave crystals melting at 93°; yield, 38%.

Anal.—Calcd. for $C_{13}H_{12}O_2S$: C, 67.25; H, 5.24; S, 13.80. Found: C, 67.32; H, 5.27; S, 13.72.

β-(α-Phenyl-α-3-thenylacetoxy) Ethyldimethylsulfonium Bromide.—Ten grams (0.04 mole) of α-phenyl-α-3-thenylacetic acid was reacted with 0.92 Gm. (0.04 mole) of sodium in 75 ml. of isopropanol. This was then refluxed for 30 minutes and 7.0 Gm. (0.06 mole) of β-chloroethylmethylsulfide added over a period of 30 minutes with good stirring. The reaction mixture was then refluxed for 24 hours; the salt formed was separated by filtration and the solvent removed by distillation. The residue was dissolved in 20 ml. of methanol and 15 Gm. (0.16 mole) of liquid methylbromide added. The reaction mixture was then allowed to stand at room temperature for 5 days in a sealed container. After boiling away both the methanol and the unreacted methylbromide, the residue was triturated with anhydrous ether. A crystalline product was formed in 72% yield; m.p. 100–101°.

Anal.—Calcd. for $C_{17}H_{21}BrO_2S_2$: C, 50.87; H, 5.27; Br, 19.19; S, 15.98. Found: C, 50.72; H, 5.36; Br, 20.10; S, 15.71.

SUMMARY AND CONCLUSIONS

Substitution of the cyclohexenyl group of compound CDS-216 with a methylthiophene grouping affected adversely the antispasmodic activity and did not alter the toxicity appreciably.

REFERENCES

(1) Protiva, K., and Exner, F., Czechoslovakian pat. 83,205 (February 3, 1955).
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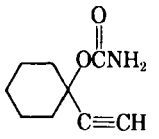
Identification of Ethinamate, Ethchlorvynol, and Methylparafynol

By W. N. FRENCH

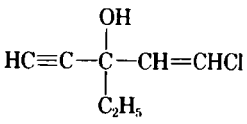
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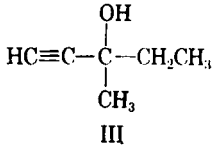
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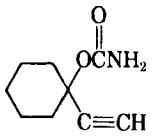
Identification of Ethinamate, Ethchlorvynol, and Methylparafynol

By W. N. FRENCH

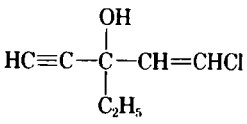
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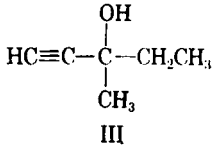
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III

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TABLE I.—2,4-DINITROPHENYLHYDRAZONE DERIVATIVES

Parent Compd.	Crude Product Yield, %	Crude Product M.p., °C.	Crystallized Product M.p., °C.	Crystallized Product $\lambda_{max.}$ (EtOH)	Crystallized Product ϵ[d]
Ethinamate	50	196–199	204–205[a]	373	26,800[b]
Ethchlorvynol	70	119–121	123–124	375	32,800
Methylparafynol	35	180–185	195–196[c]	370	26,500[b]

[a] Reported, m.p. 204° (9) and 199–201° (10). [b] Reported value, $\lambda_{max.}$ at 377 mμ, ϵ = 26,000 (9). [c] Reported, m.p. 198.5° (9). [d] In this table and subsequent discussion, ϵ is defined as the molecular extinction coefficient, *i.e.*, the absorbance of a 1 *M* solution.

acetylenic group (1), by chromatographic (2–4) and crystallographic (5) techniques, and by derivative formation (6). The infrared spectrum of ethchlorvynol has been published (7), and methylparafynol has been characterized by derivative formation (8, 9).

This paper describes some procedures permitting the identification of the above compounds both in pure form and in commercial preparations.

EXPERIMENTAL

2,4-Dinitrophenylhydrazine Reagent.—Slowly add 25 ml. of water to a solution of 2.0 Gm. of 2,4-dinitrophenylhydrazine in 20 ml. of sulfuric acid while gently swirling and cooling the flask contents in a water bath. Dilute with 50 ml. of alcohol.

Preparation of 2,4-Dinitrophenylhydrazone Derivatives.—Dissolve 100 mg. of the acetylenic compound in 10 ml. of 2,4-dinitrophenylhydrazine reagent and place in a water bath at 50–55° for 4 minutes (ethchlorvynol), 1 hour (ethinamate), or 3 hours (methylparafynol). Remove the solution and let stand at room temperature for 30 minutes. Filter off the reaction product with suction and wash with 70% ethanol. Crystallize the derivative from ethyl acetate (ethinamate) or from ethanol (methylparafynol and ethchlorvynol). The results are shown in Table I.

Preparation of *p*-Nitrobenzoate Esters of Ethchlorvynol and Methylparafynol.—Add 1 *M* equivalent of *p*-nitrobenzoic acid (for ethchlorvynol, 230 mg.; for methylparafynol, 340 mg.) to a solution of 200 mg. of the acetylenic carbinol in 5 ml. of dry pyridine and warm the mixture on a steam bath to dissolve the carboxylic acid. Add 2 *M* equivalents of *p*-toluenesulfonyl chloride (for ethchlorvynol, 525 mg.; for methylparafynol, 780 mg.) and heat the solution on a steam bath for 40 minutes. Cool the solution, and pour with stirring into 50 ml. of ice-water mixture. Filter off the brown solid, wash with water, and crystallize from aqueous alcohol. Ethchlorvynol yields 0.35 Gm. (87%) of reaction product, m.p. 87.5 to 88.0°. On recrystallization, the melting point is raised to 89.0 to 89.5°. Methylparafynol yields 0.425 Gm. (84%) of product, m.p. 64–66°. On recrystallization, the melting point is raised to 71.0 to 71.5° [reported, m.p. 72° (8)].

Preparation of 3-Ethyl-2-penten-4-ynal.—Stir a solution of freshly distilled ethchlorvynol (b.p. 72–73°/10 mm.) in 15 ml. of acid reagent (prepared by mixing 25 ml. of water, 20 ml. of sulfuric acid, and 50 ml. of ethanol) under nitrogen at 40° for 5 minutes. A yellow oil separates after about 3 minutes. Continue stirring at room temperature for 15 minutes, whereafter the mixture has become deep red. Pour into 40 ml. of ice-water and extract with petroleum ether (30–60°). Wash the extract with 5% sodium bicarbonate solution, dry over anhydrous magnesium sulfate, and evaporate under reduced pressure. Distillation of the residual oil under reduced pressure yields 2.62 Gm. (88%) of a pale yellow liquid, b.p. 48.5 to 49.5°/7 mm., η_D^{25} = 1.4851, which darkens on exposure to light and air.

The infrared spectrum of the compound in carbon tetrachloride shows absorption peaks at 3300 cm.$^{-1}$ (≡CH), 2740 cm.$^{-1}$ (—CHO), 2100 cm.$^{-1}$ (—C≡C—), 1675 cm.$^{-1}$ (unsaturated carbonyl), and 1600 cm.$^{-1}$ (conjugated double bond). The ultraviolet spectrum in ethanol shows broad absorption in the 235–260 mμ region ($\epsilon \sim 5500$) with $\lambda_{max.}$ at 240 mμ, ϵ = 5900.

Treatment of the above product with 2,4-dinitrophenylhydrazine in acidic (HCl) alcohol yields a derivative identical to that obtained directly from ethchlorvynol.

Anal.—Calcd. for $C_{13}H_{12}N_4O_4$: C, 54.14; H, 4.20; N, 19.43. Found: C, 54.59; H, 4.26; N, 18.87.

Treatment of the product with semicarbazide in aqueous alcohol yields a semicarbazone derivative, m.p. 179.5° dec.

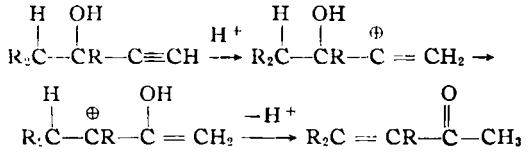
Anal.—Calcd. for $C_8H_{11}N_3O$: C, 58.16; H, 6.71; N, 25.43. Found: C, 57.80; H, 6.90; N, 25.61.

DISCUSSION

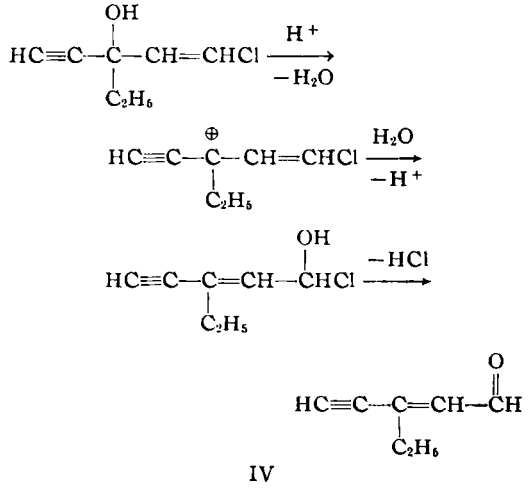
The acid catalyzed rearrangement of *tertiary*-acetylenic carbinols to α,β-unsaturated ketones is known as the Rupe reaction. The reaction appears to proceed either by a dehydration-hydration mechanism or by a straight acid-catalyzed rearrangement, depending upon reaction conditions (10–12).

TABLE II.—HOMOLOGS OF 2-PENTEN-4-YNAL

	$HC\equiv C-C(=O)-CH=CH-CH_2$ (14)	$HC\equiv C-C(=O)-CH=CH-CH_3$ (13)	$HC\equiv C-C(=O)-CH=CH-C_2H_5$
M.p. or b.p.	m.p. 19°	b.p. 53–54°/20 mm.	b.p. 48–49°/7 mm.
U. V. Spectrum $\lambda_{max.}$ (mμ); ϵ (EtOH)	257.5 18,000	261.5 12,500	240 5,900
2,4-Dinitrophenylhydrazone, m.p.	150° dec.	145°	125°
U. V. Spectrum $\lambda_{max.}$ (mμ); ϵ	...	385 33,500	375 32,800
Semicarbazone, m.p.	...	180° dec.	179° dec.
U. V. Spectrum $\lambda_{max.}$ (mμ); ϵ	...	292 32,500	289 31,000



Using an acidic 2,4-dinitrophenylhydrazine reagent (9), rearrangement and derivative formation occurs in one step. Ethinamate and methylparafynol react as expected to yield a derivative of the corresponding α,β-unsaturated methyl ketone. With ethchlorvynol, however, rearrangement to a carbonyl compound seems to proceed through an allylic 1,3-shift of the hydroxyl group with subsequent loss of hydrogen chloride leaving the acetylenic group intact



Analytical and spectral data are in agreement with structure IV. The mode of formation of the compound is analogous to that of 3-methyl-2-penten-4-ynal from 3-methyl-1-chloro-1-penten-4-yn-3-ol by allylic rearrangement (13). The properties of compound IV are compared with those of its homologs in Table II.

Using *p*-nitrobenzoic acid and *p*-toluenesulfonyl chloride in pyridine (8, 15), the tertiary alcohols are readily esterified to form the corresponding derivatives in excellent yield. With ethchlorvynol, yields of about 85% are obtained after heating the reaction solution on a steam bath for 40 minutes or more. Shorter reaction times of 10, 20, and 30 minutes give crude yields of 34, 52, and 72%, respectively.

The above procedures were used successfully for the identification of ethinamate and ethchlorvynol in pharmaceutical preparations. Ethinamate can be readily isolated from tablets by chloroform extraction, removal of the solvent, and crystallization of the residue from petroleum ether. Identification is then made by melting point determination and infrared analysis and confirmed by preparation of the 2,4-dinitrophenylhydrazone derivative. Ethchlorvynol is identified by dissolving the contents of one capsule in a small amount of alcohol and treating with 2,4-dinitrophenylhydrazine reagent. Alternatively, the contents of one capsule may be dissolved in chloroform, the chloroform solution washed with water, dried with anhydrous magnesium sulfate, and evaporated under reduced pressure. The residue readily forms the *p*-nitrobenzoate derivative.

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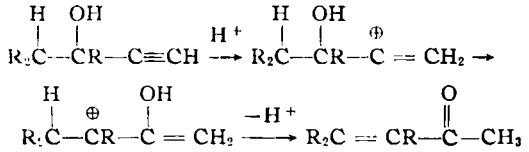
Communications

Free 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one in Maize

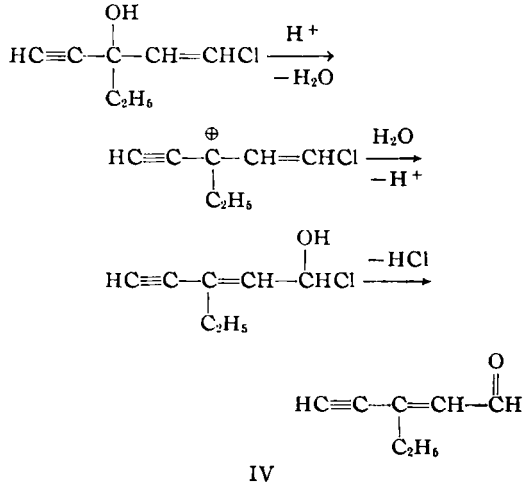
Sir:

Since the discovery that methoxybenzoxazolinone (MBOA) is formed during the extractive treatment of wheat or maize plants (1) from 2,4-dihydroxy - 7 - methoxy - 1,4 - benzoxazin - 3 - one (aglucone), which is enzymatically released from its glucoside when the fresh plant tissue is disintegrated, the presence or absence of MBOA in uninjured maize tissue has been a matter of controversy between Beck *et al.* (2) and ourselves. We found no MBOA when the enzymes of the maize seedlings (Early Albert and a sugar maize variety, grown in a greenhouse) were destroyed by immersing the intact plants in boiling water or in cold alcohol (1). Only small amounts of free aglucone were present in 1 to 2-month-old plants, and free aglucone could not be detected in young seedlings.

The large amounts of MBOA found by Beck *et al.* (2) would imply a qualitative difference in



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Communications

Free 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one in Maize

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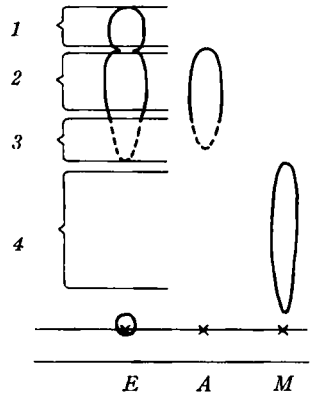


Fig. 1.—Solvent 2% AcOH in water. Key: *E*, plant extract corresponding to 1 Gm. fresh weight; *A*, pure aglucone; *M*, pure MBOA. The contoured spots were dark in U.V. light. Color of aglucone with $FeCl_3$-blue. The numbers on the chromatogram indicate the zones eluted for U.V. spectrometry. Key: *1*, unknown; *2* and *3*, spectrum identical with that of pure aglucone; *4*, no absorption, indicating the absence of MBOA. Concentration of eluate from which U.V. spectrum was measured corresponds to 1 Gm. fresh weight in 3 ml. of ether.

the maize varieties analyzed by us. It was, however, shown that the transformation of free aglucone into MBOA readily takes place in alcohol or in the presence of aluminum oxide (1, 3). The MBOA found by Beck *et al.* after treatment of the intact plants with liquid air and alcohol and chromatography on aluminum oxide could thus have been formed partly or totally from the aglucone, if the plants contained this in free form. In this case, there would be only a quantitative difference between the plant varieties.

To elucidate this problem, the following experiments were made using maize seeds (resistant maize inbred W 22).[1]

I.—The seedlings were grown on the field and harvested when about 7 cm. high.

(*a*) A sample was crushed at about 10°, allowed to warm to room temperature for enzymatic splitting of the glucoside, and extracted with ether. The extract was analyzed for MBOA and the aglucone as described earlier (1) (by paper chromatography). No MBOA was found, but the aglucone was present in an amount of about 700 mcg./Gm. fresh weight.

(*b*) Another sample was immersed in solid CO_2-ethanol mixture immediately after the plants were harvested. The amount of aglucone was about half the amount in experiment *Ia*. Also, traces of MBOA were found.

(*c*) A third sample was immersed in ethanol at room temperature and analyzed as before. This time aglucone and MBOA were found in about equal amounts; the combined amount was of the same order of size as the amount of aglucone in experiment *Ia*.

II.—A second harvest was made when the seedlings were about 20 cm. high.

A sample was crushed and extracted with ether. Aglucone was found, but not MBOA.

III.—To decide whether the MBOA found in experiments *Ib* and *Ic* was present in the plants together with the aglucone or was formed during the analysis, a sample of plants from the second harvest (kept frozen at −20° for about 3 months) was crushed in the frozen state, and the press juice collected at 0 to +3°. The juice was immediately centrifuged at 12000 g. for 30 minutes, between 0 and 2°. The supernatant was freeze-dried; the dry residue, mixed with granular calcium sulfate as a drying agent, was then extracted with dry peroxide and alcohol-free ethyl ether at +10° (270 mm. Hg) for 6 hours. The chromatogram obtained from the concentrated ether extract, with 2% acetic acid in water as solvent, showed the presence of aglucone, but no MBOA. The U.V. spectra obtained on eluting the spots with ether were consistent with this result. On repetition of this experiment, the same result was obtained. The paper chromatogram is shown in Fig. 1.

From these results it is concluded that MBOA is not present in fresh plant tissue even of the resistant maize inbred W 22, but that the free aglucone is present in appreciable concentration in this maize variety and, accordingly, may vary widely in different maize strains. The aglucone which has antifungal properties may thus be the resistance factor in maize (4).

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VOLUME 53

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JANUARY TO DECEMBER, 1964

JOURNAL OF Pharmaceutical Sciences

August 1964 volume 53, number 8

Review Article

Pharmaceutical Sciences—1963. Part II

A Literature Review

By WAYNE McKEEHAN and LEWIS C. LAPPAS

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[Contents for Part I of this review appeared in the July issue, THIS JOURNAL, 53, 695(1964).]

PHARMACEUTICAL CHEMISTRY

IN 1963, PROGRESS of the chemical aspects of pharmaceutical science was reviewed in several publications. New products released during 1962 were the topic of reviews by deHaen (599) and Tice (600). It was pointed out that the new chemical entities released by the American drug industry in 1962 were fewer than in any year of the last decade. Another writer cited 449 references in a survey of the research progress in synthetic medicinals and natural substances in 1961 (601). Bucher also reviewed new developments in pharmaceutical chemistry with examples of products marketed in Germany (602). Problems of drug research were the theme of another report (603). Sources of potential drugs were treated in other publications (604–606).

Pharmaceutical applications of azulenes (607) and antitussives (608) have been analyzed. The synthesis, properties, and uses of some new barbiturates were described (609). Vitamins and amino acids in the Japanese pharmaceutical industry were discussed in a paper on pharmaceutical nutritive preparations (610). Blazek reviewed

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phenothiazine derivatives in pharmacy (611), and Petrow surveyed steroid progress for the year 1962 (612). Consideration of the potential of enzymes for topical application also appeared in the literature during 1963 (613). In another article, the chemistry, pharmacology, and pharmacy of gold compounds were reported (614). A summary on stereochemistry with pharmaceutical illustrations was published (615); 74 references were cited.

The composition of spermaceti (616) and montan wax (617) has been determined. Christie, *et al.*, elucidated the structure of butolic acid, a hydrolytic product of shellac resin (618). Some low-boiling constituents of peppermint oil were investigated by McCarthy, *et al.* (619). A review of separation methods, structure, and uses of glycyrrhizic acid and its derivatives was published (620). The preparation of tocopherol concentrates from wheat germ by three different methods (621), and the isolation and crystallization of human insulin (622) have been reported.

David, *et al.*, noted the physicochemical characteristics of some important sulfonamide derivatives (623), and Garrett dealt extensively with the physical chemistry of complex transformations of the antibiotic, porfiromycin (624). An investigation of the stereochemistry of cycloheximide and its degradation products was printed (625). In a paper on the stereochemistry of steroids, hydrogenation of 11β-hydroxy-Δ^8-dehydroestrone was described (626). Three articles on antacids were found: Eriksen, *et al.*, investigated the antacid properties of calcium, magnesium, and aluminum salts of water-insoluble aliphatic acids (627); in another paper, the acid-neutralizing velocity of synthetic aluminum silicate and dried aluminum hydroxide gel was studied (628); the third publication was concerned with critical *in vitro* factors in the evaluation of gastric antacids (629). Mirimanoff, *et al.*, reported on the salt formation between theophylline and phenylmercuric hydroxide (630). Solution phase interaction between nicotinamide and ascorbic acid was examined by Guttman and Brooke (631). A liquid-liquid extraction method for concentration of aqueous solutions has also been described (632). The elution of vitamin B_{12} from carbon cake was considered by Kosaka (633).

Polymers.—The chemical and physical properties of polymers and their applications in pharmacy have been reviewed by Allisson (634) and Autian (635, 636). Studies of the interactions between polyvinyl alcohol and procaine hydrochloride (637), low molecular weight compounds and solid polymers (638), and polar aromatic molecules with neutral polymers and polyanions (639) were reported. The use of various water-soluble copolymers to form salts with penicillins was examined (640). Research on theoretical aspects of polymer association and hydrogen bonding has been conducted (641). Polymer-solvent interaction in polymer solutions was also noted (642). Phase equilibria for a polymer-solvent-nonsolvent system were presented by Nakagaki and Sunada (643). In another paper the acid behavior of carboxymethylcellulose was disclosed (644).

Maurer investigated particle-size distribution in polymer solutions (645). The effect of irradiation on the change in molecular weight distribution in polymers was described (646). In another publication a kinetic study of gelatin cross-linking by formaldehyde and glyoxal was revealed (647). Filtration of silica dispersions flocculated by high polymers was studied (648). Hermans reported a method for derivation of the molecular weight distribution of a polymer system from the sedimentation equilibrium (649). Also published was a procedure for the determination of sedimentation coefficients in rapidly equilibrating polymerizing systems (650).

Antibiotics.—Two general reviews of antibiotics with several references were published (651, 652). During 1963 the tetracycline antibiotics were reviewed in four different publications (653–656); these publications, citing many references, included the history, chemistry, pharmacy, and pharmacology of these drugs. New developments in the penicillins were discussed by Lhoest (657); and Uri reviewed the cephalosporins, antibiotics similar in structure to penicillin (658). A new iron-containing antibiotic, succinimycin, was reported by Haskell, *et al.* (659). Other new antibiotics disclosed in the literature last year were cirramycin (660) and monazomycin (661). Sugawara, *et al.* (662), and Sugawara (663) presented studies on protomycin, a new antibiotic of the cycloheximide group which is active against *Endameba histolytica.*

Leeson, *et al.*, recorded the thermodynamic pKa values for the three dissociation constants of three different tetracycline molecules (664). The variation of pKa′ values of tetracyclines in dimethylformamide-water solvents was also investigated (665). The crystal structure of chlortetracycline hydrochloride was confirmed (666), and the identity of cephalothin sodium was established through crystallography (667).

Huber, *et al.*, examined variation in capacity of batches of corn-steep liquor to enhance the microbiological yields of antibiotics and cyanocobalamins (668). Another article was concerned

with the manufacture of five new semisynthetic penicillins (669). Purification of cephalomycin by isoelectric precipitation and gel filtration was reported (670). The freeze-thaw method was applied for concentration of antibiotic solutions (671). Samsonov and Fleer presented a method to separate and purify erythromycin with cation-exchange resins (672), and penicillin adsorption on ion-exchange resins was the topic of two papers by Samsonov, *et al.* (673, 674). Other researchers investigated the adsorption of streptomycin from aqueous methanol solutions by carboxylic cation exchangers (675).

Radioisotopes.—Johnson, *et al.*, reviewed some applications of radioisotopes in the chemical industry (676), and Snell discussed the use of tritium-labeled compounds in pharmaceutical research (677). Another review dealt with the effects of ionizing radiation on pharmaceuticals (678). Effects of gamma-rays on aqueous solutions of *p*-aminobenzoic acid (679) and on gelation of dilute aqueous pectin solutions (680) were investigated. It has been found that radiation decomposes anion-exchange resins (681). Cornish studied ionization distribution in water irradiated with fast electrons using glass as a radiation indicator (682). One writer reviewed the potential of radio-sterilization in the pharmaceutical industry (683), and another worker explored its application to various antibiotics in ophthalmic ointment bases using Co^{60} (684). Some effects of sterilizing doses of radiation on ophthalmic solution ingredients have also been investigated (685). Clark and Swartz applied radioisotopes to research on absorption in pharmaceutical closures (686).

The use of tagged elements in the newly developed field of medical experimentation was reviewed (687). Ingrand discussed the contribution of radioisotopes to the pharmacodynamic study of proteins and polypeptides (688). Radioisotopes were also used to establish arginine as the precursor of galegine in *Galega officinalis* (689). Another paper dealt with the potential use of radioactive vitamin B_{12} as a gastrointestinal dilution indicator (690). A description was given for the preparation of radiochemicals and their formulation into parenteral dosage forms (691). Also reported was the preparation of colloidal chromic phosphate (P^{32}) for medical use (692).

Armstrong, *et al.*, proposed a tissue-equivalent chemical dosimeter capable of measuring ionizing radiation doses as low as 500 millirad (693). Methodology for simultaneous determination of H^3 and S^{35} in samples with variable quenching was disclosed (694). Another author found a linear relationship between optical density and quenching of liquid scintillation samples (695). Medium-density polyethylene vials have been compared with glass vials for use in liquid scintillation counting; lower backgrounds and increased efficiencies were observed with plastic vials (696). New techniques in 4π β-counting were presented in two papers (697, 698). Blanchard reported a computer program for automated testing and reduction of liquid scintillation counting data (699). An investigation of *in vivo* tracer techniques in drug screening studies was conducted by Rupe, *et al.* (700). Techniques and equipment for measuring total body radiation were also described in another paper (701). Various methods for the detection of radioisotopes in urine have been examined (702, 703).

BIOPHARMACEUTICS

Biopharmaceutics comprises the research effort directed toward studying the influence of pharmaceutical formulations on biological activity of medicinals. Experimental work in this area necessitates the application of knowledge evolving from other disciplines such as physical chemistry, physiology, and biochemistry. The numerous references presented here are indicative of continued interest and progress in this field.

Mechanisms of drug action appeared to be an area of experimental activity in 1963. Two publications were concerned with the dynamics (704) and chemical models (705) of drug-receptor interaction. A flux-carrier hypothesis was proposed to combine some features of the receptor theory with other features of the potential theory (706). Beckett and Youssef considered active sites in stereoselective adsorbents as drug-receptor models (707), while charge transfer, hydrogen bonding, and drug action were dealt with in a paper by Nash and Allison (708). Additional research was reported on the transport of ions across membranes in a static system (709).

Clinical evaluation of pharmacologically active compounds was the subject of a review; it was pointed out that as new techniques are developed the science of clinical evaluation will become more precise (710). In a seminar on the clinical evaluation of drugs, Ruemke discussed some of the applications of statistics to a wide variety of clinical trials (711). A generalized kinetic regression analysis, hypergeometric kinetics, was proposed for empirical curve fitting or estimating parameters of biological significance (712). An analysis of species differences and individual variations in drug metabolism was also reviewed (713).

Several researchers have observed the effects of alkali (714) and ACTH (715) on gastric secretion. Rune and Koster reported the use of a dialysis bag *in situ* for measuring gastric acidity (716). Another paper, by Nordgren, described the rate of secretion and the electrolyte content of normal gastric juice (717). A preliminary report was made on the separation of pepsins from human gastric juice (718).

Clinical significance of hydrogen-ion concentration in the duodenum was ascertained; duodenal pH was found to be influenced largely by the acidity of the stomach (719). Investigation of intestinal sensitivity to gastric juice was also conducted (720). Hunt studied the duodenal regulation of gastric emptying—the pattern of gastric emptying was found to be exponential and controlled by receptors responding to carbohydrates, osmotic pressure, and acid (721). Connell, *et al.*, used miniature radio transmitters enclosed in capsules for studying pressure of the intestinal tract during motility (722). Paper electrophoresis was employed to identify known and unknown components of pancreatic juice (723). A study of 12 enzyme systems in the mucosa of the normal human gastrointestinal tract was published (724). Dubos, *et al.*, discussed the composition, alteration, and effects of intestinal flora (725).

Current knowledge of visceral blood flow has been surveyed (726). Publications by Jacobson (727), Texter (728), and Welsh (729) were concerned with gastric blood flow, small intestinal blood flow, and colon blood flow, respectively. Blood circulation time in dogs of different ages was measured by other investigators (730).

It has been suggested that bile acids facilitate absorption of lipids by virtue of their ability to form micelles which solubilize the products of lipolysis (731). Roe pointed out a need for extensive research on the influence of food on drug absorption from the intestinal tract (732). The action of chloramphenicol on the coefficient of absorption of *p*-aminohippuric acid in man and rabbits was discussed (733). From *in vivo* studies on protein digestion, it was reported that pepsin predigestion is important to the rate of disappearance of vegetable proteins (734). Davies commented on the effect of sodium salts of organic acids on urinary pH (735).

Effects of Physicochemical Properties.—Linford observed the influence of pH on the reactivity of chlorambucil (736); the effect of pH on membrane permeability was discussed by Ariens and Simonis in relation to pH and drug action (737). In a review article, the influence of hydrogen-ion concentration on ophthalmic solutions, disinfectants, mold-inhibiting agents, antibiotics, and the control of urine pH was summarized (738).

Diffusion of organic electrolytes in hydrogen-bonding systems has been investigated (739). McGowan discussed partition coefficients and biological activity (740), and Leonards found the rate of gastrointestinal absorption of aspirin to be markedly influenced by the drug's solubility (741). The efficiency of absorption of several cetyl sulfate salts of drugs was evaluated (742). In a study of 40 dextran samples it was ascertained that renal excretion was dependent on molecular weight, regardless of the method of preparation of the dextran sample (743). Insulin binding properties in human serum were studied by a combination of immuno-electrophoresis and autoradiography (744). Calcium binding compounds were found to increase the gastrointestinal absorption of heparin (745). Interactions of steroids with human plasma proteins were reported by Slaunwhite, *et al.* (746).

Effects of Formulation.—Morrison and Campbell presented a review article on physiological availability of drugs in oral dosage forms (747). Their discussion of various types of formula modifications included sugar coatings, enteric coatings, and prolonged-release products. The effect of dosage form on biological activity was the subject of another publication (748). Two papers by Walker and Kennedy dealt with the physiology of the skin as applied to formulation (749) and factors influencing the rate and extent of percutaneous absorption from various dosage forms (750). The permeability of excised human keratin to lipid-soluble substances (751) and the absorption of phenylbutazone ointment through the skin of rats and humans (752) were examined. Cascorbi studied emulsified anesthetics for intravenous injection, finding that high potency and high boiling point are required for the most satisfactory preparations (753). Clinical trial of a new aerosol dosage spray for nasal application was described in another paper (754). Two references were found which dealt with absorption of sulfonamides and their sodium salts from various suppository bases (755, 756). Glycerinated gelatin was proposed as a base for prolonged-action vaginal suppositories (757).

An *in vivo* method for the determination of disintegration time of orally administered medicinals was reported (758). Urinary excretion in humans was employed to analyze the disintegration of protective-coated tablets (759). Sunkes studied the effect of selected granulating agents on the release of medication from granules—both *in vitro* and *in vivo* methods were used (760).

Spironolactone blood and urine levels were compared after administration of micronized powder with a surfactant and tablets (761). In a review article, Lees discussed the influence of clinical and pharmaceutical aspects of fine particles on drug absorption; preparation of powders, tablets, and suspensions; drug solubility and stability; and manufacturing, milling, and polymorphism (762). Levy also considered the effects of particle size on dissolution and gastrointestinal absorption rates of pharmaceuticals (763).

Pharmaceutical aspects of a *p*-aminosalicylate–dialdehyde starch compound were studied by Campbell (764). Aspirin-caseinate was found to be effective and less irritating to patients with gastric sensitivity to plain aspirin (765). The effect of some antispastic drugs on the absorption of antituberculosis inhalation preparations was reported (766). Poole and Gardocki investigated the prolongation of chlorzoxazone plasma levels in mice and dogs by zoxazolamine (767). Adams explored the effect of drugs and differing routes of administration on the urinary excretion of parenteral vitamin B_{12} (768). Corneal permeation rate and length of action of ophthalmic ointments with dissolved and crystalline chloramphenicol were examined (769). The influence of stearic acid on penicillin levels in blood after oral administration was also observed (770). Kharizanova noted the effects of certain vitamins on serum levels of some tetracyclines and penicillin (771). The individual contribution of sodium tartrate and tartaric acid to blood levels of tetracycline hydrochloride was investigated (772, 773).

Absorption Control.—Numerous references are included in several reviews of sustained-release dosage forms. Speiser (774), Peters (775, 776), and Simoons (777) discussed various factors in the development of sustained-release dosage forms—passive diffusion, methods of obtaining sustained release of medication, and problems in evaluating this type of dosage form were mentioned. In another review, Eriksen considered some of the specific constants describing absorption, distribution, and excretion of drugs as they apply to sustained-release medication (778). A dissertation on the theoretical aspects of prolonged-action drugs in tablet form was presented (779). Nelson analyzed some of the problems complicating evaluation and usefulness of delayed-action pharmaceuticals (780).

Rosen reported on the relationship of *in vitro* release to human urinary recovery for a sustained-release preparation of S^{35}-prochlorperazine (781). Oral prolonged-action products of penicillin (782) and ephedrine and chlorpheniramine (783), based on reaction or complexation with ion-exchange resins, have been prepared and investigated. Partial insolubilization of gelatin with formaldehyde was proposed as a basis for development of sustained-release products (784). Wynn and Landon studied the alimentary absorption of some enteric-coated sodium and potassium chloride tablets (785). Three papers published on the absorption of iron dealt with utilization of iron from a controlled-release dosage form (786), iron absorption in mice as modified by various agents (787), and regulation of intestinal iron absorption (788).

The evaluation of a long-acting, oral, antihistaminic decongestant for nasal allergies was reported (789). A double-blind, controlled study was used to compare slow-release and regular forms of thioridazine (790). Serum levels and clinical effects were observed in a comparative study of pentobarbital sodium in prolonged-action and conventional dosage forms (791). An evaluation of a long-acting oral preparation of methyl prednisolone was published (792), and Cavallito, *et al.*, employed urinary excretion in studying a sustained-release principle (793).

Oldham has presented a review on the chemical modification of sulfonylureas and related materials, including the development of long-acting sulfonamides (794, 795). Another discussion of sulfonamides dealt with rapid-acting, slow-absorbable, long-acting, and various addition products; several forms of penicillin were also discussed (796). Ballard and Nelson have investigated absorption and excretion of sulfadiazine after subcutaneous implantation of disks in rats (797).

The use of drugs as inhibitors of drug metabolism was reviewed (798). It has been found that the administration of a xanthine oxidase inhibitor concurrently with 6-mercaptopurine resulted in a marked decrease in metabolic oxidation of the latter *in vivo* (799). Other studies revealed the effects of a metabolic inhibitor, SKF 525-A, on metabolic rates of various drugs (800, 801). Oral hexobarbital, in the presence of *p*-aminosalicylic acid, increased sleeping time due to decreased elimination (802). Ruemke investigated the influence of several drugs on duration of hexobarbital and hydroxydione narcosis (803), and Kato, *et al.*, studied the mechanism of potentiation of barbiturates and meprobamate by imipramine (804). The influence of dextran for sustaining the action of orally administered drugs was evaluated (805). Increased streptomycin levels in rat skeletal muscle tissue were observed after administration of neostigmine methylsulfate alone and in combination with atropine (806).

Absorption Mechanisms.—A technique for making dose-response curves and their use in evaluation of drug parameters were described in two papers (807, 808). Hunt explored the relationship of gastric-emptying rate to drug absorption (809). Several references were cited in a review of the passage of drugs across body membranes (810). Another review was published about the physiology, biochemistry, and energetics of active transport (811). Ghosh also commented on active-transport and energy-transfer mechanisms (812), and Wilbrandt described active transport through interfaces (813). A model for permeation of living membranes was proposed by Shanes (814). Clearance experiments at different urine pH values were used to demonstrate the importance of pH-dependent diffusion processes for kidney function (815). Molecular size and the transfer of substances through the partition of blood capillaries were discussed by another writer (816). Parsons quantitated some aspects of pinocytosis in relation to intestinal absorption (817). The question of penetration through pores was considered in relation to the passive permeability of cell membranes (818). A study of the route and rate of absorption of inhaled vapors was published (819).

Active transport of quaternary ammonium compounds into bile was investigated by Schanker and Solomon (820). In another paper, Schanker, *et al.*, demonstrated the interaction of purines with the pyrimidine transport process of the small intestine (821). Chelation by lactose was suggested as a possible mechanism for enhancing intestinal absorption of calcium (822). Other researchers studied the development of mechanisms for intestinal absorption of vitamin B_{12} in growing rats (823). Vitamin B_{12} binding capacity of saliva and gastric secretions has also been ascertained (824).

Wilson reviewed principles concerned with percutaneous absorption (825), and Carson and Goldhamer described a tracer procedure for studying skin absorption (826). Percutaneous absorption of various animal or vegetable oils has been investigated (827). *In vitro* techniques were employed in an exploration of the effect of temperature and humidity on penetration of C^{14}-acetylsalicylic acid in excised human skin (828). Swelling response of skin disks was used to measure the effects of aqueous anionic, cationic, and nonionic surfactant solutions on human and calf skin (829). The influence of some surfactants on the cutaneous absorption of potassium iodide, sodium salicylate, and ammonium thiocyanate has also been examined (830).

Kinetic Studies.—The application of a newly derived composite radiocalcium reference standard in normal calcium kinetics was reported (831). Two papers were published describing the use of doubly labeled iron in evaluating the kinetics of iron absorption and metabolism (832, 833). By means of a steady-state perfusion technique, the longitudinal absorption gradient of L-methionine absorption was shown to decline progressively in the normal human small intestine (834). Andersen has stated that the rate of elimination of isonicotinic acid hydrazide in humans was proportional to plasma concentration (835). A presentation was made of the comparative pharmacodynamics, urinary excretion, and half-life determinations of nitrofurantoin sodium (836). Garrett, *et al.*, used an analog computer to examine the kinetics of steroid effects on Ca^{47} dynamics in dogs (837). Metabolism of acetaminophen by humans was the subject of another pharmacokinetic study (838). Nelson and Levy commented on the relationship of plasma salicylate concentration to urinary salicylate excretion rate observed at an elevated urinary pH (839).

A mathematical model of drug distribution and the solution of differential-difference equations have been reported (840). Higuchi analyzed the rate of release of solid drugs randomly dispersed in solid matrices (841). Kinetic studies and human metabolism and excretion of drugs from various dosage forms were reviewed (842). The subject of multiple-dose excretion kinetics was investigated by Wiegand, *et al.* (843)—general mathematical equations describing expected blood and urine drug concentrations were derived. Another manuscript on the theory of drug action disclosed the mathematics of dosage transfer (844). Mathematical models were used to determine the nature and extent of effect of experimental procedures on analysis of reaction curves (845). Per cent absorbed–time plots derived from blood level and/or urinary excretion data were presented by Wagner and Nelson (846). Wagner also discussed some possible errors in the plotting and interpretation of semilog plots of blood level and urinary excretion data (847). The validity of polynomial approximations in urinary excretion-rate studies was discussed in another paper (848); biological half-life and tissue concentrations were the subject of a presentation by Swintosky (849). The excretion and accrual of drug metabolites have also been considered (850).

Drug Absorption: Antibiotics.—In a review and discussion of the mechanisms of action of antibiotics, 246 references were cited (851). Sureau investigated variations in blood levels of

different antibiotics as a function of route of administration and the vehicle used (852). Single-disk and tube-dilution techniques for determining antibiotic sensitivities of Gram-negative pathogens were compared (853).

Blood levels and urinary excretion of some soluble esters of chloramphenicol in rat and man were reported (854). Distribution and excretion patterns were analyzed after oral administration of griseofulvin to rats (855). Karasaki and Matsubara found that trichomycin given orally to rabbits was rapidly decomposed by gastric acid (856). Various types and quantities of adsorbents were used in a study of the absorption of biomycin from the gastrointestinal tract of the chicken (857). The effect of orally and parenterally administered neomycin on plasma lipids of humans was the subject of another monograph (858). Two publications dealt with various aspects of malabsorption produced by neomycin (859, 860).

Tetracycline derivatives were evaluated by studying excretion and distribution in body fluids after intravenous administration to dogs; the relationship between serum concentration and lipid solubility was discussed (861). Using a new method for determination of tetracycline in urine, Kakemi, *et al.*, found the biological half-life of tetracycline to be about 8 hours (862). Another paper by Kakemi, *et al.*, reported some investigations of the absorption of tetracycline from the gastrointestinal tract (863). *In vitro* data were presented which support the theory that *in vivo* retention of tetracycline is due to calcium binding (864).

Single oral-dose blood levels of four phenoxypenicillins were compared with *in vitro* antibacterial activity (865). Absorption from the gastrointestinal tract of humans and *in vitro* activity against Gram-positive cocci were reported in studies with sodium diphenicillin (866). An investigation was published on the absorption, metabolism, and excretion of a new semisynthetic penicillin, 6-(2-ethoxy-1-naphthamido)-penicillanic acid (867, 868). Penicillinase interaction was considered in a discussion of chemotherapeutic aspects of penicillin (869). Smith and Hamilton-Miller commented on the differences between penicillinases from Gram-positive and Gram-negative bacteria (870). In an investigation of a new sulfonamide-penicillin combination, 5-methylsulfadiazine was reported to inhibit penicillinase (871). Papapanos, *et al.*, concluded that solution pH controlled the permeation of penicillins V and G into rabbit eyes (872). These authors also discussed the influence of blood concentrations on the diffusion of penicillins into the aqueous and vitreous humors of the human eye (873).

Drug Absorption: Inorganic.—Brown, in a review, noted the lack of agreement concerning both the precise mechanism and the regulation factors for iron absorption (874). Various aspects of iron therapy—including dosage forms, absorption, and mode of administration—have been disclosed (875). Another experiment showed decreased iron absorption in healthy aged humans (876). In two reports of mechanisms of iron absorption, Brown and Rother studied iron uptake by the normal rat and the influence of iron deficiency and other conditions on iron uptake by rats (877, 878). The role of transferrin in iron absorption has been evaluated (879), and radioactive iron (Fe^{59}) was used to study absorption of iron from the human large intestine (880). In a study of histamine-fast achlorhydria and iron absorption, it was found that Fe^{59} was absorbed three times greater in patients with acid gastric fluid (881).

Several papers were evident concerning the influence of various agents on the absorption of iron. Cleton, *et al.*, determined the influence of synthetic chelating agents on iron metabolism (882). An isolated-loop technique was used in studying absorption, distribution, and the effects of various chelating agents on radioiron in rats (883). Princeps has reported on the influence of the calcium disodium salt of ethylenediaminetetraacetic acid on iron metabolism (884), and Masuhara and Migicovsky noted the influence of vitamin D on the intestinal absorption of iron and cobalt (885). A dissertation on the influence of salicylate administration in iron metabolism was also published (886). Other investigators studied the effect of reducing agents on the absorption of iron (887).

Experiments by Code, *et al.*, were concerned with the influence of acid on the gastric absorption of water, sodium, and potassium (888). The gastrointestinal absorption of radioactive magnesium in the rat has been examined (889). Another paper disclosed a quantitative relationship between the absorption of calcium and phosphorous (890). Akagi, *et al.*, investigated variation of boron and phosphorus levels in some organs after oral administration of a single dose of borate (891).

Drug Absorption: Organic.—Schedl and Clifton found that absorption of cortisol from Ringer's solution in the normal human gut was characterized by a distally decreasing gradient (892). The absorption of castor oil in man was inversely related to the size of the dose (893). In a toxicological investigation of a potential

missile propellant, the absorption, distribution, and excretion of 1,1-dimethylhydrazine were investigated (894). Franc, *et al.*, determined absorption, distribution in tissues, and excretion after oral administration of buthiopurine-S^{35} to mice (895). Borzelleca stated that the absorption rate of nicotine from the urinary tract of the rat was dependent on solution concentration and pH (896). Additional research was reported on the absorption of hydrocortisone from the large bowel (897), of procaine from the gastrointestinal tract (898), of paracetamol tablets (899), and of chlorhydroxyquinoline (900).

Emmerson and Miya reviewed the metabolism of phenothiazine drugs (901). In a metabolic study of secobarbital, two main metabolites were isolated in addition to unchanged secobarbital (902). McMahon, *et al.*, studied the metabolism of nortriptyline-*N*-methyl-C^{14} in rats; this drug was found to be slowly, but effectively, absorbed from the intestinal tract (903). The metabolic fate of 5-cyclohexenyl-5-alkylhydantoins was discussed in a paper on the biotransformation of drugs having a cyclohexene ring (904).

The distribution and excretion of dichlorpromazine-S^{35} were studied following doses to rats and rabbits (905); the excretion and metabolism of S^{35}-labeled thioridazine in urine, blood, bile, and feces were described in another paper (906). Radioactive nicotine was used in a distribution and excretion study in various animals (907). The urinary excretion of benzquinamide in humans was reported by Cahn, *et al.* (908). Research on the elimination of glutethimide and long-acting barbiturates after toxic and therapeutically active doses was published (909).

Nogami, *et al.*, used the perfusion method to study effects of solution pH on the *in vivo* disappearance rate of sulfonamides from the rat small intestine (910). Idiopathic ulcerative colitis did not affect rectosigmoidal absorption of phenolsulfonphthalein, sulfisoxazole diethanolamine, and radioiodine (I^{131}) (911). The amount of sulfonamide excretion from kidneys was found to be dependent on the degree of protein binding (912). Protein binding was also investigated in relation to the antibacterial activity of long-acting sulfonamides (913). Using an equilibrium dialysis method, Nakagaki, *et al.*, evaluated binding between bovine serum albumin and five kinds of sulfanilamides (914). At low sulfonamide concentrations, Scholtan demonstrated that the protein-binding ratio follows Freundlich's rather than Langmuir's adsorption isotherm (915).

Wood reviewed the absorption, distribution, excretion, uses, and toxicity of salicylates (916). The influence of 2-methyl-2-propyl-1,3-propanediol bis(isopropylcarbamate) on the absorption of salicylates and acetylsalicylic acid was discussed in another paper (917). Levy and Gagliardi investigated the gastrointestinal absorption of aspirin anhydride (918). Accumulation of certain drug anions in gastric mucosal cells following oral administration was reported (919). Efficacy and toxicity were evaluated in a comparative blood level study of choline salicylate and acetylsalicylic acid (920). *In vitro* and *in vivo* properties of a new aluminum derivative of acetylsalicylic acid were also investigated (921).

Some factors affecting the absorption of vitamins were discussed by Campbell and Morrison (922). The influence of intrinsic factor on the uptake of vitamin B_{12} by the small intestine of the Rhesus monkey has been explored—maximum absorption occurred in the lower ileum (923). Absorption, deposition, excretion, and duration of action of hydroxycobalamin were investigated, with emphasis on the treatment of vitamin B_{12} deficiencies and pernicious anemia (924). Studies were conducted on the tissue distribution and storage forms of vitamin B_{12} in dogs after injection or oral administration (925). Another worker discussed the effect of deficiencies of certain B vitamins and ascorbic acid on the absorption of vitamin B_{12} (926). It was reported that glutamic acid does not increase the absorption of vitamin B_{12} (927). In dogs, absorption of vitamin B_{12} in the presence of intrinsic factor occurs primarily in the ileum (928). Waife, *et al.*, reported on the treatment of pernicious anemia patients using oral vitamin B_{12} in the absence of intrinsic factor; evidence of absorption was measured by urinary excretion methods (929). Miller, *et al.*, discussed the possible absorption of intrinsic factor into the plasma of rats (930). The intestinal absorption of L-ascorbic acid-1-C^{14} was studied *in vitro* and *in vivo* in rats and hamsters (931).

PHARMACOGNOSY[1]

Two general reviews of pharmacognosy have been published: Evans surveyed the advances in pharmacognosy and stressed the need for more fundamental research on specific plants (932); Farnsworth also reviewed the current status of botanical drugs and discussed thin-layer chromatography as a basic research tool in pharmacognosy (933). Extensive listings of medicinal plants of Casamance (Senegal) (934) and the Soviet Union (935) were presented. In another

[1] The writers thank Dr. G. H. Svoboda for his suggestions concerning the preparation of this section.

TABLE I.—PHARMACOGNOSTIC INVESTIGATIONS

Plant	Ref.
A	
Acacia seyal	(939)
Acer palmatum	(940)
Acervus species	(941)
Achillea species	(942–945)
Acokanthera oblongifolia	(946)
Adonis vernalis	(947)
Alangium lamarckii	(948)
Albizia gummifera	(949)
Aloe species	(950)
Amaryllis hybrida	(951)
Amaryllis parkeri	(952)
Ammi visnaga	(953, 954)
Anemarrhena asphodeloides	(955, 956)
Anemone nigricans	(957)
Andropogon schienanthus	(958)
Anemopsis californica	(959)
Angelica species	(960–963)
Anthemis nobilis (*Matricaria chamomilla*)	(964, 965)
Apium graveolens	(961)
Aquilegia species	(966)
Aralia elata	(967)
Arbutus menziesii	(968)
Argemone mexicana	(969)
Arnica foliosa	(970)
Arnica montana	(970, 971)
Artemisia species	(972–974)
Ascosparassis species	(941)
Aspergillus fumigatus	(975)
Aspidosperma album	(976, 977)
Atropa belladonna	(978)
B	
Banisteria lignum	(979)
Berberis barandana	(980)
Berberis vulgaris	(981)
Betonica officinalis	(982)
Bladhia japonica	(983)
Boswellia serrata	(984)
Buxus alkaloids	(985, 986)
C	
Caccinia glauca	(987)
Caesalpinia bonducella	(988)
Callitrus drummondii	(989)
Calpurnia subdecandra	(990)
Campanula rotundifolia	(991)
Cannabis indica	(992)
Capsicum annum	(993)
Carissa carandas	(994)
Carissa spinarum	(994)
Carya pecan	(995)
Casimora species	(996)
Cassia (Senna)	(997)
Catalpa ovata	(998)
Catha edulis	(999)
Catharanthus lanceus (*Vinca lancea*)	(1000)
Cephalotaxus drupacea	(1001)
Cephalotaxus fortunei	(1001)
Chaetomorpha capillaris	(1002)
Cheiranthus allionii	(1003)
Cinchona alkaloids	(1004)
Cirsium martimum	(1005)
Cissampelos pareira	(1006)
Citrullus colocynthis	(1007)
Cladophora rupestris	(1002)
Clematis terniflora	(1008)
Cnidium officinale	(961)
Codium fragile	(1002)
Convallaria majalis	(1009, 1010)
Corchorus olitorius	(1011)
Cordyceps capitata	(1012)
Coronilla varia	(1013)
Corydalis ambigua	(1014)
Crotalaria species	(1015–1017)
Croton species	(1018, 1019)
Cyathea fauriei	(1020)
Cyclea peltata	(1006)
Cynanchum caudatum	(1021)
Cyperus rotundus	(1022)
Cytisus praecox	(1023)
D	
Dalbergia latifolia	(1024)
Daphne mezereum	(1025)
Datura species	(1026–1030)
Delphinium dictyocarpum	(1031)
Dentaria pinnata	(1032)
Dichroa febrifuga	(1033)
Digitalis species	(1034–1038)
Dioscorea species	(1039–1043)
Diplopterygium glaucum	(1044)
Discomycetes	(1045)
Dryopterus crassirhizoma	(1046)
Duboisia myoporoides	(1047)
E	
Ephedra sinica	(1048)
Ergot alkaloids	(1049)
Ericaceae alkaloids	(1050)
Ervatamia dichotoma	(1051)
Erysimum canescens	(1052)
Erythrophleum ivorense	(1053)
Eucalyptus species	(1054, 1055)
Eugenia maire	(1056)
Eupatorium maculatum	(1057)
F	
Fagara xanthoxyloides	(1058)
Ficus septica	(1059)
Flammulina velutipes	(1060)
Fraxinus rhynchophylla	(1061)
Fumaria parviflora	(1062)
G	
Galerina species	(1063)
Genista aetnensis	(1064)
Genista raetam (*Retama raetam*)	(1065)
Geranium palustre	(1066)
Ginseng	(1067–1071)
Gleditschia horrida	(1072)
Glycosmis arborea	(1073)
H	
Helenium aromaticum	(1074)
Helicostylum species	(1075)
Helleborus species	(1076–1078)
Hibiscus trionum	(1079)
Hunteria eburnea	(1080–1082)
Inula helenium	(1083)
Iris kamaonensis	(1084)
Isodon trichocarpus	(1085)
Isotoma longiflora	(1086)
J	
Jasione montana	(991)
Juniperus species	(1087–1089)
K	
Kaempferia rotunda	(1090)
Kopsia species	(1091)
L	
Lampteromyces japonicus	(1092)
Laurus nobilis	(1093)

Plant	Ref.
Lavandula species	(1094, 1095)
Leptospermum seoparium	(1056)
Leucothoe grayana	(1096)
Levisticum officinale	(961)
Ligularia tussilaginea	(1097)
Ligusticum acutiloba	(961)
Linum usitatissimum	(1030)
Lobelia urens	(1098)
Lupinus species	(1099)
Lycium chinense	(1100)
Lycopodium species	(1101–1103)
M N	
Machilus species	(1104–1106)
Magnolia kachirachirai	(1107)
Majorana hortensis	(1108)
Menispermaceae	(1109, 1110)
Mentha piperita	(1111)
Metanarthecium luteo-viride	(1112)
Michelia champaca	(1113)
Mitragyna species	(1114–1117)
Myrtus communis	(1054)
Narcissus alkaloids	(1118–1120)
P	
Paeonia albiflora	(1121)
Pancratium maritimum	(1122)
Paulownia tomentosa	(1123)
Pergularia extensa	(1124)
Peschiera affinis	(1125)
Physostigma venenosum	(1126)
Phyteuma spicatum	(991)
Pieris japonica	(1127)
Pimpinella heyneana	(1128)
Pinus species	(1129, 1130)
Piper methysticum	(1131)
Pithecolobium dulce	(1132)
Plantago major	(1133)
Platycodon grandiflorum	(991)
Podophyllum peltatum	(1134)
Polygonum aviculare	(1135)
Pongamia glabra	(1136)
Populus grandidentata	(1137)
Prangos pabularia	(1138)
Pulsatilla grandis	(1139)
R	
Rauwolfia species	(1140–1144)
Rhamnus Purshiana	(1145)
Rhazya stricta	(1146–1148)
Rheum palmatum	(1149)
Ricinocarpus stylosus	(1150, 1151)
Rosmarinus officinalis	(1152)
Rumex species	(1153–1155)
Ruta graveolens	(1156)
S	
Salix species	(1157–1159)
Salvia species	(1160)
Sarcostemma viminale	(1161)
Sargassum natans	(1162)
Scabiosa succisa	(1163)
Schoenocaulen officinale	(1164, 1165)
Sciadopitys verticillata	(1166)
Scilla maritima	(1167)
Securinega suffruticosa	(1168, 1169)
Securinega virosa	(1170)
Simaba cedron	(1171)
Sinocalamus oldhamii	(1172)
Sinomenium acutum	(1173)
Skytanthus acutus	(1174)
Smilax sieboldi	(1175)
Solanum species	(1041, 1176–1178)
Sophora species	(1179, 1180)
Sphenomeris chusana	(1020)
Stephania japonica	(1181)
Strychnos species	(1182)
Sweetia panamensis	(1183)
Symphytum species	(1184)
T	
Tabernaemontana pachysiphon	(1185)
Tacca leontopetaloides	(1186)
Tagetes species	(1187)
Tecoma stans	(1188)
Thalictrum dasycarpum	(1006, 1189)
Thymus vulgaris	(1190)
Tilia species	(1191, 1192)
Tripetaleia paniculata	(1193)
Tripterygium wilfordii	(1194)
U V	
Ulva lactuca	(1002)
Ungernia species	(1195, 1196)
Valeriana angustifolia	(1197)
Veratrum album	(1198)
Vinca minor	(1199–1201)
Vinca rosea (*Catharanthus roseus*)	(1202–1205)
Virgilia oroboides	(1206, 1207)
Vitex agnus-castus	(1208)
W X Y	
Waltheria americana	(1210)
Wistaria floribunda	(1211)
Withania species	(1212–1216)
Xanthorhiza simplicissima	(1209)
Yucca aloefolia	(1217)
Yucca guatamalensis	(1218)

paper, the development of storage and retrieval techniques for punched cards on phytochemical data was reported (936). Thirty tannin-containing drugs were classified according to their ultraviolet absorption spectra (937). A procedure utilizing fresh plant material has been described for alkaloid screening of plants in the field (938).

Pharmacognostic Investigations.—This section of the review is primarily concerned with those references pertaining to isolation and identification of plant constituents. Table I lists alphabetically each plant studied, followed by the appropriate references to the bibliography.

Methodology.—Germination, growth, and alkaloid synthesis of *Datura stramonium* after various treatments have been investigated (1219, 1220). Research on biosynthetic pathways to coumarin formation in plants was published by Brown (1221). An extensive investigation was conducted concerning the influence of environmental factors on the growth and oil production of some species of Labiatae (1222–1224). Growth characteristics of spearmint tissue were explored with a dual-carboy culture apparatus (1225). Alkaloid production by *Claviceps paspali* was studied in submerged culture (1226), while laboratory culture experiments with *C. purpurea* were

reported in two different articles (1227, 1228). Teuscher discussed the influence of amino acids on the biosynthesis of clavine alkaloids in saprophytic culture (1229). Suspension cultures of *Digitalis lanata* and *D. purpurea* were employed by Staba and Lamba in the experimental production of cardiac glycosides (1230). Microbiological hydroxylation of digitoxigenin derivatives by *Absidia orchidis* was recorded (1231).

Hoerhammer, *et al.*, used thin-layer chromatography for identification and differentiation of *Strophanthus* glycosides (1232), while other investigators quantitatively determined the principal alkaloids of opium using this technique (1233). A new two-dimensional thin-layer chromatography technique was used in phytochemical studies of the alkaloids of *Pancratium maritimum* (1234). Ion-exchange, paper and thin-layer chromatography in pharmacognostic instruction have been discussed by Reuter (1235). Another paper revealed thin-layer chromatography to be useful in detecting adulteration of *Tussilago farfara* with *Petasites* leaves (1236). Thin-layer chromatography was also employed for the separation and estimation of some plant polyhydroxy anthraquinones (1237). Schwarz effected a chromatographic separation of steroid compounds on a magnesium silicate layer without binding agents (1238).

More efficient extraction of plants was accomplished through the application of certain mathematical principles to maceration and percolation (1239). Stanev and Elenkov studied the dynamics of morphine extraction from poppies (1240). A laboratory method was developed for obtaining extracts of *Leonurus cardiaca* (1241). Other investigators evaluated effects of various factors on extraction of active ingredients from medicinal plants (1242). Various aqueous-alcoholic mixtures were employed under a variety of conditions to study the efficiency of extraction of *Digitalis purpurea* leaves (1243). Deacylation and copper complexing were used to fractionate two major water-soluble polysaccharides from *Pinus sylvestris* (1244). Decomposition of rutoside was reported when ether saturated with water was used for its extraction from rue (1245). The effect of dispersion on the extraction of active substances from medicinal plants (1246) and the extraction of belladonna herb with the aid of surfactants (1247) were investigated. Surfactants were also used in preparing fluid extracts of thyme (1248).

Tyler and Stuntz reported a qualitative test for alkaloids in studies of higher fungi (1249). A hemolytic index was described for evaluation of saponin extracted from the roots of *Gypsophila paniculata* (1250). Standardized conditions for production of good quality agar from seaweed were suggested (1251). Spray drying for the isolation of various alkaloids and glycosides has been discussed (1252). Ultrasound was employed successfully in the extraction of plants containing alkaloids (1253). It was also reported that the use of ultrasonic energy to isolate alkaloids shortens maceration and extraction times and increases the yield (1254).

CONCLUSION

The number and variety of publications in pharmaceutical science continue to demonstrate the complexities of this field of study. It was the aim of this review to facilitate presentation of some of these advances to those engaged in pharmaceutical research.

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Research Articles

Method for Evaluating Behavioral Effects of Central Depressants

By MARVIN COHEN* and JOHN W. NELSON

A new method for the evaluation of central nervous system depression is presented. It is based on the scoring of behavioral responses to subhypnotic doses of central depressants. The application of the method to the study of two different types of depressants, pentobarbital and chlorpromazine, both alone and in combination, is shown. The time-response scoring method appears to be useful as a screening method and as a tool for more theoretical studies when appropriate modifications are made for different types of pharmacological agents.

The problem of drug interaction and its evaluation is a fundamental one in the field of general pharmacology. Studies in this area, such as those of Bliss (1), Gaddum (2), Berger and Lynes (3), and Gruber (4) have led to an accumulation of considerable information. Such data, however, have largely been obtained by means of toxicity studies (1) or by the measurement of sleeping times in cases where central depressants have been employed (2–4). Gruber (4) has commented on studies of this type by stating that the data obtained in this way, being only relative values, cannot be measured and compared with any degree of accuracy. More quantitative studies, using animals given subhypnotic doses of depressants, have been performed by such workers as Swinyard (5), Lim

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TABLE I.—CRITERIA USED TO EVALUATE DEPRESSANT ACTIVITY

Degree of Depression	Numerical Equivalent	Description
LOSS OF SPONTANEOUS MOTION		
No loss	0	Behavior of animal indistinguishable from normal — exploratory and cleansing activities.
Slight loss	0.5[a]	Animal motionless (standing or lying) with head in or above plane of body. Readily responds to external stimulus by seeking source of disturbance.
Moderate loss	1	Animal motionless (standing or lying) with head below plane of body or touching floor of cage.
Marked loss	2	Partial loss of righting reflex—animal takes longer than 1 sec. to regain correct posture when placed on its back.
Loss of righting reflex	3	Failure to regain normal posture when animal is placed on its back.
RESPONSE TO STIMULI		
Response to air stimulus	0	Animal shows startle response (stiffening of body, widening of eyes, rapid movement of vibrissae) or defensive response (rises on hindlegs and faces source of air blast).
Response to pain stimulus	1	Animal responds to air blast by moving head away from direction of air, moving only a few muscles, or not moving at all. Application of pain stimulus causes animal to attack source of pain within 5 sec.
Sluggish response to stimuli	2	No satisfactory response to air stimulus. Application of pain stimulus causes aimless movements without attack within 5 sec.
No response (loss of righting reflex)	3	No response to air or pain stimuli.
DEGREE OF ATAXIA		
No ataxia	0	Animal brings hindlegs up to bar within 1 sec.
Slight ataxia	1	Hindlegs are lifted after a lapse of 1–5 sec. If legs are lifted immediately, they repeatedly slip off bar.
Moderate ataxia	2	Hindlegs not lifted within 5 sec.; animal hangs by forepaws.
Marked ataxia	3	Animal unable to hang by forepaws.
Loss of righting reflex	4[b]	Animal unable to hang by forepaws in addition to maximum response to other tests.

[a] It was found that both a normal and a slightly sedated animal could exhibit slight loss of spontaneous motion. In addition, this response was relatively fleeting and often could not be detected on successive observations. For these reasons, a value of 0.5 was given so that it would contribute less to the total response.

[b] Degree of ataxia was given a maximum value of 4 because it was found to be the response component that gave the most dramatic changes in behavior and thus contributed more to the total response.

(6), and Kinnard and Carr (7). In this type of study, certain criteria and/or numerical values were established both for different levels of sedation and different aspects of behavior. A survey of the literature revealed that the above method had not been applied to combinations of central depressants. In addition, many of the criteria used seemed to be too subjective for a detailed study. It was felt that the development of a more objective method would contribute additional information to the study of central depressants at dose levels that permitted a clearer interpretation of drug-induced responses.

EXPERIMENTAL

Chlorpromazine and pentobarbital were chosen for this study for several reasons. Preliminary studies with pentobarbital showed it to be a central depressant that produced sharply delineated responses with increasing subhypnotic doses. For this reason, pentobarbital was used as a "standard" for establishing the behavioral criteria described below. Pentobarbital is also a classical example of a cortical depressant, particularly in the doses employed in this study.

Chlorpromazine was used because it can be considered as a typical example of the class of drugs known as tranquilizers. It is believed to act predominantly on subcortical regions of the brain rather than on the cortex (8). Chlorpromazine was found to have a longer duration of action than pentobarbital, particularly in higher doses; in addition, chlorpromazine brought about a different pattern of response than did pentobarbital.

In this study, ampuls containing 25 mg./ml. of chlorpromazine HCl[1] were used as the source of this drug. The commercial solution was diluted 1:20 with saline prior to administration. Pentobarbital was obtained from ampuls containing 60 mg./ml. of pentobarbital sodium.[2] This solution was diluted

[1] Marketed as Thorazine by Smith Kline and French Laboratories.

[2] Marketed as Veterinary Nembutal by Abbott Laboratories.

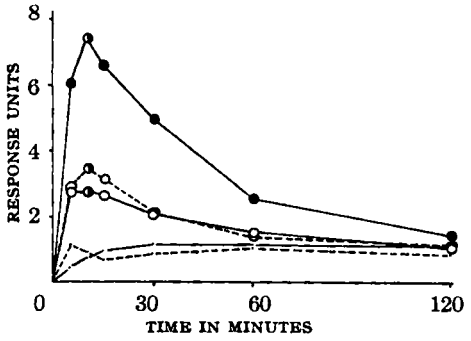


Fig. 1.—Time-response curves for pentobarbital. Key: ----, control; —·—, 5 mg./Kg. pentobarbital; O—O, 10 mg./Kg. pentobarbital; O--O, 15 mg./Kg. pentobarbital; ●—●, 17.5 mg./Kg. pentobarbital; ◑, significant difference of the maximum response from control ($P = 0.05$).

1:10 prior to administration. All drugs were administered by the intraperitoneal route. The animals used were albino male rats of the Wistar strain. They ranged in age from 3 to 5 months and in weight from 150 to 250 Gm. Environmental temperature was generally within the 20–25° range.

The method used to determine levels of central depression was called the *time-response scoring method.* It was based on the assumption that the observed behavior of experimental animals could be translated into numerical values which, when plotted on suitable coordinates, could give a meaningful description of the activity of a drug or drug combination. The time-response scoring method was used to measure depression through the observation of three components of animal behavior under the influence of depressant drugs and under control conditions. These components were loss of spontaneous motion, responses to external stimuli, and degree of ataxia. These three categories were further subdivided into 14 subcategories, each of which had distinct behavioral characteristics. These subcategories, together with their numerical equivalents and descriptions, are shown in Table I.

During experimental runs, the animals (in groups of 4, 8, or 12) were kept in individual cages. Loss of spontaneous motion was determined by direct observation. Response to stimuli was determined by presenting the animal with one or both of two stimuli, depending on its level of depression. The first stimulus was a blast of 4 ml. of air delivered from a 5-ml. syringe through a 25-gauge 0.5-inch hypodermic needle and directed at the animal's face. The second stimulus was the application of pain by pressing the tip of the animal's tail with a finger. Degree of ataxia was measured by the use of a wooden horizontal bar suspended about 4 ft. above the floor. The bar had a roughened surface and an edge for grasping; its dimensions were 8 × 1.25 × 0.5 inches (length-height-width). The animals forepaws were placed on the bar and the animal was allowed to drop from a horizontal position without having its hindlegs touch the bar. The ability of the animal to bring its hindlegs up to the level of its forepaws was used as an indication of ataxia. For a more detailed explanation of this procedure, see Table I.

The experimental design used to evaluate depression produced by chlorpromazine, pentobarbital, and combinations of the two was as follows. After transferring the animals from their home cages to the experimental cages, the animals were allowed approximately 20 minutes to become adapted to their new environment. During this time, their behavior was observed and noted. At the end of this period, the drug or drug combination being studied was administered. Observations were then made at intervals of 5, 10, 15, 30, 60, and 120 minutes after injection. The observation time did not extend beyond 120 minutes because no drug or drug combination used reached a peak of activity after this time.

When sufficient data had been obtained (an average of 12 experimental runs, with a range of 6 to 17 for each point on a graph), they were converted into numerical values and treated in three ways. (*a*) The values obtained for each response component (loss of spontaneous motion, response to stimuli, degree of ataxia) at each time period were summed. These latter values were plotted against time to produce a time-response curve (Figs. 1, 4, 6, and 7). (*b*) The values of each response components were plotted separately against time to determine the contribution of each component to the total response seen (Figs. 2, 3, and 5). (*c*) The maximum response to each drug and drug combination was determined, and this was plotted against dose to give a series of dose-response curves (Figs. 8 and 9). Interpretation of results was made from these graphs.

RESULTS

Figure 1 illustrates the total response time-response curves obtained for pentobarbital in doses of 5, 10, 15 and 17.5 mg./Kg. Figures 2 and 3 illustrate the response component time-response curves for these doses of pentobarbital. Figure 4 illustrates the total response time-response curves obtained for chlorpromazine in doses of 1 and 5 mg./Kg. Figure 5 illustrates the response component time-response curves for these doses of chlorpromazine. Figures 6 and 7 illustrate the total response time-response curves for combinations of pentobarbital with 1 and 5 mg./Kg. chlorpromazine,

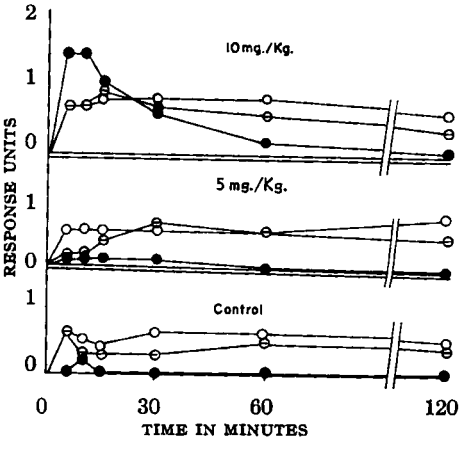


Fig. 2.—Time-response curves for pentobarbital (response components). Key: O, loss of spontaneous motion; ⊖, response to stimuli; ●, degree of ataxia.

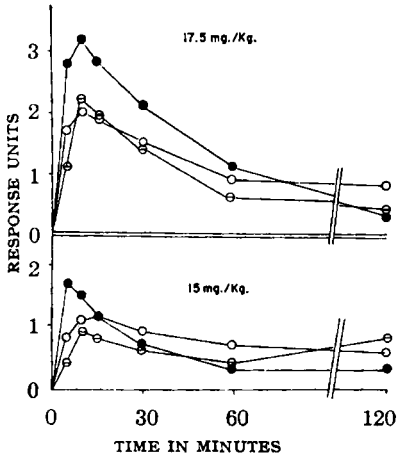


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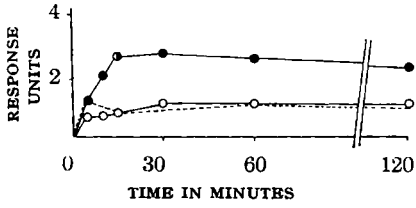


Fig. 4.—Time-response curves for chlorpromazine (total response). Key, -----, control; O—O, 1 mg./Kg. chlorpromazine; ●—●, 5 mg./Kg. chlorpromazine; ◑, significant difference from control ($P = 0.05$).

respectively. Figures 8 and 9 illustrate the dose-response relationships obtained for pentobarbital alone and in combination with chlorpromazine.

The doses used were chosen because each one brought about a particular behavioral response. In the case of chlorpromazine, only two dose levels were found where distinct behavioral responses could be elicited. Chlorpromazine in a dose of 0.5 to 3 mg./Kg. gave essentially the same response as 1 mg./Kg., while doses of 4 to 7 mg./Kg. gave the same type of response as 5 mg./Kg. Doses higher than 7 mg./Kg. did not increase the intensity of drug action, but only affected the duration of action.

Controls were run with saline (4 ml./Kg.) to determine the effect of the evaluation procedure on behavior. Control animals showed a score ranging from 0–3 with an average of 1.6. This was found to be significantly greater than an ideal response of zero for the administration of saline. Significance was determined by the Mann-Whitney U test for independent samples (9) at the 0.05 level of probability. The words "significant" and "significance" are used throughout this paper to mean statistical significance. Calculation of the regression coefficients (slopes) for the dose-response curves in Figs. 4 and 5 was accomplished using the method of least squares (10). Table II summarizes the data obtained by this method. Table III gives the average maximum response obtained with each drug and drug combination used as well as the contribution of each response component to this total.

An analysis of the response component curves for chlorpromazine used alone and pentobarbital used alone indicated that increasing doses of chlorpromazine exerted the most depressant effect on responses to stimuli. Ataxia and loss of spontaneous motion were produced to a lesser, but approximately equal, extent. Pentobarbital was found to exert its greatest depressant effect on ataxia while responses to stimuli were least affected. These data are summarized in Table III. Combinations of pentobarbital with 1 mg./Kg. of chlorpromazine produced no detectable change in the response components when compared to the responses produced by pentobarbital alone. Combinations of pentobarbital with 5 mg./Kg. chlorpromazine produced marked effects on all response components. In comparison with the responses produced by pentobarbital given alone, response to stimuli was affected to the greatest extent. Combinations of pentobarbital with 1 mg./Kg. chlorpromazine produced a duration of action that was slightly greater than the duration of action of pentobarbital given alone. Combinations of pentobarbital with 5 mg./Kg. chlorpromazine produced a greatly increased duration of action when compared with the effects of pentobarbital administered alone.

With all the combinations of pentobarbital and 1 mg./Kg. chlorpromazine, the responses of the combined drugs were not significantly greater than the responses to the predominant individual depressant,

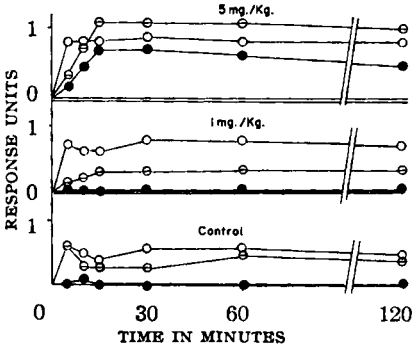


Fig. 5.—Time-response curves for chlorpromazine (response components). Key: O, loss of spontaneous motion; ⊖, response to stimuli; ●, degree of ataxia.

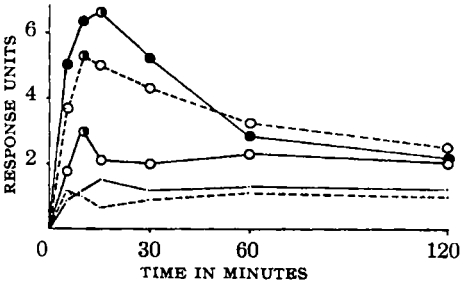


Fig. 6.—Time-response curves for combinations of pentobarbital with 1 mg./Kg. chlorpromazine. Key: ----, control; —·—, 5 mg./Kg. pentobarbital; O—O, 10 mg./Kg. pentobarbital; O----O, mg./Kg. pentobarbital; ●—●, 17.5 mg./Kg. pentobarbital; ◑, significant difference of the maximum response from control ($P = 0.05$).

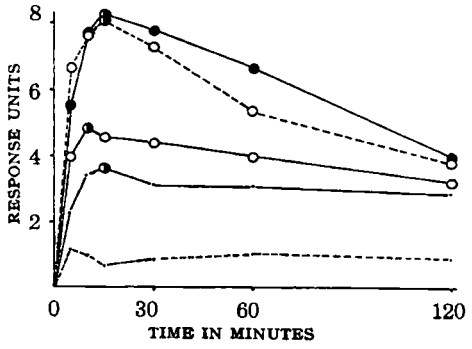


Fig. 7.—Time-response curves for combinations of pentobarbital with 5 mg./Kg. chlorpromazine. Key: ---, control; —·—, 5 mg./Kg. pentobarbital; O—O, 10 mg./Kg. pentobarbital; O---O, 15 mg./Kg. pentobarbital; ●—●, 17.5 mg./Kg. pentobarbital; ◑, significant difference of the maximum response from control ($P = 0.05$).

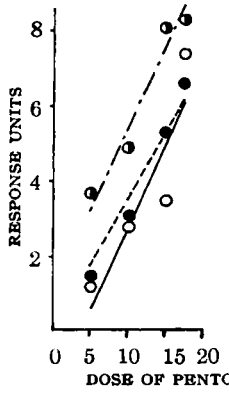


Fig. 8.—Dose-response curves for pentobarbital-chlorpromazine (combinations (total response). Key: O—O, pentobarbital; ●---●, pentobarbital + 1 mg./Kg. chlorpromazine; ◑---◑, pentobarbital + 5 mg./Kg. chlorpromazine; each point represents the maximum response seen.

i.e., the drug in the combination that produced the greater degree of depression when given alone. In two of the four combinations of pentobarbital with 5 mg./Kg. chlorpromazine (the combinations with 10 and 15 mg./Kg. pentobarbital), the responses of the combination were significantly greater than the response to the predominant individual depressant.

With combinations of pentobarbital and 1 mg./Kg. chlorpromazine, pentobarbital was the predominant depressant over the 10 to 17.5 mg./Kg. dose range. In the lowest doses used, both chlorpromazine (1 mg./Kg.) and pentobarbital (5 mg./Kg.) produced responses that were not significantly different from those obtained with saline. With the combinations of pentobarbital and 5 mg./Kg. chlorpromazine, the latter was the predominant depressant when pentobarbital was given at a dose of 5 or 10 mg./Kg., while pentobarbital became the predominant depressant in doses of 15 and 17.5 mg./Kg. These results are summarized in Table IV.

The response to the combination of 17.5 mg./Kg. pentobarbital and 5 mg./Kg. chlorpromazine was found to be not significantly greater than the response to pentobarbital alone. However, other evidence tended to modify a conclusion based solely on the statistical results. At these high dose levels, hypnosis began to appear. A comparison of the number of animals losing their righting reflex with the combination and with pentobarbital alone indicated that an increased degree of depression had occurred. After the administration of 17.5 mg./Kg. pentobarbital, 5 out of 12 animals lost their righting reflex, while the combination of 17.5 mg./Kg. pentobarbital and 5 mg./Kg. chlorpromazine caused 8 out of 12 animals to lose the righting reflex. Hypnosis was also seen with the combination of 15 mg./Kg. pentobarbital and 5 mg./Kg. chlorpromazine. In this case, 7 out of 12 animals lost the righting reflex.

DISCUSSION

The results of this study of pentobarbital and chlorpromazine interaction indicate that the time-response scoring method is capable of giving a consistent and readily interpretable visualization of the effects of two types of central depressants. The effectiveness of the method appears to be limited at the extreme ends of the subhypnotic dose-response scale, *i.e.*, at doses producing a barely detectable response and at doses that begin to cause a loss of the righting reflex. Between these two extremes, the results obtained have been satisfactory.

Pentobarbital was shown to be a rapidly acting central depressant that affected motor components of behavior more rapidly and earlier than sensory components. Chlorpromazine was shown to be a relatively slow acting depressant that affected

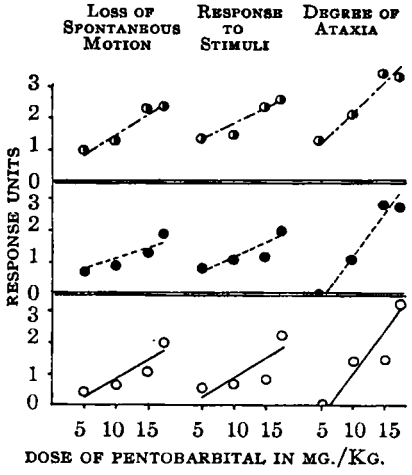


Fig. 9.—Dose-response curves for pentobarbital-chlorpromazine combinations (response components). Key: O—O, pentobarbital; ●---●, pentobarbital + 1 mg./Kg. chlorpromazine; ◑---◑, pentobarbital + 5 mg./Kg. chlorpromazine; each point represents the maximum response seen.

TABLE II.—SLOPES OF DOSE-RESPONSE CURVES FOR CHLORPROMAZINE-PENTOBARBITAL COMBINATIONS

	Pentobarbital	Pentobarbital + 1 mg./Kg. Chlorpromazine	Pentobarbital + 5 mg./Kg. Chlorpromazine
Total response	0.45	0.35	0.43
Loss of spontaneous motion	0.12	0.06	0.13
Response to stimuli	0.12	0.09	0.10
Degree of ataxia	0.25	0.26	0.20

TABLE III.—RESPONSES OBTAINED FOR CHLORPROMAZINE-PENTOBARBITAL COMBINATIONS

Pentobarbital, mg./Kg.	Chlorpromazine, mg./Kg.	Average Maximum Response	Response Components		
			Loss of Spontaneous Motion	Response to Stimuli	Degree of Ataxia
...	...	1.2	0.6	0.6	0.0
5	...	1.2	0.5	0.6	0.1
10	...	2.8	0.7	0.7	1.4
15	...	3.5	1.1	0.9	1.5
17.5	...	7.4	2.0	2.2	3.2
...	1	1.1	0.8	0.3	0.0
5	1	1.5	0.7	0.8	0.0
10	1	3.1	0.9	1.1	1.1
15	1	5.3	1.3	1.2	2.8
17.5	1	6.6	1.9	2.0	2.7
...	5	2.7	0.9	1.1	0.7
5	5	3.7	1.0	1.4	1.3
10	5	4.9	1.3	1.5	2.1
15	5	8.1	2.3	2.4	3.4
17.5	5	8.3	2.4	2.6	3.3

TABLE IV.—DEPRESSANT EFFECTS OF CHLORPROMAZINE-PENTOBARBITAL COMBINATIONS

Pentobarbital, mg./Kg.	Chlorpromazine, mg./Kg.	Response to Individual Drug	Response to Combination	Drug in Combination Exerting Predominant Response
...	1	1.1	...	...
5	...	1.2	...	...
10	...	2.8	...	...
15	...	3.5	...	...
17.5	...	7.4	...	...
5	1	...	1.5	Neither
10	1	...	3.1	Pentobarbital
15	1	...	5.3	Pentobarbital
17.5	1	...	6.6	Pentobarbital
...	5	2.7	...	...
5	5	...	3.7	Chlorpromazine
10	5	...	4.9	Chlorpromazine
15	5	...	8.1	Pentobarbital
17.5	5	...	8.3	Pentobarbital

sensory components of behavior more rapidly and earlier than motor components.

In both series of pentobarbital-chlorpromazine combinations, the increased duration of action of the combinations over the individual drugs seemed to be due to the chlorpromazine component. The data suggested that pentobarbital exerted its predominant effect within 30 minutes after the administration of low doses (5 and 10 mg./Kg.) and within 60 minutes after the administration of higher doses (15 and 17.5 mg./Kg.). Additional evidence for the predominance of chlorpromazine at these times and after is seen by the sharp break in the time-response curves at the above-mentioned times for the different chlorpromazine-pentobarbital combinations (see Figs. 6 and 7). Such breaks have been interpreted by Bliss (1) as being indicative of independent additive action in drug combinations. As applied to this study, the interpretation of Bliss would seem to indicate that chlorpromazine and pentobarbital exert independent depressant actions, the predominance of one or the other being a direct function of its depressant ability when used alone.

While many of the above conclusions are well known from numerous other studies (6, 11–13), very few attempts have been made to quantitate the responses seen. A major reason for this is inherent in the nature of the responses being studied. Quantitation to any degree, no matter how small, can give more clues to mechanisms and sites of drug action than can qualitative data. The time-response scoring method appears to accomplish this with a minimum of equipment, time, and training. It appears to be useful as a screening method and as a tool for more theoretical studies when appropriate modifications are made for different types of pharmacological agents.

SUMMARY

A new method for the evaluation of central nervous system depression is presented. It is based on the scoring of behavioral responses to subhypnotic doses of central depressants. The application of the method to the study of two different types of depressants, pentobarbital and chlorpromazine, both alone and in combination, is shown.

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Physical Chemical Study of the Distribution of Some Amine Salts Between Immiscible Solvents II

Complexation in the Organic Phase

By ROBERT LEE HULL and JOHN A. BILES

The apparent partition coefficients of some amine salts of four aromatic sulfonic acid dyes are reported. The effect of adding complexing molecules to the organic layer has been studied. The binding number and over-all stability constant for the complexation has been determined. The results indicate that the acid strength of the complexing agent and the van der Waals forces of attraction are important considerations in partition studies. The results also indicate that the analyst should consider the use of proton donor molecules to aid in an efficient extraction of amine salts of dyes.

In the preceding paper the apparent partition coefficients of some amine salts of tropaeolin OO were related to the molecular weight and branching of the aliphatic amine, the relative concentrations of the amine and dye, and the dielectric constant of the solvent (1). Methylene chloride, chloroform, and carbon tetrachloride were the solvents used. In a discussion of the choice of suitable solvents, Mukerjee suggested that the dielectric constant of the solvent was a factor which determined the amount of salt extracted (2). However, Higuchi suggested that the complexation of the salt with the solvent was a more predominating factor than was the dielectric constant (3). Therefore, we continued the investigation and now report the studies of complexation of certain azobenzenesulfonic-type acid salts with some halogenated solvents and aliphatic alcohols in a benzene rich solvent system.

EXPERIMENTAL

Reagents.—The sulfonic acid dyes used included methyl orange (Eastman Kodak), tropaeolin O (Eastman Kodak), tropaeolin OO (National Aniline), and sodium azobenzenesulfonate (Gallard Schlesinger Mfg. Corp.). The primary alcohols were purchased from Allied Chemical; the methylene chloride, chloroform, and ethylene dichloride were purchased from Baker and Adamson and Matheson Coleman and Bell.

Preparation of Solutions.—Methods of preparation were similar to those described in the first communication (1). Stock solutions of the sulfonic acid dyes and amine solutions were prepared so that 2 μm. of the acid or base were present in 5 ml. of solution. The pKa of methyl orange was sufficiently high so that the stock solution had to be buffered at a pH of 6.0 with phosphate buffer (potassium acid phosphate 0.001%, potassium phosphate 0.007%). The phosphate buffer was not added to the stock solutions of the other dyes. The pH of the final solution mixtures was such that hydrolysis of the amine dye salt in the aqueous phase was at a minimum.

The benzene solutions were prepared by adding given volumes of the complexing agent to 100 ml. of benzene. The molality (m) of the solutions was then calculated. The desired molalities were prepared after previously determining the necessary concentrations for measurable extractions of the dye salt. The complexing agents used included the normal amyl, hexyl, octyl, and lauryl alcohols as well as methylene chloride, chloroform, and ethylene dichloride.

Procedure.—The procedures previously described were modified (1). Specific volumes of the stock solution of the dye and amine were mixed in 4-ounce amber bottles and brought to a final aqueous volume of 40 ml. with distilled water. Usually 3 μm. each of the dye and amine were added. A 40-ml. quantity of the organic solvent was added to the 40 ml. of aqueous solution. Blank solutions were prepared also. All bottles were then shaken for 1 hour in an Eberbach horizontal shaker. Following the shaking the liquids were transferred to glass-stoppered separators, and the nonpolar and aqueous phases were separated.

Analysis.—The spectra of the aqueous solutions of the sulfonic acid dyes were scanned using the Cary model 15. The Beckman DU spectrophotometer was used for determining the absorbance of the dye salts in the aqueous extracts. The absorbance of the tropaeolin O solutions was determined after the solutions were made strongly basic with sodium hydroxide (pH > 12.5).

Determination of Apparent Partition Coefficient.—The apparent partition coefficient was derived in the previous paper knowing that the dye salt existed as the ion-ion pair in the organic phase and that the cation and anion were completely dissociated in the aqueous phase (1). The same approach was used in these studies in determining the apparent partition coefficient. The following chemical equation was used

$$\underset{c_1}{BH^+_{(aq.)}} + \underset{c_2}{D^-_{(aq.)}} \underset{}{\overset{K_{app.}}{\rightleftharpoons}} \underset{c_3}{S_{(benzene)}}$$

where K_{app} is the apparent partition coefficient,

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TABLE I.—APPARENT PARTITION COEFFICIENTS OF SOME DYE SALTS

Dye Salt	Apparent Partition Coefficient (L./mole)
Di-iso-amylamine salt of tropaeolin OO	1.5×10^{-3}
Di-iso-amylamine salt of methyl orange	2.3×10^{-4}
Di-*n*-octylamine salt of methyl orange	3.35×10^{-4}
Di-*n*-octylamine salt of tropaeolin O	1.3×10^{-4}
Di-*n*-octylamine salt of azobenzenesulfonic acid	2.5×10^{-4}

BH^+ (c_1) is the amine cation in the aqueous layer, D^- (c_2) is the sulfonic acid anion in the aqueous layer, and S (c_3) is the dye salt in the benzene layer. Therefore, the apparent partition coefficient was calculated using

$$K_{app} = \frac{c_3}{c_1 c_2} \qquad \text{(Eq. 1)}$$

The validity of Eq. 1 was checked in the manner previously reported. Various concentrations of amine cation and sulfonic acid dye were mixed in aqueous solution and partitioned with the organic solvent; the concentrations c_1, c_2, and c_3 were then determined. When c_3 was plotted against the product of c_1 and c_2, a straight line intercepting the origin was obtained. In addition, we worked with concentrations low enough knowing that dissociation of the dye salt in the aqueous phase was greater than 90%.

The di-iso-amylamine was the preferred amine for determination of the equilibrium constants using tropaeolin OO and methyl orange, whereas di-*n*-octylamine was used in the tropaeolin O and azobenzenesulfonic acid studies. Previously reported studies indicated that the mechanism of extraction of these two amine salts of tropaeolin OO was the same. The apparent partition coefficients (using aqueous and benzene solvents), calculated from eight or more determinations using various concentrations, are presented in Table I.

Determination of Binding Number and Over-all Stability Constant.—The complexing tendencies of primary alkanols and halogenated solvents with some dye salts are presented in Table II. The binding power of the complexing agent with the dye salt and the over-all stability constant for the complexation are also recorded. The data presented are obtained from six or more sample determinations.

The formation of the complex in the benzene solution may be expressed by $S + nA \overset{K_c}{\rightleftarrows} SA_n$, where S is the dye salt in the benzene phase, A is the complexing agent in the benzene phase, n is the binding number of the complexing agent, SA_n is the complex in the benzene phase, and K_c is the over-all stability constant. The mathematical expression for the over-all stability constant is

$$K_c = \frac{SA_n}{(S)(A)^n} \qquad \text{(Eq. 2)}$$

Equation 2 may be rearranged and expressed in the logarithmic form as

$$\log K_c + n\log A = \log \frac{SA_n}{S} \qquad \text{(Eq. 3)}$$

The concentration of A is expressed as the molality (m) of the complexing agent in the benzene layer. The amount of the dye which is not complexed in the benzene solution (referred to as S or as c_3 in Eq. 1) is calculated by (*a*) utilizing Eq. 1, (*b*) using the apparent partition coefficient listed in Table I, and (*c*) determining the concentrations of the cation and anion in the aqueous phase. Any additional increase in solubility of dye salt in the benzene solution will be attributed to complexation with the complexing agent present in the benzene. The complexed dye in the benzene solution (SA_n) is equal to the total dye salt in the organic layer minus the concentration of S. The total dye salt in the organic layer is equal to the concentration loss in the aqueous layer (1). The complexation number (n) of the complexing agent is the slope of the line obtained by plotting log (SA_n/S) *versus* log A. The complexation numbers for the various dye salts in benzene are listed in Table II. The plots of some of the data are presented in Figs. 1–5.

TABLE II.—OVER-ALL STABILITY CONSTANTS FOR INTERACTION OF COMPLEXING AGENTS WITH DYE SALTS IN BENZENE SOLUTIONS

Complexing Agent (A)	Dye Salt (S)	Binding No. Complexing Agent (n)	Over-all Stability Constant (K_c)
Amyl alcohol	Di-*n*-octylamine azobenzenesulfonate	2.23	3.5×10^2
Hexyl alcohol		2.30	4.72×10^2
Lauryl alcohol		2.23	6.69×10^2
Hexyl alcohol	Di-iso-amylamine salt of methyl orange	1.78	8.8×10^1
Octyl alcohol		1.78	9.4×10^1
CH_2Cl_2		1.15	5.5×10^{-1}
$CHCl_3$		1.11	5.4×10^{-1}
$Cl(CH_2)_2Cl$		0.96	4.2×10^{-1}
CH_2Cl_2	Di-*n*-octylamine salt of methyl orange	1.20	1.54×10^{-1}
Amyl alcohol	Di-isoamylamine salt of tropaeolin O	3.16	4.74×10^1
Octyl alcohol		3.00	8.42×10^1
Amyl alcohol	Di-iso-amylamine salt of tropaeolin OO	1.89	0.99×10^2
Hexyl alcohol		1.85	1.37×10^2
Octyl alcohol		1.81	1.67×10^2
Lauryl alcohol		1.84	1.80×10^2
CH_2Cl_2		1.68	1.24×10^{-1}
$CHCl_3$		1.97	1.21×10^{-1}
$Cl(CH_2)_2Cl$		1.88	1.01×10^{-1}

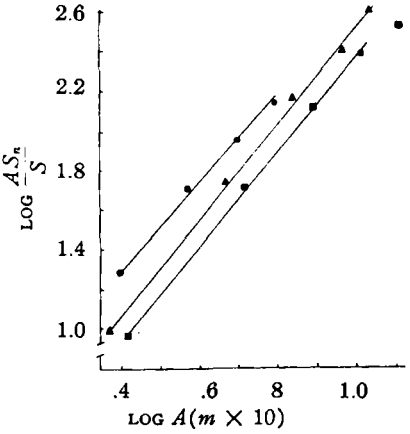
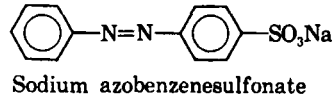


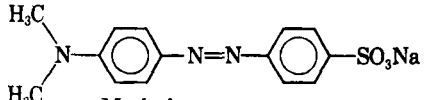
Fig. 1.—Log–log plot of the complexed (SA_n) and noncomplexed (S) amine salt of azobenzenesulfonic acid to the molality of the complexing agent. Key: ●, lauryl alcohol; ▲, hexyl alcohol; ■, amyl alcohol.

DISCUSSION

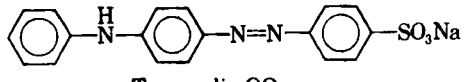
The sulfonic acid dyes were chosen because of the difference in functional groups attached to the azobenzenesulfonic acid nucleus. They were also chosen possibly to correlate the complexation of the alcohol or halogenated solvent to the structure. The formulas are



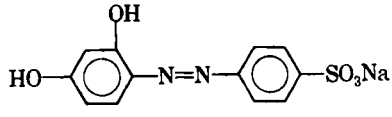
Sodium azobenzenesulfonate



Methyl orange



Tropaeolin OO



Tropaeolin O

Complexation with Primary Alkanols.—Alcohols are capable of being both acceptor and donor molecules in hydrogen bonding; the molecule or ion that serves as the proton acceptor may be termed the base (4). Pearson and Levine have stated that alcohols are "outstandingly efficient extractors of organic acids from water solution" (5). They also suggested that the sulfonate anion present in the aqueous phase would have little tendency to complex with any other structure than that of a hydrogen donor such as an alcohol.

Consequently, the presence of the amino or hydroxy group on the azobenzenesulfonate nucleus could affect the degree of complexation in the organic phase and could affect the apparent partition coefficient. In addition, the azo group may be considered to accept the hydrogen donor molecule since it is a basic functional group (6).

The binding number of the alcohols in the organic phase was determined using Eq. 3. The experimental data were plotted as shown in Figs. 1–5. The amine salt of azobenzenesulfonic acid complexed with 2 moles of the primary alcohol in benzene. Having determined this, the alcohol complexation was determined with the dyes having a substituted amine group, *i.e.*, methyl orange and tropaeolin OO. The salts of both dyes complexed with 2 moles of alcohol also. This may appear to be surprising, particularly for the salt of methyl orange, since one might predict 3 moles of alcohol to complex with the dye salt in benzene when compared to the azobenzenesulfonic acid studies. However, it is possible that the dimethylamine group hydrates, thereby carrying water into the organic layer. Investigations are now underway to determine if this does occur. Hydration has been found to occur in the partitioning of dibasic acids (7). Examination of the structure of tropaeolin O shows the presence of two phenolic groups. These groups had a positive effect in complexation with alcohol. Under the conditions of the experiments 3 moles, not 4 moles, complexed with tropaeolin O. Two moles of alcohol also complexed with the amine salt of tropaeolin OO. In considering the over-all stability constant and the apparent partition coefficient, it must be concluded that tropaeolin OO is the choice of the four dyes studied for amine salt extraction.

The presence of the phenolic groups had an over-all effect of reducing the apparent partition coefficient of the dye salt between benzene and water, whereas the dimethylamino group had a positive effect on the apparent partition coefficient.

It is significant to note that the over-all stability constants of the alcohol complexes of the dye salts increased with an increase in the molecular weight of the alcohol. This is demonstrated by examination of the values in Table II or the plots in Figs. 1, 2, 4, and (in particular) Fig. 5. The acid strength of aliphatic alcohols should not be expected to increase with an increase in molecular weight (4, 8). Therefore, the increase in the over-all stability constant for the complexation may be attributed to increased van der Waals forces of attraction as the chain length of the alcohol is increased. It is also possible that micelle formation may contribute to the over-all stability constant. Studies are underway to deter-

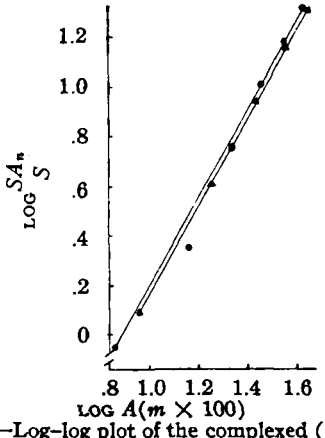


Fig. 2.—Log–log plot of the complexed (SA_n) and noncomplexed (S) amine salt of methyl orange to the molality of the complexing agent. Key: ●, octyl alcohol; ▲, hexyl alcohol.

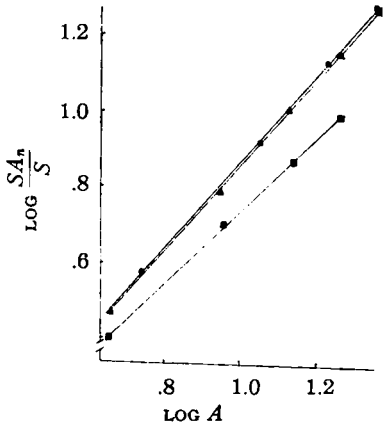


Fig. 3.—Log-log plot of the complexed (SA_n) and noncomplexed (S) amine salt of methyl orange to the molality of the complexing agent. Key: ●, methylene chloride; ▲, chloroform; ■, ethylene dichloride.

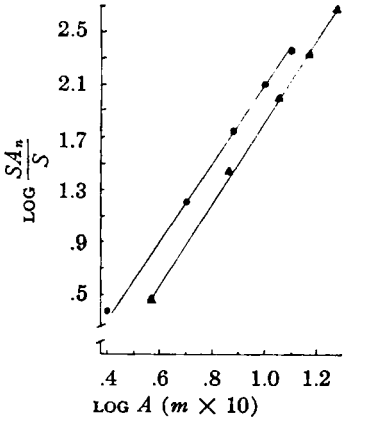


Fig. 4.—Log-log plot of the complexed (SA_n) and noncomplexed (S) amine salt of tropaeolin O to the molality of the complexing agent. Key: ●, octyl alcohol; ▲, amyl alcohol.

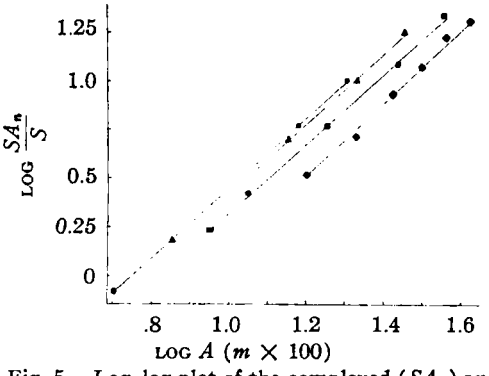


Fig. 5.—Log-log plot of the complexed (SA_n) and noncomplexed (S) amine salt of tropaeolin OO to the molality of the complexing agent. Key: ●, lauryl alcohol; ▲, octyl alcohol; ■, hexyl alcohol; ◆, amyl alcohol.

mine the thermodynamic properties (ΔF, ΔH, and ΔS) of the complexation.

When Mitchell and Clark developed a suitable method for the determination of quaternary ammonium compounds, they utilized the dye bromphenol blue. They used ethylene dichloride as the water immiscible extracting solvent. To minimize any possibility of adsorption of the dye salt from the organic solvent onto the glass surface, the investigators added 1.5% isoamyl alcohol by volume (9). As a result of our studies, one has a greater understanding of the use of isoamyl alcohol. All of the evidence provided should prove more beneficial to the analyst.

Complexation with Halogenated Hydrocarbons.—The halogenated solvents may be considered solvents whose acid strengths are less than those of the primary alcohols. Investigators recently have presented their research measuring the effectiveness of the C—H group in the halogenated solvents as a proton donor (10, 11). Complexation with these solvents is possible, but the over-all stability constant for the complexation would be less than the value for the alcohol complexation. This, as shown in Table II, was found to be true; the over-all stability constants with the halogenated solvents were about a thousandfold less than the values for the alcohol complexation. Complexation of the halogenated solvents with the dye salts of azobenzenesulfonic acid and tropaeolin O was so small that the over-all stability constant could not be determined.

The three halogenated solvents complexed with the amine salts of methyl orange and tropaeolin OO. Two moles of the hydrocarbon complexed with 1 mole of the tropaeolin OO salt. With this knowledge it was surprising to find that a 1:1 complex formed with the methyl orange salt and the hydrocarbon solvents. To verify the 1:1 complex formed with the di-iso-amylamine salt of methyl orange, studies were repeated using di-*n*-octylamine. As recorded in Table II, the di-*n*-octylamine salt of methyl orange also formed the 1:1 complex with the halogenated hydrocarbon in benzene.

The data obtained using the di-iso-amylamine salt of methyl orange are plotted in Fig. 3. On the molality basis, methylene chloride and chloroform are equally effective in extracting the dye salt. Similar results were obtained using tropaeolin OO. Since the over-all stability constants are the same or nearly so, it is quite possible to conclude that the acid strength of these halogenated hydrocarbons is the same, or the polar and nonpolar influences of the three solvents balance out to give the same over-all stability constant. These results then question the opinions of Mukerjee (2) and the authors as reported in our previous report (1) concerning the reliance on the dielectric constant in choosing a suitable solvent.

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Interaction of Sorbic Acid with an Insoluble Polyamide

By M. B. RODELL, W. L. GUESS, and J. AUTIAN

Specially treated nylon 66 strips were exposed to sorbic acid solutions at a number of different concentrations and at several temperatures and the quantity of drug sorbed determined by suitable means. Thermodynamic and kinetic treatment of the data permitted the evaluation of a number of constants such as (*a*) saturation value of drug in the plastic, (*b*) affinity of drug toward the plastic, (*c*) standard heat of sorption, (*d*) apparent diffusion coefficient, and (*e*) activation energy of diffusion. Changes in concentration of solution influenced both the apparent diffusion coefficient and the activation energy of diffusion. A nearly linear relationship was noted between the concentration of the solution and the diffusion coefficient, the diffusion coefficient decreasing with an increase in concentration of solution. The standard heat of sorption suggested that the interaction between the sorbic acid and the nylon might be due to a double hydrogen bond formation.

SORBIC ACID has been widely used in the food industry as an effective growth inhibitor for certain yeasts, molds, and bacteria. The pharmaceutical industry at times has also found important uses for sorbic acid as a preservative in certain dosage forms. The packaging of food and drug products in plastic containers has focused attention on the possible interaction between the plastic material and the preservative, reducing the concentration of the preservative to a level where the agent no longer exerts a significant effect. Marcus, *et al.* (1), noted the binding of sorbic acid by nylon. Autian and Shaikh (2) continued further work on the interaction of sorbic acid by nylon and found that solvent, temperature, and pH had an effect on the quantity of sorbic acid bound.

To gain more quantitative information on sorbic acid and nylon, which in turn could lead to more interesting results that were unobtainable in the previous studies, a series of sorption experiments was conducted with sorbic acid and one particular insoluble polyamide (nylon 66). This paper reports on the results of this particular study.

EXPERIMENTAL

Equipment and Supplies.—A Beckman DB spectrophotometer, (Beckman Instruments, Inc., Fullerton, Calif.), sorbic acid,[1] and nylon 66 (0.01 in. thick)[2] were employed.

Method of Analysis of Sorbic Acid.—In general, the analysis of sorbic acid in solution was conducted by diluting a 1.0-ml. aliquot to a volume of 500 ml. with 0.1 *N* HCl[3] and measuring the optical density at a wavelength of 264 mμ in the spectrophotometer using matched 1-cm. silica cells. Appropriate blanks were utilized throughout the investigation. The difference between the concentration of sorbic acid in the original solution and that after exposure to the plastic was assumed to have been sorbed by the nylon. Control solutions of sorbic acid without nylon were run simultaneously with the samples to detect if degradation had occurred in the solution. For the temperatures and time periods used in this study, no significant degradation of sorbic acid was noted in the control solution.

Effect of Concentration on Sorption.—Solutions of sorbic acid at seven concentrations ranging from 0.15% down to 0.0125% were prepared using distilled water as the solvent, and exactly 100 ml. of each of these solutions was pipeted into specially constructed cylindrical glass tubes, measuring 12 in. in length and 1.5 in. in diameter. The tubes were fitted with a ground glass stopper attached to the tubes by metal springs, allowing for total immersion into the water bath at the desired temperature. After a period of time adequate for equilibrium temperature to be reached in the tubes, the tubes were lifted from the water bath, the stoppers removed, and a strip of nylon[4] (approximately 2.0 Gm.) placed into each tube. The tubes were once again stoppered and carefully placed back into the water bath. Sorbic acid determinations were made on each solution after sufficient time had elapsed to ensure that equilibrium had been reached. These experiments were run in duplicate and at three different temperatures (55 ± 0.02°, 61 ± 0.02°, and 67 ± 0.02°).

The data were plotted by using a form of the Langmuir relationship

$$\frac{1}{q} = \frac{1}{KSC} + \frac{1}{S} \qquad \text{(Eq. 1)}$$

where q is the amount of solute sorbed by the nylon at equilibrium in moles/kilogram, K is a constant, C the equilibrium concentration of the solution in moles/liter, and S the saturation value in moles/

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[1] Marketed as Sorbistat by Chas. Pfizer and Co., Inc., Brooklyn, N. Y.

[2] Marketed as Polypenco 101 by the Polymer Corp. of Pennsylvania, Reading, Pa.

[3] Hydrochloric acid (0.1 *N*) was employed to suppress possible ionization of sorbic acid.

[4] In all instances the samples of nylon 66 used were washed as described in a previous paper (3).

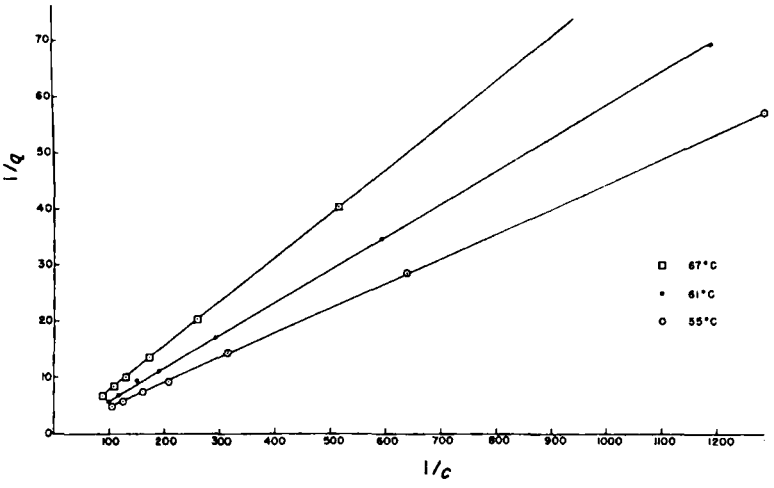


Fig. 1.—Sorption of sorbic acid by nylon 66 as a Langmuir plot at three different temperatures.

TABLE I.—EFFECT OF TEMPERATURE AND CONCENTRATION ON EQUILIBRIUM SORPTION OF SORBIC ACID BY NYLON 66

55° ± 0.02°C.		61° ± 0.02°C.		67° ± 0.02°C.	
$1/q$[a]	$1/C$[b]	$1/q$	$1/C$	$1/q$	$1/C$
4.850	104.8	5.770	97.64	6.863	87.32
5.770	124.9	6.916	116.2	8.628	108.5
7.496	161.3	9.025	151.3	10.26	129.9
9.285	207.3	11.34	190.3	13.74	173.4
14.53	314.4	17.09	293.5	20.33	259.2
28.65	641.3	34.84	594.2	40.65	518.4
57.14	1282.0	69.44	1189.0	75.18	1036.0
S[c] = 2.67		S = 5.69		S = 9.73	

[a] q = Amount of solute sorbed by nylon at equilibrium in moles/kilogram. [b] C = Equilibrium concentration of solute in solution in moles/liter. [c] S = Saturation value of solute in nylon if all sites were taken in moles/kilogram.

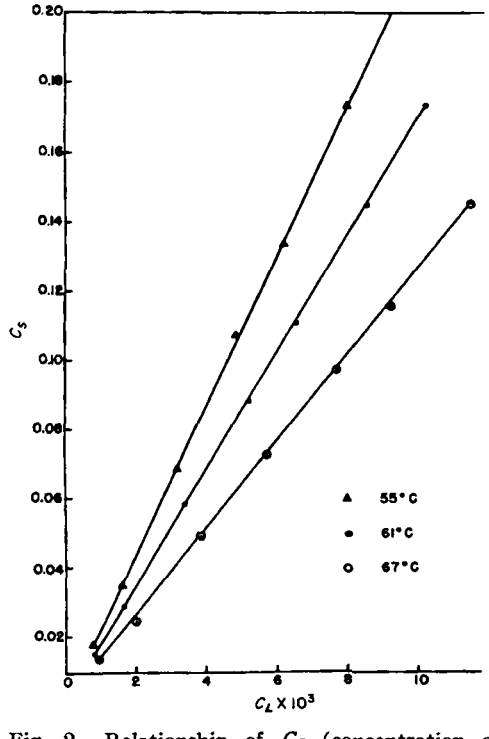


Fig. 2.—Relationship of C_S (concentration of sorbic acid in solid) and C_L (concentration of sorbic acid in liquid) at equilibrium and at three temperatures.

kilogram, or the theoretical amount of solute which the nylon would sorb if all the sites were filled. Table I presents the data as $1/q$ and $1/C$ values, while Fig. 1 illustrates the sorption isotherms at three temperatures. Each line was then extrapolated to the $1/q$ axis by least squares to find $1/S$, from which S was calculated. These values are included in Table I.

Affinity and Heat of Sorption.—To learn more of the attraction forces between the sorbic acid molecules and the nylon, it was decided to determine the difference in standard chemical potential ($-\Delta\mu°$) between the solute in the liquid phase and the solute in the solid phase. This difference, or $-\Delta\mu°$, is often referred to as the standard affinity (4) and may be approximated by using

$$-\Delta\mu° = RT \ln C_s/C_L \qquad \text{(Eq. 2)}$$

where C_s and C_L are the concentrations in moles/kilogram of solute in the solid phase and the solute in the liquid phase, respectively. The ratio C_s/C_L was determined from the slopes of the line shown in Fig. 2 where C_s is plotted against C_L. The experimental data used for the evaluation of affinity were taken from the previous experiment (*Effect of Concentration on Sorption*). Standard affinities for the three temperatures are shown in Table II.

TABLE II.—STANDARD AFFINITIES FOR SORBIC ACID AT THREE TEMPERATURES (NYLON 66)

Temp., °C.	$-\Delta\mu°$ (Kcal./mole)
55.0	2.00
61.0	1.87
67.0	1.71

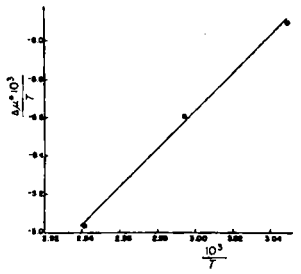


Fig. 3. — Relationship between affinity of sorbic acid (toward nylon 66) and temperature.

Standard heat of sorption ($\Delta H°$) for sorbic acid (in nylon 66) was calculated from the slope produced when $\Delta\mu°/T$ was plotted against $1/T$ as shown in Fig. 3.[5] This value ($\Delta H°$) was −9.77 Kcal./mole.

Diffusion and Activation Energy of Diffusion.—Since the sorption process of dyes and other agents in nylon is governed primarily by the diffusion step in the nylon, experiments were conducted to evaluate the apparent diffusion coefficient at three temperatures and at a number of original solution concentrations.

Exactly 300 ml. of sorbic acid solution at a definite concentration was placed in a 500-ml. three-necked, round-bottom flask. The solution was brought to the temperature of the water bath, at which time 6 Gm. of nylon (as strips) were introduced into the flask and the two outside necks sealed. A sealed stirring device was inserted in the middle neck of the flask and the glass rod adjusted so that the stirrer would fall just below the surface of the solution. The solution was stirred at a constant rate throughout all of the diffusion experiments. At various time intervals aliquots of solution were removed and sorbic acid content determined until equilibrium was reached. In each case the experiment was run in duplicate. Removal of aliquots from the solution did not materially alter the uptake of sorbic acid throughout the run.

The apparent diffusion coefficient was calculated for each original concentration at each temperature. An experimental plot of fractional uptake (M_t/M_∞) *versus* the square root of time is shown in Fig. 4 at one original concentration (0.075%). From the linear portions of these curves, D was calculated by using Berthier's method (5, 6) which has been explained in detail in a previous paper (7). These D values, as cm.²/second, are shown in Table III. Activation energy of diffusion (ΔE) was evaluated from the slope of the line when log D was plotted against $1/T$. Figure 5 shows these Arrhenius relationships, while Table III contains the actual ΔE values at the four original concentrations.

Nearly linear relationships were noted when the apparent diffusion coefficient at each temperature was plotted against the original concentration of sorbic acid in the solution. These relationships may be seen in Fig. 6.

DISCUSSION

Effect of Concentration on Sorption.—As the concentration of sorbic acid was increased, a related increase in the sorption of the solute by the nylon occurred. The Langmuir sorption isotherms for sorbic acid at three different temperatures followed the expected linear relationship for most of the points in the concentration range employed in the experiment. It will be noted that the lines do not have the same slope but do converge to one point on the $1/q$ axis (or $1/S$ as intercept) in the figure. This is as to be expected, at least theoretically, since the saturation value (S) may be thought of as the total possible quantity of solute which can interact with all of the sites in the plastic. Actual calculations by least squares, however, indicated slight differences in the intercept, which in turn gave different S values as may be seen in Table I. The difference in S values for each temperature may appear to be large (*e.g.*, at 55° the S value is 2.67, while at 67° the S value is 9.73), but part of this difference may be attributed to experimental error since a very small change in intercept can produce a much larger change in S value. One may also speculate that a difference in S values may be due to slight alterations in the nylon as the temperature is increased, producing new sites for interaction. Complete reliance on saturation values (S) for nylon or for that matter any plastic material should be discouraged since slight changes in the "same" plastic sample or the study of equilibrium sorption in other concentration ranges will often produce different S values.

Affinity and Heat of Sorption.—Standard affinity ($-\Delta\mu°$) decreased as the temperature increased (Table II) and parallels what has been seen for dyes and weak organic acids. The affinity values found for sorbic acid in this study fell below 2.0 Kcal./mole which were lower than for a group of weak organic acids sorbed by nylon 610 by a factor of 0.5 to 1.5 Kcal./mole (3, 7). The affinity values reported here, however, for sorbic acid were approximately the same (at comparable temperatures) for salicylic acid sorbed by the same type of nylon (nylon 66). For example, at 60° the standard affinity for salicylic acid (7) was equal to 1.84 Kcal./mole, while sorbic acid came to (at 61°) 1.87 Kcal./mole.

The standard heat of sorption ($\Delta H°$) was equal to

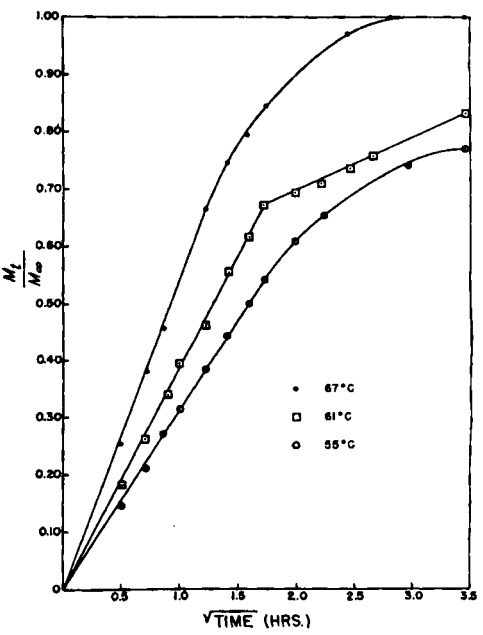


Fig. 4.—Fractional uptake (M_t/M_∞) of sorbic acid by nylon 66 *vs.* square root of time at three temperatures.

[5] Figure 3 follows the relationship: $\Delta\mu°/T = \Delta H°/T + C$.

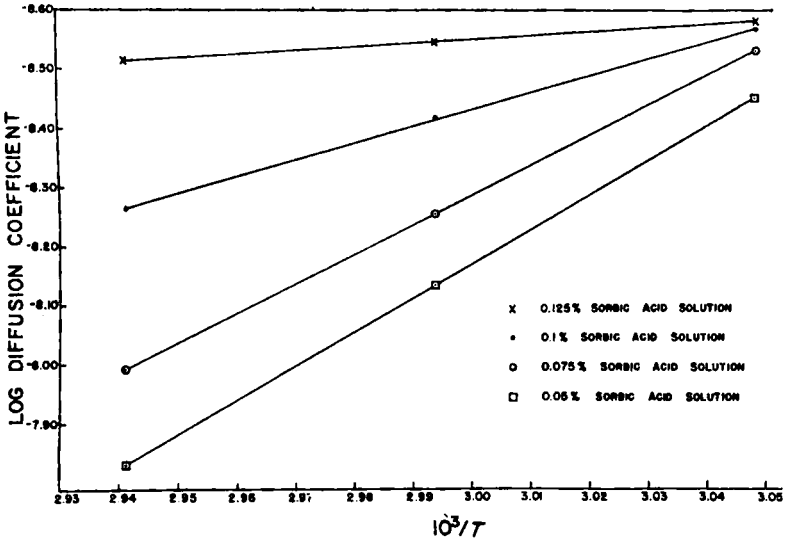


Fig. 5.—An Arrhenius plot of diffusion of sorbic acid in nylon 66 as log D *vs.* $1/T$.

TABLE III.—EFFECT OF TEMPERATURE AND CONCENTRATION ON DIFFUSION OF SORBIC ACID BY NYLON 66 AND ACTIVATION ENERGY OF DIFFUSION

55° ± 0.02°C.		61° ± 0.02°C.		67° ± 0.02°C.		
$C^a \times 10^3$	$D^b \times 10^6$	$C \times 10^3$	$D \times 10^6$	$C \times 10^3$	$D \times 10^6$	ΔE^c
11.53	0.262	10.67	0.285	10.98	0.309	3.07
9.276	0.270	8.681	0.383	8.907	0.542	12.8
6.978	0.295	7.019	0.555	6.731	1.01	22.7
4.453	0.353	4.515	0.729	4.535	1.46	26.2

[a] C = Initial concentration of solute in solution in moles/kilogram. [b] D = Diffusion coefficient in cm.²/second. [c] ΔE = Activation energy in Kcal./mole.

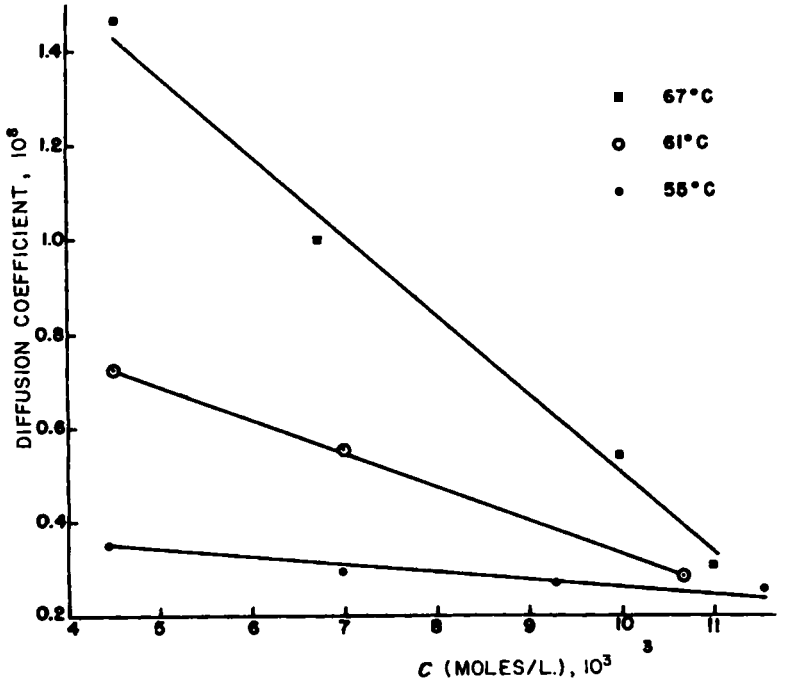


Fig. 6.—Relationship between apparent diffusion coefficient for sorbic acid in nylon 66 and original solution concentration at three temperatures.

−9.77 Kcal./mole, a value much higher than for other weak organic acids previously studied which had $\Delta H°$ values in the range of −2.0 to −3.63 Kcal./mole (3, 7). The higher $\Delta H°$ value for sorbic acid would seem to suggest that the molecule (sorbic acid) is interacting with the polyamide through two hydrogen bonds with secondary valence forces stabilizing the interaction. There may be one other possibility for the high $\Delta H°$ value for sorbic acid. A slight chemical reaction might be taking place. This possibility is now being investigated.

Diffusion and Activation Energy of Diffusion.—Table III illustrates the effect of temperature on

the apparent diffusion coefficients, an increase in temperature causing an increase in the rate of diffusion. The diffusion coefficients have an order to around 10^{-9} cm.²/second. These values were slightly less than, but not too far from, the values observed with a group of weak organic acids reported previously (3). Table III and Fig. 6 reveal that the original concentration of solution will have a direct effect on the apparent diffusion coefficient at any particular temperature or, in this case, as the original concentration in the solution is increased, the apparent diffusion coefficient decreases. These results suggest that the larger number of sorbic acid molecules (at the higher concentration) will have a tendency to reduce the mobility of the molecules as they attempt to traverse the matrix of the plastic. Conversely, in a more dilute solution of sorbic acid, less of the sorbic acid molecules will enter the plastic and less hinderance to passage or diffusion will occur.

The activation energy (ΔE) further confirms the effect concentration of the solution has on diffusion—from a low value of 3.07 Kcal./mole for the highest concentration of sorbic acid studied to a value of 26.2 Kcal./mole for the lowest concentration employed.

SUMMARY

Sorption studies were conducted on sorbic acid at a number of concentrations and at several temperatures, nylon 66 being used as the substrate. From these experiments it was possible to evaluate a number of constants such as (*a*) saturation value of solute in plastic, (*b*) standard affinity of solute for nylon 66, (*c*) standard heat of sorption, (*d*) apparent diffusion coefficient of sorbic acid in nylon 66, and (*e*) activation energy of diffusion in the plastic. The value of approximately −10.0 Kcal./mole for $\Delta H°$ suggested that there was a possibility of a double hydrogen bond formation between the sorbic acid and a reactive site in the plastic. Some evidence during the experiments also suggested that a slight chemical reaction might be taking place between the sorbic acid and the nylon 66. This point must, however, be further investigated. Results of the diffusion experiments revealed that concentrations of the original solutions influenced the rate of diffusion: the higher the concentration the slower the rate.

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Wurster Coated Aspirin II

An *In Vitro* and *In Vivo* Correlation of Rate from Sustained-Release Preparations

By JOHN H. WOOD and JOHN SYARTO

In vitro **and** ***in vivo*** **evaluations have been made of the salicylate release characteristics of aspirin coated by the Wurster process. The films used were of methyl and ethyl cellulose plasticized by glycerol. The** ***in vitro*** **release rates of such coatings were shown to be first order. The** ***in vivo*** **release may be best represented as first order. A mathematical correlation between** ***in vivo*** **and** ***in vitro*** **rates is possible, but this correlation is directly dependent on film composition. It is shown that the slower the release pattern, the less complete is the apparent plasma availability, due to incomplete physical release and insufficient time for complete biologic absorption.**

The last decade has seen an exponential increase in number and diversity of publications, both original and review, dealing with some aspect of the controlled rate of release of medicament following oral administration. These have covered both *in vivo* and *in vitro* evaluation and their possible correlations.

As part of our program of the study of dosage forms suitable for proprietary use, we were interested in release performance of crystalline drugs coated in the Wurster apparatus (1) to yield delayed release characteristics. The first paper of this series (2) describes the preparation of granules and their incorporation into a rapidly disintegrating tablet. The coatings were made of various ratios and amounts of ethyl and methylcellulose plasticized with glycerol.

Since it has been shown that stomach pH's may be quite variable (3) and since the normal stomach residence time and average emptying time may range up to several hours (4, 5), it is desirable for more uniform release between individuals

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Sorption studies were conducted on sorbic acid at a number of concentrations and at several temperatures, nylon 66 being used as the substrate. From these experiments it was possible to evaluate a number of constants such as (*a*) saturation value of solute in plastic, (*b*) standard affinity of solute for nylon 66, (*c*) standard heat of sorption, (*d*) apparent diffusion coefficient of sorbic acid in nylon 66, and (*e*) activation energy of diffusion in the plastic. The value of approximately −10.0 Kcal./mole for $\Delta H°$ suggested that there was a possibility of a double hydrogen bond formation between the sorbic acid and a reactive site in the plastic. Some evidence during the experiments also suggested that a slight chemical reaction might be taking place between the sorbic acid and the nylon 66. This point must, however, be further investigated. Results of the diffusion experiments revealed that concentrations of the original solutions influenced the rate of diffusion: the higher the concentration the slower the rate.

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Wurster Coated Aspirin II

An *In Vitro* and *In Vivo* Correlation of Rate from Sustained-Release Preparations

By JOHN H. WOOD and JOHN SYARTO

In vitro **and** ***in vivo*** **evaluations have been made of the salicylate release characteristics of aspirin coated by the Wurster process. The films used were of methyl and ethyl cellulose plasticized by glycerol. The** ***in vitro*** **release rates of such coatings were shown to be first order. The** ***in vivo*** **release may be best represented as first order. A mathematical correlation between** ***in vivo*** **and** ***in vitro*** **rates is possible, but this correlation is directly dependent on film composition. It is shown that the slower the release pattern, the less complete is the apparent plasma availability, due to incomplete physical release and insufficient time for complete biologic absorption.**

The last decade has seen an exponential increase in number and diversity of publications, both original and review, dealing with some aspect of the controlled rate of release of medicament following oral administration. These have covered both *in vivo* and *in vitro* evaluation and their possible correlations.

As part of our program of the study of dosage forms suitable for proprietary use, we were interested in release performance of crystalline drugs coated in the Wurster apparatus (1) to yield delayed release characteristics. The first paper of this series (2) describes the preparation of granules and their incorporation into a rapidly disintegrating tablet. The coatings were made of various ratios and amounts of ethyl and methylcellulose plasticized with glycerol.

Since it has been shown that stomach pH's may be quite variable (3) and since the normal stomach residence time and average emptying time may range up to several hours (4, 5), it is desirable for more uniform release between individuals

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TABLE I.—COMPOSITION AND CHARACTERISTICS OF TEST DELAYED-RELEASE ASPIRIN PRODUCTS USED IN THIS STUDY

	Code			
	134B	134C	152	138
Ratio ethyl to methylcellulose	75/25	25/75	82.5/17.5	100/0
Aspirin mesh size	−20	−40	−20+40	−20+40
Amount of coating (wt. %)	2.7%	4.8%	6%	6%
Tablet disintegration time, sec.	40–60	25–35	3	6
Aspirin content	5 gr.	5 gr.	5 gr.	5 gr.
Cornstarch	0.87 gr.	0.90 gr.	0.96 gr.	0.96 gr.
Talc	...	...	0.13 gr.	0.13 gr.

that the coatings not be significantly dependent upon ambient pH for their rate of release. This criterion is met by the coatings chosen here.

From a series of preparations of various *in vitro* release rates, it was desirable to establish the extent to which these could be differentiated *in vivo*. In this study acetylsalicylic acid was the drug of choice. Normally, salicylate would not be considered as a likely candidate for sustained release since its biologic half-life in a normal population is 6.2 hours by plasma studies (6) and 6 hours by urinary recovery (7). However, sustaining is practical and, in the case of the chronic arthritic under salicylate therapy, can be highly desirable for minimizing the excursions in plasma level (8) that are a necessary consequence of the usual periodic massive dosages. The hydrolysis *in vivo* of aspirin is very rapid so that the apparent plasma half-life is short (9). Thus, the permissible rate of release to maintain an aspirin level is limited by the residual free salicylate levels.

In vivo evaluation of aspirin release was, therefore, monitored in this report by the plasma salicylate levels rather than by urinary recovery.

EXPERIMENTAL

Preparation of Dosage Forms.—The preparation of the granules and tablets is described elsewhere (2). The specific composition of the test products is given in Table I. In each case the cellulose derivatives are plasticized with 17% glycerol.

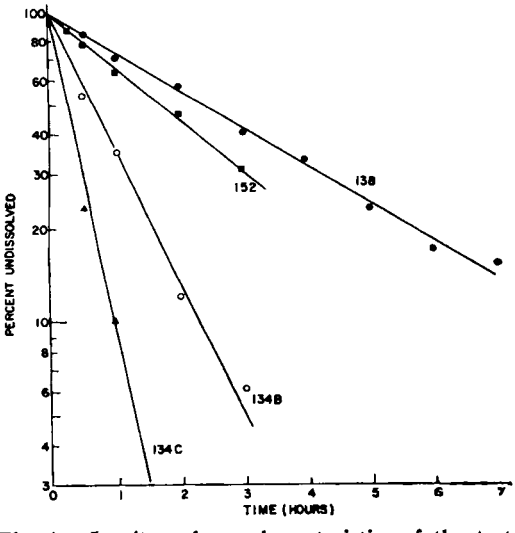


Fig. 1.—*In vitro* release characteristics of the test tablets.

In Vitro Evaluation.—Because tablet environmental conditions *in vivo* were felt to be variable and indeterminate, a test medium of pH 4 was arbitrarily chosen for use. Initially, McIlvaine standard buffer solution (12.29 ml. of 0.1 *M* citric acid plus 7.71 ml. of 0.2 *M* Na_2HPO_4) was used. Since the pH shifted, in test, from 4.00 to 3.80, the Na_2HPO_4 concentration was arbitrarily raised to 0.2125 *M* so that the pH shift was 4.05 to 3.95 during test. To 500 ml. of test solution in a 1-L. round-bottom flask thermostated at 37°, 15 gr. of coated aspirin product was added, either as granules or tablets. Stirring was with a 1/4-in. stainless steel rod, bent to a 1/2-in. loop off the axis of rotation, the end being on the axis. This was rotated at 420 r.p.m. using an Eberbach 7065 three-speed stirring motor.[1] This was a similar physical assembly to that which has been in use since 1957 and found valuable in study of normally fast-releasing tablets (10).

Aliquots were removed periodically and analyzed for salicylate following alkaline hydrolysis by reading on a Beckman DU at 298.5 mμ.

Release characteristics are shown graphically in Fig. 1. In Table II, replicability of the test procedure is demonstrated.

In Vivo Evaluation.—Plasma salicylate levels were determined as a function of time over a 24-hour period using the Brodie (11, 12) procedure after allowing the whole blood to stand for several hours to permit complete enzymatic hydrolysis of any aspirin (ASA) to the salicylate (SA)[2] (13). Thus, the procedure measures total plasma salicylate (and salicylurate, SUA, if any) present. It does not detect salicyl glucuronides (SG).

Administration was 20 gr. ASA at one time. As the control, buffered aspirin[3] was used to provide rapid complete absorption (13). The plasma levels obtained for the various dosage preparations tested are given in Table III.

It has recently been emphasized (14) that, because of the kinetics involved, a metabolite cannot *per se* be used directly to follow the rate of uptake of a parent drug. In the case of a sustained-release aspirin preparation it can be shown that plasma salicylate levels do meet the requirements of the simplified kinetic picture, where K_A and K_E are the apparent rate constants for absorption and elimination.

$$\text{Solid Drug} \xrightarrow{K_A} \text{Plasma Salicylate} \xrightarrow{K_E} \text{Organs of Elimination}$$

$$\text{Plasma Salicylate} \rightleftarrows \text{Apparent Volume of Distribution}$$

[1] Eberbach Corporation, P.O. Box 1024, Ann Arbor, Mich.

[2] We wish to acknowledge that these levels were obtained by Drs. W. D. Paul and J. I. Routh, School of Medicine, State University of Iowa, Iowa City.

[3] Marketed as Bufferin by the Bristol-Myers Co.

TABLE II.—REPLICABILITY OF *In Vitro* TEST PROCEDURE, PERCENTAGE DISSOLVED AS A FUNCTION OF TIME

Time	Sample 138 Run 1	Sample 138 Run 2	Sample 138 Run 3	Sample 152 Run 1	Sample 152 Run 2	Sample 152 Repeat Run 1	Sample 152 Repeat Run 2
5 min.	0	0	..	7	7	6	6
15	6	6	..	13	11	13	13
30	17	16	..	22	20	22	23
1 hr.	28	29	31	36	36	37	37
2	44	42	42	51	55	57	61
3	58	58	60	69	69	74	74
4	68	64	69	77	79	80	85

TABLE III.—SALICYLATE PLASMA LEVELS FOR INDIVIDUALS FOR THE VARIOUS FORMS AS FUNCTION OF TIME (20 GR. ASPIRIN ADMINISTERED)

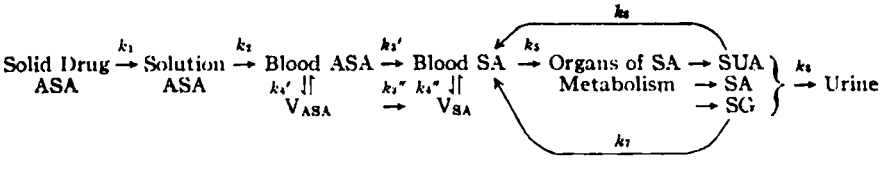
Sample	Subject[a]	Plasma Levels, mg./ml. 1/3 hr.	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
Buffered aspirin	1	87.0	120.0	125.0	108.0	69.3	36.5	0.0
	2	30.5	79.2	111.0	87.0	60.5	40.0	5.3
	3	20.7	64.2	103.4	104.4	64.4	38.2	0.4
	4	54.5	84.0	86.0	70.0	46.4	24.4	4.0
	5	105.7	112.2	104.7	94.7	58.2	31.7	0.6
134B	1	38.7	65.9	95.9	99.2	69.5	41.7	0.3
	2	3.0	8.0	44.7	77.2	59.7	37.1	2.0
	3	0.7	6.2	38.4	91.2	75.4	41.4	0.4
	4	9.3	17.3	58.0	62.5	40.4	23.4	0.3
134C	1	56.5	98.2	131.2	116.2	78.0	50.9	6.5
	2	6.3	20.3	55.2	81.7	56.7	36.7	1.7
	3	9.0	12.8	24.0	87.2	75.5	43.0	0.0
	4	34.0	57.5	59.0	45.4	21.3	8.0	1.0
138	2	6.0	13.3	25.3	36.4	35.0	29.0	9.3
	3	1.7	6.0	17.7	29.8	37.8	37.4	7.4
	4	2.0	10.3	19.4	24.8	27.0	23.4	1.3
	5	7.7	17.4	22.0	32.4	45.4	48.4	5.4
152	2	1.0	6.4	20.0	42.9	49.0	29.2	1.0
	3	2.6	11.3	25.0	49.3	57.5	53.5	5.3
	4	6.6	13.0	30.8	42.5	37.5	24.3	0.0
	5	20.0	36.8	60.0	65.0	64.0	45.0	2.0

[a] Subject 1, female, age 30, 110 lbs. Subject 2, male, age 48, 155 lbs. Subject 3, male, age 39, 150 lbs. Subject 4, male, age 32, 205 lbs. Subject 5, female, age 40, 139 lbs.

TABLE IV.—ACCUMULATED SALICYLATE DELIVERY TO THE PLASMA WITH TIME AS A PERCENTAGE FUNCTION OF THE TOTAL AMOUNT DELIVERED FROM A FAST-RELEASING TABLET

Sample	Subject	Percentage of Possible Total Delivery 1/3 hr.	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
134B	1	21	37	58	72	89	93	90
	2	3	7	42	82	89	86	80
	3	1	5	30	73	91	87	80
	4	7	14	48	62	74	77	77
	Av.	8	16	44	72	86	86	82
134C	1	27	48	69	75	92	97	100
	2	6	25	59	95	88	87	80
	3	6	9	18	69	88	86	73
	4	26	46	53	52	55	52	53
	Av.	16	32	50	73	81	81	77
138	2	5	11	22	36	46	52	54
	3	1	4	13	35	42	55	59
	4	2	8	17	26	40	59	59
	5	5	11	16	28	50	69	72
	Av.	4	8	17	31	44	59	61
152	2	1	5	17	41	61	57	51
	3	2	8	19	41	65	82	80
	4	5	11	27	44	59	63	61
	5	12	25	44	58	79	90	89
	Av.	5	12	27	46	66	73	70

A more realistic approximation of the various processes involved is given by



where V_{ASA} and V_{SA} are the apparent volumes of distribution of both ASA and SA, respectively.

It has been shown by Leonards (15, 16) that ASA is absorbed intact and very rapidly. Therefore, in a sustained-release preparation k_2 will be very much faster than k_1 and hence k_1 will be the rate determining step, equivalent to K_A of the simplified version. Although there is speculation concerning the site of ASA hydrolysis, the measured apparent *in vivo* blood half-life is appreciably less than 10 minutes and the half-life for k_4'' is of the same magnitude (6). Judging by Leonards' dog data (15), k_4' is of the same order as k_4''. Thus, it may be concluded that there is rapid hydrolysis and rapid dynamic equilibration of both species for their respective volumes of distribution. Both Leonards (15) and Smith (17) have reported that ASA yields slightly lower plasma levels than SA, presumably because of serum albumen binding of SA implying that the relative volumes of distribution are not greatly dissimilar. However, by 1 hour essentially no ASA remains in the plasma from a fast releasing form (15), and trivial percentages may be calculated to be present from a sustained form so that the consideration of an average volume of distribution V may be reasonably assumed.

Under dosage there is some tubular resorption of SUA and SG (18), totaling 1% at 2 hours and 6% at 24 hours of the total salicyl then present in plasma. Almost half of this is the SUA, which will be detected by the Brodie procedure. The correction then necessary to consider net k_5 equivalent to K_E of the simplified version is then negligible.

Thus, the Brodie procedure as used to measure the summation of drug and essentially all plasma metabolite gives a direct measure of total drug absorption and loss as required (14) for simplified kinetics. Internal self-consistency of the data thus obtained then becomes a verification of the assumptions.

From the plasma levels obtained from buffered aspirin, the apparent biologic half-life for each subject was determined. The range and average (6.1 hours for $t_{1/2}$), for the group was within that normally to be expected (6). The rate constants for elimination from the plasma were for subjects one to five 0.147, 0.099, 0.129, 0.150, and 0.145 hours^{-1}, respectively. Certain assumptions must then be made to calculate percentage recoverable from the delayed-release forms. First, for the time intervals used, plasma levels are in dynamic equilibrium with the tissues, and no appreciable quantity is irreversibly removed by a depot. For salicylates, this would appear to be the case (6). Second, it is assumed that first-order elimination rates are not dependent on dosage form. In this case, the kinetics are as already shown. The elimination is, at any time, proportional to the level present, and the total amount eliminated up to any time is readily obtained by mathematical or graphical integration. Thus, the amount absorbed to the plasma, x_t, at any time t is given by a summation of the amounts eliminated and the level C_t at that time. One of the possible forms of the equations representing this process is that given by Wagner, *et al.* (19), and was

$$X_t = V\left\{k\int_0^t C\,dt + C_t\right\} \qquad \text{(Eq. 1)}$$

where V is the effective equivalent distribution volume, and k is the salicylate elimination constant. For each individual the value of X_t may be calculated as a function of time in units of the individual V.

The value of X at infinite time—here 8, 12, and 24 hours—for the fast-releasing tablet is then considered to represent complete biologic recovery for 20 gr. of aspirin. Thus

$$X_\infty = Vk\int_0^\infty C\,dt \qquad \text{(Eq. 2)}$$

Then, for each of the test drugs, X_t may be evaluated as a function of time for each person. The ratio X_t/X_∞ then constitutes the fraction absorbed at that time, and this fraction becomes independent of the distribution volumes. The individual percentages of theoretical maximum are tabulated in Table IV, and the averages shown graphically in Fig. 2.

DISCUSSION

The general validity of the use of Eq. 1 is demonstrated by the good plateauing of total delivery values occurring for the later time periods for all the drug forms as shown in Table IV.

Careful minor reassignment of half-lives of the individuals would prevent the slight apparent fall off in total recovery tabulated for 24 hours; but this violates the calculation criterion set in the beginning of this report—namely, that the fast-release form of aspirin would be used as the sole criterion against which to judge the delayed forms.

As first pointed out by Wiegand and Taylor (20), the *in vitro* release of many sustained preparations is exponential and usually displays first-order kinetics. This is clearly seen in Fig. 1 for the dosages used in this study and was found for all preparations tested (2). It is surprising that no induction period appears to exist, since some initial limited destruction of the film might reasonably be expected. Any major cracking of the film is evidenced by rapid initial release, *i.e.*, a curve showing two release rates with time.

Plots of the cumulative *in vivo* release by both zero and first order gave somewhat better fits for the first order as shown in Fig. 2.

The literature contains frequent reference (21)

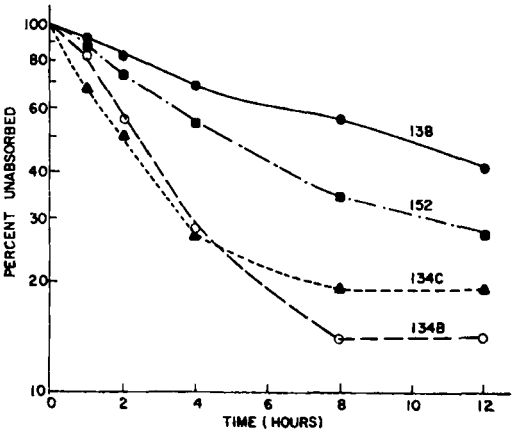


Fig. 2.—*In vivo* absorption characteristics of test tablets.

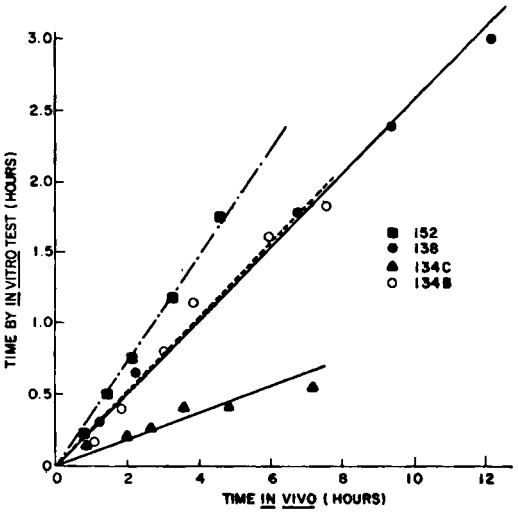


Fig. 3.—Correlation between *in vivo* absorption and *in vitro* release rates for corresponding fractions of total salicylate considered.

to the fact that no *in vitro* test can *per se* directly predict precise *in vivo* release. It was therefore of interest to determine for this study the extent to which a correlation might exist. Figure 3 was constructed by taking corresponding times for *in vivo* and *in vitro* release of the same fraction of the total salicylate used. Since both the *in vitro* test and the *in vivo* release could be represented by first-order plots, the lines of the correlation should be expected to be linear. This was so. It is immediately apparent that two lines are coincident, a third is close, and the fourth is somewhat divergent. The grouping of upper three lines represent the coatings of 75 to 100% ethylcellulose of various thicknesses, and there is no obvious correlation existing within this ranking. Probably the best average of the three would be most valid. The *in vivo* release between four individuals is so different that a larger panel would probably facilitate more rigid correlation. The obviously divergent line is for the 25% ethylcellulose.

As may be seen from a comparison of Figs. 1 and 2, the reason for this duality of correlation lines lies in the rapid *in vitro* release from the 25% ethyl-75% methylcellulose film which was not paralleled *in vivo*. Thus, it is apparent that *in vitro* testing may be used in this case to choose modifications of release rate patterns from high content ethylcellulose films by one correlation line. However, it is inferred that intermediate ratios of ethyl to methylcellulose between the limits studied here would display intermediate release rate correlations. Considering the relative similarities of these two cellulose derivatives, this is a striking confirmation of the earlier literature that *in vitro* data alone cannot predict *in vivo* performance.

One other observation should be noted. Since the integrated plasma levels may be considered a measure of the completeness of delivery of a drug, these levels clearly demonstrate that there is a price for delayed action—namely, incomplete recovery. In Table IV, it will be noted that for the fastest acting preparations, 134B and 134C, the release of salicylate to the plasma essentially ends at 8 hours, and that about 15 and 20%, respectively, are not apparently available for release. In the slowest releasing preparation, 138, all subjects show uptake to the 12-hour period. It is suggested, since so much aspirin is still unaccounted for, that this may signal the end of the period of ready biologic adsorption.

Thus, for release to occur over an 8-hour period, there is some loss in yield to the plasma. This loss increases with the slowing of the release rate.

It is then evident that integrated plasma levels can serve as a very convenient method for the study of drug release characteristics, both for the establishment of rates of absorption and for completeness of recovery.

As a result of the incomplete plasma recovery from delayed release and the long salicylate half-life in plasma, at no point does the sustained form provide superior plasma levels to those obtained from a fast-releasing tablet. However, where the elimination rate has been accelerated, as for arthritics under salicylate therapy (6), it is apparent that prolonged levels may be attained by suitable choice of dosage release rate.

SUMMARY

For aspirin coated with films of ethylcellulose-methylcellulose plasticized with glycerol, *in vivo-in vitro* correlations of release rates have been made. High ratios of ethyl to methylcellulose would appear to have one correlation curve. Variations in ratio to higher methylcellulose content are reflected in individual divergent correlations.

In vitro release rates from the film coatings used obeyed first-order kinetics.

The plasma absorption rate in this study would appear to closely obey first-order kinetics. The slower the sustained release, the less complete is the absorption recovery of the drug, probably due to incomplete release in all preparations and insufficient time for biologic absorption in the slower releasing preparations.

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Evaluation of Polymeric Materials III

In Vitro and *In Vivo* Testing of Granules Coated with the *n*-Butyl Half Ester of Poly(Methyl Vinyl Ether)/Maleic Anhydride

By ROBERT JAY NESSEL*, H. GEORGE DEKAY, and GILBERT S. BANKER

A sustained-action dosage form was developed in which film coated granules designed for independent distribution in the stomach were compressed into tablets for subsequent release in an intact condition upon disintegration of the tablet. The polymeric coating developed contained the *n*-butyl half ester of poly(methyl vinyl ether)/maleic anhydride, diethylphthalate, sorbitan monooleate, and precipitated calcium carbonate. The polymeric coating was applied to medicinally active granules of *d*-amphetamine sulfate which were assayed by *in vitro* and *in vivo* methods for drug release. The administration of the coated granules to a series of rats produced a statistically significant duration of action of 11.5 hours as compared to 7 hours for the uncoated granules. The coated granules were tableted utilizing polyethylene glycol 6000 as the matrix. The tablets of coated granules continued to produce increased motor activity in the test animals after 12 hours, while the tablets of uncoated granules showed no increased activity after 7.5 hours.

MANY INVESTIGATORS (1, 2) have studied various chemical and physical methods of controlling drug release from solid dosage forms intended for oral administration. One of the most prominent physical methods of achieving this goal has been the use of coatings.

In the last decade, a large number of products has been introduced that are designed to provide a prolonged duration of drug action. One of the most prominent of these consists of encapsulated coated pellets or beads. In the coating of these pellets mixtures of various fatty materials and/or waxes have been the most widely used materials, however, various synthetic polymers (3, 4) have also been used as coatings in the preparation of sustained-release dosage forms.

The tableted sustained-release products have been compressed ion-exchange resins, or drug-wax or drug-resin fusion products or similar mixtures. Coated particles designed for sustained release are conventionally placed in hard gelatin capsules. The virtual absence of coated particles designed for sustained release and placed in tablet form, with the dosage form designed to release the granules in the stomach, may be due to inadequately plasticized films, the use of less pliable films (cellulosics), the lack of a suitably elastic tablet matrix, or a combination of these factors.

The preparation of coated pellets in a tablet dosage form would be advantageous over the conventional encapsulated form from the standpoints of expense, patient convenience, accuracy of dosage, and ease of manufacture. This investigation was undertaken to study the feasibility of preparing a sustained-release dosage form in which coated particles designed for independent distribution in the stomach would be compressed in tablet form for subsequent intact release upon tablet disintegration.

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EXPERIMENTAL

Polyvinylpyrrolidone-d-Amphetamine Sulfate Dialysis.—Polyvinylpyrrolidone[1] was selected as the granulation binding agent because it produces very hard granules. This characteristic is necessary in the formation and coating of granules by the pan method, and was considered vital to the objectives of the study.

Since polyvinylpyrrolidone has been reported to interact with a variety of medicinals (5), it was necessary to determine if any such interaction occurred with *d*-amphetamine sulfate and if the polymer interfered with the assay procedure.

Twenty milliliters of a 3.5×10^{-4} *M* solution of polyvinylpyrrolidone was accurately pipeted into bags prepared from Nojax Visking casing. The bags were placed in bottles containing 70 ml. of a 5×10^{-3} *M* solution of *d*-amphetamine sulfate. Four such bottles were prepared, tightly sealed, and rotated end over end at 41 r.p.m. in a water bath maintained at 37 ± 2°. Two bottles were removed after 24 hours and the others after 48 hours. Control bottles were similarly prepared by (*a*) substituting distilled water for the polyvinylpyrrolidone, and (*b*) replacing the drug solution with distilled water. These samples served to determine drug-membrane binding and polymer permeability of the membrane. The assay for drug content was conducted using the baseline technique, as described by Royal (6), on the internal and the external solutions. The Bausch & Lomb spec-

[1] Antara Chemicals, Division of General Aniline and Film Corp., New York, N. Y.

tronic 505 recording spectrophotometer[2] was used to obtain the data on the polyvinylpyrrolidone–distilled water samples. The assay for drug content was conducted on the Beckman DU model 2400 spectrophotometer.[3]

Preparation of Granules.—The granulation used for the coating procedure was

d-Amphetamine Sulfate	3%
Lactose	92%
Polyvinylpyrrolidone K-30	5%
Ethyl Alcohol 75%	*q.s.*

The dry ingredients (3 Kg.) were mixed and granulated with the alcohol. The mass was passed through a 20-mesh screen by hand and the resulting wet granules were placed in a standard 12-inch stainless steel coating pan revolving at 36 r.p.m. The granules were allowed to rotate until they were rounded and the granule surfaces smoothed, after which an infrared lamp was directed into the revolving pan for drying. The dried granules were sized and those passing through a 20-mesh but not a 30-mesh screen were selected for coating.

Coating Procedure.—In the preparation of a coated granulation, 150 Gm. of 20/30-mesh granules was placed in a 6-inch copper coating pan.[4] Coating solutions containing 5% w/v of the *n*-butyl and isobutyl half esters and the 2-ethylhexyl partial ester of PVM/MA [poly(methyl vinyl ether)/maleic acid], one or the other, and 1% w/v of sorbitan monooleate plasticizer in the acetone-alcohol solvent system, were separately applied to the granules. Application was achieved by spraying the various solutions on the moving bed of granules, with a No. 15 DeVilbiss[5] atomizer. Coating was continued until 10% polymer, based on granulation weight, was applied. The acrylic/methacrylic copolymer was similarly applied, but due to its high molecular weight and high viscosity could be applied in a solution concentration of only 1%, and was thus added as a coating at a lower level; 1% polymer to granulation weight.

In addition to coating the granules with the polymer films described above, the granules were also coated with a combination of polymer material and dusting powder. In this study the coating procedure involved spraying 150 Gm. of 3% *d*-amphetamine sulfate granules, as previously described, with Fully Plasticized Coating Solution (No. 1)

n-Butyl Half Ester of PVM/MA	5%
Sorbitan Monooleate	3%
Diethylphthalate	1.5%
Acetone	
Ethyl Acetate	aa *q.s.* 100%

A sufficient quantity of solution was sprayed on the moving granule bed to completely wet the granules to the point that they began to adhere to each other. One of the three dusting powders (talc, magnesium stearate, or precipitated calcium carbonate) was then applied in a quantity sufficient to restore free bed movement. An infrared lamp was used to thoroughly dry the coating before repeating the cycle. Each dusting powder was used with the coating solution at a 5% and at a 10% level of polymer to uncoated granulation starting weight (150 and 300 ml. of coating solution). In coating with the dusting powder, the coating solution containing the highest concentration of sorbitan monooleate and phthalate plasticizer which would produce an intact film was used, in order that the final film containing a high level of dispersed solid would retain some degree of flexibility.

In Vitro Assay and Sampling Procedure.—The assay procedure of Royal (6) was modified in the case of the coated granules, due to polymer and plasticizer interference. The modification entailed the use of carbon tetrachloride as a selective solvent for the plasticizer and an ion-exchange resin, Amberlite IRC-50,[6] to separate the polymer and the drug.

Approximately 1.0 Gm. of coated granules was accurately weighed and placed in a 90-ml. bottle to which was added 60 ml. of simulated gastric fluid. The bottle was tightly sealed and rotated at 41 r.p.m. in a water bath maintained at 37 ± 2°. Samples of the test solution were periodically withdrawn and replaced with other test solutions as shown in Table I.

TABLE I.—SAMPLING TIME AND FLUID REPLACEMENT FOR *In Vitro* ASSAY

Time, hr.	Sample Withdrawn, ml.	Replacement Fluid	ml.	pH of Test Solution
0.0	..	...	..	1.2
0.5	20	Gastric	20	1.2
1.5	30	Intestinal	30	2.4
2.5	40	Intestinal	40	6.8
4.5	30	Intestinal	29	...
		NaOH T.S.	1	7.6
6.5	30	Intestinal	29	...
		NaOH T.S.	1	7.6
8.0	30	...	..	...

A 10-ml. aliquot of each sample which was withdrawn after the time intervals shown in the table was pipeted into a separator containing 10 ml. of carbon tetrachloride, and the mixture was agitated until equilibrium was obtained. The aqueous layer was then quantitatively transferred to an ion-exchange column and allowed to filter at the rate of 1 ml. per minute. To each of the 10-ml. aliquots removed from the samples withdrawn after 0.5, 1.5, 2.5, and 4.5 hours, sodium hydroxide test solution was added to neutralize the acid present and thus prevent premature elution of the drug. The column was then washed with approximately 30 ml. of distilled water. Elution of the drug was carried out using 0.5 *N* sulfuric acid and the eluate was collected in a 50 ml. volumetric flask at the rate of 1 ml. per minute. The acid was passed through the column until the eluate measured 50 ml. Each solution was then assayed for drug content on the Beckman DU model 2400 spectrophotometer.

In Vivo Assay of Granules.—The method used in the determination of the *in vivo* release data involved the use of the Williamson activity cage[7] to measure increased spontaneous motor activity.

Female Holtzman rats were used throughout the study. Their initial weight in each case was between 150 and 175 Gm. In all cases, the rats were fasted 18 to 24 hours prior to dosing and were precondi-

[2] Bausch & Lomb Optical Co., Rochester, N. Y.
[3] Beckman Instrument Co., Fullerton, Calif.
[4] Arthur Colton Co., Detroit, Mich.
[5] The DeVilbiss Co., Somerset, Pa.
[6] Rohm & Haas Co., Philadelphia, Pa.
[7] Williamson Development Co., Inc., West Concord, Mass.

TABLE II.—TWENTY-FOUR HOUR DIALYSIS STUDY OF POLYVINYLPYRROLIDONE AND *d*-AMPHETAMINE SULFATE

Solution Inside Permeable Sac	Solution Outside Permeable Sac	Sample Assayed	Drug Concn., mg./ml.
PVP[a] solution	Drug	Inside sac	1.5
PVP solution	Drug	Outside sac	1.4
Water (control)	Drug	Inside sac	1.5
Water (control)	Drug	Outside sac	1.5

[a] Polyvinylpyrrolidone.

tioned 30 minutes to acquaint the animal with the cage. In all portions of this study, three groups of animals were used simultaneously. One group served as controls, one received uncoated granules of the drug, and the third group received coated *d*-amphetamine sulfate granules. Ten animals were employed in each group. All doses were administered by stomach tube as 5 ml. suspensions in 5% hydroxypropyl methyl cellulose[8] solution. The control animals each received 5 ml. of the suspension vehicle.

Each dose was calculated on the basis of 10 mg. of drug per kilogram of body weight, taking into consideration the weight of the coating. Five milliliters of the suspension vehicle was poured into the barrel of a 5 ml. hypodermic syringe, to which was attached a No. 10 French catheter by means of a collar. The granules were added to the barrel and mixed with the suspension vehicle. The catheter was then forced down the esophagus of the preconditioned rat into the stomach, and the suspension was introduced with the aid of the syringe plunger. The animals were placed in the Williamson activity cages, the room darkened, and the recorder started. The door to the test room was closed and other steps were taken to reduce external animal stimulation due to noise. The test period lasted 12 hours, at which time the total number of responses in each successive half-hour period was determined. For each run of the activity cages at least one animal received coated granules, one received uncoated material, and one (the control) received the vehicle only. According to this method, any condition resulting in external stimulation of the animals would affect all three test groups and would thus have a minimal effect on the end results. As stated above, ten animals received coated granules, ten received uncoated granules, and ten served to provide the control activity line.

The hydroxypropyl methyl cellulose suspension vehicle for the coated and uncoated granules, was selected from a series of suspension vehicles studied, and was virtually the only vehicle of the group which did not appear to affect (retard) the absorption of the drug studied. The vehicles studied included solutions or dispersions of starch, Methocel 4000,[8] sodium carboxymethylcellulose,[9] and Cab-O-Sil,[10] as well as Tween 80,[11] and corn oil.

The *in vivo* assay procedure utilized the granulation coated with 10% w/w of *n*-butyl half ester of PVM/MA [poly(methyl vinyl ether)/maleic acid, low molecular weight grade], plasticized with sorbitan monooleate 60% and diethylphthalate 33% based on polymer weight, and using precipitated calcium carbonate as the dusting powder. A series of ten trials was conducted and the responses per half-hour interval were averaged. Statistical analysis was accomplished using the Student Newman Kuels test as described by Winer (7).

Preparation of Tablets.—The formulas for the tablets were

TABLETS OF COATED GRANULES

Polyethylene Glycol 6000[12] (20-mesh flakes)	90 mg.
Coated Granules	40 mg./tablet

TABLETS OF UNCOATED GRANULES

Polyethylene Glycol 6000 (20-mesh flakes)	90 mg.
Lactose	18 mg.
Uncoated Granules	22 mg./tablet

CONTROL TABLETS

Polyethylene Glycol 6000 (20-mesh flakes)	90 mg.
Lactose	40 mg.

Tablets were designed to contain the correct drug dose (10 mg./Kg.) for each rat according to weight. The quantity of each ingredient per tablet was weighed separately, mixed, and transferred to a 1/4-inch die for compression by hand on a single punch tablet machine, to a hardness of 5 to 7 Kg.

In Vivo Assay of Tablets.—Administration of the tablets to the test animals was accomplished by surgical procedure. Female rats were fasted as previously described and were then anesthetized. An incision was made in the area of the greater curvature in the cardiac region of the stomach, and three tablets of one of the three series were introduced into the stomach. The incision was then sutured and the animals were transferred to activity cages. Three groups of animals were used as before (one group for tablets containing coated drug granules, one for uncoated granules, and one for the control tablets), but in this case only three or four animals were used per group. Statistical analysis of the results was conducted in the same manner as that used for the granules.

RESULTS AND DISCUSSION

Polyvinylpyrrolidone-d-Amphetamine Sulfate Dialysis.—Prior to the preparation of the *d*-amphetamine sulfate granulation, it was necessary to determine the existence or extent of drug-polymer granulating agent interaction. The results of the dialysis study relating to this portion of the work are shown in Table II. Other dialysis samples which were run for 48 hours demonstrated the same results as those shown in the table, *i.e.*, free and equilibrated drug exchange across the membrane indicating no significant polymer drug binding.

Drug Release from Coated Granules.—The *in vitro* release rates of the 3% *d*-amphetamine sulfate granulation coated with 10% w/w polymer are given in Table III. Only 1% w/w of acrylic/methacrylic copolymer coating was used as previously discussed. The rapid release rates found for these coated granules was at first regarded as surprising, but on more careful analysis was concluded to be the result of inadequate coating.

[8] Methocel 60HG, 50 cps., Dow Chemical Co., Midland, Mich.
[9] CMC-7HG, Hercules Powder Co., Wilmington, Del.
[10] Cabot Corp., Boston, Mass.
[11] Atlas Chemical Co., Wilmington, Del.
[12] Carbowax, Union Carbide and Carbon Co., New York, N. Y.

TABLE III.—RELEASE OF *d*-AMPHETAMINE SULFATE FROM COATED GRANULES

Polymer Coating	% w/w of Polymer Coating	% Release with Time ½ Hr.	1½ Hr.
PVM/MA 2-ethylhexyl ester	10	84	100
PVM/MA *n*-butyl half ester	10	82	100
PVM/MA isobutyl half ester	10	85	100
Acrylic/methacrylic copolymer	1	96	100

One gram of uncoated 20/30-mesh drug granulation was found to contain about 1750 granules. Since the rounded granules passed a No. 20 U. S. Sieve Series standard sieve and were retained on a No. 30 sieve, the mean diameter of the granules might be taken as the mean open dimension of these sieves, or 0.72 mm., as a sieve analysis approximation. The surface of 1750 such particles if they were true spheres, would be 28.2 cm.^{2}. Since many of the "rounded" granules were observed to be elongated the actual surface was probably considerably larger than this. If a thin film such as the 0.003-inch dry film which was the thinnest studied in the permeability study, was applied to completely coat 1 Gm. of granulation, 0.214 cm.^{3} of polymer would be required (28.2 cm.^{2} area × 0.0076 cm. thickness). This corresponds to 0.278 Gm. of polymer film material needed to coat 1 Gm. of granulation (the film density was 1.30 Gm./cm.^{3}). In other words if the granules had been truly spherical, nearly 30% of polymer composition would be required, based on the weight of the uncoated granulation, to produce a thin drug impermeable film about the granules. Since the granules were not truly smooth surfaced and spherical, the actual percentage of polymer coat material needed to produce the necessary protective film, might be closer to 50 to 100% of the granulation weight being coated at a 20/30-mesh uncoated particle size range.

Selection of a Filler-Coating Dusting Powder.—Since the application of more than 10% w/w of polymer film composition could not be conveniently or economically applied to the granules, various dusting powders were studied as coating adjuvants in order to increase effective coat thickness without having to resort to long coating times and high polymer concentrations in the dosage form. The materials used as dusting powders included two agents which are substantially insoluble in the pH environment of the alimentary canal—talc and magnesium stearate—and one material, precipitated calcium carbonate, which is reactive and soluble in the acid environment of the stomach.

Eight granulations were coated with the aid of the three dusting powders and the average amount of each coating constituent per gram was calculated (Table IV). Utilizing the extraction procedure for the removal of the plasticizer from solution, each granulation was assayed with time and pH change to determine the drug release rate (Table V).

In reference to Table V it will be noted that the granulations coated with the aid of magnesium stearate as a dusting powder, showed some promise as prolonged-action coatings as the concentration of dusting powder was increased. However the low density of the material resulted in more than doubling the granule size when 0.20 Gm. of magnesium stearate was added to the uncoated granule weighing 0.40 Gm. The coatings containing magnesium stearate were also friable.

The granulations coated with the aid of talc preliminarily released the drug *in vitro* even at the higher dusting powder concentrations.

Precipitated, calcium carbonate even though it was the only dusting powder used which was not inert in the test solutions, most effectively prolonged drug release *in vitro*, of the three dusting powders used. More recent work with other polymers, as well as the data reported by Wagner (4), suggests that each polymeric coating must be separately considered to determine the best dusting powder filler. It might be hypothesized that the various polymer film surfaces vary in their affinity for the various dusting powders, and that this affinity is an important consideration in this type of dosage form development.

TABLE IV.—AVERAGE AMOUNT OF COATING CONSTITUENT APPLIED TO EACH GRANULE[a]

Coating Number	Polymer,[b] mg.	Diethylphthalate, mg.	Magnesium Stearate, mg.	Precipitated Calcium Carbonate, mg.	Talc, mg.
1	0.0284	0.0085	...	...	...
2	0.0568	0.0171	...	...	...
3	0.0284	0.0085	...	...	0.185
4	0.0568	0.0171	...	...	0.368
5	0.0284	0.0085	0.1014	...	...
6	0.0568	0.0171	0.2041	...	...
7	0.0284	0.0085	...	0.2230	...
8	0.0568	0.0171	...	0.3860	...

[a] Calculation: 0.402 Gm. of uncoated granules = 707 granules on the average; therefore, 150 Gm. = 263.806 granules. Thus, (total quantity of material added/263.806) = quantity/granule. [b] *n*-Butyl half ester of PVM/MA.

TABLE V.—*In Vitro* ASSAY OF PER CENT DRUG RELEASE FROM GRANULES COATED WITH THE DIFFERENT DUSTING POWDERS

Coating Number[a]	Per Cent Release After (Hr.) 0.5	1.5	2.5	4.5	6.5	8
1	69.48	1.56	0.00	Dissolved	...	...
2	44.41	24.67	0.49	Dissolved	...	...
3	64.34	3.28	0.00	Dissolved	...	...
4	60.96	7.54	0.00	Dissolved	...	...
7	48.16	16.05	0.00	5.55	Dissolved	...
8	25.69	35.60	0.00	0.00	9.91	Dissolved

[a] Constituents listed in Table IV.

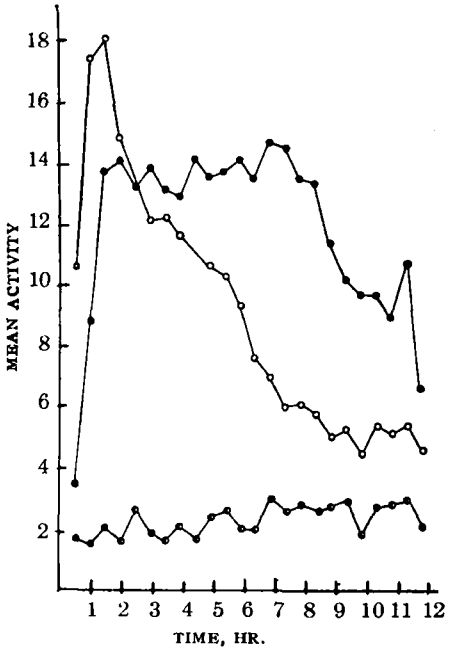


Fig. 1.—*In vivo* comparison of coated and uncoated granules. Key: ● coated, O uncoated, ◐ control.

In Vivo Activity of Coated Granules.—The *in vivo* activity of the coated granules was determined using the 3% *d*-amphetamine sulfate granulation coated with 10% of PVM/MA *n*-butyl half ester applied from the fully plasticized coating solution (No. 1), with the aid of the calcium carbonate dusting powder (101.8 Gm. of powder/150 Gm. of uncoated granulation). The coated granules were then administered to the test animals as previously described. The *in vivo* results of administering the coated granules, the uncoated granules, and the control material are presented in Fig. 1. Each line in Fig. 1 represents the average results from the responses of ten animals, with the data being averaged and plotted at half-hour intervals.

The results of the Newman Kuels significance tests showed that the uncoated granules had an immediate onset of action (within the first half-hour), and that the duration of action lasted until the seventh hour. At this time there was no significant difference between the control and the uncoated granules (2 × 3). The coated granules caused a significant response within 1 hour after administration and provided a duration of significant therapeutic response lasting 11.5 hours. This is an increase in duration of activity of the coated granules over the uncoated material of 4.5 hours. In addition to the longer duration of activity the coated granules eliminated the high response peak in the first hour. This was statistically shown by the significant difference between the coated and uncoated granules during that time period.

Tableting of Coated Granules.—Polyethylene glycols with and without starch or lactose were evaluated as tablet matrices for the coated granules. The major criterion for the selection of the tablet matrix was the prevention of rupture of the granule coating upon compression.

The tablets were prepared by mixing various ratios of polyethylene glycol 6000, 9000, and 20,000 and coated granules. The results of the disintegration tests on the tablets prepared with different polyethylene glycols showed that the addition of starch and lactose had no effect on the disintegration time as compared to that of tablets using polyethylene glycol alone. During the compression of the coated granules the pressure was adjusted to produce tablets of a hardness of 5 to 7 Kg., so as to maintain a disintegration time of 15 to 20 minutes and prevent rupture of the granule coating. Disintegration time increased considerably and the coating ruptured when the hardness of the tablets was 9 Kg. or higher. Analysis of the disintegrated tablets for ruptured granules showed that polyethylene glycol 6000, 20-mesh flakes, was the best matrix.

As a result of the tableting investigation, a maximum of approximately 30% coated granules was found to produce the most satisfactory tablets. Concentrations of coated granules exceeding this resulted in ruptured coatings due to an inadequate amount of "cushioning" material. Granules with ruptured coatings were identified by their ability to float, upon tablet disintegration, as compared to the denser intact granules. The floating granules were found to be empty shells. The pattern of granule release upon tablet disintegration is shown in Fig. 2.

Figure 3 shows the time-response curves for each group of tablets. The onset of action of the tablets containing coated granules occurred 2 to 3 hours after administration, which was considerably slower than the onset of the coated granules. This is due to the fact that the tablets must disintegrate before the granules are released. Although the disintegration time *in vitro* was between 15 and 20 minutes, this time period was probably considerably longer *in vivo* due to the smaller volume of fluid present in the animal's stomach. In the formulation of a complete product, it would thus be desirable to combine coated granules with some uncoated granules in order to provide the initial dose.

The release of the *d*-amphetamine from the uncoated granules compares favorably to the release obtained from tablets of the same granules. Significant reponses were obtained from the tablets for 7.5 hours as compared to 7 hours for the independent granules. Tablets of the coated granules continued to show increased activity after 12 hours, while the independent granules showed no increased activity after 11.5 hours.

Sustained action was obtained from these coated granules by two mechanisms. The first involved the random entry of the granules into the intestinal tract. The active constituent was then released by dissolution of the coating at the various pH levels of the intestine due to the small variation in coating

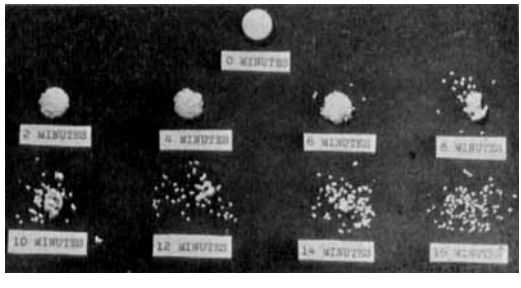


Fig. 2.—Granule release upon tablet disintegration.

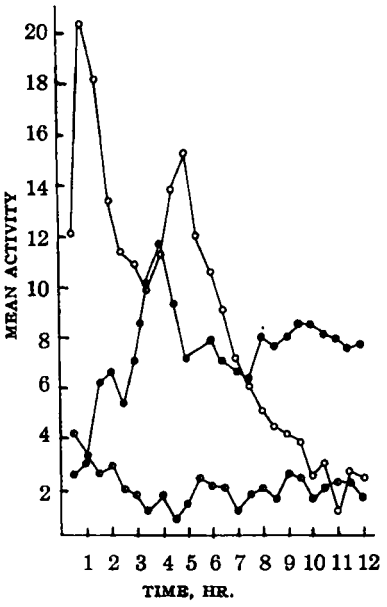


Fig. 3.—*In vivo* comparison of tableted coated and uncoated granules. Key: ● coated, O uncoated, ◐ control.

thickness, which occurs in pan coating. This was shown by the *in vivo* procedure.

An added advantage to this approach to obtaining sustained release is the use of a single coating composition which is applied in one concentration to an entire batch of granules. Therefore the entire coating process is accomplished in one easy operation without additional coatings and mixing of the coated granules.

SUMMARY

d-Amphetamine sulfate granules were successfully coated with a plasticized film of the *n* butyl half ester of PVM/MA 119 using each of three dusting powders: talc, magnesium stearate, and precipitated calcium carbonate. *In vitro* release rates were determined for each coating and the granules coated with the aid of precipitated calcium carbonate produced the most satisfactory results. *In vitro* tests indicated that the coated granules could be recovered intact from compressed tablets.

In vivo release rates were determined using the Williamson activity cage to measure spontaneous motor activity. The results obtained showed that the activity of the rats receiving the coated granules was 4.5 hours longer than those receiving uncoated granules.

In vivo analysis of tablets prepared by compressing the coated granules in a polyethylene glycol 6000 matrix, showed a continued increase in motor activity after 12 hours, while the tablets containing uncoated granules showed no increase after 7.5 hours.

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Gas Chromatography of Some Antihistamines

By ALEXANDER MACDONALD, JR., and RONALD T. PFLAUM

The gas chromatographic behavior of 16 antihistamines was investigated using packed 0.010-in. diameter capillary and 0.065-in. open tubular columns. All packed columns were 6 ft. long, with a low phase to support ratio and were operated at 175°. The liquid phases used were Carbowax 20M, SE-30, XF-1150, and PDEAS. Of the seven solid supports evaluated for use in low loaded columns, only glass beads, Gas Chrom-P, and Chromosorb W-HMDS proved to be satisfactory. The most successful column for the separation of these antihistamines was a 6-ft.–0.08% PDEAS on 120/170 glass-bead column operated at a temperature of 175°. This column provided good separation and symmetrical peaks. Instrument limitations prevented any valid evaluation of the 0.010-in. capillary column and of the 0.065-in. open tubular column for antihistamine separation.

Gas chromatography has proved to be a valuable tool for the separation and identification of many types of compounds. In pharmaceutical analysis, barbiturates (1–4), sympathomimetic amines (5–7), and tranquilizers (8, 9) have been successfully separated by gas chromatography. Toxicological screening of alkaloids and the above classes using gas chromatographic separation on a single column has been reported by Parker (10). Gas chromatographic data on 14 antihistamines using a 5-ft. × 1/8-in. diameter–2% Carbowax 20M on 10% KOH

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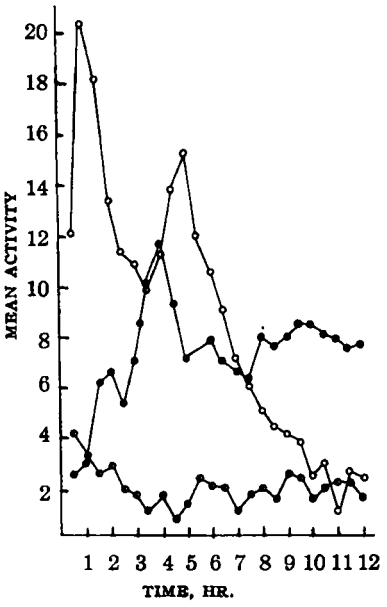


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TABLE I.—RETENTION TIMES OF ANTIHISTAMINES ON FOUR PHASES

Compd.[d]	SE-30,[a] Min.	PDEAS,[a] Min.	XF-1150,[a] Min.	Carbowax 20M,[a] Min.
Antazoline (Ciba Pharmaceutical Co.)	...[b]	65.8	...[b]	...[b]
Carbinoxamine (McNeil Laboratories)	4.3	8.5	10.6	10.6
Chlorcyclizine (Burroughs Wellcome)	6.2	12.9	14.9	12.0
Chlorothen (Lederle Laboratories)	5.3	10.5	13.5	10.6
Chlorpheniramine (Schering Research)	3.0	5.2	6.6	6.0
Cyclizine (Burroughs Wellcome)	3.2	4.9	6.2	4.8
Diphenhydramine (Parke, Davis & Co.)	1.7	2.5	3.9	2.9
Doxylamine (Wm. S. Merrell Co.)	2.3	3.9	4.2	4.5
Meclizine (Chas. Pfizer & Co.)	N.R.[c]	N.R.[c]	N.R.[c]	N.R.[c]
Methapyrilene (Irwin Neisler & Co.)	2.8	5.8	7.7	5.5
Pheniramine (Dorsey Laboratories)	1.4	2.1	2.8	2.4
Promethazine (Wyeth Laboratories)	7.2	21.7	27.9	17.1
Pyrilamine (Dorsey Laboratories)	7.6	20.3	23.6	20.2
Thenyldiamine (Sterling Winthrop Research Institute)	2.6	6.9	9.7	6.1
Thonzylamine (Warner-Lambert Research Institute)	6.2	18.0	20.6	17.2
Tripelennamine (Ciba Pharmaceutical Co.)	2.7	5.0	6.5	5.0
Support	Glass beads	Glass beads	Chromosorb W-HMDS	Gas Chrom P
Mesh	120/170	120/170	100/120	100/120
Liquid phase, w/w%	0.07	0.08	1.07	1.08

[a] Column length, 6 ft.; column temperature, 175° C.; detector temperature, 230° C.; injection point temperature, 260° C.; argon flow rate, 60 ml./minute. [b] Slight response due to decomposition product observed. [c] No response. [d] Supplier of compound.

coated 60/80 Chromosorb W has been reported by Fontan (11).

Present interest was concerned with the gas chromatographic behavior of a series of 16 antihistamines. Optimum conditions for packed column separation of these antihistamines on four different phases were established after evaluating the effect of phase loading, solid support, and column operating conditions. These groups of antihistamines were also investigated using a 0.010-in. diameter capillary column and a 0.065-in. diameter open tubular coated column.

EXPERIMENTAL

Apparatus and Reagents.—A Barber-Colman model 10 dual column gas chromatograph equipped with an argon ionization detector and a hydrogen flame ionization detector was used in this work. All packed columns were prepared from 6-ft. glass U-tubes having an inside diameter of 3 mm. A 100-ft. stainless steel capillary column of 0.010 in. inside diameter and a 100-ft. copper open tubular column of 0.065 in. inside diameter were also used. Each of these columns was wound on a 5-ft. section of $^1/_4 \times 1^1/_2$-in. steel bar designed to fit conveniently into the column oven of the chromatograph.

Seven solid supports were investigated for use in column packings. Gas Chrom-P was obtained from Applied Science Laboratories, State College, Pa. Chromosorb W-HMDS, Fluoropak-80, and glass beads were obtained from Wilkins Instrument and Research, Inc., Walnut Creek, Calif. Gas Pack-F was obtained from Chemical Research Services, Inc., Addison, Ill. Crushed firebrick and acid washed crushed firebrick were prepared in this laboratory from G-30 insulating firebrick obtained from A. P. Green Firebrick Co., Mexico, Mo.

The following four liquid phases were used in the various columns: polyethylene glycol (Carbowax 20M, Union Carbide Chemicals Co., New York, N. Y.), methyl silicone rubber gum (SE-30, Applied Science Laboratories), nitrile silicone polymer liquid (XF-1150, Wilkins Instrument and Research), and phenyldiethanolamine succinate polymer (PDEAS, Wilkins Instrument and Research).

The 16 antihistamines were generously furnished by the pharmaceutical companies listed in Table I. All were used as received without further purification.

PROCEDURES

The column packings were prepared by adding a weighed amount of the liquid phase in a suitable solvent to a weighed amount of the solid in a 1-L. creased round bottom flask (12). The solvent was removed using a rotary evaporator and the phase coated support dried at 110° for 4 hours. The glass columns were filled by the slow addition of packing to the tube which was settled by vibration (vibrating spatula) and tapping (rubber mallet). The prepared columns were equilibrated for 12 hours at a temperature of 175° and a flow rate of 20 ml./minute.

The procedure outlined by Averill was followed in coating the capillary and open tubular column with a liquid phase (13).

Two procedures were used for the preparation of chromatographic samples. In the first, a stock solution of 0.5 Gm. of the antihistamine salt in 50 ml. of distilled water was prepared. A 5-ml. aliquot of this solution was placed in a 30-ml. separator, 1 ml. of 6 *N* NH_4OH added, and the free amine extracted with 0.5 ml. reagent grade chloroform.

In the second procedure, a weighed sample of 0.01 Gm. of the salt was dissolved in 5.0 ml. of distilled water. One milliliter of 6 *N* NH_4OH was added to the 5-ml. solution in the separator and the free amine extracted with 0.5 ml. of reagent grade chloroform.

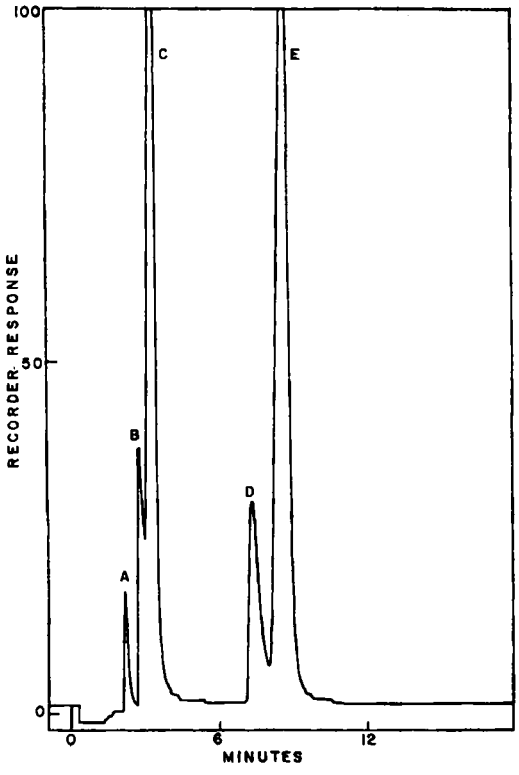


Fig. 1.—Separation using a 6-ft.—0.07% SE-30 on 120/170 glass bead column at 175° C. Key: *A*, diphenhydramine; *B*, doxylamine; *C*, methapyrilene, thenyldiamine; *D*, chlorcyclizine, thonzylamine; and *E*, promethazine, pyrilamine.

Synthetic mixtures of the antihistamines were prepared either by combination of the individual extracts or by the treatment and extraction of a mixture of the antihistamine salts.

A Hamilton 10-μl. syringe was used to inject the chloroform extract into the chromatographic column.

The behavior of several antihistamines was used to evaluate the following column parameters over the indicated ranges: liquid phase per cent (1–5%), solid supports (seven listed), column length (6 to 8 ft.), column temperature (155 to 189°), injection port temperature (185 to 260°), and column flow rate (10 to 85 ml./minute). The maximum number of theoretical plates calculated was used as the criteria to determine the optimum value for each of the above variables.

RESULTS AND DISCUSSION

Separation of the antihistamines with minimal tailing in a short analysis time was desired for each of the four liquid phases. The optimum operating conditions determined for each phase and the antihistamine retention times on them are listed in Table I. The separation obtainable with each of these columns is shown in Figs. 1–4. Figures 1–4 indicate that Carbowax 20M, PDEAS, and XF-1150 are much better phases for the separation of these compounds than SE-30. The PDEAS and XF-1150 columns offer comparable separation to the Carbowax 20M column but with a decrease in tailing. The 0.8% PDEAS on 120/170 glass-bead column was the most efficient column used because it provides adequate separation and symmetrical peak shapes. The response of the argon ionization detector, using the PDEAS on glass-bead column, was linear over the range of 1–20 mcg. of antihistamine. The minimum identifiable amount was arbitrarily chosen as the amount which would give a 10% scale unattenuated response using the argon ionization detector. The minimum amount determined for each antihistamine is shown in Table II.

Samples of antazoline yield very small peaks with short retention times on all columns, which

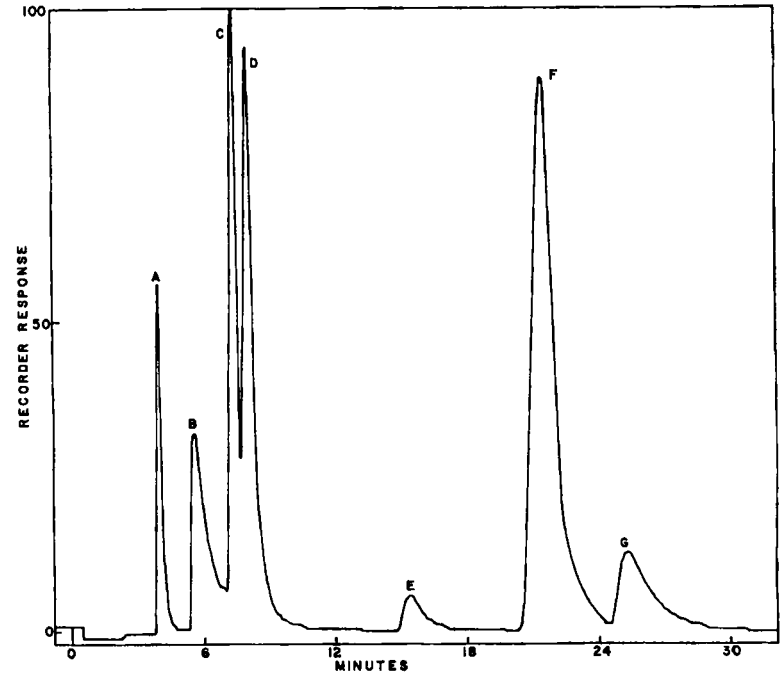


Fig. 2.—Separation using a 6-ft.—1.08% Carbowax 20M on 100/120 Gas Chrom-P column 170° C. Key: *A*, diphenhydramine; *B*, doxylamine; *C*, methapyrilene; *D*, thenyldiamine; *E*, chlorcyclizine; *F*, promethazine, thonzylamine; and *G*, pyrilamine.

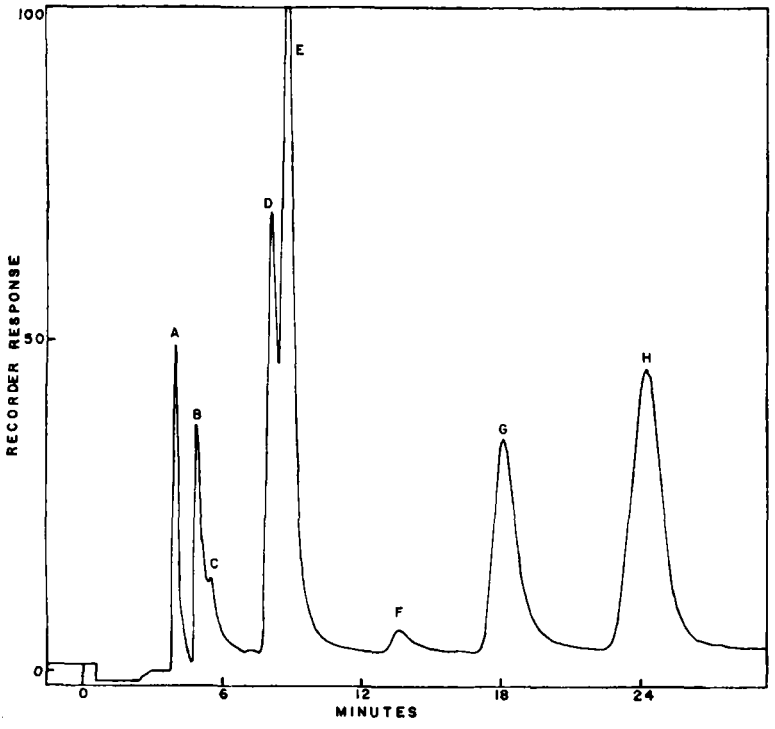


Fig. 3.—Separation using a 6-ft.—1.07% XF-1150 on 100/120 Chromosorb W-HMDS column at 174° C. Key: *A*, diphenhydramine; *B*, doxylamine; *C*, antazoline (decomposition product); *D*, methapyrilene; *E*, thenyldiamine; *F*, chlorcyclizine; *G*, thonzylamine; and *H*, pyrilamine.

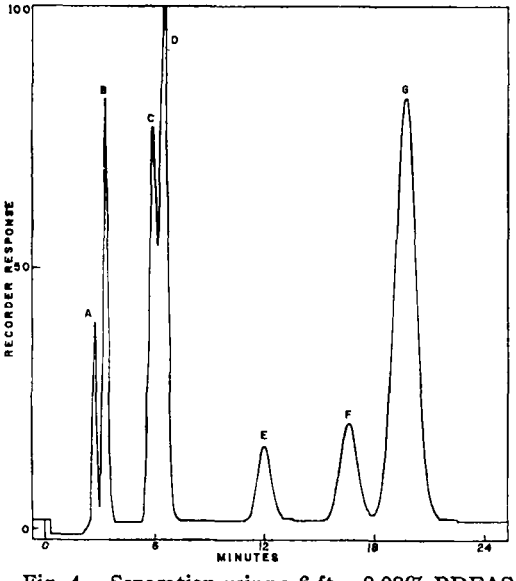


Fig. 4.—Separation using a 6-ft.—0.08% PDEAS on 120/170 glass bead column at 175° C. Key: *A*, diphenhydramine; *B*, doxylamine; *C*, methapyrilene; *D*, thenyldiamine; *E*, chlorcyclizine; *F*, thonzylamine; and *G*, promethazine, pyrilamine.

corresponds to the results reported by Fontan (11). However, during the evaluation of the last column investigated, 0.07% PDEAS on 120/170 glass beads, the first small peak was obtained, and the chromatograph was allowed to run for an extended period of time. A very strong peak was obtained with the retention time of 65.8 minutes. This indicated that the small peak obtained with the other columns was not antazoline, but an impurity or decomposition product. A retention time of 41.0 minutes was obtained by Kirk (8) using a 4 ft.-0.05% SE-30 on 60/80 mesh glass beads at 165°. No response for meclizine was obtained with the columns tested.

The first sample procedure in which aliquots of a stock solution of the antihistamine salt in water were made basic and extracted with chloroform often produced multiple peaks, indicating decomposition when injected into the chromatograph (14). The second procedure used a single weighed amount of the antihistamine salt which was dissolved in water, made basic, and extracted with chloroform. Injected samples using this method produced single peaks and showed that the decomposition experienced with the first method occurred in the stock solution of the antihistamine salt in water.

Since all column packings used in this investigation were lightly loaded, the solid support used has a

TABLE II.—MINIMUM AMOUNT NECESSARY FOR GAS CHROMATOGRAPHIC IDENTIFICATION USING A 6 FT.—0.077% PDEAS ON 120/170 GLASS-BEAD COLUMN AT 175° C.

Compd.	Amount, mcg.
Antazoline	5.4
Carbinoxamine	0.4
Chlorcyclizine	1.7
Chlorothen	0.7
Chlorpheniramine	0.4
Cyclizine	0.2
Diphenhydramine	0.2
Doxylamine	0.3
Meclizine	NR[a]
Methapyrilene	0.3
Pheniramine	0.1
Promethazine	0.2
Pyrilamine	0.8
Thenyldiamine	0.2
Thonzylamine	0.9
Tripelennamine	0.2

[a] No response.

definite bearing on the usefulness of any column packing prepared. The performances of the seven supports mentioned previously were examined under the same operating conditions. The supports that can be used for lightly loaded packings are: glass beads, Gas Chrom-P, and Chromosorb W-HMDS. The other four supports cannot be used for lightly loaded column packing since their interaction with the antihistamines causes excessive peak tailing.

The hydrogen flame detector used in conjunction with the 0.010-in. stainless capillary column would not respond to compounds with boiling points above 330°. This limitation prevented evaluation of this column for the analysis of these antihistamines.

The 100-ft. 0.065-in. copper open tubular column was coated with XF-1150 and evaluated using the above group of antihistamines. The Sr^{90} ionization detector was used with a column flow of 36 ml./minute. The retention times obtained were comparable to the 6-ft.-XF-1150 packed column, but the peak base widths were considerably wider. Because of this increase in base width, the 0.065-in. column was less efficient than the 6-ft. packed column.

A 250-ft. 0.065-in. column wound on a $1^1/_4$-in. diameter mandrel has been reported to be more efficient than a packed column (15). There are two possible reasons why efficiency was less than previously reported: (*a*) the column was shorter (100 ft.), and (*b*) the winding configuration was markedly different. The column was wound on a $1^1/_4 \times {}^1/_4$-in. bar which resulted in a definite flattening of the tube around the edge of the bar.

CONCLUSIONS

The antihistamines investigated, except for meclizine, can be separated, identified, and concentration estimated using the Carbowax 20M, PDEAS, and XF-1150 columns described. The PDEAS column is the most efficient of the three for the analysis of antihistamines.

The usefulness of the 0.010-in. capillary and the 0.065-in. open tubular columns cannot be properly evaluated until the mentioned limitations are removed.

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By C. RIFFKIN, R. HUBER, and C. H. KEYSSER

Steroid hormones may be administered parenterally in high concentrations as oil solutions. In this form they exhibit a prolonged action and reduce the number of injections required. To accommodate the demand for increasingly greater concentrations of hormones in solution, castor oil in combination with other suitable oil-miscible solvents, has been found to fulfill a need. The development of several formulations together with the results of animal testing, as well as clinical trials in humans, attest to the acceptability of this oil for the purposes intended.

FIXED OILS are included in the "United States Pharmacopeia XVI" as nonaqueous vehicles for injection and are characterized as being of vegetable origin, essentially odorless, and without suggestion of rancidity. They must also comply with certain measurable physical limits specified for the saponification, acid, and iodine values.

Received September 9, 1963, from the Pharmacology and Pharmaceutical Research & Development Sections, Squibb Institute for Medical Research, New Brunswick, N. J.
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After subcutaneous injection, Deanesly and Parkes (1) observed the persistence of olive oil and castor oil in animal tissue. Comparing other oils Brown, *et al.* (2), reported that sesame and corn oils were superior to cottonseed and peanut oils because they were less irritating, less antigenic, more quickly released from tissue, and possessed superior physical properties.

More recently the use of steroid hormone medication has expanded considerably. Due to limited water solubility, hormones have been administered as aqueous suspensions or solutions in oil. It has been claimed that the latter provided the slow release preferred in cyclical

definite bearing on the usefulness of any column packing prepared. The performances of the seven supports mentioned previously were examined under the same operating conditions. The supports that can be used for lightly loaded packings are: glass beads, Gas Chrom-P, and Chromosorb W-HMDS. The other four supports cannot be used for lightly loaded column packing since their interaction with the antihistamines causes excessive peak tailing.

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More recently the use of steroid hormone medication has expanded considerably. Due to limited water solubility, hormones have been administered as aqueous suspensions or solutions in oil. It has been claimed that the latter provided the slow release preferred in cyclical

TABLE I.—ANALYSIS OF COMMERCIAL OILS AND COMPARISON TO U.S.P. XVI SPECIFICATIONS

Oil	Lot No.	ml. 0.02 *N* NaOH Equiv. to Free Fatty Acid in 10-Gm. Sample	Sapon. Value	Iodine Value
Castor Oil	U.S.P. specs.	35.0[a]	179–185	83–88
	23946	14.0	183.3	84.8
	25589	4.6	179.8	87.0
	23463	7.9	182.7	84.5
	33742	9.2	180.4	84.2
Sesame Oil	U.S.P. specs.	3.0	188–195	103–116
	23549A	0.5	189.6	106.9
	26953	1.4	194.0	111.8
	33646	0.75	189.6	104.7
	29981	0.45	191.7	108.2
Cottonseed Oil	U.S.P. specs.	2.0	190–198	109–116
	49684	...	195.9	111.8
	44441	...	196.3	113.1
Corn Oil	U.S.P. specs.	2.0	187–193	102–128
	52148	1.0	194.5	119.1
	36716	1.2	191.4	124.4
	33436	1.2	189.3	125.0
	33715	1.0	189.3	123.0
Peanut Oil	U.S.P. specs.	2.0	185–195	84–100
	22160	1.2	192.0	94.4
	20993	1.4	191.7	93.2
	33622	0.8	193.1	87.8
	26147	1.2	190.4	93.9

[a] The U.S.P. specifies that the titration of free fatty acids in oral grade castor oil shall not exceed 7 ml. of 0.1 *N* NaOH which is equal to 35.0 ml. of 0.02 *N* NaOH.

TABLE II.—SOLUBILITY OF STEROIDS IN U.S.P. OILS AT 25°

Steroid	mg./ml. Castor Oil	mg./ml. Sesame Oil	mg./ml. Peanut Oil
17-Hydroxyprogesterone caproate	55.6	23.4	27.9
Testosterone	38.6	5.4	8.1
Estradiol valerate	60.6	16.1	18.8
Progesterone	52.0	22.9	23.5

therapy (3). Using withdrawal bleeding in human females as the criterion, Master, *et al.* (4), compared the duration of action of an aqueous suspension of progesterone with an oil solution, and confirmed the superiority of the latter. The prolongation of activity was generally related to storage in the fatty depots of the body (5).

In 1952 Junkmann (6) determined that a testosterone ester dissolved in sesame oil prolonged the androgenic effects in castrated rats. Davis and Wied (7) demonstrated that prolonged activity was also obtained in humans when oil solutions of a progesterone derivative were injected. There was still a limiting factor, however, in that only a relatively small amount of hormone could be dissolved in the traditional oils. To increase the solvent power of the oil it was necessary to add compatible and nonirritating cosolvents. Such additions consisted of benzyl benzoate, benzyl alcohol, ethyl lactate, ethyl oleate, etc. The U.S.P. recognized the need for such "other vehicles," with the restrictions that they must be safe in the volume of injection administered, and that they should not interfere with the therapeutic efficacy of the preparation or its testing.

Demand for increased hormone concentrations per dose, furthered the search for an acceptable oil with greater solubilizing power *per se*. Boschann (8) in 1954, observed that 17-hydroxyprogesterone caproate in a castor oil–ethyl lactate vehicle was well tolerated. In addition, private communications from clinicians in West Germany[1] reported good tolerance to Proluton-Depot containing a castor oil–benzyl benzoate vehicle. Since then other hormones have been used as solutions in ricinoleic acid esters, as well as in castor oil (9–11). Accordingly, an investigation was undertaken into the suitability of castor oil as a vehicle for parenteral administration of steroid hormones.

METHODS AND RESULTS

Representative samples of U.S.P. oils obtained from commercial sources were tested in accordance with the official method for free fatty acid content, saponification, and iodine values. The results are listed in Table I along with the U.S.P. XVI specifications for these oils.

Solubility of selected steroids in various oils was determined in the following manner. An excess of steroid was stirred for 4 hours at room temperature (25°) in the test oil, after which the undissolved solids were removed by filtration, and the clear solution assayed for steroid content. Table II shows the results obtained.

An attempt was made to reduce the free fatty acids in castor oil by treatment with alumina and anhy-

[1] Dr. Napp, Universitats-Krankenhaus, Hamburg; Dr. Pots, Humboldt-Universitat-Charite Frauenklinik, Berlin; Dr. Prill, Universitats-Frauenklinik, Wurzburg; and Dr. Rauscher, Universitats-Frauenklinik, Vienna.

TABLE III.—ABSORPTION OF OIL FROM ANIMAL MUSCLE[a]

Days after Injection	Oil	ml. 0.02 N NaOH Equiv.	Residual Oil in Muscle (estd.)
1–3	Castor oil (aged)	50	1 day —50% 3 days—20%
1–3	Castor oil U.S.P.	13	1 day —30% 3 days—10%
1–3	Sesame oil U.S.P.	1.4	1 day —30% 3 days—30%
7–60	All oils	...	Declining 10 to 2%

[a] 1 ml. injected into back muscle of rabbit.

drous sodium sulfate. Three grams of dried, powdered, amorphous aluminum oxide (Merck No. 1097) and 6 Gm. of anhydrous sodium sulfate, reagent grade, were suspended in 120 ml. of oil and heated at 80° under a blanket of nitrogen for 1.5 hours. After allowing the oil to cool to room temperature, the solids were filtered off and the acids titrated in the usual manner. A significant reduction in free fatty acid was not obtained.

The absorption characteristics of oils with varying fatty acid content were examined and compared on a biological basis. Aged castor oil with a high free fatty acid content was compared to fresh U.S.P. castor oil with a low acid content and U.S.P. sesame oil by injecting 1 ml. of oil into the back muscles of rabbits, approximately 2 in. from the iliac crest. A rotational pattern of injection was used and the oil samples were stained to aid visibility in the tissues. The animals were sacrificed and the muscles excised and examined grossly. The results were averaged and appear in Table III.

The test disclosed that oil migrated or was carried to the fascia, and very small amounts remained for 60 days. Localized degeneration produced by the high acid value castor oil was essentially healed in 7 days, and the low acid value castor oil appeared to be no more irritating than sesame oil.[2]

In a specific test for irritation 0.25 ml. of the above oil samples were also injected into the *vastus lateralis* muscles of rabbits. After 2 days the animals were sacrificed and the injected muscles examined grossly for evidence of irritation. It was found that the castor oil containing a high level of free fatty acid produced a lesion size measuring approximately 121 mm.[2]. The lesion itself was characterized mainly by degeneration of local tissue without necrosis. Castor oil with low free fatty acid and sesame oil, on the other hand, produced no measurable lesion at the injection site.

Combinations of benzyl alcohol and benzyl benzoate with both castor oil and sesame oil were also injected into the *vastus lateralis* muscles of rabbits and Table IV lists the lesion sizes produced.

Solutions which were formulated for clinical trials in humans were prepared by dissolving the steroid hormones in appropriate vehicles at 60° under nitrogen. The solutions were then filtered through a coarse sintered-glass filter with the aid of nitrogen pressure, filled into vials, and sterilized by autoclaving for 2 hours at 121° (15 lb. steam pressure). The products were then submitted for assay, safety, and animal muscle irritation testing prior to release for clinical investigation.

[2] Due to the apparent increase in free fatty acids with aging, subsequent work utilized fresh oils which required for neutralization less than 3 ml. of 0.1 N NaOH (15 ml. of 0.2 N NaOH) per 10 Gm. of sample.

DISCUSSION

Throughout the investigation it was desirable to have a reference oil to serve as a basis for comparison. Since sesame oil is universally accepted as a parenteral oil vehicle, it was chosen as the "standard" vegetable oil to be compared to castor oil, with and without other cosolvents. The physical, chemical, and biological properties of sesame oil are well documented and require no comments here.

Chemically, castor oil consists of the triglycerides of ricinoleic acid, together with small quantities of glycerides of other acids. The quantitative composition is given by Eckey (12) as follows: ricinoleic acid 87%, oleic acid 7.4%, linoleic acid 3.1%, dihydroxyricinoleic acid 0.6%, and miscellaneous acids 2.4%. Two grades are commonly recognized in this country—U.S. No. 1 which is cold pressed oil, and U.S. No. 3 which is oil extracted from the pressed cake. Only the former is used for medicinal purposes.

The high viscosity of castor oil compared to other vegetable oils is undoubtedly related to hydrogen bonding and it is probably the hydroxy groups which contribute to the greater polarity and superior solvent power of the oil. As indicated in Table I, the saponification and iodine values of commercial castor oil appear to be slightly lower than the U.S.P. XVI limits for oils used for injection. On the other hand, the content of free fatty acids even in fresh oil, varies considerably and exceeds the traditional limits for injectable oils. The significance of this is somewhat obscure, although "Remington's Practice of Pharmacy, 12th edition," page 387, states "a low free fatty acid content is essential since it indicates a fresh and pure product and not one that is likely to have become old and heavily contaminated with bacterial products."

Despite better solubility of steroids in castor oil, other cosolvents were necessary to dissolve the

TABLE IV.—LOCAL IRRITATION PRODUCED IN RABBIT MUSCLE BY INJECTION OF VARIOUS OIL VEHICLES[a]

Identification	Composition	Lesion size, mm.[2]
SHY-47-2	Sesame oil 98% Benzyl alcohol 2%	61
SHY-47-4	Castor oil 98% Benzyl alcohol 2%	Too small to measure
SHY-47-3	Sesame oil 95% Benzyl alcohol 5%	506
SHY-47-5	Castor oil 95% Benzyl alcohol 5%	106
SHY-14-2	Sesame oil 65% Benzyl benzoate 35%	291
SHY-14-5	Castor oil 65% Benzyl benzoate 35%	184
SHY-47-6	Sesame oil 63% Benzyl benzoate 35% Benzyl alcohol 2%	207
SHY-47-7	Castor oil 63% Benzyl benzoate 35% Benzyl alcohol 2%	262
SHY-14-3	Sesame oil 50% Benzyl benzoate 50%	291
SHY-14-6	Castor oil 50% Benzyl benzoate 50%	158

[a] A 0.25-ml. quantity of the oil vehicle was injected into the *vastus lateralis* muscle of the rabbit. Two days later the muscle was excised and the lesion size measured in mm.[2].

increasingly higher concentrations required by therapeutic regimens. Often these materials contributed additional advantages. For example, the addition of benzyl alcohol or benzyl benzoate to castor oil resulted in a lower and more favorable viscosity, making it easier to inject. Also, benzyl alcohol was an effective preservative and local anesthetic.

The nature of the irritative response depended on the particular hormone, its concentration in the formulations, and/or the composition of the vehicle. Although rabbit muscles are more sensitive than human muscles, they were selected primarily because local changes in the muscle were observed easily. It was not always possible, however, to correlate muscle irritation in animals to that of humans.

A numerical assignment to lesion size was used solely as a convenience for grading response. The numbers alone do not adequately describe the nature of the response, however. More completely it is characterized by the amount of hemorrhage and edema and the incidence, degree, and extent of local degeneration produced by the injection. A slight, reversible irritative response may cover a large area and a severe irreversible one may be comparatively small. A decrease in the size of the degenerated area indicates a reversible condition. The presence of necrosis, which is the most damaging situation, means that the cellular structure was destroyed and repair must take place. The debris must be removed and the original cellular mass in the area replaced with fibrous connective tissue. The extent of this fibrosis or formation of scar tissue gives an index of the amount of irreversible damage. Fortunately necrosis was not encountered, indicating the lack of permanent muscle damage. Since these changes take time, final assessment of the effects of an injection in the muscle frequently required observation for 7 days or longer.

It is unfortunate that pain cannot be measured by any known method of animal testing. The animal usually does not respond unless the painful stimulus is marked. Furthermore, the pain caused by injection into human muscle is not usually proportionate to the irritation produced either in animal muscle or in human muscle. Realizing that these limitations are inherent in animal test methods, it remained for final acceptability to be determined in man.

When it was discovered that 17-hydroxyprogesterone caproate possessed high progestational activity, potencies of the order of 65 mg./ml. were used. By increasing the dose, additional prolongation of action was obtained, and eventually concentrations of the order of 250 mg./ml. were required. Such a solution in sesame oil produced acceptable animal muscle tolerance, but the pain and local reaction in humans was so great as to prohibit the adoption of the formulation as a commercial product (see Table V, Lot Pr. 142-53/15-10).[3] Solutions were also prepared using castor oil as the vehicle, and Table V lists the formulations tested and the results obtained. Information obtained from the clinical trials (14–21) attested to the acceptability and safety of the adopted formulations.

Inherent in the development of an acceptable formulation of 17-hydroxyprogesterone caproate was the required development of a suitable assay method. This was accomplished by Roberts and Florey (13) using paper-strip chromatography.

Since estrogens are more potent than progestogens and require less per dose, an acceptable formulation of estradiol valerate was easier to prepare. Besides use in estrogen therapy, estradiol valerate has found utility in the treatment of carcinoma, and for that purpose high dosages were required. Concentrations were increased from 10 to 40 mg./ml. and

[3] Reactions in excess of 5–6% were considered unacceptable.

TABLE V.—EVALUATION OF 250 MG./ML. 17-HYDROXYPROGESTERONE CAPROATE SOLUTIONS IN VARIOUS OIL VEHICLES

Vehicle Composition	Animal Muscle Lesion Size, mm.[a]	Lot Number and Remarks on Clinical Testing
Sesame oil 50% Benzyl benzoate 50%	1049	Pr.142-53/15-7—238 injections, 20.6% reactions, rejected
Castor oil 58% Benzyl benzoate 40% Benzyl alcohol 2%	691	Pr.142-53/15-8—270 injections, 23.2% reactions, rejected
Sesame oil 60% Benzyl benzoate 35% Benzyl alcohol 5%	697	Pr.142-53/15-10—189 injections, 10.7% reactions, rejected
Castor oil 54% Benzyl benzoate 46%	258	Pr.142-53/15-11—503 injections, 4.2% reactions, accepted
Castor oil 52% Benzyl benzoate 46% Benzyl alcohol 2%	633	Pr.142-53/15-13—924 injections, 1.3% reactions, accepted

[a] Injection of 0.25 ml. into *vastus lateralis* muscle of rabbits and lesion size determined 2 days after injection.

TABLE VI.—EVALUATION OF ESTRADIOL VALERATE IN VARIOUS OIL VEHICLES

Composition	Animal Muscle Lesion Size, mm.[a]	Lot Number and Remarks
20 mg./ml. in Castor oil 78%, Benzyl benzoate 20%, Benzyl alcohol 2%	197	Es.31-53/15-B—Commercially available
30 mg./ml. in Sesame oil 60%, Benzyl benzoate 40%	306	DEK-98-2—Not tested clinically; dosage increased to 40 mg./ml.
30 mg./ml. in Castor oil 80%, Benzyl benzoate 20%	194	Es.31-53-V—Not tested clinically; dosage increased to 40 mg./ml.
40 mg./ml. in Sesame oil 65%, Benzyl benzoate 30%, Benzyl alcohol 5%	803	SHX-94-4—Too irritating; not tested clinically
40 mg./ml. in Sesame oil 58%, Benzyl benzoate 40%, Benzyl alcohol 2%	496	Es.31-53-8—201 injections, 23.2% reactions, rejected
40 mg./ml. in Castor oil 58%, Benzyl benzoate 40%, Benzyl alcohol 2%	250	Es.31-53-A—826 injections, 2.67% reactions (all mild), accepted

[a] Injection of 0.25 ml. into *vastus lateralis* muscle of rabbits and lesion size determined 2 days after injection.

again formulations containing castor oil in the vehicle proved to be less irritating than similar preparations containing sesame oil. Physically and chemically both oil solutions were stable. Based on acceptable preliminary data, formulations such as those listed in Table VI were prepared and tested. Acceptability in humans was confirmed by clinicians and described in the literature (22, 23) and in case reports.[4]

SUMMARY

1. The development and testing of parenteral steroid hormone formulations has been described, using castor oil as a vehicle.

2. After ascertaining stability and animal muscle irritation, selected formulations were evaluated in humans. They exhibited a prolonged action, were effective and well tolerated.

3. Examples of commercially available products are the estrogen, estradiol valerate[5] at 20 mg./ml. and 40 mg./ml., and the progestogen, 17-hydroxyprogesterone caproate[6] at 250 mg./ml.

[4] Case reports: estradiol valerate, 20 mg./ml., in castor oil 78%, benzyl benzoate 20%, benzyl alcohol 2%—90 injections in 46 patients. Two mild local reactions. Estradiol valerate 40 mg./ml. in castor oil 58%, benzyl benzoate 40%, benzyl alcohol 2%—51 patients. Number of injections not completely tabulated. One report is in press.

[5] Marketed as Delestrogen by E. R. Squibb & Sons, New York, N. Y.

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By HAROLD J. NICHOLAS*

A simple column chromatographic method for isolating the bicyclic diterpene marrubiin from acetone and ethanol extracts of *Marrubium vulgare* L. is described. An unsaturated sterol of the stigmastanol series, present in esterified form, and a sesquiterpene ($C_{15}H_{22}O_2$) have been isolated from the extracts.

IN PREPARATION for radioactive tracer work on the biosynthesis of marrubiin it was necessary to examine extracts of the plant for associated terpenoid substances. A convenient column chromatographic method was therefore devised for separating relatively pure marrubiin from crude acetone extracts. Two new terpenoid substances were detected in the extracts.

EXPERIMENTAL

Materials and Methods.—Ground *M. vulgare* L. was obtained from the Wunderlich-Diez Corp., Hasbrouck Heights, N. J.[1] This material was exhaustively extracted with hot acetone or hot ethanol. Either solution on removal of solvent by distillation (the last stages *in vacuo*) yielded black, viscous material which was used for further examination. Melting points were determined on a Fisher-Johns melting point apparatus. Optical rotations (in $CHCl_3$) and C—H analyses were determined by Drs. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England. An infrared spectrum of the unidentified diterpene was determined on a Perkin-Elmer spectrophotometer by the KBr disk method.[2] An infrared spectrum of the sterol was determined in chloroform solution in a 0.1-mm. sealed cell, compensated with $CHCl_3$, on a Beckman IR-4 recording infrared spectrophotometer,[3] and by the KBr disk method. The

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TABLE I.—CHROMATOGRAPHY OF CRUDE *M. vulgare* ACETONE EXTRACTS

Fraction[a]	Eluting Solvent[b]	Material Eluted, Gm.
1	...	11.8, reddish, crystalline wax-like substance
2	Petroleum ether	0.2, orange wax-like substance
3	5% ethyl ether	0.5, orange wax-like substance
4	5% ethyl ether	0.6, orange wax-like substance
5	10% ethyl ether	0.4, orange wax-like substance
6	10% ethyl ether	0.3, orange wax-like substance
7	20% ethyl ether	0.2, orange wax-like substance
8	20% ethyl ether	1.8, brown wax-like substance
9	40% ethyl ether	3.2, blackish, crystalline wax-like substance
10	40% ethyl ether	1.3, blackish, crystalline wax-like substance
11	40% ethyl ether	0.7, black glass-like substance
12	80% ethyl ether	1.3, black glass-like substance
13	100% ethyl ether	3.6, blackish-green, crystalline solid
14	100% ethyl ether	5.0, blackish-green, crystalline solid
15	100% ethyl ether	1.4, dark green glass-like substance
16	100% ethyl ether	0.8, black wax-like substance
17	100% ethyl ether	1.4, black wax-like substance
18	100% ethyl ether	1.5, black wax-like substance
19	100% ethyl ether	1.1, black wax-like substance
20	100% ethyl ether	0.5, black wax-like substance
21	100% ethyl ether	1.2, black wax-like substance
22	100% ethyl ether	0.8, dark green, semicrystalline solid
23	100% ethyl ether	0.2, dark green, semicrystalline solid
24	100% ethyl ether	0.1, dark green, semicrystalline solid
25	5% ethanol in benzene	0.1, greenish solid
26	5% ethanol in benzene	2.0, black glass-like substance
27	5% ethanol in benzene	2.2, black glass-like substance
28	5% ethanol in benzene	1.9, black glass-like substance
29	1:1 ethanol-benzene	1.6, black wax-like substance

[a] Each fraction 800 ml. in volume. [b] The percentages of ethyl ether refer to mixtures with petroleum ether (v/v). Ethanol-benzene mixtures are also expressed as v/v.

alumina used was Merck, acid-washed. The silicic acid was Mallinckrodt A.R. grade, 100 mesh, heated to 100–110° for 1 hour just prior to use. Except for material used to perform the initial extraction of plant material, all solvents were distilled before use. The petroleum ether had a boiling point of 30–60°.

Chromatography of Crude Extract.—Acetone or ethanol proved satisfactory for extracting marrubiin, sterol, and sesquiterpene from the plant, but a comparison of yields from each solvent was not made. The black viscous concentrate from either solvent contained some benzene-insoluble tar[4] which interfered with chromatography. Since hot benzene effectively separated the materials under investigation from the tar, a preliminary separation was made, based on this differential solubility in hot benzene. A description of isolation from acetone concentrates follows.

Benzene soluble material (78 Gm.) in 200 ml. of benzene was diluted with an equal volume of petroleum ether and poured onto a 5.5-cm. diameter column containing 1000 Gm. of alumina. After the solvent was all on the column, an additional 600 ml. of petroleum ether was added and the eluate was collected as Fraction *1*. Additional 800-ml. fractions containing varying quantities of ethyl ether or ethanol-benzene were subsequently collected. Table I gives the composition of the eluting fractions and a description and yield of material eluted after removal of solvent. Only about 55% of the original benzene-soluble material was recovered from the column.

Isolation of Marrubiin.—Fractions *13* to *15* were combined and crystallized to constant melting point from absolute ethanol, giving rods, m.p. 160–161°; $[\alpha]_D^{21°} = +42.9°$ (1). Yield: 8.0 Gm. Marrubiin also is slowly removed from alumina with 80% ethyl ether in petroleum ether. An additional small amount was recovered from Fraction *12*. In a control experiment 50 mg. of pure marrubiin in 30 ml. of 1:1 benzene-petroleum ether[5] was placed on a 1.2-cm. diameter column containing 15 Gm. of alumina. The eluate was collected as Fraction *1*. An additional 30 ml. of petroleum ether was poured through as Fraction *2*. Three 30-ml. fractions each of 5, 10, 20, 40, and 80% ethyl ether in petroleum ether (v/v), respectively, were poured through, followed by three ethyl ether fractions, each also 30 ml. in volume. A small amount of solid was removed by the second and third 80% ethyl ether fractions. The bulk of the material was recovered from the 100% ethyl ether fractions. Total recovery from the column: 37.4 mg. (74.8%).

ISOLATION OF STEROL FRACTION AND PREPARATION OF DERIVATIVES

The material in Fractions *9* and *10* (40% ethyl ether fraction) had the characteristics of a steroid ester. It readily became semifluid at steam bath temperature and gave no precipitate with digitonin. On saponification with 10% KOH in ethanol, a nonsaponifiable fraction was obtained, representing a large portion of the original wax. After several crystallizations of the nonsaponifiable fraction from acetone-methanol, crystals melting in the range 100–300° were obtained; these slowly gave characteristic colors with the Liebermann-Burchard reagent and gave a precipitate with digitonin. Since a nonsaponifiable fraction of the original acetone or ethanol concentrates proved a more ready source of the sterol mixture, purified sterol was obtained

[4] Examination of this hot benzene-insoluble fraction yielded no products of interest.

[5] At benzene-petroleum ether ratios, less than 1:1 the marrubiin precipitated.

in the following manner. Black gummy extractive from ethanol concentrates of *M. vulgare* was refluxed for 2 hours in 2 L. of 10% alcoholic KOH. After cooling, the mixture was diluted with H_2O and extracted thoroughly with ethyl ether. The latter was washed with H_2O and distilled, leaving 104 Gm. of orange, semisolid nonsaponifiable fraction. This was shaken thoroughly with 1.5 L. of petroleum ether and decanted from 22.5 Gm. of insoluble red oily substance which was not further examined. The petroleum ether solution was poured onto a 5.5-cm. diameter column containing 1000 Gm. of alumina. The column was washed with petroleum ether until only traces of yellow gummy substance were eluted. It was then washed with 3200 ml. of 2% ethanol in petroleum ether which yielded on distillation 32.8 Gm. of a red wax-like substance. This proved to be the source of the sterol and unidentified diterpene. On trituration with acetone-methanol and standing overnight, the solid precipitated. This was crystallized from acetone, yielding 2.3 Gm. of yellow solid as crude sterol. The filtrate on removal of solid gave viscous, red oily substance with a faintly fragrant odor, the crude diterpene fraction.

Purification of Sterol.—Crude sterol (9.9 Gm.) was converted to the acetate with acetic anhydride and anhydrous pyridine under reflux. The acetylated crystalline product so obtained could not be purified to a constant melting point by chromatography on silicic acid and crystallization from several solvents. It was therefore saponified with 5% alcoholic KOH. The free sterol was dissolved in 20 ml. of warm benzene, diluted with 100 ml. of petroleum ether, and poured onto a 2.2-cm. diameter column containing 100 Gm. of alumina. The eluate was collected as Fraction *1*. Seven 50-ml. fractions of petroleum ether were taken (yielding a small amount of colorless oily substance: discarded), followed by sixty 50-ml. fractions of benzene. Of the latter, Fractions *15* to *60* yielded white crystalline solid, combined weight 4.0 Gm.

Sterol Acetate.—The above product was acetylated with pyridine and acetic anhydride under 0.5-hour reflux, then processed in the usual manner. After three crystallizations from acetone-methanol, 2.6 Gm. of scales were obtained, m.p. 133–134°. Four additional crystallizations from the same solvent mixture or acetone-ethanol gave scales, m.p. 134–136°; $[\alpha]_D^{21°} = -45.61°$.

Anal.—Calcd. for $C_{31}H_{50}O_2$: C, 81.88; H, 11.08. Found: C, 81.81; H, 11.11.

Free Sterol.—Saponification of the above acetate with 5% alcoholic KOH (steam bath, 2 hours) gave rods after five crystallizations from either acetone, ethyl acetate–methanol, or acetone-methanol, m.p. 158–160°. An additional crystallization from acetone-methanol gave needles, m.p. 159–160°; $[\alpha]_D^{21°} = -46.52°$. The compound exhibited only nonspecific end absorption in the region 220–300 mμ. In the Liebermann-Burchard test ($CHCl_3$–HAc underlayed with concentrated H_2SO_4) the compound gave no immediate color in either liquid phase, although a pink interface developed within a few minutes. After 30 minutes, a light green developed in the $CHCl_3$ layer, remaining several hours. The substance, therefore, falls in the category of a "slow-reacting" sterol (2). It gave a negative Tortelli-Jaffe reaction (4).

Anal.—Calcd. for $C_{29}H_{48}O$: C, 84.40; H, 11.73. Found: C, 84.45; H, 11.51.

The infrared spectrum obtained in chloroform solution showed a close similarity to that of β-sitosterol, but was characterized by general poor resolution of the bands. The spectrum obtained by the KBr disk method was identical with that of β-sitosterol, except for the appearance of an additional band at 14.3 μ.

Sterol Benzoate.—Free sterol (290 mg.), benzoyl chloride (1 ml.), and 3 ml. of anhydrous pyridine were refluxed 1 hour. The cooled mixture was diluted with H_2O, filtered, and washed thoroughly with 5% Na_2CO_3 and H_2O, respectively. The product was then crystallized to a constant m.p. of 151–153° from benzene-ethanol, acetone-methanol, or chloroform-acetone; $[\alpha]_D^{21°} = -21.67°$.

Anal.—Calcd. for $C_{36}H_{52}O_2$: C, 83.68; H, 10.15. Found: C, 83.42; H, 9.90.

Reduction of Sterol Acetate in Neutral Medium.—The sterol acetate (70 mg., m.p. 134–136°) was shaken for 8 hours under 60 p.s.i. hydrogen pressure in the presence of 50 mg. of PtO_2 and 30 ml. of ethyl acetate. After three crystallizations from acetone-methanol, the product so obtained gave scales, m.p. 122–124°, unchanged by an additional crystallization from chloroform-methanol; $[\alpha]_D^{21°} = \pm 0°$. Yield: 45 mg.

Reduction of Sterol Benzoate in Neutral Medium.—The sterol benzoate (93 mg., m.p. 151–153°) was hydrogenated as described for the above acetate. The product crystallized readily from acetone-methanol giving microscopic needles, m.p. 122–124°, after four crystallizations; $[\alpha]_D^{21°} = 26.74°$. Yield: 80 mg.

Free Sterol After Reduction in Neutral Medium.—The benzoate (m.p. 122–124°, 60 mg.) was saponified with 5% KOH in ethanol. The product was recovered by dilution with H_2O, extraction with ethyl ether, etc. After two crystallizations from acetone-methanol, needles (m.p. 136–137°) were obtained, unchanged after an additional crystallization from acetone-ethanol; $[\alpha]_D^{20°} = +23.5°$. The compound gave a melting point depression of 3–5° on admixture with stigmastane-3β-ol.

Reduction of Sterol Acetate in Acidic Medium.—Sterol acetate (107 mg.) in 15 ml. of glacial acetic acid was shaken under 60 p.s.i. hydrogen pressure in the presence of 50 mg. of PtO_2 for 8 hours. After filtration, acetic acid was removed *in vacuo* and the product crystallized four times from either aqueous acetone or acetone-methanol giving needles, m.p. 134–135°; $[\alpha]_D^{21°} = +15.36°$. The melting point was undepressed on admixture with stigmastanol acetate.

Free Sterol After Reduction in Acidic Medium.—Saponification of the above acetate with 10% alcoholic KOH (steam bath, 1 hour) and work-up of the product gave needles, m.p. 137–139°, after four crystallizations from acetone-methanol; $[\alpha]_D^{21°} = +19.06°$. The melting point was undepressed on admixture with stigmastane-3β-ol.

Examination of Mother Liquors.—Extensive examination of the combined filtrates from crystallization of the original sterol mixture revealed free sterol, m.p. higher than 142°, acetate 133°+. It appeared likely that little or no β-sitosterol was present in the nonsaponifiable fraction of the plant.

TABLE II.—CHROMATOGRAPHY OF CRUDE ACETYLATED SESQUITERPENE PREPARATION

Fraction[a]	Eluting Solvent[b]	Material Eluted, Gm.
1 to *5*	Petroleum ether	None
6 to *20*	Petroleum ether	1.1, colorless oily substance
21 to *25*	1% ethyl ether	0.8, light yellow oily substance
26 to *39*	1% ethyl ether	0.5, partially crystalline yellow oily substance
40 to *44*	2% ethyl ether	5.0, semicrystalline oily substance
45 to *50*	2% ethyl ether	11.6, yellow crystalline solid
51 to *64*	2% ethyl ether	5.2, yellow gummy substance
65 to *70*	5% ethyl ether	0.2, yellow gummy substance
71 to *75*	10% ethyl ether	1.2, yellow gummy substance
76 to *80*	20% ethyl ether	3.0, yellow gummy substance
81 to *90*	50% ethyl ether	17.0, red oily substance
91	100% ethyl ether	1.5, yellow gummy substance

[a] Each fraction 100 ml. in volume. [b] The percentages of ethyl ether refer to admixture in petroleum ether.

TABLE III.[a]—MOLECULAR ROTATION DIFFERENCES BETWEEN DERIVATIVES OF STEROL FROM *M. vulgare* AND Δ^5- AND Δ^7-STENOLS

	Molecular Rotation (M_D) of—			—M_D (deriv.)	$-M_D$ (sterol)—
	Free Sterol	Acetate	Benzoate	Acetate	Benzoate
From *M. vulgare*	−192	−207	−113	−15	+79
Δ^5-Stenols[b]	−153 ± 8	−190 ± 12	−145	−35 ± 16	+81 ± 16
Δ^7-Stenols[c]	+35 ± 8	+31 ± 8	+68 ± 8	−15 ± 15	+20 ± 14

[a] Data, other than for *M. vulgare* sterol, largely from References *5* and *6*. [b] Δ^5, 11(?)-Stigmastadiene-dβ-ol (6). [c] Δ^7,11(?)-Stigmastadiene 3β-ol (6).

ISOLATION OF TERPENES

A quantity of the viscous red oily substance obtained from the filtrates in the preliminary crystallization of the crude sterol mixture was refluxed with excess acetic anhydride and anhydrous pyridine for 1 hour. The cooled mixture was diluted with H_2O and extracted thoroughly with ethyl ether. The latter was washed alternately with dilute HCl, H_2O, 5% NaOH and H_2O, then distilled, yielding 59 Gm. of viscous red oily substance. In 100 ml. of petroleum ether this oily substance was poured onto a 4.5-cm. diameter column containing 300 Gm. of silicic acid. The column was subsequently washed with 100-ml. volumes of petroleum ether and ethyl ether in petroleum ether. Yields and a description of the products are given in Table II.

Most of the crystalline solids (Fractions *40* to *50*) proved to be sterol acetate retained in the original filtrates. The unidentified diterpene was found in Fractions *81* to *90*. This material was heated on the steam bath in 5% KOH in ethanol and a neutral fraction obtained with ethyl ether in the usual manner as 15.9 Gm. of deep red oily substance. On distillation at 0.6 mm. Hg, a fraction boiling at 200–205° was obtained as a pale yellow, viscous gummy substance.[6] The color was not removed by further distillation. Yield: 2.8 Gm.; $n_D^{25°} = 1.5231$.

Anal.—Calcd. for $C_{15}H_{22}O_2$ (bicyclic, 2 double bonds): C, 76.88; H, 9.42. Found: C, 76.92; H, 9.63. The product exhibited only nonspecific end absorption in the region 230–300 mμ. Molecular weight (Rast): 244,257; no optical rotation in chloroform. The presence of a hydroxyl group(s) was substantiated by an infrared curve of the compound (absorption band, 3.0 μ).

In the Liebermann-Burchard test the substance gave colors in the H_2SO_4 layer ranging from orange to pink. A faint green $CHCl_3$ developed after about 1 hour. These reactions were interpreted as additional evidence for unsaturation. Attempts to form a crystalline phenyl urethane, acetate, or benzoate were unsuccessful.

Reduction of Unidentified Sesquiterpene.—Purified sesquiterpene (1.25 Gm., b.p. 200–205°/0.6 mm. Hg) in 50 ml. of ethyl acetate was shaken for 7 hours under 60 p.s.i. hydrogen pressure with 100 mg. PtO_2. The product was recovered and distilled under 0.6 mm. Hg pressure. The bulk of the material (0.80 Gm.) was received as a pale yellow oily substance at 268–270°; $n_D^{24°} = 1.5134$.

Anal.—Calcd. for $C_{15}H_{26}O_2$: C, 75.58; H, 10.91. Found: C, 75.66; H, 11.00.

No optical rotation was found in $CHCl_3$. Oxidation of 520 mg. of this product with CrO_3 in glacial acetic acid gave 211 mg. of neutral material and 234 mg. of acidic product, both as yellowish gummy substances. Neither product yielded an identifiable substance.

DISCUSSION

In addition to the bicyclic diterpene marrubiin (1) and ursolic acid (3), two additional substances may now be included as major lipid constituents of *M. vulgare* L. One of these, a sterol, is present in the plant in esterified form. Although the presence of other sterols was not excluded, the unidentified sterol appeared to be the major one present in *Marrubium.* β-Sitosterol appeared to be the major sterol of several other members of the *Labiatae* family (4). Reduction of the sterol in acidic medium gave stigmastane-3β-ol, proving the relationship of the substance to members of the sitosterol series.

[6] An additional viscous oily substance boiling unsharply at this pressure and at a considerably higher temperature (250°+) was obtained from the residue remaining after distillation of the sesquiterpene. It was not further examined.

It appears that two double bonds are present in the sterol, although unequivocal evidence must await further data. One double bond is at position 5(6)-; rotational differences between the new sterol and its acetate and benzoate coincide with established differences for Δ^5-stenols (Table III). This is substantiated by the similarity of the infrared spectrum to β-sitosterol and the rate of the Liebermann-Burchard reaction. The exact position of the second double bond cannot be ascertained with the available data, which were limited by the supply of sterol on hand. However, nuclear positions 7(8)-, 8(9)-, 8(14)-, and 9(11)- can be ruled out because of the rotational values in Table III and because of the negative Tortelli-Jaffe reaction (Reference 5, p. 101). Positions 7(8)- and 3(4)- can be eliminated since no conjugation was indicated in the ultraviolet spectrum, and position 11 can be eliminated because of the lack of identity with the $\Delta^{5,(11^7)}$-stigmastadienol of Idler *et al.* (6). Hydrogenation in neutral medium converted the sterol to a compound (unidentified) that was not Δ^5-stigmastenol (β-sitosterol). Therefore, the second (presumed) double bond must have been more resistant to hydrogenation than Δ^5. This eliminates the side chain positions 22(23)-, 24(25)-, or 24(28)- (6). It also eliminates position 14(15)- (7). With these eliminations ring A, positions 1(2)- or 2(3)- become possibilities, since some "vicinal" effect (Reference 5, p. 209) is indicated by the M_D for both the free sterol and acetate (Table III). The significance of the peak at 14.3 μ in the infrared shows promise for later identification; at the moment its significance is unknown.

The other substance new to *M. vulgare*, a strongly polar sesquiterpene which probably contains two nonconjugated double bonds, was not isolated from the crude extracts but was readily isolated from nonsaponifiable fractions of extracts of the plant. Neither the sterol nor the sesquiterpene has yet been equated with known substances; we are describing them as "unidentified," pending further experimentation.

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Preservatives for Poliomyelitis (Salk) Vaccine III

2-Phenoxyethanol

By HILLIARD PIVNICK, J. M. TRACY, A. L. TOSONI, and D. G. GLASS

Poliomyelitis vaccine contains antibiotics, but the antibiotics are inadequate for preventing the growth of heavy contamination with bacteria or light contamination with fungi. The addition of 0.375 per cent v/v of 2-phenoxyethanol to poliomyelitis vaccine furnished a stable mixture of preservatives (streptomycin, neomycin, and 2-phenoxyethanol) which was inhibitory to both bacteria and fungi. This mixture was also effective when poliomyelitis vaccine was mixed with diphtheria and tetanus toxoids and pertussis vaccine.

VACCINE packaged in multiple-dose vials must contain a preservative to prevent the growth of microbial contaminants which may be introduced when samples are withdrawn. In studies of preservation of poliomyelitis vaccine and DPT polio vaccine (1, 2) we showed that antibiotics, benzethonium chloride, formaldehyde, and esters of *p*-hydroxybenzoic acid (parabens) alone, or in various combinations, had deficiencies as preservatives.

The present report concerns 2-phenoxyethanol[1] (ethylene glycol monophenyl ether) (2-POE) as a preservative for poliomyelitis vaccine. This compound was chosen for study because of its activity against *Pseudomonas aeruginosa* (3–6) a potential pathogen which may grow in vaccines and other medicinals (7–9).

MATERIALS AND METHODS

2-Phenoxyethanol was sterilized by Seitz filtration, added to vaccines or media and the mixture was stirred slowly for a few hours to effect solution.

A method of assay for 2-POE in poliomyelitis

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TABLE I.—ANTIBACTERIAL ACTIVITY OF 2-PHENOXYETHANOL IN HB597 MEDIUM[a]

Challenge Culture	No. of Culture	2-Phenoxyethanol, % 0	0.5	0.75	1.0
Pseudomonas species	C-1	+[b]	—	—	—
Pseudomonas species	C-2	+	—	—	—
Pseudomonas species	C-3	+	—	—	—
Pseudomonas species	C-5	+	—	—	—
Pseudomonas aeruginosa	C-4	+	—	—	—
Pseudomonas aeruginosa	C-6	+	+	—	—
Pseudomonas aeruginosa	C-13	+	+	—	—
Pseudomonas aeruginosa	C-16	+	+	+	—
Shigella flexneri	C-8	+	+	—	—
Proteus species	C-9	+	+	—	—
Salmonella paratyphi C	C-12	+	+	—	—
Staphylococcus aureus	C-10	+	+	—	—
Staphylococcus aureus	C-11	+	+	+	—

[a] No antibiotics present. [b] + = growth within 60 days at room temperature; — = no growth.

vaccine was developed. A standard curve of 2-POE, distilled in a fractionating column, was prepared. The relation between the absorbance at 271 mμ in reagent grade ethylene chloride compared to a solvent blank was found to be linear in the range from 0 to 0.005% v/v. To assay poliomyelitis vaccine, a suitable volume of sample was extracted with an equal volume of ethylene chloride by thoroughly shaking in a separator for 1 minute. The ethylene chloride extract was diluted 1:100 with ethylene chloride and the absorbance read on a Beckman DK-2 spectrophotometer at 271 mμ. Centrifugation was usually necessary to obtain clear extracts when working with DPT polio vaccine. Poliomyelitis vaccine which did not contain 2-POE gave no absorbance and it was found that one extraction gave essentially quantitative recovery.

All other materials and methods have been previously described (1, 2).

RESULTS

All inoculated media and vaccines were incubated at room temperature, unless otherwise stated, and examined after 4, 11, and 60 days of incubation. When HB597 medium without preservatives was inoculated, growth was visible within 4 days. When medium or vaccine containing preservatives was inoculated, only about two-thirds of all tubes which eventually contained visible growth did so by the fourth day. Most of the remainder were positive by the eleventh day. Subcultures were usually done on the eleventh day after inoculation and about 5–10% of those tubes which did not show macroscopic evidence of growth contained viable cells. Rarely did these viable cells proliferate sufficiently to give macroscopic evidence of growth within 60 days after inoculation.

Table I shows that all 13 bacterial challenge cultures grew when approximately 25×10^6 cells were added to each milliliter of HB597 medium. This medium with added antibiotics is used in monkey kidney tissue culture for growing poliomyelitis virus and is a major part of the finished vaccine. This table also shows that 0.75% v/v of 2-POE did not inhibit growth of all the cultures but 1% did.

Table II shows the effect of challenging, with three concentrations of 12 different bacterial cultures, poliomyelitis vaccine containing 2-POE. Poliomyelitis vaccine is mainly HB597 medium with killed virus and antibiotics. This vaccine contained 135 p.p.m. of streptomycin and 2 p.p.m. of neomycin (1). The vaccine itself had considerable antibacterial activity and this was greatly increased by 0.375% but not by 0.25% of 2-POE. A concentration of 0.5% of 2-POE gave the same results as 0.375% but 0.75% prevented growth of all cultures. For further work we used 0.375% v/v of 2-POE.

The preservative activity of the mixture of antibiotics and 2-POE was stable. Vaccine containing 0.375% of 2-POE was challenged with the 13 bacterial cultures using the three concentrations of cells previously employed. After 60 days at room temperature those tubes in which growth was not evident were rechallenged with freshly prepared inoculum. As a result of the first challenge, growth occurred in 3 of the 39 tubes. When the tubes without growth were rechallenged 60 days later, growth occurred in only one.

2-Phenoxyethanol was effective as a preservative when challenged poliomyelitis vaccine was

TABLE II.—ANTIBACTERIAL ACTIVITY OF 2-PHENOXYETHANOL IN POLIOMYELITIS VACCINE[a]

2-Phenoxyethanol, %	Approximate Number of Cells per ml. of Vaccine	No. of Tubes with Growth / No. of Tubes Challenged	
0	25×10^5	12/12	
	25×10^3	4/12	(C1, C2, C3,
	25	0/12	C13)[b]
0.25	25×10^5	9/12	
	25×10^3	5/12	(C2, C3, C4, C6,
	25	0/12	C13)
0.375	25×10^5	3/12	(C4, C6, C13)
	25×10^3	0/12	
	25	0/12	

[a] Formaldehyde neutralized. [b] Numbers in brackets refer to cultures listed in Table I and identify the cultures which grew.

TABLE III.—PRESERVATIVE EFFECT OF 2-*para*-CHLOROPHENOXYETHANOL[a] AND 2-PHENOXY-1-METHYLETHANOL[b] IN POLIOMYELITIS VACCINE

Vaccine	Approximate Number of Cells per ml. of Vaccine	No. of Tubes with Growth / No. of Tubes Challenged
Poliomyelitis	25×10^5	12/13
	25×10^3	8/13
	25	8/13
Poliomyelitis + 0.2% of 2-*para*-[a]	25×10^5	0/13
	25×10^3	0/13
	25	0/13
Poliomyelitis + 0.3% of 2-phenoxy-[b]	25×10^5	10/13
	25×10^3	8/13
	25	6/13

TABLE IV.—PRESERVATIVE ACTIVITY OF 2-PHENOXYETHANOL IN VACCINE CONTAINING NONNEUTRALIZED FORMALDEHYDE

Preparation Tested	Bacteria: Approximate Number of Cells per ml. of Vaccine	Bacteria: Growth[b] Challenged	Yeasts and Molds[a]: Approximate Number of Cells per ml.	Yeasts and Molds[a]: Growth Challenged
HB597	25 × 10^6	13/13	2.5 × 10^6	7/7
	25 × 10^3	13/13	2.5 × 10^3	6/7
	25	13/13	2.5	4/7
DPT polio[c] vaccine	25 × 10^6	1/13	2.5 × 10^6	6/7
	25 × 10^3	0/13	2.5 × 10^3	6/7
	25	0/13	2.5	3/7 (C28, C33, C35[d])
DPT polio vaccine + 0.375% of 2-phenoxyethanol	25 × 10^6	0/13	2.5 × 10^6	3/7 (C28, C33, C35)
	25 × 10^3	0/13	2.5 × 10^3	0/7
	25	0/13	2.5	0/7

[a] C27—*Saccharomyces ellipsoideus;* C28—*Debaromyces kloeckeri;* C29—*Saccharomyces rouxii;* C30—*Rhodotorula glutinis,* C32—*Rhizopus oryzae;* C33—*Fusarium lini;* C35—*Circinella spinosa.* [b] No. of cultures with growth/No. of cultures used for challenge. [c] DPT polio vaccine was made by combining 100 volumes of poliomyelitis vaccine with 4 volumes of a mixture of diphtheria and tetanus toxoids and pertussis vaccine. [d] Bracketed figures identify cultures which grew.

stored at 4°. When the vaccine without 2-POE was challenged with 12 different bacterial cultures, 8 of the 12 grew within 10 weeks at 4° but when the vaccine contained 0.375% of 2-POE, none grew within 15 weeks.

Two other compounds similar to 2-POE were also tested briefly as preservatives in poliomyelitis vaccine in which the formaldehyde had been neutralized. These were *2-para*-chlorophenoxyethanol (Para-phenoxetol) and 2-phenoxy-1-methylethanol (Propylene phenoxetol). Table III shows that the former, at a concentration of 0.2% v/v had excellent antibacterial activity while the latter was useless at 0.3% v/v. The high antibacterial activity of 2-*para*-chlorophenoxyethanol indicates that further study of this material may be rewarding.

Formaldehyde may be added to poliomyelitis virus to inactivate the virus in order that it may be used for Salk vaccine. The residual formaldehyde (70–80 p.p.m.) may be left or it may be neutralized by bisulfite (2). If not neutralized the formaldehyde supplements the antibacterial activity of the antibiotics in the vaccine but has little antimycotic activity (2). Table IV shows that 2-POE provided antifungal activity for DPT polio vaccine which contained nonneutralized formaldehyde and already posessed adequate antibacterial activity. This table also gives evidence that the 2-POE was not inactivated by the toxoids and pertussis vaccine in DPT polio vaccine or by the formaldehyde.

Studies were done to determine the stability of 2-POE in DPT polio vaccine stored for 11 months at 4°. During this time the 2-POE decreased from 0.375 to 0.33% but the antimicrobial activity of the vaccine was not diminished.

DISCUSSION

2-Phenoxyethanol has not been used widely as a preservative for biologicals and would not be useful by itself unless about 1.0% were added. Its solubility at 20° in water is only 2.4%. As part of a mixture of preservatives in poliomyelitis and DPT polio vaccine it does, however, appear to be useful. It supplements the antibacterial activity of the antibiotics, provides adequate antifungal activity, and has excellent stability. Moreover, it is not inactivated by components of DPT polio vaccine nor, unlike parabens, does it cause a change of pH in the vaccine during prolonged storage. Besides its preservative activity, 2-POE has other necessary attributes of a preservative for vaccines—it is nontoxic and it does not destroy antigenicity of any of the components of DPT polio vaccine when used at a concentration of 0.375% and stored at 4° (10). Thus, 2-POE appears to be a satisfactory preservative for this vaccine.

2-Phenoxyethanol may have further application as a preservative for biologicals which contain high concentrations of proteins. Berry (3) has stated that the bacteriostatic concentration is not greatly increased by 20% of serum. It must be emphasized, however, that the bacteriostatic concentrations are about 0.8% for *S. aureus.* We believe that the best method of using 2-POE is in conjunction with stable antibiotics such as streptomycin and neomycin, in which mixtures it may be satisfactory for a variety of products.

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Synthesis of Some α,β-Unsaturated β,δ-Disubstituted δ-Lactones

By CLAUDE PIANTADOSI and VILHJALMUR G. SKULASON

A series of 18 new α,β-unsaturated β,δ-disubstituted δ-lactones was synthesized as possible therapeutic agents. The majority of the compounds described contain an aryloxy group in the β-position of the lactone ring, while those naturally occurring in *Piper methysticum* all contain an alkoxy group in this position. Some of the lactones were hydrogenated by means of palladium-on-charcoal to the corresponding dihydro derivatives. The infrared absorption spectra of most of the synthesized compounds were also studied.

PIPER METHYSTICUM Forst. of the family *Peiperaceae* is a shrub common to the islands of Indonesia where it is known in various dialects as kawa, kava, or ava. The rest of the kawa plant has been held in high esteem by the Polynesians from ancient times for its use in preparing an extract, which upon drinking, is reported to reduce fatigue and produce complete freedom from anxiety (1).

"The National Standard Dispensatory" of 1909 (2) describes *P. methysticum* and lists as a pharmaceutical preparation fluid extract of kava N.F. The same edition has a long list on action and uses of the drug.

Marpmann demonstrated in 1905 (3) that the constituent kawain possesses bacteriological properties, especially for the gonococcus, but also for the *Coli bacillus*.

The remarkable physiological action of *P. methysticum* has prompted numerous chemical investigations, dating from an article by Gobley in 1860 (4). The most extensive work on the constituents of this plant was carried out by W. Borsche and his co-workers, who in a series of 14 papers extending over a period of 19 years (1914–1933) reported the isolation and structure of two new compounds, kawain (Gonosan) and dihydrokawain (DHK). These workers also elucidated the structures of other crystalline components, methysticin, dihydromethysticin (DHM), and yangonin, which had previously been isolated from this plant. At the conclusion of his work, Borsche stated that all of these compounds failed to exhibit the typical physiological action of the kawa root (5). In a later study, using chromatographic techniques, Van Veen isolated a substance "marindinin"[1] which when administered in a lecithin-water emulsion in the amount of 50–70 mg. to pigeons and in the amount of 500 mg. to monkeys (weighing 1 Kg.), exhibited the characteristic action of kawa (6). This compound was shown to be identical with dihydrokawain previously isolated by Borsche. Hansel and Beiersdorff (7) showed that dihydromethysticin is another active ingredient of the root. Besides the compounds already mentioned, Klohs and co-workers (8) succeeded in isolating a small quantity of a new compound which was designated as "compound A" and later shown to be desmethoxyyangonin (9).

As can be seen from Fig. 1 all the naturally occurring lactones of known structure are α-pyrones except for yangonin and desmethoxyyangonin which belong to the γ-pyrone series.

The structural determination of the kawa lactones was exclusively determined by degradation of the molecule and the final proof for kawain was accomplished by synthesis in 1950 and for methysticin and dihydromethysticin in 1959 (10, 11). Another synthesis of kawain by a different method was announced in 1951 (12).

EXPERIMENTAL[2]

Ethyl β-Chlorocrotonate.—This compound was prepared by a modification of the method used by Thomas-Mamert (13) resulting in an improved yield. In a dry 1-L. round-bottom, three-necked flask equipped with a reflux condenser and placed under the hood 200 Gm. (0.154 mole) of ethyl acetoacetate and 400 ml. of petroleum ether (b.p. 30–60°) were added. To this mixture 170 Gm. (0.816 mole) of phosphorus pentachloride was gradually added. Upon completion of the reaction, 500

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Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1963.

[1] Named after the Marindines tribe of Netherlands New Guinea.

[2] All melting points were taken with the Mel-Temp apparatus and are uncorrected as are the boiling points. The microanalyses were performed by Drs. Weiler and Strauss, Microanalytical Laboratories, Oxford, England. The infrared spectra were determined on a Perkin-Elmer model 137B spectrophotometer using 2.5% solution in chloroform and sodium chloride cell.

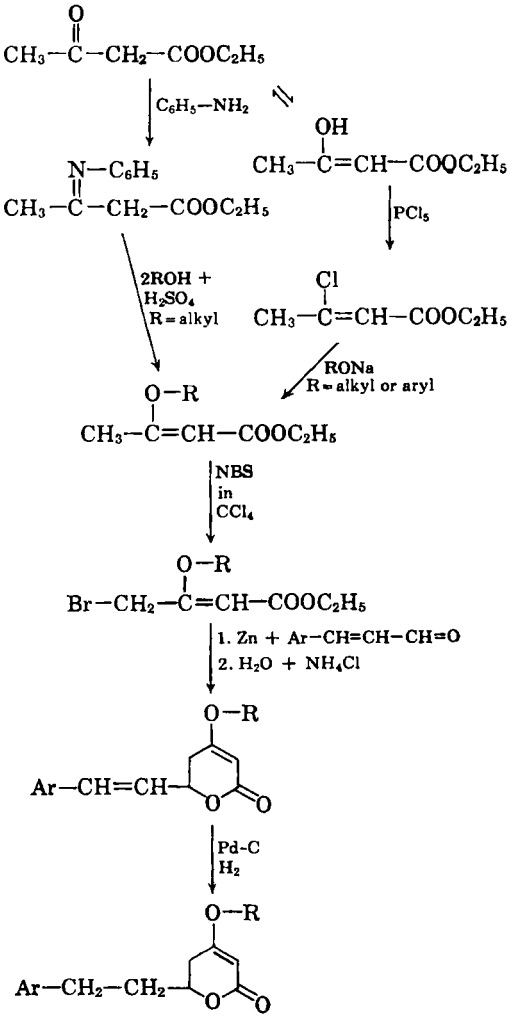
ml. of water was added in small portions, transferred to a separator, and the layers separated, followed by washing the aqueous layer with three 100-ml. portions of petroleum ether (b.p. 30–60°). The combined yellow petroleum ether extracts were washed with a 20% potassium carbonate solution, dried over anhydrous sodium sulfate, then evaporated *in vacuo*. Distillation of the residue gave 48% of a colorless liquid with a pungent odor, which on standing turns green, b.p. 163–176°.

Anil of Ethyl Acetoacetate.—This compound was prepared by a method previously described in the literature and used without further purification for the preparation of ethyl β-methoxycrotonate and ethyl β-ethoxycrotonate (14).

Ethyl β-n-Propoxycrotonate and Ethyl β-Isopropoxycrotonate.—These compounds were prepared essentially by the method of Koll (15).

A general method was employed in the preparation of all compounds listed in Tables I and II. The method used is illustrated below for the synthesis of ethyl β-*p*-chlorophenoxycrotonate and ethyl-γ-bromo β-*p*-chlorophenoxycrotonate, respectively.

Ethyl β-p-Chlorophenoxycrotonate.—This compound was prepared by a modification of a method



Reaction Sequence Used in Present Synthesis

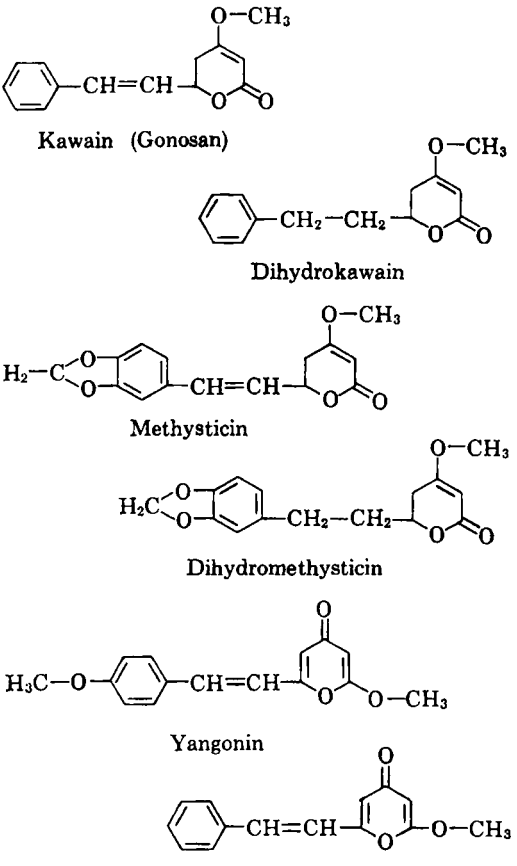
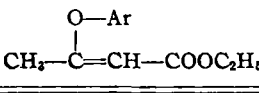


Fig. 1.

described in the literature (14). To 11.5 Gm. (0.5 Gm. At.) of sodium dissolved in 200 ml. of ethanol, 65 Gm. (0.505 mole) of *p*-chlorophenol, and 85 Gm. (0.572 mole) of ethyl β-chlorocrotonate were added. This mixture was heated on the steam bath for 2 hours, at which time the mixture was almost neutral to litmus. The sodium chloride was filtered, the alcohol removed by distillation, and the resulting product triturated with water and extracted with ether. Unreacted phenol was removed from the ether layer by shaking it twice with 100-ml. portions of 5% solution of sodium hydroxide. The ether solution was dried over anhydrous sodium sulfate and evaporated. Distillation of the residue gave 53% of a colorless oil, b.p. 110–117° (0.8 mm.)

Ethyl γ-Bromo β-p-Chlorophenoxycrotonate.—This compound was prepared by a modification of the procedure described by Kogl and deBruin (16). In a dry 1-L. round-bottom flask fitted with a reflux condenser, 200 ml. of carbon tetrachloride and 98 Gm. (0.407 mole) of ethyl β-*p*-chlorophenoxycrotonate were added. To this solution 73 Gm. (0.410 mole) of *N*-bromo-succinimide and 8 Gm. (0.033 mole) of benzoyl peroxide were added and the mixture heated on the water bath for 3 hours. The reaction mixture was then placed in the refrigerator overnight, the succinimide removed by filtration, and the solvent distilled *in vacuo*. Distillation of the residue gave 71% of a faint greenish liquid, b.p. 140–160° (0.15 to 0.25 mm.). After several

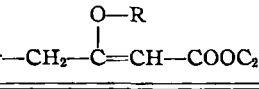
TABLE I.—ETHYL β-ARYLOXYCROTONATES



Ar	Formula	B. p.	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	Yield, %
p-Cl—C_6H_4—	$C_{12}H_{13}ClO_3$	110–117 (0.8 mm.)	59.88	59.80	5.44	5.41	53[a]
o-CH_3—OC_6H_4—	$C_{13}H_{16}O_4$	120–132 (0.8 mm.)	66.09	66.00	6.82	6.79	55
p-CH_3—C_6H_4—	$C_{13}H_{16}O_3$	70– 98 (0.1 mm.)	70.88	70.73	7.32	7.26	51
p-*n*-C_4H_9—O—C_6H_4—	$C_{16}H_{22}O_4$	139–147 (0.05 mm.)	69.04	68.94	7.97	7.90	47

[a] Calcd. for Cl: 14.73. Found: 14.68.

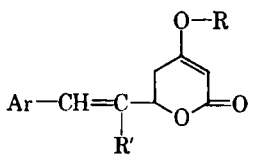
TABLE II.—ETHYL γ-BROMO β-ALKOXY (OR ARYLOXY) CROTONATES



R	Formula	B. p.	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	Br, % Calcd.	Br, % Found	Yield, %
CH_3—	$C_7H_{11}BrO_3$	68– 72 (0.1 mm.)	37.69	37.65	4.97	4.93	35.82	35.78	78[a]
C_2H_5—	$C_8H_{13}BrO_3$	79– 84 (0.15–0.05 mm.)	40.52	40.49	5.53	5.47	33.70	33.66	75[a]
n-C_3H_7—	$C_9H_{15}BrO_3$	165–170 (33 mm.)	43.04	42.95	6.02	5.98	31.82	31.73	[c]
i-C_3H_7	$C_9H_{15}BrO_3$	150–154 (35 mm.)	43.04	42.99	6.02	5.95	31.82	31.69	[c]
C_6H_5	$C_{12}H_{13}BrO_3$	108–124 (0.3 mm.)	50.55	50.51	4.59	4.56	28.02	28.00	[c]
p-CH_3—C_6H_4—	$C_{13}H_{15}BrO_3$	127–140 (0.3 mm.)	52.19	52.08	5.05	5.00	26.71	26.68	39
o-CH_3—O—C_6H_4—	$C_{13}H_{15}BrO_4$	123–154 (0.35 mm.)	49.54	49.50	4.80	4.78	25.36	25.27	58
p-Cl—C_6H_4—	$C_{12}H_{12}BrClO_3$	140–160 (0.15–0.25 mm.)	45.10	45.08	3.78	3.75	25.00	24.93	71[a,b]
p-*n*-C_4H_9—O—C_6H_4—	$C_{16}H_{21}BrO_4$	190–193 (0.40–0.55 mm.)	53.79	53.73	5.92	5.89	22.37	22.29	42

[a] Benzoyl peroxide was used as a catalyst. [b] Calcd. for Cl: 11.09. Found: 11.00. [c] Yields not calculated.

TABLE III.—α,β-UNSATURATED β,δ-DISUBSTITUTED δ-LACTONES



Ar	R′	R	M.p., °C.	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	C, % Calcd.	C, % Found	Yield, %
C_6H_5—	H	CH_3—	145–146[a,b]	...	...	...	...	...	...	17
C_6H_5—	H	C_2H_5—	99–100	73.77	74.03	6.56	6.37	...	...	5
C_6H_5—	H	*n*-C_3H_7—	77– 78	74.42	74.25 74.29	7.05	7.13 7.16	...	...	6
C_6H_5—	H	*i*-C_3H_7—	94– 95	74.42	74.14	7.05	7.25	...	...	5
C_6H_5—	H	C_6H_5—	135–136	78.08	77.83 77.44	5.48	5.58 5.50	...	...	12
C_6H_5—	H	*p*-CH_3—C_6H_4—	128–129	78.43	78.45	5.88	5.75	...	...	20
C_6H_5—	H	*o*-CH_3—O—C_6H_4—	144–145	74.54	74.26 74.32	5.65	5.71 5.86	...	...	13
C_6H_5—	H	*p*-Cl—C_6H_4—	150–151	69.81	69.71 69.75	4.64	4.59 4.73	10.86	11.05 11.20	17
C_6H_5—	CH_3—	C_2H_5—	74– 76	74.39	74.28 74.41	7.02	7.32 7.40	...	...	8
	H	C_2H_5—	86– 87	66.65	67.16 67.04	6.02	5.84 5.89	...	...	2
C_6H_5—	CH_3—	*p*-Cl—C_6H_4—	167–168	70.44	69.66 69.86	5.03	4.69 4.85	10.40	11.22 11.25	45
	H	*p*-Cl—C_6H_4—	122–124	64.46	64.13 64.31	4.14	4.11 4.26	11.19	11.11 11.15	8
C_6H_5—	CH_3—	CH_3—	133–135	73.75	73.51 73.67	6.60	6.57 6.66	...	...	17
C_6H_5—	Br—	*p*-Cl—C_6H_4—	173–174	56.25	56.51 56.70	3.48	3.76 3.88	...	...	27
C_6H_5—	CH_3—	*p*-*n*-C_4H_9—O—C_6H_4—	107–108[c]	76.16	76.13 76.48	6.93	6.93 6.95	...	...	10

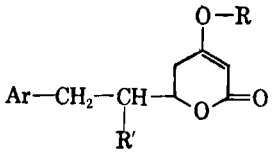
[a] Melting point reported in the literature is 146–147° (6). [b] All the compounds listed in Table III gave a strong characteristic red when a few crystals were dropped on concentrated sulfuric acid in a test tube. [c] The synthesis of this compound required the addition of magnesium and iodine to initiate the reaction.

days at room temperature the compound turned to a solid, m.p. 30°.

4 - p - Chlorophenoxy - 6 - β - methylstyryl-5,6-dihydro-2-pyrone.—To a completely dry 500-ml. round-bottom flask fitted with a reflux condenser and a calcium chloride drying tube, 6.5 Gm. of completely dry and activated zinc metal granules (40-mesh) was added.[3] To this was added a solution of 32 Gm. (0.100 mole) of ethyl γ-bromo-β-*p*-chlorophenoxy-crotonate and 15 Gm. (0.102 mole) of

[3] The activated zinc was obtained by allowing 10% hydrochloric acid to act on it for 10 minutes, washing it thoroughly with water, acetone, alcohol, and anhydrous ether, respectively. Finally, it was dried in a vacuum oven at 100°.

TABLE IV.—α,β-UNSATURATED β,δ-DISUBSTITUTED δ-LACTONES



Ar	R′	R	M.p., °C.	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	C, % Calcd.	C, % Found	Yield, %
C_6H_5—	H	CH_3—	74–75[a]	72.39	72.56 72.74	6.86	7.26 7.35	...	...	82
C_6H_5—	H	*p*-CH_3—C_6H_4—	120–122	77.89	77.66 77.75	6.55	6.73 6.84	...	...	92
C_6H_5—	CH_3—	*p*-Cl—C_6H_4—	135–137	70.07	69.79 69.93	5.59	5.66 5.88	10.34	10.48 10.61	74
C_6H_5—	H	*p*-Cl—C_6H_4—	119–120	69.41	69.46 69.53	5.21	5.31 5.62	10.78	10.48 10.50	100
C_6H_5—	H	*o*-CH_3—O—C_6H_4—	103–105	74.06	73.85 73.95	6.21	6.67 6.82	...	...	69

[a] Melting point reported in the literature is 76° (3).

freshly distilled α-methylcinnamaldehyde in 200 ml. of tetrahydrofuran which had been previously distilled over mineral oil. This mixture was heated to its boiling point, whereupon a small crystal of iodine was introduced to help initiate the reaction. It was then refluxed with occasional shaking for 5 hours. At the end of the reflux time, 5.9 Gm. of the zinc had dissolved. The mixture was cooled to room temperature and added with stirring to 600 ml. of a saturated solution of ammonium chloride. After extracting three times with chloroform (200 ml. and 2 times 100 ml.), the combined chloroform extracts were washed once with 200 ml. of water, dried over and filtered through anhydrous sodium sulfate, and concentrated *in vacuo* to a small volume. The residue was placed in the refrigerator overnight causing crystals to separate. After washing with methanol and drying, the crystals had m.p. 164–166°. A second crop of crystals was obtained by evaporating the methanol solution and placing it in the refrigerator. Further recrystallization from a mixture of chloroform-ether afforded 45% of white crystals, m.p. 167–168°. The compounds listed in Table III were prepared in an analogous manner.

4-p-Tolyloxy-6-phenethyl-5,6-dihydro-2-pyrone.—A solution of 4.3 Gm. (0.013 moles) of 4-*p*-tolyloxy-6-styryl-5,6-dihydro-2-pyrone in 30 ml. of tetrahydrofuran was treated with 0.2 Gm. of 15% palladium-carbon catalyst and hydrogenated at 30 p.s.i. After 2.5 hours, the mixture had taken up one molecular equivalent of hydrogen. The catalyst was removed by filtration, and the solvent was distilled *in vacuo*. Recrystallization of the residue from tetrahydrofuran gave 92% of product, m.p. 48–49°. Table IV lists the compounds prepared by the above method.

Infrared Absorption Spectra.—The infrared absorption spectra of all the lactones with a saturated side chain are similar to those with an unsaturated side chain, except for the absence of the absorption band at 10.38 μ, which is assigned to the *trans* configuration of the unsaturated lactones, and consequently is absent in the saturated ones. The strong absorption bands at 5.90 and 6.17 μ are common to all the lactones and are assigned to the C=O group in conjugation with the C=C grouping, respectively.

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Antiradiation Compounds V

α-Amino Acid Esters of 2-Mercaptoethylamine

By WILLIAM O. FOYE and ROBERT H. ZAIM

A series of *S*-α-amino acid esters of 2-mercaptoethylamine (MEA) has been prepared in an attempt to obtain less toxic and longer-acting derivatives of MEA with radiation-protective properties. The method of preparation consisted of a dry fusion of the amino acid chloride hydrochloride with MEA hydrochloride. It was found that the thioesters liberate MEA on acid hydrolysis; hydrolysis constants have been determined.

BETA - MERCAPTOETHYLAMINE (cysteamine, MEA), one of the more effective of the radiation-protective compounds in animals (1), has shown toxic symptoms in mice (2) and dogs (3) with doses close to those necessary for radioprotection. In humans, its use in moderate doses (200–400 mg. i.v.) over periods of several weeks without causing unfavorable effects has been reported (4). To provide less toxic and longer-acting derivatives of MEA, thioesters with α-amino acids have therefore been prepared. The α-amino acids themselves, except for cysteine which gives good protection against ionizing radiation, have shown but slight radioprotective action (5). Previously, we have prepared α-amino acid derivatives to lower the toxicity of bis-(4-aminophenyl)sulfone (6) and various nitro and halogenated benzene compounds with antibacterial activity (7). Acylation of MEA with aliphatic acids has already been shown to lower the intraperitoneal toxicity of MEA (8).

It was anticipated that the α-aminoacyl thioesters of MEA would liberate MEA *in vivo* over a period of time, or that possibly the thioesters would function as more active thiolation agents *in vivo* than the mercaptan. In regard to the hydrolysis of thioesters of MEA, however, some question exists concerning the nature of the hydrolysis products that might be expected *in vivo*. Wieland (9) has shown that in strong alkali the acyl group shifts to the nitrogen exclusively, giving amides which generally show little or no radioprotective capacity. Chromatographic tests conducted at the Walter Reed Army Institute of Research have shown that at least three species are present after hydrolysis of *S*-acetyl MEA at pH 7.4 (10), whereas Purdy (11) has found that only straight hydrolysis to MEA occurs on hydrolysis of this compound over a wide pH range in dilute solutions.

The method of preparation of the α-amino acid thioesters was patterned after that of Wieland (9), which consisted of a dry fusion of the α-amino acid chloride hydrochloride (12) and MEA hydrochloride. An excess of MEA hydrochloride was more effective in obtaining products of good purity than an excess of the acid chloride as recommended by Wieland. With heat labile acid chlorides, such as those from L-proline and DL-methionine, the reaction proceeded satisfactorily at room temperature. Infrared absorption spectra of the compounds showed no absorption band near 2550 $cm.^{-1}$ attributable to a free mercapto group, nor did their aqueous solutions reduce iodine solution until after standing for varying periods of time. Physical properties of the thioesters prepared are recorded in Table I.

Hydrolysis rate constants were determined for the thioesters in acetate buffer at pH 3.7 and a temperature of 15°. In alkaline solution, the esters undergo an immediate shift to the *N*-acyl derivatives (9), and in neutral solution the hydrolysis was too rapid for accurate measurements; therefore, the comparison was made in acid solution. The liberated thiol was titrated with standard iodine solution. Kuhn *et al.* (13) have shown that in 90% acetic acid thiol esters are oxidized readily to disulfides with iodine in stoichiometric amount, using very small quantities of material and without excluding atmospheric oxygen. Values for k were obtained from the slope of the plot of log C (concentration of unhydrolyzed ester) *versus* time. The data agreed with first-order kinetics, as would be expected from acid hydrolysis of esters, and the k values are listed in Table II. Representative data for a hydrolysis are presented in Table III.

The following sequence of hydrolytic reaction rates in decreasing order was: L-prolyl > glycyl >

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Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

The authors are indebted to Dr. W. E. Lange for infrared absorption curves.

TABLE I.—α-AMINO ACID ESTERS OF MEA

$$R\text{—}\overset{O}{\overset{\|}{C}}\text{—}SCH_2CH_2NH_2\cdot 2HCl$$

R	M.p., °C.	Yield, %	Formula	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	S, % Calcd.	S, % Found
NH_2CH_2	175–179 dec.[a]	55	$C_4H_{12}Cl_2N_2OS$	23.19	23.06	5.84	5.99	13.53	13.85	15.47	16.19
DL-$CH_3CH(NH_2)$	187–189.5 dec.	21	$C_4H_{14}Cl_2N_2OS$	27.15	26.89	6.38	6.27	12.67	12.52	14.50	14.85
DL-$CH_3CH_2CH(NH_2)$	192–196 dec.	32	$C_5H_{16}Cl_2N_2OS$	30.64	30.87	6.86	7.15	11.91	12.19	13.63	13.93
DL-$(CH_3)_2CHCH(NH_2)$	190–194 dec.[b]	30	$C_7H_{18}Cl_2N_2OS$	33.74	33.03	7.28	7.18	11.24	11.51	12.87	13.31
L-$(CH_3)_2CHCH_2CH(NH_2)$	178–181 dec.	44	$C_8H_{20}Cl_2N_2OS$	36.48	36.61	7.65	7.38	10.70	10.80	12.17	12.26
DL-$C_4H_9CH_2CH(NH_2)$	184–186 dec.	26	$C_{11}H_{16}Cl_2N_2OS$	44.44	44.98	6.10	6.06	9.43	9.30	10.78	10.81
L-	178–184 dec.	27	$C_7H_{16}Cl_2N_2OS$	34.01	33.85	6.53	6.63	11.34	11.08	12.97	13.55
DL-$CH_3SCH_2CH_2CH(NH_2)$	168–172 dec.	25	$C_7H_{18}Cl_2N_2OS_2$	29.89	29.92	6.45	6.67	9.96	9.95	22.80	23.10

[a] Lit. (9) m.p. 184° dec. [b] Lit. (9) m.p. 197° dec.

TABLE II.—HYDROLYSIS RATE CONSTANTS OF MEA ESTERS[a]

Ester Dihydrochloride	$k \times 10^4$
S-L-Prolyl MEA	16.00
S-Glycyl MEA	14.40
S-DL-Methionyl MEA	6.86
S-DL-β-Phenylalanyl MEA	6.70
S-DL-Alanyl MEA	4.60
S-L-Leucyl MEA	2.56
S-DL-α-Amino-*n*-butyryl MEA	1.80
S-DL-Valyl MEA	0.23

[a] Observed at pH 3.7 and 15° in acetate buffer solution.

DL-methionyl > DL-β-phenylalanyl > DL-alanyl > L-leucyl > DL-α-aminobutyryl > DL-valyl. It is apparent that neither molecular weight nor chain length are determinant factors in the ease of hydrolysis of these esters.

A paper chromatographic determination of the hydrolysis products of one of the thioesters, the valyl derivative, was carried out in a butanol-formic acid-water system at 25°. Spots were developed with ninhydrin and ammonia, and after remaining overnight in this medium, *S*-valyl MEA liberated MEA (R_f = 0.23), the disulfide of MEA (R_f = 0.06), and *N*-valyl MEA (R_f = 0.52). Wieland (9) had previously observed these compounds in the same solvent system at an unspecified temperature, and listed corresponding R_f values of 0.24, 0.05, and 0.50. It is unlikely that the $S \rightarrow N$ acyl shift occurred at the low pH (3.7) of the hydrolysis rate determinations, but it appears quite probable that some *N*-acyl derivative would be formed *in vivo* or in the solutions (pH 7.4) used for antiradiation testing.

Antiradiation testing results have not yet become available for these compounds. Bonati and Nuvulone (14) have tested several *N*-substituted α-amino acid derivatives of MEA, however, and claimed that *N*-glutamyl cysteamine and *N*-cysteaminemonoacetic acid were as effective against 600 r of X-radiation as cysteamine itself. Tank (15) has also claimed that *S*-2-aminoisobutyryl cysteamine has no radioprotective ability.

EXPERIMENTAL

Melting points were taken on a Mel-temp apparatus and are uncorrected. Carbon, hydrogen, and nitrogen analyses were done by Weiler and Strauss, Oxford, England.

α-Amino Acid Chloride Hydrochlorides.—The following procedure is representative. To a suspension of 5.0 Gm. (0.038 mole) of L-leucine in 100 ml. of reagent grade acetyl chloride was added 8.0 Gm. (0.038 mole) of phosphorus pentachloride. The suspension was shaken for 2 hours at room temperature. An additional 1.5 Gm. of phosphorus pentachloride was added, and the shaking period

TABLE III.—HYDROLYSIS OF *S*-DL-α-AMINO-*n*-BUTYRYL MEA·2HCl[a]

t, Sec.	0.01 *N* I_2 Consumed	SH, %	*S*-Acyl, %	C, mole/L.	Log *C*
0	0	0	100	0.0150	$\bar{2}$.1761
620	2.30	15.34	86.67	0.0127	$\bar{2}$.1038
1825	5.60	37.34	62.66	0.0094	$\bar{3}$.9731
3660	8.22	54.81	45.19	0.0068	$\bar{3}$.8312
6870	10.37	69.14	30.86	0.0046	$\bar{3}$.6656
10,170	11.30	75.34	24.66	0.0037	$\bar{3}$.5682

[a] Observed at pH 3.7 and 15° in acetate buffer solution.

was continued for 1.5 hours. The resulting white solid was collected in a sintered-glass crucible and washed several times with anhydrous ether. It was fused immediately with cysteamine hydrochloride as described below. The yield of L-leucyl chloride hydrochloride was 3.4 Gm. (47% of theoretical). Modifications were followed in the case of DL-valine (16), DL-alanine (17), L-proline (18), and DL-methionine (19).

Thioesters of Cysteamine Hydrochloride.—The following procedure is representative. In a mortar placed in a drying oven maintained at 80–90°, 4.5 Gm. (0.04 mole) of cysteamine hydrochloride (Chemicals Procurement Laboratories, Inc.) was melted. Then 3.4 Gm. (0.018 mole) of L-leucyl chloride hydrochloride was added all at once. The mass was triturated constantly; a large amount of hydrogen chloride was evolved. When hydrogen chloride evolution had ceased, the mortar was removed from the oven, and a small amount of absolute ethanol was added to the hot mass. The mass was triturated with the hot alcohol to remove excess MEA hydrochloride and the mortar was placed in an ice bath. After being cooled, a white solid crystallized. This was collected and washed several times with absolute ethanol. The residue was dissolved in a minimum amount of ice-cold 2*N* hydrochloric acid, and the solution was filtered into 150 ml. of acetone. A white solid crystallized and was isolated in a sintered-glass crucible; it was washed several times with absolute ethanol and stored in a desiccator over Drierite. The yield was 2.0 Gm. (44% of theoretical).

The ester was extremely water soluble, and its aqueous solution did not immediately decolorize iodine solution, but in approximately 30 minutes the solution was decolorized. The ester darkened at 174° and melted at 178–181° with decomposition.

Anal.—Calcd. for $C_8H_{20}Cl_2N_2OS$; C, 36.48; H, 7.65; N, 10.70; S, 12.17. Found: C, 36.61; H, 7.38; N, 10.80; S, 12.26.

Determination of Hydrolysis Rate Constants.—The following procedure is representative. A buffer solution having a pH of 3.7 was prepared by mixing equal volumes of 1 *N* hydrochloric acid solution and 1.25 *N* sodium acetate solution. Throughout the hydrolysis the temperature was maintained at 15 ± 0.1°. To 100.00 ml. of buffer solution contained in a stoppered iodine flask was added 0.3528 Gm. (0.0150 mole) of *S*-DL-α-amino-*n*-butyryl cysteamine dihydrochloride. At exactly determined times, aliquot portions were removed and added to 25 ml. of 2 *N* hydrochloric acid solution to attain a pH of 1 and stop further hydrolysis. Twice the theoretical amount of iodine was added to this acid solution; after 1 minute, the volume was doubled by the addition of distilled water. The excess iodine was back titrated with standard sodium thiosulfate solution. A *k* value was obtained by determining the slope of the straight line from the plot of log *C versus* time and multiplying this value by 2.303. The data from this determination are shown in Table III.

Chromatographic Analysis.—*S*-DL-Valyl cysteamine dihydrochloride (5 mg./ml.) in phosphate buffer solution (pH 7.4) was placed on Whatman No. 1 paper, dried, and allowed to remain in contact with *n*-butanol-85% formic acid-water (75:15:10 parts by volume) overnight at 25°. Spots were developed by treating with ninhydrin in *n*-butanol, drying, and suspending over ammonia. R_f values were taken at the center of the spots. Authentic samples of cysteamine and its disulfide (cystamine) (8) gave R_f values of 0.23 and 0.06, respectively. *N*-Valyl cysteamine hydrochloride was obtained by making strongly alkaline an aqueous solution of *S*-valyl cysteamine dihydrochloride followed by acidification (9).

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Free Radicals in Alkaloidal Color Identification Tests

By DAVID W. SCHIESER*

A number of color tests for alkaloids, some appearing in the U.S.P. and N.F., have been studied by electron paramagnetic resonance (e.p.r.) spectroscopy with the ultimate aim of ascertaining the chemical species responsible for the colors. In the case of the Marquis test for opium alkaloids, in which an intense purple is produced at once with morphine and quickly changes to deep blue-violet, it is shown that a different free-radical spectrum appears concurrently with each of these colors. Data are presented on the e.p.r. spectra of radicals thus formed from various alkaloids and several synthetic compounds. These conditions also produced free radicals from phenol and benzene; therefore, it is suggested that the site of radical formation in the alkaloids studied is the aromatic group. No proposal for a chemical mechanism is made, but it is suggested that a common mechanism is involved when aromatic compounds are treated with the Marquis reagent. Free radicals have also been observed in other color tests for alkaloids, such as the Mecke test for morphine. Like the Marquis test, the latter also uses concentrated sulfuric acid as solvent.

AN IDENTIFICATION test for morphine sulfate appearing in the U.S.P. XVI (1) involved treatment of morphine sulfate with a reagent consisting of a dilute solution of formaldehyde in concentrated sulfuric acid. An intense purple is produced at once which quickly changes to deep blue-violet. The reagent is sometimes referred to as the Marquis reagent for opium alkaloids (2–4). The test is described in other references (5) dealing with the identification of morphine. Conversely, the application of this color reaction with opium alkaloids is used as a very sensitive test for formaldehyde (6).

The test is not entirely specific for morphine or modified morphine type alkaloids. Colors are obtained with many aromatic compounds. For example, phenol and benzene give a qualitative red to brick-red color with this reagent. The test is referred to by Feigl (7) as the "Le Rosen Test for Aromatic Compounds," where the proposed reaction for the test involves the condensation of 1 mole of formaldehyde with 2 moles of the aromatic compound. Following the condensation step, oxidation by sulfuric acid occurs to produce unidentified colored quinoidal products.

Previous work in this laboratory has shown that free radicals can be stabilized in concentrated solutions of sulfuric acid (8). Quinoidal-type free radicals are known to occur in alkaline solution (9) and have also been reported to occur in acid solution (10). Hence, it was decided to examine several color tests by e.p.r. spectroscopy to determine if the colors were due to free radicals.

This work reports free radicals to be present in colored mixtures produced by the Marquis test as well as examples of free radicals occurring in colored mixtures produced by other tests in which sulfuric acid is the reaction solvent. In most cases the observed e.p.r. spectra exhibited a time dependency and indicated mixtures of free radicals.

EXPERIMENTAL

The e.p.r. spectrometer used in this study was previously described (11). This was modified by addition of a Varian 100-kc. field modulation and control unit, a Varian multipurpose cavity, and a flat sample cell (0.04 ml.). Good reviews of the technique and theory of e.p.r. spectroscopy are available (12).

The Marquis reagent was prepared by adding 1 ml. of 40% aqueous formaldehyde (Baker analyzed reagent) to 25 ml. of concentrated sulfuric acid (Baker and Adamson reagent). A dilute Marquis reagent was prepared using one-tenth of the above amount of formaldehyde in the same quantity of concentrated sulfuric acid. The Marquis reagents were freshly prepared every 2 or 3 days during the course of the e.p.r. measurements.

The Mecke reagent was prepared by adding 0.1 Gm. of selenous acid (Fischer analyzed reagent) to 20 ml. of concentrated sulfuric acid.

The Froehde reagent was prepared by adding 0.1 Gm. of sodium molybdate (Baker and Adamson reagent) to 100 ml. of concentrated sulfuric acid.

The Erdmann reagent was prepared by adding 10 drops of concentrated nitric acid (Baker and Adamson reagent) to 20 ml. of concentrated sulfuric acid. This reagent was prepared immediately before use.

The perhydrol reagent was prepared by adding 1 ml. of 30% hydrogen peroxide (Baker analyzed reagent) to 20 ml. of concentrated sulfuric acid. This reagent was prepared immediately before use.

Three general methods were used for the preparation of solutions for e.p.r. spectral determinations. (*a*) Approximately 1 ml. of dilute Marquis reagent or other color reagent was added to 0.01 mmole of

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TABLE I.—RESULTS FROM E.P.R. SPECTRA OF COLORED MIXTURES RESULTING FROM TREATMENT BY MARQUIS REAGENT OF ALKALOIDS AND SYNTHETIC COMPOUNDS

Parent Compd.	Final Concn., *M*	Time After Mixing and Color	Lines, No.	Splitting in Gauss	*g* Value
Morphine	0.01	10 min. red-violet	7	4.5	2.00305
Morphine	0.01	130 min. blue	4	9.0	2.00345
Morphine	0.005	Mixture of solns. violet to red-brown	37+	Varied	2.00345
Codeine	0.01	10 min. red-violet	7	4.6	2.00301
Codeine	0.01	48 hr. red-brown	3	...	2.0031
Codeine	0.005	Mixture of solns. blue-violet to reddish brown	3	6.2	2.00261
Pseudomorphine[a]	0.01	Brick-red or burgundy	Unresolved	...	2.0030
Apomorphine	0.01	5 min. green to blue	More than 4	...	...
Apomorphine	0.01	20 min. blue-green	4	9.9	2.0032
Apomorphine	0.005	Mixture of solns. blue-green	4	10.6	2.0030
Papaverine	0.02	Red-violet	Unresolved	...	2.0030
Meperidine	0.005	Mixture of solns. red or brick-red	Unresolved	...	...
Benzene	0.5	Brick-red	Unresolved	...	...
Salicylic acid	0.5	Red	Unresolved	...	...
Phenol	0.3	Brick-red	Unresolved	...	...
2 6-Dimethyl phenol	0.1	2 hr. deep brick-red	15 Doublets	1.92, 1.36, 0.3	2.00267
2,6-Dimethyl phenol	0.1	24 hr. deep brick-red	15	1.32	2.00266
2,6-Dichloro phenol	0.1	Red-violet	8 or 10	7.5	...

[a] This sample was of questionable purity.

solid alkaloid or synthetic compound. With respect to the dilute Marquis reagent, this represents a five-fold molar excess of formaldehyde. (*b*) Solutions of morphine sulfate U.S.P., codeine sulfate N.F., apomorphine hydrochloride, U.S.P., and 2,6-dimethylphenol (Eastman White Label) were prepared in concentrated sulfuric acid at 0.01 *M* concentrations. The solutions did not exhibit color formation even when allowed to stand for 1 week at room temperature. In an attempt to avoid complications due to the presence of a solid, the solutions were mixed with an equal volume of the dilute Marquis reagent or with a few drops of the previously mentioned color reagents immediately before the e.p.r. spectral determinations. (*c*) In a few instances radicals were not detectable at the above concentrations. In these cases the problem was overcome by either increasing the concentration of the solute or color reagent or both.

As soon as practical after the colored solutions were prepared e.p.r. spectra were obtained. Five to 10 minutes were usually required to measure the first spectrum. The spectral measurements were repeated at time intervals for several hours in those instances where a change in spectrum was apparent within 1 hour.

The *g* values[1] and hyperfine splittings of the spectra were obtained by simultaneously observing the spectrum of a 0.01 *M* solution of peroxylamine disulfonate (Aldrich Chemical Co.) in concentrated ammonia in a capillary fastened to the outside of the sample cell. The e.p.r. spectrum of a solution of peroxylamine disulfonate has been shown (13) to consist of three sharp peaks split by 13 gauss and with a *g* value of 2.00569.

[1] The *g* value is a property of a free radical relating the center of its e.p.r. spectrum to the magnetic field and the spectrometer frequency. See Blois, M. S., Jr., Brown, H. W., and Maling, J. E., *Arch. Sci. (Geneva)*, **13**, 243(1960); and "Free Radicals in Biological Systems," Proc. Symposium, Stanford, Calif., (1960), pp. 117–131. The true *g* value cannot be determined in cases where there are mixtures of free radicals present, but it is nevertheless a convenient notation for the location of the center of the spectrum and has value in comparing spectra in this report.

RESULTS AND DISCUSSION

The e.p.r. spectral data in Table I indicate that the action of the Marquis reagent produces free radicals with a variety of compounds. At room temperature no e.p.r. signals were observed in the Marquis reagent or in the sulfuric acid solutions of the compounds studied. The e.p.r. spectra of several of the colored solutions were observed to change with time (Table I). In each of several instances, a mixture of free radicals was indicated by the asymmetry of the e.p.r. spectrum. Normally, a single free radical shows an e.p.r. spectrum which is approximately symmetrical about a central point (12). In addition to the information given in Table I, measurements of concentration of free radicals were determined for a number of the spectra by double integration and comparison with a standard free radical spectrum (1,1-diphenylpicrylhydrazyl). The results from these calculations showed some variation, but in all cases it was within the range of 0.1 to 10% of the molar concentration of the parent compound.

Figure 1-A shows the e.p.r. spectrum of a solution prepared by adding dilute Marquis reagent to solid morphine sulfate. The same solution after a lapse of 2 hours yielded the spectrum in Fig. 1-B. When a solution of morphine sulfate prepared by method (*b*) was treated with the dilute Marquis reagent, a different spectrum was obtained, as shown in Fig. 2. The lack of symmetry of the spectrum probably indicates the presence of two or more radicals. While differing in appearance from Fig. 1-B, the spectrum in Fig. 2 has the same *g* value and could be a better resolved spectrum of the same free radicals. On the other hand, the dissimilarity in color of the two morphine solutions (Table I) would indicate the possibility of different free radicals.

The e.p.r. spectrum obtained 10 minutes after addition of dilute Marquis reagent to solid codeine sulfate is apparently identical with that obtained from morphine under the same conditions. After a lapse of time the two compounds no longer have

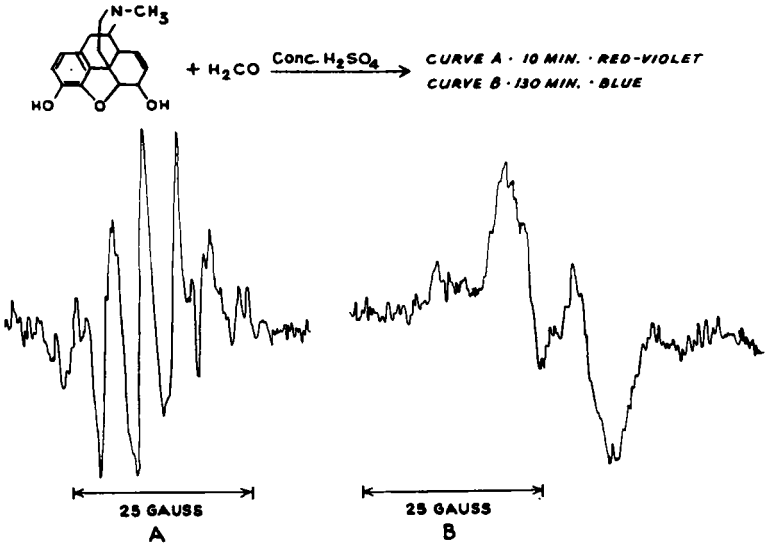


Fig. 1.—E.p.r. spectra of a solution prepared by the action of dilute Marquis reagent on *solid* morphine sulfate.

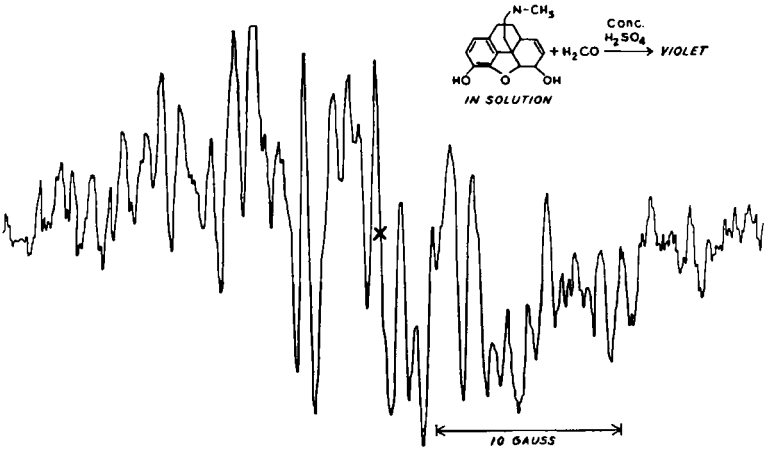


Fig. 2.—E.p.r. spectrum of a solution prepared by the action of dilute Marquis reagent on a sulfuric acid *solution* of morphine sulfate. The center of the spectrum is marked (×).

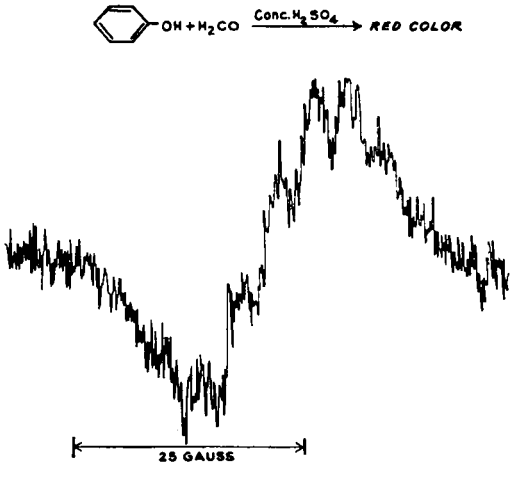


Fig. 3.—E.p.r. spectrum of a mixture prepared by the action of Marquis reagent on phenol. The sample contained a precipitate.

identical spectra, showing a difference in g values and in the number of hyperfine lines (Table I). The spectrum of the solution prepared from codeine by method (*b*) is similar to the secondary codeine spectrum from the solid.

The action of dilute Marquis reagent on apomorphine also produces a time-varying e.p.r. spectrum, but the initial spectrum was so transient that a complete graph was not obtained. The initial multilined spectrum changed rapidly to a broadened four-line spectrum. A solution of apomorphine in concentrated sulfuric acid treated with dilute Marquis reagent yields an e.p.r. spectrum which is comparable to the broad four-line spectrum having the same g value as that obtained from the solid.

The e.p.r. spectrum of the solution resulting from the treatment of papaverine with undiluted Marquis reagent consists of a single broad line which has the same g value as the initial spectra of solutions produced by the application of the same reagent to morphine, codeine, and pseudomorphine.

The action of the undiluted Marquis reagent on both benzene and phenol produced colored solutions with precipitation. Adjustments of concentrations

to produce highly colored solutions without precipitation and also a well-resolved e.p.r. spectrum were unsuccessful. Solutions which were sufficiently dilute to eliminate precipitation showed no detectable concentration of free radicals. Hence, the spectra reported in Table I and illustrated in Fig. 3 were obtained from solutions which contained precipitates. The lack of resolution in these spectra (Fig. 3) is probably due to the presence of mixtures of free radicals, some of which may be in the solid state.

The e.p.r. spectra obtained from solutions formed by the action of the undiluted Marquis reagent on meperidine and salicylic acid consist of broad single lines. Because of the unresolved nature of these spectra, the only possible inference is that free radicals are also formed in these two cases.

Since the only similarity in chemical structure of all the compounds shown above to produce free radicals by the action of the Marquis reagent is the aromatic ring, it is concluded that the ring is the site of the odd electron. The role of aromaticity in stabilizing free radicals is well known (12).

The reactions of the Marquis reagent on aromatic compounds, including alkaloids containing aromatic groups, may be similar to the acid-catalyzed reactions of the phenol-formaldehyde polymerization to resins as discussed by Walker (14). Since unsubstituted phenol produced a precipitate with the Marquis reagent, the 2,6-disubstituted compounds were tested in the hope of modifying the reactions by blocking the two reactive *ortho* positions.

2,6-Dimethylphenol, when treated with the Marquis reagent, exhibits a symmetrical e.p.r. spectrum (Fig. 4) consisting of at least 15 doublets. After standing 24 hours, the solution yielded a spectrum of the same g value and essentially the same basic 15 lines, but with the doublets unresolvable. The resulting solutions were deep brick-red and contained no precipitate, indicating decreased polymer formation. A higher concentration of free radicals was present than in the cases of benzene and phenol. The presence of the two methyl groups causes such a great number of hyperfine lines that the spectrum is difficult to analyze. The change in spectrum over a period of time also complicates the analysis of the hyperfine splitting.

2,6-Dichlorophenol was treated with the Marquis reagent in an attempt to simplify the e.p.r. spectrum while still blocking the two reactive *ortho* positions. This sample exhibited a spectrum of perhaps eight or ten lines, but so poorly resolved that conclusive analysis was not possible.

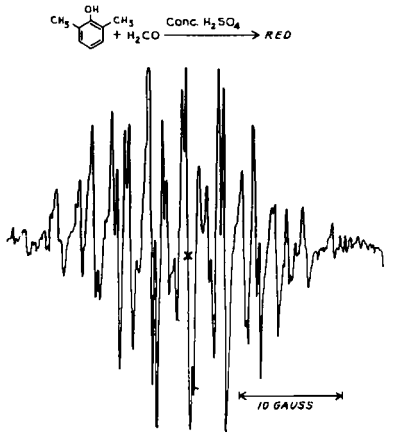


Fig. 4.—E.p.r. spectrum of a solution prepared by the action of dilute Marquis reagent on 2,6-dimethylphenol. The center of the spectrum is marked (×).

It is hoped that continued studies with other substituted phenols will lead to a better understanding of the hyperfine structure of the free radicals produced by reaction with the Marquis reagent and subsequently to the elucidation of the free radical structures. Once these spectral-structural relationships are established, the role of free radicals in the color tests will be clarified as will their role in the mechanism of acid-catalyzed phenol-formaldehyde polymerization.

Table II shows some e.p.r. data of compounds treated with several additional alkaloidal color reagents. The common feature of these reagents is the presence of an oxidizing agent in concentrated sulfuric acid. If only oxidation is involved, the action of any of these reagents on a given compound may result in the formation of identical radicals. There is sufficient similarity in the hyperfine structures and g values of morphine treated with the various reagents (Table II) to indicate that the principal free radical formed is the same in each case. Minor variations in the spectra may be due to (*a*) subtle differences in the relative concentration of minor radicals, (*b*) paramagnetic substances present as impurities in the color reagents, and (*c*) the presence of a paramagnetic transition element as a component of the reagent (Froehde reagent). In the latter case the observed spectrum consisted of the sum of the free radical spectrum and the paramagnetic spectrum of molybdenum.

TABLE II.-E.P.R. SPECTRAL DATA OF COLORED MIXTURES RESULTING FROM THE ACTION OF SEVERAL COLOR REAGENTS ON ALKALOIDS

Compd.	Reagent	Color	Lines, No.	Splitting in Gauss	g Value
Apomorphine	Mecke	Brown-green	Unresolved	...	...
Codeine	Mecke	Blue-green	12+	Uneven av. 0.28	2.00376
Morphine	Mecke	Blue-green	6	5.3 2.74	2.00363
Morphine	Froehde	Violet-green	6	4.06 2.56	2.00365
Morphine	Erdmann	Green-brown	6	4.77 2.65	2.00349
Morphine	Perhydrol	Green-brown	3	7.9	2.00355

The studies reported in this paper do not show conclusively that the colors are due to free radicals. In a few cases addition of water caused a marked decrease in the e.p.r. signal while the color became more intense; a striking example of this phenomenon is 2,6-dimethylphenol. It appears that the colors are due to a mixture of materials, some of which may be free radicals. This is supported by concentration measurements, which showed that the free radicals comprised less than 10% of the parent compound. The changes in color and e.p.r. spectrum with time provide evidence that several reactions are occurring simultaneously in the tests.

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Powdered Particle Interactions: Suspension Flocculation and Caking IV

By ROBERT G. WILSON* and BERNARD ECANOW

The incompatibility of bismuth subnitrate with tragacanth mucilage has been reported in the literature. In this study, the phenomenon is discussed in terms of the chemical reaction that occurs between reactive bismuth sites on the bismuth subnitrate particle surface and the acidic groups of the D-galacturonic acid units of the complex acid polysaccharide present in the tragacanth gum.

AN AQUEOUS SUSPENSION containing bismuth subnitrate and one of a number of polysaccharide or carbohydrate derivatives such as tragacanth will exhibit a well known incompatibility (1, 2). The substances coagulate on mixing to form soft curd-like aggregates which settle to the bottom of the container. If the compounds are introduced at higher levels of concentration, the aggregates may set to hard, intractable masses.

The phenomenon has been explained (3, 4) as the interaction of positive bismuth or bismuth ions with the negatively charged tragacanth. It has been shown that the incompatibility does not appear in the presence of phosphate, citrate, or arsenate ions (3, 5). The negatively charged phosphate ion was thought to be strongly adsorbed on the tragacanth micelle and thus provided a protective action against the adverse effect of the positively charged bismuth. Nitrate ion was thought to be similarly adsorbed, but due to a weaker protective action could not prevent the occurrence of the incompatibility.

The chemistry of bismuth subnitrate in aqueous suspension has been explored in our recent work (6). The phosphate ion was shown to be a flocculating agent for an aqueous bismuth subnitrate suspension because strong interparticle bonds were formed by chemisorption of the phosphate ions at the surface of the bismuth subnitrate particles. An interaction of this type provides a basis for the discussion of the bismuth subnitrate-tragacanth or similar suspension systems.

EXPERIMENTAL

Materials.—The same lot of a commercial grade of bismuth subnitrate N.F. was used throughout the series of tests. It was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Two types of tragacanth U.S.P. were used in the work.[1] They will be more simply described as gum or ribbon

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* Fellow of the National Science Foundation.

[1] Tragacanth Aleppo Ribbons No. 1 and Fine Powdered Gum Tragacanth Extra Grade No. 1, supplied by S. B. Penick and Co., New York, N. Y.

The studies reported in this paper do not show conclusively that the colors are due to free radicals. In a few cases addition of water caused a marked decrease in the e.p.r. signal while the color became more intense; a striking example of this phenomenon is 2,6-dimethylphenol. It appears that the colors are due to a mixture of materials, some of which may be free radicals. This is supported by concentration measurements, which showed that the free radicals comprised less than 10% of the parent compound. The changes in color and e.p.r. spectrum with time provide evidence that several reactions are occurring simultaneously in the tests.

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tragacanth in the balance of this report. Each experiment involving tragacanth was thus run in duplicate—first with the gum tragacanth, then with the ribbon form of the suspending agent. The monobasic potassium phosphate was of reagent grade, supplied by Merck & Co., Rahway, N. J.

Settling Data.—The methods of Ward and Kammermeyer (7) were used to obtain and analyze the sedimentation data. Where necessary, the suspensions were shaken on a Birrell wrist-action shaking machine, model DD.

Suspension Formulation.—Meaningful results could be obtained from suspensions which contained 10 Gm. of bismuth subnitrate and 100 ml. of the suspending medium. The tragacanth was prepared as a stock mucilage and was introduced as required to give a final concentration of 0.1% tragacanth in the suspending medium. The monobasic potassium phosphate was also prepared as a stock solution. This contained 100×10^{-4} meq. of phosphorus per milliliter and was added as required to give the final concentration of 50×10^{-4} meq. of phosphorus per milliliter of the suspending medium. After being prepared, the suspensions were allowed to settle for 5 days before being photographed.

RESULTS

Suspension I.—The bismuth subnitrate was suspended in distilled water and the mixture shaken by hand to disperse the solid evenly. The supernatant liquid was still slightly cloudy after 5 days and the dense, compacted sediment could be resuspended only with difficulty. However, the particles resuspended as individuals in the suspension medium.

Suspension Ia.—This suspension was the same as *Suspension I*, but was placed on the shaker for 24 hours before being allowed to settle. It was visibly comparable to Suspension I in its settling characteristics and resuspended with the same difficulty.

Suspension II.—The bismuth subnitrate was suspended in phosphate solution, promptly became flocculated, and rapidly settled to a bulky sediment. The supernatant liquid was clear and colorless. At the end of 5 days, the sediment was easily resuspendable, with particles in both the flocculated and deflocculated states.

Suspension IIa.—This suspension was the same as *Suspension II*, but was placed on the shaker for 24 hours before being allowed to settle. During this period, it became partially deflocculated and was visibly comparable to Suspensions I and I*a* in its settling characteristics. However, its final sediment height was slightly greater than that of Suspensions I or I*a*, and the solids were a little more easily resuspendable.

Suspension III.—The bismuth subnitrate was suspended in water, and tragacanth was added from the stock mucilage. On mixing, the solids coagulated and settled rapidly to a coarse, lumpy sediment which bulked to a high suspension height. The supernatant liquid was cloudy and failed to clear completely during the 5 days of settling. The sediment was slightly gelatinous and could be easily resuspended in the form of large, coarse aggregates.

Suspension IIIa.—This suspension was the same as *Suspension III*, but was placed on the shaker for 24 hours before being allowed to settle. In settling, the sediment of this suspension bulked to a lesser degree than did that of Suspension III. In addition, it was seen that the sediment was more gelatinous and the shaking had acted to smooth out the suspension and to eliminate the coarse, lumpy appearance of the sediment of Suspension III. The solids were easily resuspendable in the form of small floccules.

Suspension IV.—The bismuth subnitrate was suspended in a medium containing tragacanth and phosphate ion. The order of addition was: (*a*) bismuth subnitrate, (*b*) distilled water, (*c*) phosphate ion, and (*d*) tragacanth mucilage. The solids flocculated and settled rapidly to a bulky sediment. The supernatant liquid was clear and colorless, although not so clear as when phosphate ion alone was the flocculating agent. The sediment had a marked gel structure, but this could easily be broken by shaking. The material was resuspended in the form of fine floccules.

Suspension V.—This suspension was the same as *Suspension IV*, but the order of addition was: (*a*) phosphate solution, (*b*) tragacanth mucilage, (*c*) bismuth subnitrate, and (*d*) distilled water. The solids settled rapidly leaving a cloudy supernatant liquid which failed to clear within 5 days. The sediment showed a marked gel structure; it seemed to be slightly more difficult to resuspend the material than it was to resuspend the solids of Suspensions IV. However, both sediments could be described as easily resuspendable. The floccules of the suspended material were larger than those seen with Suspension IV and settled more rapidly on standing.

Suspension Va.—This suspension was prepared in the same order as *Suspension V*, but the mucilage containing phosphate ion and tragacanth was shaken for 24 hours before the bismuth subnitrate was added. As far as could be seen, Suspensions V and V*a* were identical in their settling characteristics and in their response to resuspension. Any slight difference in their sediment heights may be explained as being due to further hydration of the tragacanth during the shaking period.

Suspension VI.—This suspension was the same as *Suspension V*, but the order of addition was: (*a*) phosphate solution, (*b*) tragacanth mucilage, and (*c*) bismuth subnitrate suspended in distilled water. Suspension VI was quite comparable to Suspensions V and V*a* in its settling characteristics, the only apparent difference being that the final sediment height was slightly greater than that of the other two suspensions.

DISCUSSION OF RESULTS

The study was begun with a suspension of 5 Gm. of bismuth subnitrate in a quantity of 1% tragacanth mucilage sufficient to give 60 ml. of final suspension. Confirming prior work (5), no apparent differences could be seen when the order of mixing was changed. Visual inspection showed that a gelatinous network of flocculated material had formed throughout the suspension. The aqueous medium could be drained from the aggregates; when these were spread on a slide and viewed under low magnification, an open network of gelatinous floccules could be seen. The suspension was prepared again, but was shaken for

24 hours before being examined. The particles were uniformly suspended in a smooth gel, both when viewed macroscopically or under low magnification.

These initial findings showed that the interaction of bismuth subnitrate and tragacanth mucilage was qualitatively similar to the chemical reaction that occurs between bismuth subnitrate and various flocculating anions (6). When the highly viscous tragacanth mucilage was added to a bismuth subnitrate suspension, the bismuth subnitrate could react to set the mixture to a coarse, granular, gelatinous network before complete mixing could be achieved. Twenty-four hours of continuous shaking served to break this network into a more homogeneous gel structure.

The above interaction seemed analogous to the reaction in which bismuth subnitrate was flocculated in a medium containing a large excess of the phosphate ion (6). It was shown that continuous shaking for a 24-hour period would reduce the size of the flocs but would not give rise to deflocculation. On the other hand, a bismuth subnitrate suspension flocculated by a solution containing a relatively low concentration of phosphate ion became deflocculated after 24 hours of shaking. The same type of phenomenon was demonstrated for the bismuth subnitrate-tragacanth mucilage suspension system. An aqueous 4% bismuth subnitrate suspension was reacted with an equal volume of 0.02% tragacanth mucilage to form a flocculated suspension. The solids settled rapidly and bulked to a relatively high suspension height at the bottom of the container. The suspension was then shaken for 24 hours and became deflocculated. After deflocculation, the particles settled slowly, as individuals, to form a dense, compact precipitate with a relatively low sediment height.

Our recent study (6) has shown that a bismuth subnitrate suspension is strongly electrolytic because hydrogen and nitrate ions are released to the suspension medium. The hydrogen ions appear in greater number than do the nitrate ions, and the bismuth subnitrate particle surface becomes correspondingly more basic as it yields this excess number of hydrogen ions to the medium. Bismuth subnitrate or its more basic forms are all quite insoluble, and do not yield trivalent bismuth ions to the suspension medium in an appreciable amount. The possible interaction of bismuth ion with tragacanth mucilage was tested by adding an acidic 0.1% bismuth nitrate solution to an equal volume of 0.1% tragacanth mucilage. There was no visible coagulation or flocculation of the tragacanth. It seems definite that the small amount of ionic bismuth present in a bismuth subnitrate suspension would not give rise to the incompatibility with tragacanth, as has been suggested (3, 4).

Tragacanth has been shown to be a mixture of the salt of a complex acid polysaccharide, a neutral polysaccharide composed principally of L-arabinose, and small amounts of a glycoside and of starch (8). The acidic character of the gum is due to the presence of units of D-galacturonic acid. The D-galacturonic acid units could act somewhat like the acid phosphates in a reaction with the bismuth subnitrate particle surfaces. The official tragacanth mucilage (9) was unsuitable for use in the current studies due to its benzoic acid content. The slightly soluble benzoic acid is also a flocculating agent for an aqueous bismuth subnitrate suspension.

A series of suspensions was prepared in accordance with the formulations described under the heading *Experimental*. Suspensions I and I*a* (see Fig. 1) do not contain a flocculating agent and can be seen to be of the same low sediment height. Thus, a freshly prepared bismuth subnitrate suspension settles to form a compact sediment, and the same end result is obtained either with or without a shaking step. Suspension II illustrates the sediment height obtained by flocculating a bismuth subnitrate suspension with phosphate ion, and Suspension II*a* shows what happens when the same suspension is prepared but shaken continuously for 24 hours before being allowed to settle. Suspensions III and III*a* present the same general picture for the tragacanth-flocculated bismuth subnitrate suspension.

Husa and Plaxco (5), in discussing Schmitz and Hill's work (3) on the bismuth subnitrate-tragacanth incompatibility, suggested that the addition of stabilizing agent to the tragacanth mucilage prior to the final mixing with bismuth subnitrate should afford a greater degree of protection than if the stabilizing agent were first added to the bismuth subnitrate in suspension and the tragacanth mucilage then mixed in to form the final suspension. Husa and Plaxco conducted a few experiments on this point; at the concentrations used, they were unable to show that differences in the order of mixing had significant effect upon the end results. With more dilute suspensions, it has been possible to show differences due to the order of mixing. Four suspensions were prepared, using the three different orders of mixing outlined in Table I.

Suspensions V and V*a* were mixed in the same order and proved to have the same sedimentation characteristics. The only difference between the two was that during its preparation Suspension V*a* was shaken for 24 hours following the mixing of the tragacanth mucilage and the phosphate solution. Hereafter, the two suspensions will be referred to as Suspension V since the shaking step had no apparent effect upon the performance of the suspension formulation.

Suspension IV gave the bulkiest sediment. This seems due to the fact that the bismuth subnitrate

TABLE I.—SUSPENSION NUMBERS WITH ORDERS OF MIXING

Suspension No.	Order of Mixing			
	1	*2*	*3*	*4*
IV	Bismuth subnitrate	Distilled water	Phosphate solution	Tragacanth mucilage
V and V*a*	Tragacanth mucilage	Phosphate solution	Bismuth subnitrate	Distilled water
VI	Bismuth subnitrate	Distilled water	A mixture of the phosphate solution and tragacanth mucilage	

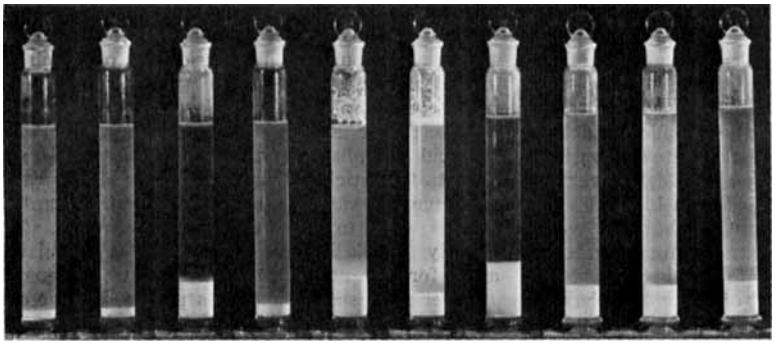


Fig. 1. — Suspensions prepared from the gum tragacanth mucilage.

Suspension No.	I	Ia	II	IIa	III	IIIa	IV	V	Va	VI
Suspension Ht. (as per cent of total suspension volume)	4.5%	4.5%	19.0%	6.0%	20.5%	12.0%	26.0%	15.0%	15.0%	17.5%

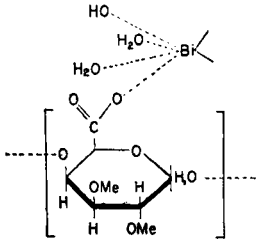


Fig. 2. — Chemical bond between acidic groups of hydrated tragacanth and the hydrated, charged surfaces of the bismuth subnitrate particles.

was first flocculated by the phosphate ion. The flocculated sediment could then be given added volume by the bulking action of the tragacanth without the formation of bonds between the bismuth subnitrate and the tragacanth. The comparable clarity of the supernatant liquids of Suspensions II and IV indicates this to be a proper explanation. In Suspension V, the bismuth subnitrate was placed into a medium containing both phosphate ion and tragacanth. Thus, it could interact with either material, forming bonds with the phosphate ions or with the D-galacturonate units of the tragacanth polysaccharides. It is of interest that the final sediment height of Suspension V was smaller than that of Suspension III, which had been flocculated by tragacanth alone. Two explanations may be offered. First, the gelatinous curd-like aggregates of the tragacanth-flocculated suspension left many open spaces in the sedimented material. These added to the height of the sediment of Suspension III. Second, in Suspension V the dry bismuth subnitrate could interact and become trapped within the floccules before becoming evenly dispersed throughout the suspension. In Suspension VI, some of this lost height was recovered by dispersing the bismuth subnitrate particles more evenly before allowing them to react with the mixture of the phosphate solution and the tragacanth mucilage.

A portion of the experiments were run in duplicate, first with the gum tragacanth and then with ribbon tragacanth. The photographs of Fig. 1 show suspensions prepared from the gum tragacanth mucilage. The ribbon tragacanth series could have been shown and described in the same terms, except that the ribbon tragacanth generally produced bulkier sediments than did the gum tragacanth mucilage.

The results obtained have been explained as being due to the chemical reaction that occurs between acidic groups of hydrated tragacanth and the hydrated, charged surfaces of the bismuth subnitrate particles. The reaction is analogous to that which occurs between phosphate ions and bismuth subnitrate (6). The chemical bond may thus be drawn as shown in Fig. 2.

SUMMARY

The incompatibility that is seen when an aqueous suspension of bismuth subnitrate is mixed with a tragacanth mucilage has been touched upon in many places in the pharmaceutical literature. Most authors have been satisfied to report the phenomenon without discussing the basic reasons for the incompatibility. A few investigators have worked on this aspect of the problem, and their conclusions may be found in the references that have been cited.

In this report, a new interpretation has been made concerning the nature of the incompatibility. The interaction is analogous to the chemical reaction that gives rise to the flocculation of a bismuth subnitrate suspension in the presence of phosphate ion. In the bismuth subnitrate-tragacanth suspension system, the chemical interaction occurs between reactive sites on the bismuth subnitrate particle surfaces and the acidic groups of the D-galacturonic acid units of the complex polysaccharides present in the tragacanth.

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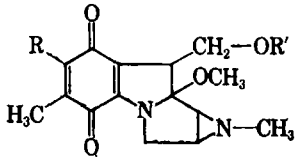
Prediction of Stability in Pharmaceutical Preparations XIII

Stability, Spectrophotometric, and Biological Assay of the Antibiotic Porfiromycin in Pharmaceutically Useful pH Ranges

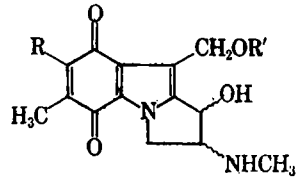
By EDWARD R. GARRETT and WILLIAM SCHROEDER

The kinetics of solvolysis of the antibiotic porfiromycin are quantified in the pharmaceutically useful pH ranges in acetate, phosphate, and borate buffers. The devised ultraviolet spectrophotometric assays of solution degraded antibiotic are correlated with the plate-disk assay against *S. aureus* and the paper chromatographic assay against *S. lutea*. General acid catalyzed solvolysis is indicated as well as specific acid-base. In acetate buffers, the primary degradative mechanism is the hydrolysis of the fused ring aziridine group, whereas mild alkali substitutes a hydroxyl for the quinoid ring amine. In the intermediate pH ranges characterized by phosphate buffers reaction at both sites is implied and maximum stability at pH 8 at 30° can be predicted. Conditions for isolation of solvolytic products and their characterizations are given.

THE NEW BROAD SPECTRUM antibiotic porfiromycin has been assigned the fused ring aziridine structure, α, by Webb, *et al.* (1–3).



	R	R′
α	$-NH_2$	$-CONH_2$
β(B)	$-OH$	$-CONH_2$
γ(B,B)	$-OH$	$-COOH$
δ(B,B,B)	$-OH$	$-H$



	R	R′
β(A)	$-NH_2$	$-CONH_2$
γ(A,A) γ(B,A)	$-OH$	$-CONH_2$
γ(A,B) δ(A,A,B)	$-OH$	$-H$

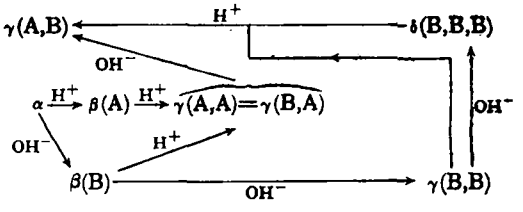
Properties, biological activities and assay methods have been studied (4–8). The kinetics of the acidic and basic catalyzed transformations of porfiromycin in solution have been evaluated and quantitative expressions have been established for their kinetics (9). The products of acidic and alkaline catalyzed solvolysis have been spectrophotometrically identified and their dissociation constants and apparent equivalent weights determined. In addition, these physicochemical and kinetic data have been correlated (9) with structural transformations of the proposed structure for porfiromycin, α (1, 2).

The purpose of this paper is to consider the correlation of biological and spectrophotometric assays of porfiromycin, to evaluate the antibiotic's stability in the pharmaceutically useful pH ranges and to provide additional physicochemical data for characterization.

In this paper the products of porfiromycin degradation are identified by an operational nomenclature. In this scheme porfiromycin is α and distinct spectrophotometric identities resulting from acid or basic solution degradations are represented as β, γ, and δ in their chronological order of appearance. A subscript represents the solution (A = acid, B = basic) of the distinct product. The order of B's or A's in the subsequent parentheses represents the sequence of basic or acid degradation.

For example, δ_B(A,B,B) indicates that this is the third distinct product of porfiromycin degradation in basic solution from firstly, acid, *i.e.*, β(A); then, secondly, basic γ(A,B); and a final degradation in basic solution, δ(A,B,B).

As has been shown previously (9) the possible acid and alkaline transformations of porfiromycin, α, with their isolable intermediates, are



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TABLE I.—CONDITIONS AND OBSERVED FIRST-ORDER RATE CONSTANTS (k IN SEC.$^{-1}$) FOR THE HYDROLYSIS OF PORFIROMYCIN IN AQUEOUS BUFFERS

Run	° C.	Observed pH at 30° C.	Porfiromycin (mcg./ml.)	Buffer Composition		$10^5 k$ (sec.$^{-1}$)
				[CH_3COOH]	[CH_3COO^-]	
1	30.2	3.60	30	0.1756	0.0122	28.4[a]
2	30.2	4.53	26	0.05	0.05	3.03[a]
3	30.2	4.54	24	0.10	0.10	3.24[a]
4	30.2	4.51	26	0.20	0.20	4.26[a]
5	30.2	4.55	25	0.40	0.40	5.00[a]
				[KH_2PO_4]	[NaOH]	
6	30.2	5.91	16	0.05	0.0057	0.158[a]
7	30.2	6.90	15	0.05	0.02963	0.0216[a]
8	60.0	5.91	28	0.05	0.0057	3.24[a]
9	60.0	6.96	32	0.05	0.02963	0.484[a]
10	60.0	7.91	27	0.05	0.0468	0.179[a]
11	69.7	6.93	26	0.05	0.02963	0.883[b]
12	69.7	6.97	25	0.025	0.01482	0.695[b]
				[H_3BO_3]	[NaOH]	
13	60.0	8.95	28	0.05	0.0213	0.185[c]
14	60.7	8.95	500	0.05	0.0213	0.229[c]
15	60.7	8.95	500	0.05	0.0213	0.946[d]
16	60.0	9.89	29	0.05	0.0439	0.250[c]
17	60.0	9.89	536	0.05	0.0439	0.300[c]
18	60.0	9.89	536	0.05	0.0439	6.16[e]
19	60.0	9.89	536	0.05	0.0439	3.21[f]
20	30.2	...	30	0.00	0.10	81.9[g]
21	30.2	...	30	0.00	0.10	0.352[h]

[a] Rate constant calculated from loss of absorbance at 363 mμ ($\alpha \rightarrow \beta$). [b] Aliquots were submitted for plate-disk assay (7) at two different phosphate concentrations for both runs 11 and 12 to test the effect of phosphate concentration on the sensitivity of the assay. It had no apparent effect. Rate constants for $\alpha \rightarrow \beta$ were calculated from U.V. spectra. [c] Rate constant calculated from loss of absorbance at 360 mμ ($\beta \rightarrow \gamma$). [d] Same run as No. 14, but rate constant calculated from loss of potency by plate-disk assay ($\alpha \rightarrow \beta$) (7). [e] Same run as No. 17, but rate constant calculated from loss of potency by *S. lutea* papergram ($\alpha \rightarrow \beta$) (4). [f] Same run as No. 17, but rate constant calculated from loss of absorbance at 367.5 mμ for time interval during which aliquots showed potency by bioassays ($\alpha \rightarrow \beta$). [g] Rate constant calculated from loss of potency by plate-disk assay ($\alpha \rightarrow \beta$) (7). [h] Same run as No. 20, but rate constant calculated from loss of absorbance at 360 mμ ($\beta \rightarrow \gamma$).

EXPERIMENTAL

The isolation and characterization of the porfiromycin used in these studies has been reported by Herr, *et al.* (5).

Kinetic Studies.—The appropriate amount of porfiromycin or isolated degradation product was weighed into a tared volumetric flask, a few drops of methanol were added to dissolve the sample, and the solution was diluted up to volume with the appropriate aqueous buffer. The buffer solutions had been previously equilibrated at the temperatures of the degradation studies. The flasks were covered with aluminum foil to prevent possible photolytic degradation, put in a constant temperature bath, and at recorded time intervals a spectrum was run on a Cary model 11 or 14 recording spectrophotometer after appropriate dilution, usually to 12 mcg./ml.

The conditions for the degradation of porfiromycin in acetate buffers at 30° are given in Table I, runs 1–5. The pH was measured by glass-saturated calomel electrodes by a Cambridge pH meter and recorded. The average is reported. Aliquots of these solutions were taken at various times. The aliquots were diluted 1:1 with sufficient alkali to adjust to pH 7 and the spectra of the resulting 12–15 mcg./ml. solutions were recorded on the Cary recording spectrophotometer. Run 1 was also diluted 1:1 with the same acetate buffer and the spectra observed. Aliquots of the same acetic acid–acetate buffered solution, runs 2–5, Table I, were taken at the same intervals and neutralized 1:1 with 0.1 *M* phosphate buffer, pH 7.8, which also included sufficient alkali to neutralize the excess acetic acid. These samples were assayed by the plate-disk method against *S. aureus* (7).

The conditions for the degradation of porfiromycin in phosphate and borate buffers are given in Table I, runs 6–12 and runs 13–21, respectively. The procedure for spectrophotometric assay was the same for runs 6–12, 13, 16, and 20, Table I, as that given for the acetate buffers except that the 1:1 dilution before reading on the spectrophotometer was made with the same buffer used in the kinetic study.

Runs 11 and 12 were also used to study the effect of different phosphate buffer concentrations on the biological assay.

Simultaneously with the removal and treatment of aliquots from runs 11 and 12 for spectrophotometric assay, two other aliquots were removed from each run for biological assay by plate-disk against *S. aureus* (7), each to be assayed under different phosphate buffer concentrations so that the effect of this variable could be evaluated. Also, solutions of porfiromycin of 500 mcg./ml. were prepared in borate buffers, maintained at desired temperature, and aliquots removed at timed intervals (see runs 14, 15, 17, 18, and 19, Table I). For plate-disk assay against *S. aureus* (7), 0.1-ml. aliquots were diluted to 5 ml. with 0.1 *M* phosphate buffer, pH 7.9, and assayed directly. For spectrophotometric assay, 0.2-ml. aliquots were diluted to 10 ml. with buffer. For paper chromatographic assay (4) by ultraviolet scanning and against *S. lutea* 1-ml. aliquots were adjusted to the appropriate pH with concentrated reagents so that no significant change in concentration occurred.

Studies were conducted in borate buffers at 60° and at pH values 9 (runs 14, and 15) and 10 (runs 17–19). The aliquots of the pH 10 study (run 18) were adjusted to pH 7 and submitted for papergram assay by both ultraviolet scanning and against *S. lutea* (4).

The aliquots of the pH 9 study (runs 14 and 15) were adjusted to pH 8 and 9 and both adjusted samples were submitted for papergram assay by ultraviolet and against *S. lutea* (4).

In 0.1 *M* NaOH solution the porfiromycin absorbance at 363 mμ dramatically decreased with time and a new chromophore appeared with a $\lambda_{max.}$ at 333 mμ. The half-life of the porfiromycin chromophore was 55 hours at 30° (run 21). However, when an attempt was made to correlate the half-life of the porfiromycin biological activity by plate-disk assay (7), the biological activity was found to disappear at a much faster rate (half-life of 14 minutes) under these conditions (run 20) than did the porfiromycin 365-mμ chromophore.

When this apparent inconsistency was observed, the changes in spectra were followed carefully for the first half-hour and a slight but significant change in chromophore was determined. The 363 mμ (a = 63.8) maximum shifted to 360 mμ (a = 67.1) and the absorbance *increased.*

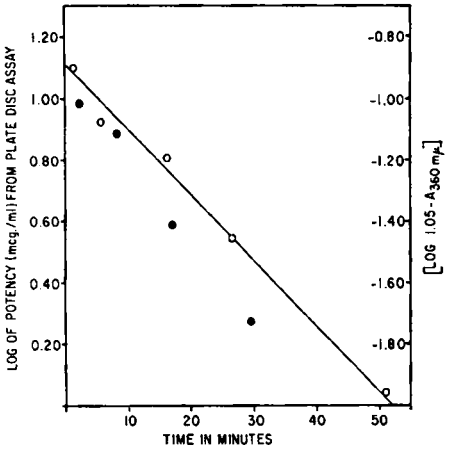


Fig. 1.—Correlation of rate of loss of biological potency by plate-disk assay with rate of increase of porfiromycin absorbance at 360 mμ in 0.10 *M* NaOH at 30°. Key: O, logarithm of potency by plate-disk assay plotted against time; ●, logarithm of the difference between 1.05 and the absorbance at 360 mμ plotted against time for a 15 mcg./ml. solution of porfiromycin.

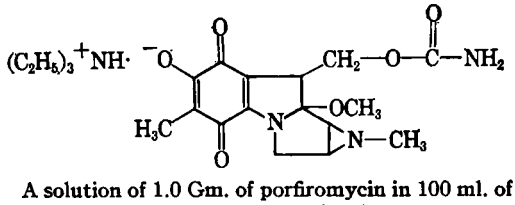
Even considering the small changes in absorbance which would introduce relatively large error into a first-order plot, the coincidence of the slopes of such plots for absorbance changes and bioassay values was apparent (Fig. 1). Thus, the correlation of the rates of this chromophoric enhancement with loss in biological activity was conclusively demonstrated. This was considered as the transformation of porfiromycin $\alpha_B \rightarrow \beta_B(B)$ (9).

Visible Spectra of Porfiromycin and Its Acid and Alkaline Degradation Products.—The visible spectra of porfiromycin and its products of acid and alkaline degradation were also run on the Cary 11 spectrophotometer at several pH values ranging from acid to alkaline solutions. The changes in color of these products as a result of changes in pH are noted in Table II.

The conditions for their preparation were determined from the kinetics described previously (9). The β(B), γ(B,B), and δ(B,B,A) were all prepared *in situ*, starting with the triethylamine salt of β(B). The γ(B,A) and δ(B,A,B) were prepared *in situ*, starting with isolated γ(B,A). All other materials were prepared from porfiromycin in solution as previously specified (9) and were analyzed spectrophotometrically *in situ*.

Isolation and Characterization of Solvolytic Products of Porfiromycin.

Preparation of β (B) Triethylamine Salt



A solution of 1.0 Gm. of porfiromycin in 100 ml. of 0.1 *N* sodium hydroxide was stirred at room temperature for 75 minutes and then allowed to stand at 5° for an additional 45 minutes. Cold methylene chloride (100 ml.) was then added and the mixture acidified with 12 ml. of 1 *N* sulfuric acid. The cold mixture was shaken and the organic layer quickly

TABLE II.—VISIBLE PHENOMENA OF PORFIROMYCIN AND ITS ACID-BASE DEGRADATION PRODUCTS

Compound	In Alkaline Solution: $\lambda_{max.}$	In Alkaline Solution: a[a] at $\lambda_{max.}$	In Alkaline Solution: Color of Solution	In Acid Solution: $\lambda_{max.}$	In Acid Solution: a at $\lambda_{max.}$	In Acid Solution: Color of Solution	Method of Preparation
Porfiromycin	572	0.78	Purple	Degrades rapidly to β(A)			
β(B)	575	0.47	Purple	Degrades rapidly to γ(B,A)			Dissolution of Triethylamine salt of β(B)
γ(B,B)	530	0.53	Slight pink	Degrades rapidly to δ(B,B,A)			Alkaline Degradation of β(B), Triethylamine Salt
β(A)	548	3.61	Red-purple	532	3.17	Red-purple	Acid Degradation of Porfiromycin
δ(B,B,A)	556[b]	1.45	Yellow and turbid	430	2.75	Yellow and turbid	Acid Degradation of γ(B,B)
γ(B,A)	556	3.61	Purple	457	1.52	Yellow	Dissolution of Isolated γ(B,A)
γ(A,A)	556	4.03	Purple	457	1.70	Yellow	Acid Degradation of β(A)
δ(A,A,B)	537	0.104	Light pink	475[b]	0.069	Light pink	Alkaline Degradation of γ(A,A)
γ(A,B)	537	0.11	Pale yellow	475[b]	0.092	Pale yellow	Alkaline Degradation of β(A)
δ(B,A,B)	537	0.041	Yellow	475[b]	0.034	Pale yellow	Alkaline Degradation of γ(B,A)

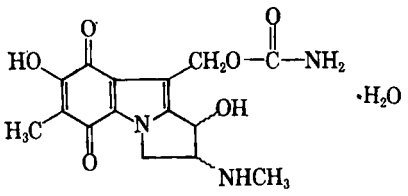
[a] Absorptivity is in Absorbance/Gm./L. calculated on the basis of a concentration of the original undegraded material of 0.5 mg./ml. [b] This was not a distinct maximum.

separated. The aqueous phase was re-extracted with another 50-ml. portion of methylene chloride. The methylene chloride extracts were combined and washed with ice water. After drying over magnesium sulfate for 10 minutes, the methylene chloride solution was concentrated to a purple gum on the Rinco rotating evaporator at 20°. The gum was dissolved in 175 ml. of ether and stirred at room temperature while 2 ml. of triethylamine were added. The crystals which separated were collected and washed with ether. The yield of air dried material was 830 mg. Recrystallization could be effected by adding ether containing some triethylamine to a chloroform solution of the crude product. The salt lost birefringence at 180–185° but did not melt below 320°.

Anal.—Calcd. for $C_{22}H_{24}N_4O_6$: C, 58.7; H, 7.55; N, 12.4; O, 21.3; OCH_3, 6.9. Found: C, 58.48; H, 7.59; N, 12.21; O, 21.12; OCH_3, 6.17.

I.R. spectra: $\lambda_{max.}$ at 3570, 3320, 3270, 3170, 2600, 2450, 1715, 1620, 1580, 1567, 1510 sh, 1500 sh, 1485, 1340, 1327, 1320, 1220, 1102, 1050, 695 cm.$^{-1}$.

Preparation of $\gamma(B,A)$

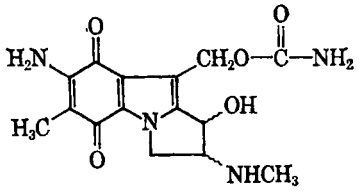


A solution of 1.0 Gm. of porfiromycin in 100 ml. of 0.1 *N* sodium hydroxide was stirred at room temperature for 1 hour. The resulting solution of β(B) was then acidified with 25 ml. of 1 *N* hydrochloric acid and allowed to stand at room temperature overnight. The color of the solution changed from blue through brown to orange. Neutralization of the orange solution with ammonia deposited deep blue crystals of the desired product. The yield was 600 mg. Recrystallization from wet dimethylformamide-methanol afforded the analytical sample. The somewhat hygroscopic material analyzed best for a monohydrate.

Anal.—Calcd. for $C_{15}H_{17}N_3O_6 \cdot H_2O$: C, 51.0; H, 5.4; N, 11.9. Found: C, 51.65; H, 5.78; N, 11.82; OCH_3, none.

I.R. Spectra: $\lambda_{max.}$ at 3600, 3500 sh, 3300, 3160, 2730, 2440, 1717, 1660, 1580, 1535, 1493 sh, 1485, 1335, 1102, 1085, 780, 745 cm.$^{-1}$.

Preparation of $\beta(A)$



Two grams of porfiromycin were dissolved in a small amount of methanol and diluted to a liter volume with 0.01 *N* HCl. The pH of the solution (2.65) was lowered to 2.0 with sufficient 2 *N* HCl and was allowed to stand at room temperature for 20 minutes to allow time for completion of $\alpha_A \rightarrow \beta$(A) reaction. The pH of the solution was adjusted to 10.5 with 1.0 *N* NaOH, and saturated with anhydrous Na_2SO_4. A heavy precipitate appeared and was filtered through a fine glass frit under suction and the residue washed with acetone until most of the β(A) had been extracted. (I.R. analysis of the residue showed over 90% Na_2SO_4.) The acetone solution was taken to dryness by evaporation at room temperature. The filtrate from the original solution was resaturated with Na_2SO_4 and extracted once with acetone in a separator. Most of the color left the water phase after one extraction, so only one was deemed necessary. The acetone extract was combined with the acetone washings of the filter residue and taken to dryness by evaporation at room temperature. The β(A) precipitated out as a hard, glassy plate on the bottom of the evaporating dish and was broken up and bottled for use.

Anal.—Calcd. for $C_{15}H_{18}N_4O_5$: C, 53.9; H, 5.41. Found on correction for ash: C, 53.2, 54.2; H, 5.35, 5.68.

I.R. spectra $\lambda_{max.}$ at 3420, 3320, 3270 OH/NH; 1710, 1665 (weak) C=O; 1600, 1495 C=C/C=N/N—H deformation; and 1120, 1085, 1050 cm.$^{-1}$ C—O/C—N.

Preparation of $\gamma(A,A)$.—Further subjection of the porfiromycin to strong acid resulted in a material which precipitated at pH 5. This material was filtered off, washed, and considered γ(A,A). Its infrared and titration curves were the same as for γ(B,A).

RESULTS AND DISCUSSION

Effect of Buffers on the Degradation of Porfiromycin. $\alpha \rightarrow \beta$**(A).**—The rates of degradation of porfiromycin in acetate buffers were studied by the loss of absorbance at 363 mμ of initially 24–30 mcg./ml. solutions at 30°. The rate constants and conditions were listed as runs 1 through 5 in Table I.

Figure 2 is a plot of the rate constant *vs.* acetate ion concentration or acetic acid concentration for runs 2 through 5 maintained at constant pH, where $[C_2H_3O_2^-] = [HC_2H_3O_2]$, but where ionic strength was not maintained constant. The relationship is given by the equation

$$k = k_{HC_2H_3O_2}[HC_2H_3O_2] + k_0 \quad \text{(Eq. 1}a\text{)}$$

$$= 5.69 \times 10^{-5}[HC_2H_3O_2] + 2.75 \times 10^{-5} \quad \text{(Eq. 1}b\text{)}$$

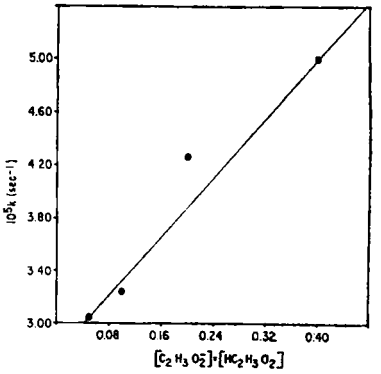


Fig. 2.—Dependence of rate of porfiromycin degradation at 30° and pH 4.54 on acetate ion and/or acetic acid concentration.

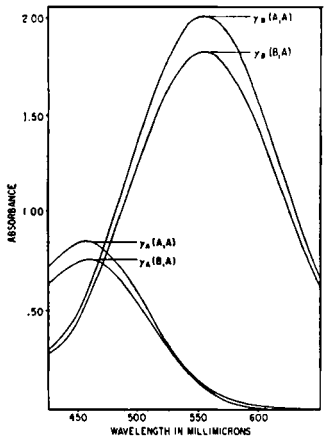


Fig. 3.—Visible spectra of γ(A,A) and γ(B,A) in acid (pH <3), subscript A, and base (pH >9), subscript B. The γ(B,A) was 0.5 mg./ml. The γ(A,A) was derived *in situ* from porfiromycin at 0.5 mg./ml.

at 30° and at pH 4.55, k in sec.$^{-1}$. Of course, [$k_{HC_2H_3O_2}$] is kinetically equivalent to $k_{C_2H_3O_2^-}$[$C_2H_3O_2^-$] so that whether rate acceleration by general base or general acid catalysis is due to acetate ions or undissociated acetic acid molecules, respectively, cannot be concluded on kinetic grounds. However, since the product of solvolysis in these acetate buffers can be spectrophotometrically identified as β(A) (9) which is associated with the opening of the fused aziridine ring of porfiromycin Iα (1, 2, 9) and since the aziridine ring is not attacked by even strong nucleophile hydroxyl ion (9), the most plausible catalytic species is the undissociated acetic acid and general acid catalysis is implicated in the transformation $\alpha \rightarrow \beta$(A).

If in Eq. 1, $k_0 = k_{H^+}$ [H^+] and [H^+] = 10^{-pH} = $10^{-4.55}$ = 2.8 × 10^{-5}, then k_{H^+} at 30° is 1.0 L./mole/sec. This is consistent with k(sec.$^{-1}$) at pH 3.60 at 30° of 2.84 × 10^{-4} (run No. 1, Table I) where [H^+] = 2.51 × 10^{-4}. Thus $k_{H^+} \sim 1$ L./mole/sec. is confirmed. This, of course, is the bimolecular rate constant for the hydrogen ion catalyzed solvolysis of undissociated porfiromycin, α, where $k[\alpha] = k_{H^+}[\alpha][H^+]$.

Run 6, Table I, in phosphate buffers has a $k_{5.9pH}$ = 1.58 × 10^{-6} sec.$^{-1}$ based on the loss of the 363 mμ band at 30°; the assumed route is $\alpha \rightarrow \beta$(A). Thus a calculated value where the rate constant depends solely on hydrogen ion catalyzed solvolysis could be $k = k_{H^+}[10^{-pH}] = k_{H^+}[H^+] = 1 \times 1.4 \times 10^{-6} = 1.4 \times 10^{-6}$ sec.$^{-1}$ which is reasonably consistent with the observed value. Similarly, for run 7, Table I, $k_{8.9pH}$ = 2.16 × 10^{-7} at 30°. The calculated rate constant would be $k = k_{H^+}[10^{-pH}] = 1.4 \times 10^{-7}$.

Although in both of these cases the k(sec.$^{-1}$) values calculated on the basis of hydrogen ion attack on uncharged porfiromycin are of the proper order of magnitude as the observed value, it is indicated that the observed value exceeds the calculated. This could be due to two possible phenomena, the possible catalytic effect of $H_2PO_4^-$ ion and the superimposition of an additional degradation sequence, *i.e.*, $\alpha \rightarrow \beta$(B) $\rightarrow \gamma$(B,B) which is enhanced with the higher pH and which gives an apparent faster change in the spectra used to evaluate rate constants (9).

A plot of log k (k in sec.$^{-1}$) *vs.* pH for studies in phosphate buffers at 60° for runs 8, 9, and 10 (see Table I) is not truly linear with a slope of unity. The slope of unity would be expected if $k = k_{H^+}[H^+]$ and log $k = -pH + \log k_{H^+}$. Neither is the plot of k *vs.* [$H_2PO_4^-$] completely linear. Thus, since porfiromycin has no titratable groups in this region it must be concluded that porfiromycin degradation in the intermediate pH ranges, pH 5.9 to 8, is catalyzed by hydrogen ion [$\alpha \xrightarrow{H^+} \beta$(A)], may be catalyzed by $H_2PO_4^-$ concentration and may be catalyzed by hydroxyl ion [probably $\alpha \xrightarrow{OH^-} \beta$(B) $\xrightarrow{OH^-} \gamma$(B,B)] (9).

The bimolecular rate constant for the hydroxyl ion catalyzed solvolysis of porfiromycin to β(B) can be estimated from the rate of loss of biological assay (run 20, Table I) as k_{OH^-} = 8.2 × 10^{-3} L./mole/sec. at 30°.

Prediction of Stability.—Estimates of the stability of porfiromycin, α, in unbuffered aqueous solution at 30° may be based on the expression.

$$k(\text{sec.}^{-1}) = 1.0\,[H^+] + 8.2 \times 10^{-3}[OH^-] \quad \text{(Eq. 2}a\text{)}$$

$$= 1.0 \times 10^{-pH} + 8.2 \times 10^{-3}\,10^{-(pK_w - pH)} \quad \text{(Eq. 2}b\text{)}$$

where the first quantity on the right in Eq. 2 estimates the apparent first-order transformation, $\alpha \rightarrow \beta$(A), and the second quantity on the right estimates the apparent first-order transformation, $\alpha \rightarrow \beta$(B). Approximate values for the half-life of porfiromycin are: 0.2 hours at pH 3, 2 hours at pH 4, 20 hours at pH 5, 200 hours at pH 7, 11,000 hours at pH 8, 2300 hours at pH 9, 250 hours at pH 10, 25 hours at pH 11, 2.5 hours at pH 12, and 0.25 hours in 0.1 *M* NaOH. The maximum stability of porfiromycin at 30° is *ca.* pH 8 with an estimated half-life of 1.3 years.

The estimated half-life at pH 3 of 0.21 hours indicates that porfiromycin should be significantly degraded in the stomach on oral ingestion.

Visible Spectra of Porfiromycin and Its Acid and Alkaline Degradation Products.—The similarities of (B,A) and (A,A) have already been pointed out as based on ultraviolet spectroscopy and spectrophotometric and potentiometric titrations (9). The coincidence of these two compounds is very clearly demonstrated by the visible spectra in Fig. 3.

The similarities of γ(A,B), δ(B,A,B), and δ(A,A,B) have also been pointed out as based on ultraviolet spectroscopy (9). The similar shift in visible spectra from acid to basic conditions (see Table II)

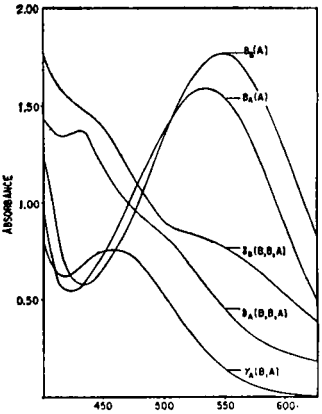


Fig. 4.—Visible spectra of β(A), δ(B,B,A), and γ(B,A) in acid (pH <3), subscript A, and base (pH >8), subscript B. The γ(B,A) was 0.5 mg./ml. The β(A) was derived *in situ* from porfiromycin at 0.5 mg./ml. The δ(B,B,A) was derived *in situ* from β(B), triethylamine salt at 0.5 mg./ml.

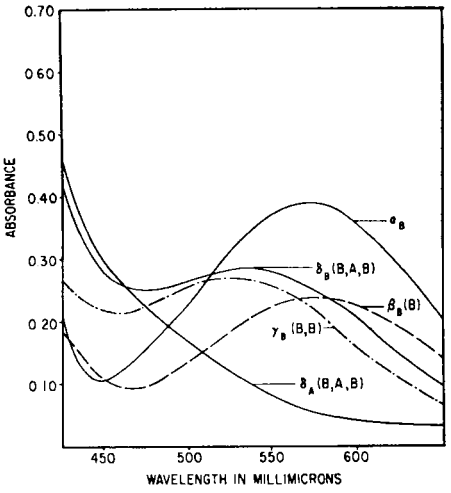


Fig. 5.—Visible spectra of porfiromycin, α, β(B), γ(B,B), and δ(B,A,B) in acid, subscript A, and basic solutions, subscript B. The δ(B,A,B) was derived *in situ* from γ(B,A) at 6.7 mg./ml. The porfiromycin and β(B) triethylamine salt were at 0.5 mg./ml. and the γ(B,B) was derived *in situ* from β(B) triethylamine salt.

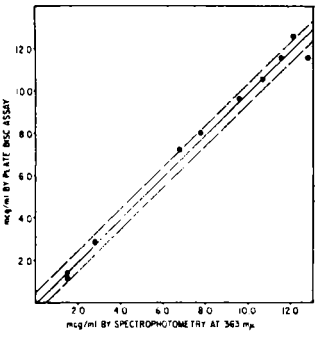


Fig. 6.—Correlation of bioassay, plate disk *vs. S. aureus*, and spectrophotometric assay at 363 mμ for porfiromycin degrading at 30° in 0.05 *M* acetic acid–0.05 *M* sodium acetate. Key: — — —, standard deviation in bioassay; ———, regression fitted by least squares.

tends to confirm these similarities. Several anomalies do exist although some explanation may be made on the fact that the apparent absorptivities are calculated on the basis of the weight of the starting materials, which materials as stated above, differed in the several cases. The δ(B,A,B) has 0.37 the absorbance at the maxima of γ(A,B) in the acid and alkaline regions. However, the ratios are maintained under both conditions as are the qualitative spectral shifts. Typical examples of the visible spectra of these products of porfiromycin solvolysis are given in Figs. 4 and 5.

Correlation of Spectral and Biological Assays in Acetate Buffers.—The undegraded porfiromycin, α (runs 2–5, Table I), after adjustment to pH 7 was calculated on the basis of the following equations:

$$A_t = a_\alpha[\alpha] + a_\beta[\beta] \qquad \text{(Eq. 3)}$$

where A_t is the absorbance at any time and a_α and $a\beta$ are the absorptivity of porfiromycin, α, and of β(A), respectively, at 363 mμ. The initial absorbance at $t = 0$, is $A_0 = a_\alpha[\alpha]_0$; the final absorbance at $t = \infty$ is $A_\infty = a_\beta[\beta]$.

Thus, the concentration of α, *i.e.*, $[\alpha]$, can be calculated from

$$[\alpha] = \frac{A_t - A_\infty}{A_0 - A_\infty}[\alpha]_0 \qquad \text{(Eq. 4)}$$

where

$$[\alpha]_0 = [\beta]_\infty = [\alpha] + [\beta] \qquad \text{(Eq. 5)}$$

Aliquots of the same acetic acid-acetate buffered solution which were taken at the same intervals and neutralized 1:1 with 0.1 *M* phosphate buffer, pH 7.8, were assayed by the plate-disk method against *S. aureus* (7).

A typical example of the regression of the microbiological plate-disk assay against the estimates obtained by spectrophotometry is given in Fig. 6. The line of best fit by least squares is the solid line whereas the dashed lines represent the plus or minus standard deviations and are estimates of the error in the biological assay.

The statistics of the regression are given in Table III. The average standard deviation of the assay is 10%. Although two out of the four runs are consistent with a 1:1 relation of the assays, *i.e.*, the regression slope is approximately unity and the intercept of regression approximately zero, two show differences from these values. Overall, however, it can be concluded that the spectrophotometric assay is a valid measure of porfiromycin's biological activity in its transformation to β(A) in acetate buffers at pH 4.5.

Correlation of Spectral and Biological Assays in Phosphate Buffers.—The spectrophotometric assay was performed on porfiromycin degrading in phosphate buffered solutions at 70°. These were the

TABLE III.—STATISTICS OF REGRESSION ($y = mx + b$) OF BIOASSAYS[a] (y) ON SPECTROPHOTOMETRIC ASSAYS[b] (x) IN MCG./ML. FOR PORFIROMYCIN IN VARYING ACETATE CONCENTRATION BUFFERS (pH 4.5) AT 30° C. (RUNS 2–5, TABLE I)

Buffer Composition [CH_3COOH]	Buffer Composition [CH_3COO^-]	pH	Run[c]	n[d]	Least Squares Fit	σ_b[e]	σ_m[f]	σ_y[g]	Remarks
0.05	0.05	4.53	2	11	$y = 0.979X + 0.062$	0.262	0.031	0.471	The slope, m, is not significantly different from 1.0; intercept, b, not significantly different than 0.
0.10	0.10	4.54	3	8	$y = 1.332X - 1.372$	0.658	0.076	0.797	Both slope and intercept are significantly different from theoretical values 1.0 and 0.0.
0.20	0.20	4.51	4	7	$y = 1.015X + 0.762$	0.640	0.068	0.503	Slope and intercept not significantly different from theoretical.
0.40	0.40	4.55	5	8	$y = 1.234X - 1.222$	0.496	0.059	0.714	Slope and intercept are significantly different from theoretical.

[a] Plate disk against *S. aureus*. [b] As estimated from the 353 mμ absorbance. [c] From Table I. [d] Number of assays. [e] Standard deviation of intercept, b. [f] Standard deviation of slope, m. [g] Standard deviation about regression, presumably of bioassay, y.

TABLE IV.—STATISTICS OF REGRESSION ($y = mx + b$) OF BIOASSAYS[a] (y) ON SPECTROPHOTOMETRIC ASSAYS[b] (x) IN MCG./ML. FOR PORFIROMYCIN IN VARYING PHOSPHATE CONCENTRATION BUFFERS AT 70° (RUNS 11 AND 12, TABLE I)

Buffer Composition of Solution in 70° Bath		Buffer Composition of Aliquot Submitted for Bioassay							
(KH_2PO_4)	(NaOH)	(KH_2PO_4)	(NaOH)	pH	n[c]	Least Squares Fit	σ_b[d]	σ_m[e]	σ_y[f]
0.050	0.0296	0.0500	0.0296	6.93	12	$y = 0.741X + 0.632$	1.11	0.119	1.55
0.050	0.0296	0.0375	0.0222	6.93	12	$y = 0.819X + 0.151$	1.19	0.127	1.66
0.025	0.0148	0.0250	0.0148	6.97	12	$y = 0.660X + 1.658$	0.997	0.105	1.25
0.025	0.0148	0.0125	0.0074	6.97	12	$y = 0.598X + 2.101$	1.43	0.151	1.80

[a] Plate disk against *S. aureus*. [b] As estimated from the 353 mμ absorbance. [c] Number of assays. [d] Standard deviation of intercept, b. [e] Standard deviation of slope, m. [f] Standard deviation about regression, presumably of bioassay, y.

TABLE V.—STATISTICS OF REGRESSION ($y = mx + b$) OF PAPERGRAMS (y) ON PLATE-DISK ASSAYS[a] (x) IN MCG./ML. FOR PORFIROMYCIN IN pH 9 AND pH 10 BORATE BUFFERS AT 60° (RUNS 14 AND 17, TABLE I)

Buffer Composition		Type of Papergram Analysis	pH of Aliquots Submitted for Papergrams	n[b]	Least Squares Fit	σ_b[c]	σ_m[d]	σ_y[e]	Run[b,f]
[H_3BO_3]	[NaOH]								
0.05	0.0439	U.V. paper strip	*ca.* 7	8	$y = 2.499X - 182.3$	37.2	0.106	44.5	17, 18, 19
0.05	0.0439	*S. lutea*	*ca.* 7	8	$y = 1.326X + 40.08$	29.1	0.083	34.8	17, 18, 19
0.05	0.0213	*S. lutea*	7.85	12	$y = 1.572X - 169.3$	75.4	0.190	88.2	14, 15
0.05	0.0213	U.V. paper strip	7.85	13	$y = 1.335X - 67.7$	36.6	0.0960	51.6	14, 15
0.05	0.0213	*S. lutea*	8.95	4	$y = 2.192X - 328.1$	167.0	0.386	84.0	14, 15
0.05	0.0213	U.V. paper strip	8.95	5	$y = 1.640X - 91.5$	93.7	0.241	88.1	14, 15

[a] Against *S. aureus*. [b] Number of assays. [c] Standard deviation of intercept, b. [d] Standard deviation of slope, m. [e] Standard deviation about regression. [f] See Table I.

same runs as 11 and 12 of Table I. Simultaneously with the removal and treatment of aliquots for spectrophotometric assay, two aliquots were removed for biological assay under two different phosphate buffer concentrations. Variation of phosphate buffer from 0.0125 to 0.05 *M* had no apparent effect on the plate-disk assay against *S. aureus*. The statistics of the regressions of bioassay *vs.* spectrophotometric assay are given in Table IV. Although the intercepts of all four regressions are not significantly different from zero, the slopes are significantly different from and are less than unity. This implies that destruction of spectrophotometric absorbance is correlated with destruction of activity but that absorbance may register a higher value than activity. This is readily explainable if it is assumed that at pH 7 two alternate degradation pathways exist:

$$\beta(B) \leftarrow \alpha \rightarrow \beta(A) \qquad \text{(Eq. 6)}$$

However, $\beta(B)$ has a very similar spectrum to α and thus destruction of the measured biological activity may proceed slightly faster than the diminution of the absorbance.

Correlation of Spectral and Biological Assays by Paper Chromatogram of Porfiromycin in Borate Buffers.—Studies were conducted in borate buffers at 60° and at pH values 9 and 10. The aliquots of the pH 10 study were adjusted to pH 7 and submitted for papergram assay by both ultraviolet scanning and against *S. lutea* (4). In both cases, the correlation of papergram assay with plate-disk (see Table V) was good.

The aliquots of the pH 9 study were adjusted to pH 8 and 9 and both adjusted samples were submitted for papergram assay by ultraviolet and against *S. lutea* (4). Again the correlations of papergram assay with plate-disk assay (7) (see Table V) were good.

However, in almost all cases the papergram assay decreased at a greater rate with time than did the paper-disk assay until at low assay values the former demonstrated no porfiromycin whereas the latter did. This can be explained (*a*) by a negative bias in paper chromatography assay at low concentrations or (*b*) by the fact that the papergram is more specific than the plate-disk assay, that porfiromycin degrades to intermediates which still exhibit plate-disk potency but not papergram activity or ultraviolet absorbance at the spot assigned to porfiromycin.

Both $\beta(B)$ and $\gamma(B,B)$ prepared *in situ* were submitted for bioactivity on papergrams and were shown to have biological activity.

Consistency of Isolated Intermediates with Proposed Scheme for the Solvolytic Transformations of Porfiromycin.—The isolations of $\beta(B)$, $\gamma(B,A)$, $\beta(A)$, and $\gamma(A,A)$ were based on the observed kinetics reported previously (9) and in this paper. The elemental analysis and physicochemical characterizations of these isolates are consistent with the reported structural assignments given in the introduction.

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Schardinger Dextrin Interaction IV

Inhibition of Hydrolysis by Means of Molecular Complex Formation

By JOHN L. LACH and TING-FONG CHIN

Kinetic data presented substantiates the postulated 1:1 stoichiometric ratio for the benzocaine-cyclodextrin interaction. Data presented also show that the degree of retardation of benzocaine hydrolysis is considerably greater than that observed for the caffeine-type complexes indicating an interaction involving inclusion formation and other attractive forces.

THE APPLICATION of molecular complex formation as a means of drug solubilization and stabilization has received considerable attention in recent times. The complexing agents used in these studies include a variety of various types of molecules. Several reports have appeared in the literature concerning the application of such interaction in the stabilization of medicinal agents.

Recent studies (1–3) in these laboratories have indicated that the cyclodextrins[1] (homogeneous cyclic molecules containing 6, 7, or 8 D-glucopyranose units linked in a 1,4 position) also exhibit a marked tendency to solubilize various compounds. It was noted that the formation constants of these interactions were considerably greater than those reported for other interactions involving complex formation. The high values obtained for these formation constants indicate a higher degree of stability presumably due to an interaction involving inclusion formation and other attractive forces. It was of interest to study this interaction with respect to its ability to retard hydrolysis and to compare it to previously reported systems. The present study deals with the influence of beta-cyclodextrin on the base catalyzed degradation of benzocaine. Data from this kinetic study have been employed to verify the stoichiometric relationship of the cyclodextrin-benzocaine interaction and to compare the hydrolytic inhibition with other previously studied complexing agents.

EXPERIMENTAL

Reagents

Beta-cyclodextrin was prepared and purified by the modified method of French (4). $[\alpha]_D^{25}$ in water = +162.5 ± 0.5. Benzocaine N.F. recrystallized; m.p. 87–88°.

Apparatus

A constant temperature water bath set at 30 ± 0.5° with rotating spindle; a "Tacan" thermostat water bath set at 30 ± 0.1, 35 ± 0.1, 40 ± 0.1, and 45 ± 0.1°; 20-ml. capacity vials with gum-rubber stoppers and aluminum caps; and a Beckman DU spectrophotometer, with 1-cm. silica cells were used.

Procedures

Benzocaine Interaction.—The solubility method of Higuchi and Lach (5) was used to study the interaction of benzocaine with cyclodextrin.

Benzocaine Hydrolysis.—An accurate amount of benzocaine was dissolved in hot distilled water. It was then allowed to cool down to a definite temperature in a constant temperature water bath. To this solution, a calculated amount of standard barium hydroxide solution was added and immediately brought up to a definite volume and separated into 20-ml. capacity vials (approximately 8 ml. was used for each vial). All of these procedures were conducted under nitrogen. Another series of samples was made under the same procedures and same conditions, containing various concentration of beta-cyclodextrin.

A 5-ml. sample solution was withdrawn from a vial at a designated time interval and immediately extracted with 3 × 10 ml. of chloroform. The chloroform extracts were combined and made up to 50 ml. with chloroform. The unhydrolyzed benzocaine was extracted by the chloroform and the hydrolyzed product, *para*-aminobenzoic acid, remained in the alkaline solution. The concentration of unhydrolyzed benzocaine was determinated spectrophotometrically at 285 mμ.

RESULTS AND DISCUSSION

The interaction of benzocaine with beta-cyclodextrin in aqueous solution is shown in Fig. 1. The straight-line relationship obtained indicates a first-order dependancy with respect to benzocaine concentration in the system. Since the concentration of benzocaine in this system studied is an invariant, a 1:1 stoichiometric relationship has been assigned to this interaction based on the slope obtained in Fig. 1.

The kinetic phase of this investigation was carried out at constant pH [0.04*N* $Ba(OH)_2$ solution] and at various temperatures. As has been reported previously benzocaine hydrolysis in aqueous solution follows a pseudo first-order reaction (6). The results of benzocaine hydrolysis in aqueous solution

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[1] The toxicity of the cyclodextrins has not been established.

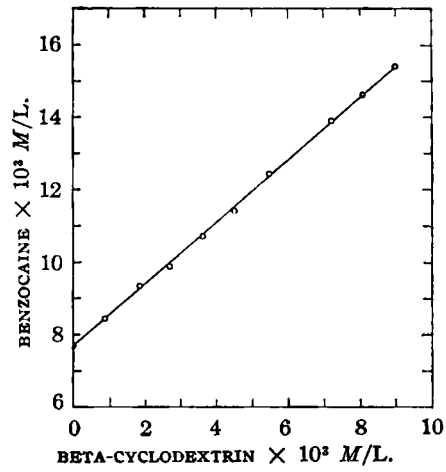


Fig. 1.—Interaction of benzocaine with beta-cyclodextrin at 30°.

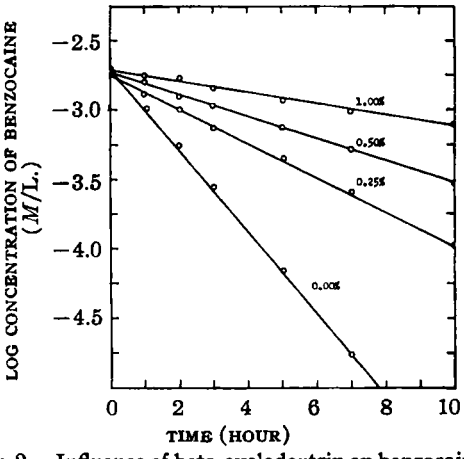


Fig. 2.—Influence of beta-cyclodextrin on benzocaine hydrolysis in 0.04 N $Ba(OH)_2$ at 30°.

with and without beta-cyclodextrin, shown in Fig. 2, demonstrate the marked ability of the cyclodextrin in inhibiting this hydrolytic reaction. It is interesting to point out here that this decrease in rate is proportional to the cyclodextrin concentration in the system. This is illustrated in Fig. 3 which shows the increase in half-life as a function of cyclodextrin in solution. The effect of beta-cyclodextrin on half-life and rate constant of benzocaine hydrolysis in aqueous solution is summarized in Table I.

From Table I it is clearly evident that addition of beta-cyclodextrin to this system produces a significant decrease in the hydrolytic rate of benzocaine. In a system containing 1% cyclodextrin a fivefold increase in the half-life of this reaction is achieved. This can be compared to the earlier work of Higuchi (6) in which he showed a 2.5-fold increase in the half-life with the addition of 1% caffeine. Pauli and Lach (7) reported smaller increases in the half-life of benzocaine with various complexing agents used in their study. This greater half-life observed in this cyclodextrin-benzocaine interaction resulting from inclusion formation is considerably stronger than those observed for the caffeine-type complex. It is reasonable to expect that inclusion formation would provide a better shield for the benzocaine ester linkage from hydroxyl ion attack. Rate constants and half-life of the hydrolysis of benzocaine with and without the cyclodextrin as a function of temperature are listed in Table II and Fig. 4.

This temperature dependancy for benzocaine degradation can also be illustrated by an Arrhenius plot as shown in Fig. 5. Plotted here are rate constants obtained at different temperatures for systems free of the cyclodextrin and those obtained at corresponding temperatures containing 1% beta-cyclodextrin. Interestingly the same slope is obtained in both cases, indicating that the degradation mechanism in systems containing the cyclodextrin is the same as that in systems without complexing agent, although the rate constants are different. The amount of free benzocaine existing in the two systems—the stoichiometric concentration or total benzocaine in each system being the same—causes the difference. In a benzocaine solution containing the cyclodextrin, the amount of free benzocaine is small and is governed by the dissociation constant of the complex. Since only one mechanism of degradation in the two systems studied

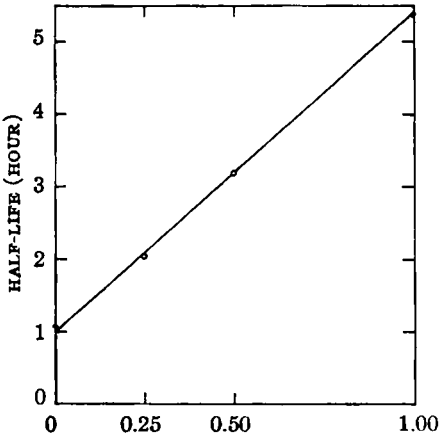


Fig. 3.—Beta-cyclodextrin influence on the half-life of benzocaine hydrolysis of 0.04 N $Ba(OH)_2$ at 30°.

TABLE I.—INFLUENCE OF BETA-CYCLODEXTRIN (β-CD) ON RATE CONSTANT AND HALF-LIFE OF BENZOCAINE HYDROLYSIS IN 0.04 N $Ba(OH)_2$ SOLUTION AT 30°

Per Cent of β-CD	Rate Constant (hr.$^{-1}$/M);	Half-life (hr.)
0.00	.666	1.04
0.25	.358	2.03
0.50	.229	3.19
1.00	.129	5.39

TABLE II.—INFLUENCE OF TEMPERATURE ON RATE CONSTANT AND HALF-LIFE OF BENZOCAINE HYDROLYSIS IN 0.04N $Ba(OH)_2$ IN A SYSTEM WITH AND WITHOUT BETA-CYCLODEXTRIN (β-CD)

	Rate Constant (hr.$^{-1}$/M)		Half-life (hr.)	
Temp.	No β-CD	1% β-CD	No β-CD	1% β-CD
30°	0.666	0.129	1.040	5.37
35°	0.849	0.170	0.828	4.14
40°	1.190	0.228	0.579	3.04
45°	1.576	0.339	0.439	2.05

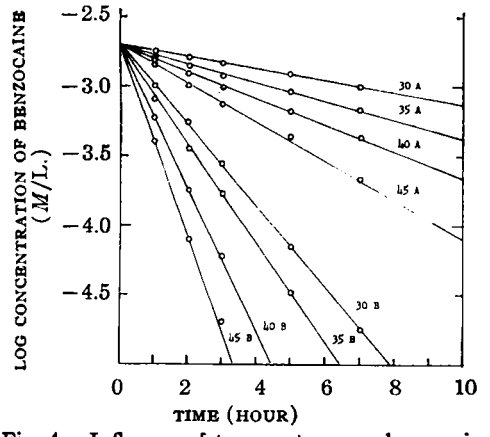


Fig. 4.—Influence of temperatures on benzocaine hydrolysis in 0.04 *N* $Ba(OH)_2$. Key: A, with beta-cyclodextrin; B, without beta-cyclodextrin.

is operative, one can conclude that this rate is dependent on the amount of free benzocaine in solution and not the total concentration present. The degradation rate of the complexed benzocaine species is negligible.

Based on the data obtained in this study and on the work of Guttman (8), the following degradation mechanism is proposed:

$$(\text{BZA}:\beta\text{-CD}) \underset{K_2}{\overset{K_1}{\rightleftharpoons}} (\text{BZA})f + (\beta\text{-CD})f$$

$$K_3 \downarrow \text{HOH}$$

$$p\text{-Aminobenzoic acid} + \text{Ethanol}$$

where K_1 is the dissociation constant, K_2 is the association constant, K_3 is the hydrolysis constant, $(\text{BZA})f$ is free *benzocaine*, $(\beta\text{-CD})f$ is free beta-cyclodextrin, $(\text{BZA})t$ is total benzocaine, and $(\text{BZA}:\beta\text{-CD})$ is the complexing compound.

Dissociation constant K_1 can be calculated from Eq. 1, if a 1:1 stoichiometric ratio is assumed for

$$K_1 = \frac{(\text{BZA})f \cdot (\beta\text{-CD})f}{(\text{BZA}:\beta\text{-CD})} \qquad \text{(Eq. 1)}$$

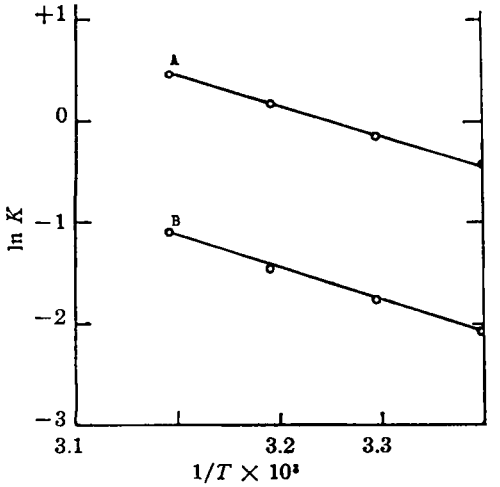


Fig. 5.—Arrhenius plot of hydrolysis of benzocaine in 0.04 *N* $Ba(OH)_2$ at different temperatures. Key: A, without beta-cyclodextrin; B, with beta-cyclodextrin.

this interaction. From experimental data, K_1 has been calculated to be 2.34×10^{-3}. The free benzocaine in the system containing beta-cyclodextrin can be calculated from Eq. 2. The molar

$$(\text{BZA})f = \frac{k_1 \cdot (\text{BZA}:\beta\text{-CD})}{(\beta\text{-CD})f} \qquad \text{(Eq. 2)}$$

concentration of the (benzocaine:beta-cyclodextrin) complex is equal to the molar concentration of benzocaine present in the complexed form which in turn is equal to the difference of total benzocaine added to the system minus free benzocaine in solution. Since $(\text{BZA}:\beta\text{-CD}) = (\text{BZA}) = (\text{BZA})t - (\text{BZA})f$, therefore

$$(\text{BZA})f = \frac{K_1[(\text{BZA})t - (\text{BZA})f]}{(\beta\text{-CD})f}$$

$$(\text{BZA})f \cdot (\beta\text{-CD})f = K_1 \cdot [(\text{BZA})t - (\text{BZA})f]$$

$$= K_1(\text{BZA})t - K_1(\text{BZA})f$$

$$(\text{BZA})f \cdot (\beta\text{-CD})f + K_1 \cdot (\text{BZA})f = K_1 \cdot (\text{BZA})t$$

$$(\text{BZA})f[K_1 + (\beta\text{-CD})f] = K_1(\text{BZA})t$$

$$(\text{BZA})f = \frac{K_1}{K_1 + (\beta\text{-CD})f}(\text{BZA})t \qquad \text{(Eq. 3)}$$

Table III.—Comparison of Calculated and Experimental Apparent Rate Constants of Benzocaine Hydrolysis in a System Containing Beta-cyclodextrin (β-CD) in 0.04 *N* $Ba(OH)_2$ Solution at 30°

Per cent of β-CD	Experimental (hr.$^{-1}$/*M*)	Calcd. (hr.$^{-1}$/*M*)
0.00	0.666	
0.25	0.358	0.342
0.50	0.229	0.228
1.00	0.129	0.139

Equation 4 is rate equation for benzocaine hydrolysis in basic solution. Equation 5 is rate equation for

$$-d(\text{BZA})f/dt = K_3(\text{BZA})f \qquad \text{(Eq. 4)}$$

benzocaine hydrolysis in basic solution containing beta-cyclodextrin. The apparent rate constants,

$$-d(\text{BZA})t/dt = K_3(\text{BZA})f = K_3 \frac{K_1}{K_1 + (\beta\text{-CD})f}(\text{BZA})t \qquad \text{(Eq. 5)}$$

evaluated for the reaction in a system containing beta-cyclodextrin are thus related to the true rate constant by Eqs. 6 and 7. From Eq. 6, the apparent

$$K_{app.} = K_3 \frac{K_1}{K_1 + (\beta\text{-CD})f} \qquad \text{(Eq. 6)}$$

$$K_3/K_{app.} = 1 + \frac{(\beta\text{-CD})f}{K_1} \qquad \text{(Eq. 7)}$$

rate constants can be calculated and can be compared with the experimental rate constant data obtained in a system containing beta-cyclodextrin. These comparison data are given in Table III.

The close agreement of the calculated and experimental apparent rate constant gives additional support to the assumption made of the 1:1 type of interaction between benzocaine and beta-cyclodextrin. If the interaction was not of this type, Eq. 1 would not be valid. Equation 7 shows a linear

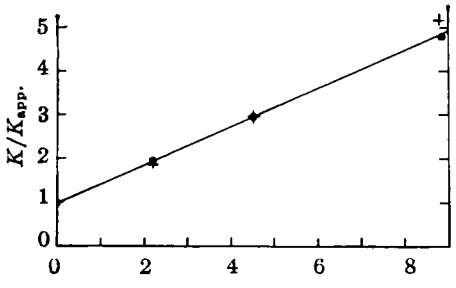


Fig. 6.—Effect of beta-cyclodextrin on the rate of benzocaine hydrolysis in 0.04 N $Ba(OH)_2$ at 30°. Key: +, experimental; ●, calculated.

relationship between the ratio of $K_s/K_{app.}$ and the concentration of free beta-cyclodextrin. This linear relationship is shown in Fig. 6, and further substantiates this 1:1 stoichiometry and the benzocaine hydrolysis in system containing the beta-cyclodextrin is dependant on the uncomplexed benzocaine in solution.

Studies involving inclusion formation and molecular complexation are of importance in the area of drug stabilization and solubilization. Since inclusion formation plays a role in the chemistry of living cells, studies of this type are also important in that data obtained may provide information with respect to the mechanism of drug absorption, transport, and metabolism in biological systems.

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Behavior of Erythrocytes in Various Solvent Systems II

Effect of Temperature and Various Substances on Water–Glycerin and Water–Propylene Glycol Solutions

By D. E. CADWALLADER, B. W. WICKLIFFE, and B. L. SMITH

Hemolytic behavior of rabbit and human erythrocytes in water–glycerin and water–propylene glycol solutions was studied at 25 and 37°, and the effect of various added substances to these systems was investigated. Human *i* values obtained for sodium chloride in aqueous glycerin or propylene glycol solutions at 37° were slightly greater than the corresponding *i* values at 25°. Increase in temperature from 25 to 37° decreased the concentrations of propylene glycol in 0.9 per cent saline solution needed to cause hemolysis of erythrocytes. Mono-monovalent salts, sugars and sugar alcohols, magnesium chloride, and sulfate and sodium succinate afforded essentially the same degree of protection as sodium chloride against hemolysis by propylene glycol. Isotonic concentrations of sodium or potassium sulfate, potassium sodium or disodium tartrate, or trisodium citrate afforded greater protection to erythrocytes than 0.9 per cent sodium chloride. The order in which the anions of the above salts appeared to protect human erythrocytes against propylene glycol hemolysis is citrate > tartrate > gluconate > sulfate; for rabbit erythrocytes the order is sulfate, tartrate > citrate. Much lower concentrations of propylene glycol were required to hemolyze erythrocytes in solutions containing isotonic concentrations of calcium chloride.

THE PREVIOUS PAPER in this series (1) discussed the behavior of erythrocytes in water–glycerin and water–propylene glycol systems in experiments carried out at 25°. Hemolytic *i* values were obtained for sodium chloride in the presence of various concentrations of glycerin and propylene glycol. It was observed that complete hemolysis took place in most glycerin solutions, but the addition of suitable amounts of sodium chloride prevented hemolysis of rabbit and human erythrocytes. Complete hemolysis occurred in all propylene glycol solutions, and in solutions containing 45–50% or more of propylene glycol the addition of iso- and hypertonic quantities of sodium chloride failed to prevent complete hemolysis of rabbit and human red blood cells.

The presently reported experiments are in three areas. (*a*) The behavior of rabbit and human red blood cells was compared at 37 and 25° in aqueous polyhydric alcohol systems,

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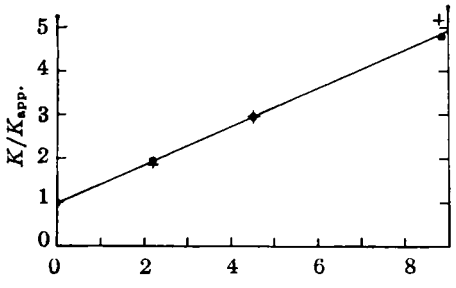


Fig. 6.—Effect of beta-cyclodextrin on the rate of benzocaine hydrolysis in 0.04 N $Ba(OH)_2$ at 30°. Key: +, experimental; ●, calculated.

relationship between the ratio of $K_s/K_{app.}$ and the concentration of free beta-cyclodextrin. This linear relationship is shown in Fig. 6, and further substantiates this 1:1 stoichiometry and the benzocaine hydrolysis in system containing the beta-cyclodextrin is dependant on the uncomplexed benzocaine in solution.

Studies involving inclusion formation and molecular complexation are of importance in the area of drug stabilization and solubilization. Since inclusion formation plays a role in the chemistry of living cells, studies of this type are also important in that data obtained may provide information with respect to the mechanism of drug absorption, transport, and metabolism in biological systems.

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Behavior of Erythrocytes in Various Solvent Systems II

Effect of Temperature and Various Substances on Water–Glycerin and Water–Propylene Glycol Solutions

By D. E. CADWALLADER, B. W. WICKLIFFE, and B. L. SMITH

Hemolytic behavior of rabbit and human erythrocytes in water–glycerin and water–propylene glycol solutions was studied at 25 and 37°, and the effect of various added substances to these systems was investigated. Human *i* values obtained for sodium chloride in aqueous glycerin or propylene glycol solutions at 37° were slightly greater than the corresponding *i* values at 25°. Increase in temperature from 25 to 37° decreased the concentrations of propylene glycol in 0.9 per cent saline solution needed to cause hemolysis of erythrocytes. Mono-monovalent salts, sugars and sugar alcohols, magnesium chloride, and sulfate and sodium succinate afforded essentially the same degree of protection as sodium chloride against hemolysis by propylene glycol. Isotonic concentrations of sodium or potassium sulfate, potassium sodium or disodium tartrate, or trisodium citrate afforded greater protection to erythrocytes than 0.9 per cent sodium chloride. The order in which the anions of the above salts appeared to protect human erythrocytes against propylene glycol hemolysis is citrate > tartrate > gluconate > sulfate; for rabbit erythrocytes the order is sulfate, tartrate > citrate. Much lower concentrations of propylene glycol were required to hemolyze erythrocytes in solutions containing isotonic concentrations of calcium chloride.

THE PREVIOUS PAPER in this series (1) discussed the behavior of erythrocytes in water–glycerin and water–propylene glycol systems in experiments carried out at 25°. Hemolytic *i* values were obtained for sodium chloride in the presence of various concentrations of glycerin and propylene glycol. It was observed that complete hemolysis took place in most glycerin solutions, but the addition of suitable amounts of sodium chloride prevented hemolysis of rabbit and human erythrocytes. Complete hemolysis occurred in all propylene glycol solutions, and in solutions containing 45–50% or more of propylene glycol the addition of iso- and hypertonic quantities of sodium chloride failed to prevent complete hemolysis of rabbit and human red blood cells.

The presently reported experiments are in three areas. (*a*) The behavior of rabbit and human red blood cells was compared at 37 and 25° in aqueous polyhydric alcohol systems,

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TABLE I.—ISOTONIC VALUES OF VARIOUS SUBSTANCES

Compound	Isotonic Values — Rabbit Blood: *i* Value[a]	Rabbit Blood: % Compn. (anhydr.) equiv. to 0.9% NaCl	Human Blood: *i* Value[a]	Human Blood: % Compn. (anhydr.) equiv. to 0.9% NaCl
Calcium chloride	2.85	1.11	2.76	1.15
Dextrose	...	...	0.55[b]	9.39
Glycine	...	...	1.21	1.78
Lactose	1.24	7.91	1.20	8.16
Magnesium chloride	2.97	0.92	2.90	0.94
Magnesium sulfate	...	...	1.99	1.73
Mannitol	...	...	1.37(3)	3.83
Potassium bromide	1.81	1.88	1.79	1.91
Potassium gluconate	...	...	2.28(4)	2.94
Potassium sodium tartrate	...	...	3.30	1.82
Potassium sulfate	...	...	3.06	1.63
Sodium benzoate	...	...	1.85	2.24
Sodium chloride	1.86	0.90	1.86	0.90
Sodium citrate	4.18	1.77	4.02	1.84
Sodium iodide	1.92	2.21	1.93	2.20
Sodium nitrate	1.83	1.33	1.86	1.30
Sodium tartrate	3.36	1.61	3.37	1.60
Sodium succinate	...	...	3.27	1.35
Sodium sulfate	3.20	1.26	3.19	1.27
Sorbitol	1.28(3)	4.07	1.36(3)	3.83
Sucrose	...	...	1.37	7.16

[a] All *i* values were calculated from hemolysis data obtained at 37° and represent an average of at least two blood samples. Except for dextrose, these *i* values proved to be the same as those previously reported at 25°. [b] Hemolytic *i* value at 25°, 1.17 (2).

especially water–propylene glycol. (*b*) Because of the inability of 0.9% sodium chloride to prevent complete hemolysis in 45–50% propylene glycol solutions, experiments were conducted to determine the critical hemolytic concentrations of propylene glycol in 0.9% saline for rabbit and human erythrocytes. (*c*) Experiments were also carried out to observe what effect the addition of isotonic quantities of various compounds had in preventing hemolysis of erythrocytes in glycerin and propylene glycol systems.

EXPERIMENTAL

Materials.—All chemicals were U.S.P., N.F., or reagent grade.

Collection of Blood.—Rabbit and human blood samples were collected and defibrinated in the manner described by Husa and co-workers (2–4). Human blood was obtained from several female and male donors.

Solutions.—All solutions were weight-in-volume preparations. Isotonic concentrations of the various compounds used in this study are shown in Table I. The hemolytic *i* value of each compound was used to calculate the concentration in water of anhydrous compound equivalent to 0.9% sodium chloride. Previously reported *i* values of most compounds in Table I had been calculated from data obtained at 25°. Since hemolysis experiments at 37° were planned, *i* values were determined for this temperature. It was found that *i* values were the same at 37 and 25° for all compounds in Table I except dextrose. Human *i* values are reported for the first time for lactose and sucrose.

Quantitative Determination of Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in various solutions was described in the first paper of this series (1).

Hemolysis experiments were carried out at 25 ± 1° and 37 ± 1°. In obtaining data for the calculation of *i* values at 37°, the procedure was essentially the same as that referred to except that four tubes of each concentration were used: two each were kept at 37° for 45 minutes, while two were maintained at 25° for the same period of time.

In determining the critical concentrations of propylene glycol in the presence of 0.9% sodium chloride that caused hemolysis of rabbit and human erythrocytes, blood was added to aqueous solutions of 0.9% sodium chloride containing various amounts of propylene glycol. The blood mixtures were allowed to stand 45 minutes—two tubes at 37° and two tubes at 25°. The results are shown in Fig. 1. Similar experiments in which isotonic equivalents of various substances were added to propylene glycol solutions were carried out at 37°. The results are depicted in Figs. 2 and 3.

RESULTS

Water–Glycerin Solution.—Complete hemolysis of rabbit and human erythrocytes occurred in 0.0

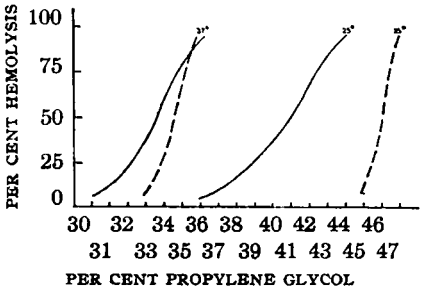


Fig. 1.—Hemolysis of erythrocytes after 45 minutes in propylene glycol solutions containing 0.9% sodium chloride. Key: – – – –, human blood; ———, rabbit blood.

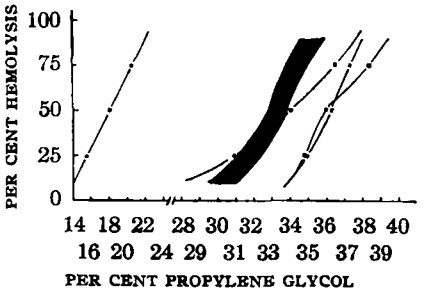


Fig. 2.—Hemolysis of rabbit erythrocytes at 37° in propylene glycol solutions containing isotonic concentrations of various substances. [Isotonic concentrations of various substances refer to isotonic concentrations of these substances in water (Table I).] Key: shaded area includes lactose, $MgCl_2$, KBr, NaCl, NaI, $NaNO_3$, and sorbitol; ●, $CaCl_2$; O, Na_3citrate; ×, Na_2SO_4; □, Na_2tartrate.

to 100% glycerin solutions after 45 minutes at 37°.

Human i values for sodium chloride calculated from data obtained at 37° were slightly greater than the corresponding 25° i values, including the i values previously reported at 25° (1); the increases were not more than 5%.

Compounds in Table I, when added in isotonic concentrations to aqueous solutions containing 0.0 to 60% glycerin (solubility permitting), prevented hemolysis of rabbit and/or human erythrocytes at 37 and 25°.

Water-Propylene Glycol Solutions.—Rabbit and human erythrocytes were completely hemolyzed in 0.0 to 100% propylene glycol after 45 minutes at 37°.

Human i values obtained for sodium chloride in 5, 10, 20, and 30% propylene glycol solutions at 37° were slightly greater (2–5%) than the corresponding i values at 25°, including i values previously reported at 25° (1).

Inclusion of 0.9% sodium chloride in 40% propylene glycol solution did not protect rabbit and human erythrocytes from complete hemolysis at 37°; nor did addition of up to 10% sodium chloride prevent complete hemolysis.

The exact concentrations of propylene glycol in 0.9% sodium chloride that caused hemolysis of rabbit and human red blood cells after 45 minutes at 25 and 37° are shown in Fig. 1. The increase in temperature from 25 to 37° greatly decreased the concentrations of propylene glycol in 0.9% saline solution needed to cause hemolysis of rabbit and human erythrocytes. The concentration of propylene glycol needed to produce 50% hemolysis of rabbit red blood cells decreased from 41% (at 25°) to 33.7% (at 37°). For 50% hemolysis of human blood the concentration dropped from 46% (at 25°) to 34.6% (at 37°), a decrease of more than 11%. At 25° rabbit red corpuscles were hemolyzed by much lower concentrations of propylene glycol than were human red blood cells. However, these differences were markedly decreased at 37°.

Hemolysis experiments were also carried out in which isotonic equivalents of various compounds were added to propylene glycol solutions at 37°. Hemolysis curves plotted from the data obtained are shown in Figs. 2 and 3. The mono-monovalent salts, sugars and sugar alcohols, magnesium chloride and sulfate, glycine, and sodium succinate had sigmoid hemolysis curves for rabbit and/or human blood that fell within the boundaries of the wide, shaded curves in Figs. 2 and 3. Thus, these compounds afforded essentially the same degree of protection as sodium chloride against hemolysis by aqueous propylene glycol solutions. Sodium benzoate gave slightly less protection to human erythrocytes than the above-mentioned compounds.

Isotonic concentrations of sodium or potassium sulfate, potassium sodium or disodium tartrate, or trisodium citrate afforded greater protection to rabbit and/or human red blood cells than 0.9% sodium chloride; in their presence higher concentrations of propylene glycol were necessary to initiate and bring about complete hemolysis of erythrocytes. The order in which the anions of the above salts appeared to protect human erythrocytes against propylene glycol hemolysis was citrate > tartrate > gluconate > sulfate. The curves were not as well defined for rabbit blood; however, the order of protection against hemolysis appeared to be sulfate, tartrate > citrate.

When blood was added to propylene glycol solutions containing isotonic concentrations of calcium chloride, hemolysis of erythrocytes took place in much lower concentrations of propylene glycol than solutions containing isotonic amounts of other compounds. Eight to ten per cent less propylene glycol was required to hemolyze human erythrocytes in aqueous solutions containing 1.15% calcium chloride than in solutions containing isotonic concentrations of other chloride salts. The decrease in propylene glycol concentrations for hemolysis of rabbit erythrocytes in 1.11% calcium chloride solutions was approximately 15%.

DISCUSSION

Current experiments showed that at 37° complete hemolysis of rabbit erythrocytes occurred in all aqueous glycerin solutions, whereas in previous work at 25° (1) hemolysis was incomplete in 50 to 70% glycerin solutions. This is consistent with the observations that for most permeants containing hydroxyl groups the usual hemolytic response to an increase in temperature is increased hemolysis.

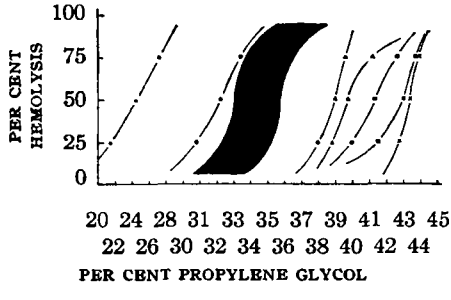


Fig. 3.—Hemolysis of human erythrocytes at 37° in propylene glycol solutions containing isotonic concentrations of various substances. [Isotonic concentrations of various substances refer to isotonic concentrations of these substances in water (Table I).] Key: shaded area includes dextrose, glycine, lactose, $MgCl_2$, $MgSO_4$, mannitol, KBr, NaCl, NaI, $NaNO_3$, Na_2succinate, sorbitol, and sucrose; ●, $CaCl_2$; O, Na benzoate; ▲, K_2SO_4 and Na_2SO_4; △, K gluconate; ■, KNa tartrate; □, Na_2tartrate; ×, Na_3citrate.

Eisenberg (5) found that hemolysis caused by water, ethanol, and glycerin was hastened and increased by a rise in temperature.

It appeared that all the hemolytic phenomena encountered in glycerin solutions were of an osmotic character since hemolysis was prevented by inclusion of isotonic concentrations of sodium chloride or of other salts and nonelectrolytes.

In a series of papers (6–11) concerning the hemolysis of human erythrocytes in relation to the lattice structure of water, Good established the view that the effect of a solute upon the structural properties of the water lattice is basic to the mechanism of hemolysis. A nonpenetrating, structure-promoting substance stabilizes the extracellular water lattice, and by thus reducing the fluidity of the extracellular phase, it slows the rate of movement of water into the cells. Hemolysis is therefore inhibited to an extent dependent upon the stabilizing power of the solute. Conversely, a nonpenetrating, structure-breaking substance reduces the stability of the extracellular lattice, thereby facilitating the flow of water into the cell. Its inhibition is therefore less than that of a structure former. Good found that glycerin, propylene glycol, and ethylene glycol did not greatly influence the stability of a malonamide–water lattice, and that hemolysis was essentially unaffected in the presence of small concentrations of these substances. He also reported that isosmotic concentrations of glycerin and glycols took longer to induce hemolysis than water alone, the longest time for complete hemolysis being reported for glycerin.

In the present study and in the first paper of this series (1) the hemolytic i values of sodium chloride in various glycerin and propylene glycol solutions at 37 and 25° were found to be slightly higher than 1.86, the i value for sodium chloride in water. This indicated that neither glycerin nor propylene glycol contributed much to the tonicity of the extracellular aqueous solutions, or employing the semantics of the above discussion, they did not promote stability of the extracellular water lattice to any significant extent. The slightly higher i values for sodium chloride in glycerin solutions indicated only a slight stabilizing effect of glycerin on the water lattice.

An interesting observation in the present study was an abrupt change in behavior of erythrocytes in concentrated propylene glycol solutions containing 0.9% sodium chloride. At a propylene glycol concentration of about 30%, a sudden initiation of hemolysis occurred, and over the next 2 to 3% range complete hemolysis took place. The exact concentrations where hemolysis occurred varied and were dependent on temperature and the species origin of erythrocytes.

This sudden initiation of hemolysis occurred unexpectedly as the addition of 0.9% sodium chloride protects against hemolysis over 5 to 30% concentrations of propylene glycol in aqueous solutions. Furthermore the hemolytic i value of sodium chloride increases slightly as the propylene glycol concentration increases from 5 to 30% (1) so that theoretically, even less sodium chloride should have been needed to prevent hemolysis at the higher propylene glycol concentrations.

The hemolysis of red blood cells in aqueous solutions containing less than critical hemolyzing concentrations of propylene glycol was apparently of an osmotic character since it was inhibited by 0.9% or less sodium chloride. That hemolysis was not prevented above critical propylene glycol concentrations would indicate some other hemolytic mechanism at these levels.

The behavior of rabbit and human erythrocytes in aqueous propylene glycol solutions containing isotonic concentrations of substances other than sodium chloride (Table I) was similar to that of erythrocytes in glycol solutions containing 0.9% sodium chloride. For solutions containing isotonic concentrations of most compounds, the propylene glycol concentrations causing 50% hemolysis of rabbit erythrocytes were between 32.8 to 33.8%, and for human erythrocytes the range was 33 to 35.8%.

If the same hemolyzing concentration of propylene glycol had been observed with all added compounds, it probably would have been proper to assume that propylene glycol was cytotoxic and therefore caused a complete breakdown of the cell membrane at this particular strength. However, differences in hemolyzing concentrations of propylene glycol were observed upon the addition of other substances, particularly upon the addition of salts having di or trivalent anions. Of the polyvalent organic salts employed in this study, all except sodium succinate provided increased protection of erythrocytes to hemolysis in propylene glycol solutions. From these observations it would appear that some other phenomenon was responsible for the above-mentioned behavior rather than destruction of the membrane.

Stein (12) has shown that the theory of simple diffusion fails at higher concentrations of ethylene glycol, diethylene glycol, 1,2-dihydroxypropane, and 1,3-dihydroxypropane. Experimental results were accounted for by the assumption that interactions occur at these high concentrations between glycol molecules in the main bulk of the permeant solution. These interactions lead to the formation of hydrogen-bonded dimers and oligomers which penetrate the membrane at a different rate from that of the monomer.

The increased inhibition of propylene glycol hemolysis by salts having di and trivalent anions might be explained by classical behavior of erythrocytes to these polyvalent anions. Although permeable to anions (7), the red blood cell is generally thought of being essentially impermeable to cations. However, many cations are able to diffuse slowly and in small proportions into the interior of the cell (13). If a sodium ion can traverse the membrane, then a chloride ion could follow, or a potassium ion could come out of the cell in order to maintain electrical neutrality. If a sodium and chloride ion entered the cell, then there would be an influx of water. The sodium salts of succinic, tartaric, fumaric, and citric acids are known to be fairly indifferent to various cells and tissues (14). In the case of sodium citrate, the anion cannot diffuse through the membrane, so that there can be no immediate change in concentration. This phenomenon could account for sodium citrate and other similar salts providing increased protection against propylene glycol hemolysis.

Isotonic concentrations of calcium chloride considerably reduced the propylene glycol concentrations necessary to induce and complete hemolysis of rabbit and human erythrocytes. This behavior was

probably due to some effect of calcium ion on the red blood cell which made the cell more permeable to extracellular permeants. Calcium does not cross the red cell membrane under normal conditions (15). However, Davson and Danielli (16) have shown that calcium ion in concentrations greater than 0.01 *M* accelerates the penetration of extracellular potassium into the cell and increases loss of intracellular sodium from the cat erythrocyte. They also point out that unusual reponses of cell membranes take place in the presence of alkaline earth ions. Ruyssen and Croes (17) found that alkaline earths appear to thicken the wall of the red blood cell by forming insoluble salts with phosphatide acids. Dentzer (18) reported that calcium ion increased saponin hemolysis of human and cattle erythrocytes. These literature reports emphasize the fact that the calcium ion is capable of producing unusual responses in hemolysis studies.

The hemolysis behavior of the other divalent cation, magnesium, was similar to that of the majority of compounds employed in this investigation.

Jacobs, *et al.* (19), found that the rate of osmotic hemolysis of ox erythrocytes in solutions of penetrating nonelectrolytes (glycerin and ethylene glycol) were considerably increased by the addition of low concentrations of certain electrolytes. Salts with bivalent cations were more effective than those with univalent cations, while salts with bi- or trivalent anions usually had a retarding effect. In most instances, the effect of these ions on the rate of hemolysis are analogous to our present findings for the protective effect of similar ions against hemolysis of rabbit and human erythrocytes in concentrated propylene glycol solutions; calcium afforded the least protection while polyvalent anions gave the most protection.

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Inclusion Compounds of α-Lipoic Acid Methyl Ester with Urea and Thiourea

By HIROYUKI MIMA and MASAO NISHIKAWA

Recently several works have been reported on stabilization of various pharmaceuticals by formation of urea, thiourea, or deoxycholic acid adducts. Methyl α-lipoate, a relatively unstable hepatotonic, was found to form adducts both with urea and thiourea. X-ray diffraction patterns and infrared spectra showed that these adducts were typical inclusion compounds. Methyl α-lipoate in the urea adduct was relatively stable under exposure to sunlight or to ultraviolet light, whereas it was not so stabilized in the thiourea adduct. It was also found that in these adducts methyl α-lipoate became its free radical under such irradiation and that this radical remained stable in these channels for several hours to one day even after the irradiation was stopped.

Inclusion compounds are described as crystalline compounds which consist of two or more distinct components and in which one of the components fits into cavities provided by the other. The component which forms the cavity is designated as the host molecule, and the component which is included in the cavity as the guest molecule.

Urea and thiourea form such adducts or inclusion compounds with various other organic molecules such as hydrocarbons, acids, and esters. These interesting properties, found by Bengen (1), Angla (2), and Schlenk (3) in the 1940's, have been the subject of many investigations. X-ray studies have revealed the general details of the structure of these adducts. Ordinary urea has a tetragonal crystal structure but when crystallized from methanol containing normal paraffins, normal fatty acids, or other straight-chain molecules, it adopts a hexagonal crystal

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structure. In this structure, long hexagonal channels are formed with the lattice of urea molecules and straight-chain molecules are included in these channels. The diameter of urea channels is about 5 Å. Consequently branched molecules or cyclic molecules are too large to be included in urea channels.

On the other hand, ordinary thiourea has a rhombic structure. On inclusion of cyclohexane, carbon tetrachloride, or other molecules of similar sizes, it changes its crystal lattice to a rhombohedral form. The principle of the adduct formation of thiourea and urea is the same but the diameter of thiourea channels is a little larger. Though branched-chain molecules or cyclic molecules can be included, straight-chain molecules cannot form stable adducts with thiourea because they are too thin to remain in the channels.

The compounds included in urea or thiourea channels will be stabilized, as there is no room to react within channels with other molecules such as oxygen. When adducts are dissolved in water or extracted with ether, they decompose into their component host and guest molecules. Therefore, these adducts will be utilized as "molecular coating" of otherwise unstable pharmaceuticals. With these ideas, one of us has studied the stabilization and extractive crystallization of some pharmaceuticals (4–6). For example, 9-oxocamphor is used as a cardiotonic in Japan but it is extremely unstable and loses its potency almost completely on standing overnight in the air. However, its thiourea adduct protects 9-oxocamphor from oxidation for as long as one year and by dissolving the adduct in water, effective 9-oxocamphor is readily regenerated at any time (4).

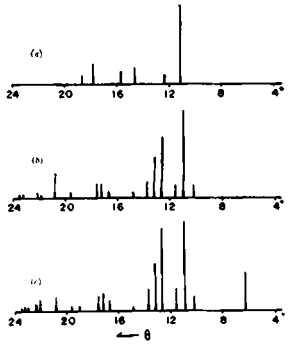


Fig. 1.—X-ray powder diffraction patterns of methyl α-lipoate urea adduct and its related compounds. (*a*) Urea (tetragonal); (*b*) methyl α-lipoate urea adduct; (*c*) methyl stearate urea adduct.

In the present work, we tried to stabilize methyl α-lipoate by the same technique. Methyl α-lipoate is a widely used hepatotonic in Japan but it is also unstable and polymerizes rapidly on standing in the air, especially under sunlight or ultraviolet light. The polymerization would proceed through the mechanism as shown below, which was assumed from the studies conducted by Barltrop, *et al.* (7) and Thomas and Reed (8).

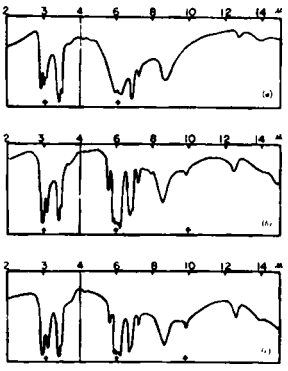
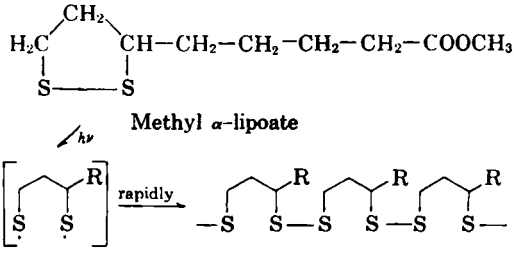


Fig. 2.—Infrared spectra of methyl α-lipoate urea adduct and its related compounds. (*a*) Urea (tetragonal); (*b*) methyl α-lipoate urea adduct; (*c*) methyl stearate urea adduct.



Methyl α-lipoate

If methyl α-lipoate is included in urea or thiourea channels, its trimethylene disulfide rings will be forced to be separated from each other so that such polymerization cannot take place and therefore the compound will be stabilized.

METHODS

Preparation of Adducts.—Methyl α-lipoate was prepared by esterification of commercially available α-lipoic acid with diazomethane in ether. On several careful redistillations under a reduced pressure, a yellow liquid with low viscosity was obtained, the boiling point ranging from 137 to 140° under 0.4 mm. Hg (9).

Preparation of its urea adduct was conducted as follows: 5 Gm. of freshly distilled methyl α-lipoate was dissolved (by heating if necessary) in 50 ml. of methanol containing 15 Gm. of urea. The resulting clear solution was then allowed to stand

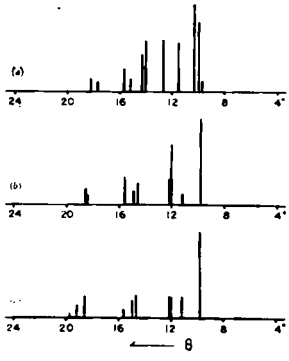


Fig. 3.—X-ray powder diffraction patterns of methyl α-lipoate thiourea adduct and its related compounds. (*a*) Thiourea (rhombic); (*b*) methyl α-lipoate thiourea adduct; (*c*) cyclohexane thiourea adduct.

overnight at room temperature. About 10 Gm. of the needle crystals separated were filtered, washed with isooctane to eliminate excess methyl α-lipoate, and dried on filter paper. This adduct decomposed at 63° and melted at 130°.

The preparation method of the thiourea adduct was almost the same as that of the urea adduct. Nine grams of thiourea and 8 Gm. of methyl α-lipoate in 40 ml. of methanol gave about 8 Gm. of the yellow adduct crystals. Acetone was used as washing solvent in this case. The decomposition point of the thiourea adduct was 106° and the melting point was 176°. Further purification was not conducted in both cases since these adducts partly decomposed into their components on recrystallization.

Stability Test.—About 50 mg. each of liquid methyl α-lipoate and the crystals of its urea and thiourea adducts was spread on a watch glass. A mere mechanical mixture of methyl α-lipoate and ordinary urea was also prepared and was spread in the same way. As a source of ultraviolet light, 15 W Blacklite blue (Sylvania) was used. The watch glasses were placed at the distance of 5 cm.

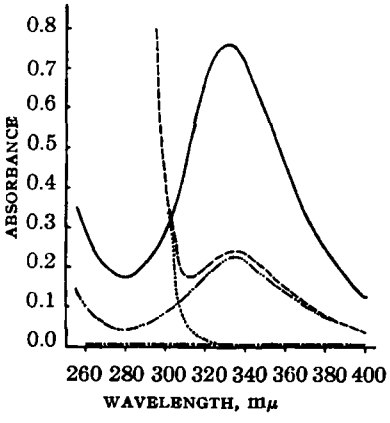


Fig. 4.—Ultraviolet spectra at methyl α-lipoate adducts and their related compounds. Key: ———, methyl α-lipoate; –·–·–·, urea; –··–··, methyl α-lipoate urea adduct; ....., thiourea; - - - - -, thiourea adduct.

from the light source and exposed to the light for 9 hours. The amount of unpolymerized methyl α-lipoate was determined by measurement of the intensity of the ultraviolet absorption at 334 mμ in methanolic solution and by the height of the reduction wave of polarography due to the —S—S— bond at pH 6.5.

Tests of stability under the exposure to sunlight were conducted by a similar method. In this case, the intensity of the sunshine was measured by a Matsuda integrating heliograph model IL-1-A.

RESULTS AND DISCUSSION

For confirmation that the crystals obtained as above are really urea and thiourea inclusion compounds, X-ray powder diffraction patterns and infrared spectra are very useful. As shown in Fig. 1, the X-ray pattern of methyl α-lipoate urea adduct is different from that of urea and is almost the same as that of methyl stearate urea adduct, one of the typical urea inclusion compounds.

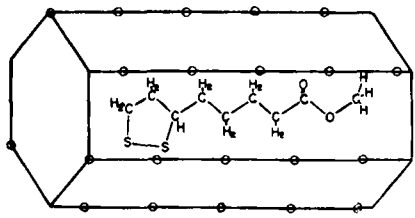


Fig. 5.—A possible structure of methyl α-lipoate in a urea channel. Key: O, urea molecules.

Comparison of the infrared spectra (Fig. 2) would also lead to the same results. Therefore, methyl α-lipoate urea adduct was definitely identified as an inclusion compound. Similarly, methyl α-lipoate thiourea adduct was also identified as a thiourea inclusion compound (Fig. 3). This would be the first time that a molecule which forms stable inclusion compounds both with urea and thiourea at room temperature was found, at least in the case of such a small molecule, since molecules to be included in urea and thiourea channels have been thought to be essentially different owing to the different diameters of the two channels (4).

Ultraviolet spectra of methyl α-lipoate urea and thiourea adducts in methanolic solution are shown in Fig. 4. Since these adducts dissociate into their components on dissolution in solvents, their spectra will be just the superposition of those of methyl α-lipoate and urea or thiourea. From the extinction coefficient of the absorption at 334 mμ which arises from the trimethylene disulfide ring in methyl α-lipoate, the molar ratios of [urea]:[methyl α-lipoate] and [thiourea]:[methyl α-lipoate] were determined to be 8.8:1 and 6.2:1, respectively. The molar ratios were also determined by elementary analysis and polarography and the values of 8.2:1 and 8.4:1 were given to the urea adduct and those of 5.9:1 and 6.2:1 were given to the thiourea adduct. Some discrepancy of these data may be attributed to the difficulty in purification of the adduct crystals. How methyl α-lipoate is included in urea or thiourea channels will be elucidated from these molar ratios without difficulty. Taking the value of 8.5:1 for the urea adduct as an average, the number of the atoms which form a zigzag chain in urea channels was calculated to be 10.2 by Smith's equation (10). If a methyl α-lipoate molecule is assumed to be enclosed in urea channels in such a state as suggested in Fig. 5, the number of the atoms in the zigzag chain including three carbon atoms in the trimethylene disulfide ring will be 10, which is in good accordance with the value calculated above. In the case of the thiourea adduct the molar ratio of about 6:1 indicates that one methyl α-lipoate molecule is enclosed only in one unit cell or, in other words,

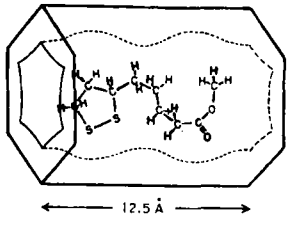


Fig. 6.—A possible structure of methyl α-lipoate in a thiourea channel.

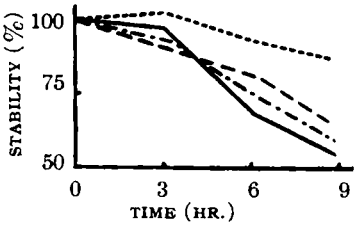


Fig. 7.—Stability of methyl α-lipoate and its adducts under the exposure to ultraviolet light. Key: ———, methyl α-lipoate; - - - -, urea adduct; – – – –, thiourea adduct; –·–·–·, mechanical mixture of methyl α-lipoate and urea.

in two relatively wide parts of thiourea channels (3). Therefore, the methyl α-lipoate molecules may be twisted to some extent in channels as suggested in Fig. 6.

The results of stability tests of methyl α-lipoate in its urea and thiourea adducts under the exposure to ultraviolet light are shown in Fig. 7 in comparison with those of liquid methyl α-lipoate itself. From the figures it is clear that the urea adduct stabilizes methyl α-lipoate considerably whereas the thiourea adduct has no such effect. As the adducts exist as fine crystals, scattering of light on the surface may be stronger than liquid methyl α-lipoate itself and this may make α-lipoate in the adduct stable. However, this effect seems to be insignificant since the mere mechanical mixture of methyl α-lipoate and ordinary urea powder does not stabilize α-lipoate as shown in Fig. 7. Stabilizing effect of the urea adduct must be, therefore, attributed to the suppression of polymerization reaction of methyl α-lipoate molecules by the hexagonal crystal lattice of urea. In the case of the thiourea adduct, such effect became apparent if sunlight was used as an irradiation source, although it was small compared with that of the urea adduct (Fig. 8).

These results may be explained as follows. Methyl α-lipoate may be excited by a wide range of ultraviolet and visible light, since it has absorption maximum in the near ultraviolet region. Thiourea also absorbs ultraviolet light having wavelengths shorter than 300 mμ and therefore it can react with methyl α-lipoate under exposure to such short wavelength light. However, light with wavelengths longer than 300 mμ may have little power to give rise to such reaction. On the other hand, urea does not absorb light up to 260 mμ, and thus it has a stronger stabilizing effect.

In the course of this work, we observed that the

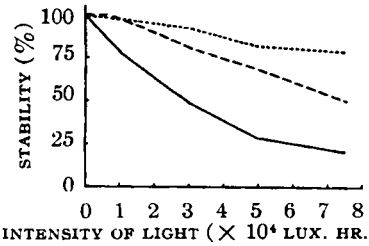


Fig. 8.—Stability of methyl α-lipoate and its adducts under the exposure to sunlight. Key: ———, methyl α-lipoate; - - - -, urea adduct; – – – –, thiourea adduct.

original crystals of urea or thiourea adducts of methyl α-lipoate were yellow, but when they were irradiated with sunlight or ultraviolet light, the color gradually changed to pink. This pink remained unchanged for several hours to 1 day even after the irradiation was stopped and then returned to the original yellow or a slightly faded color. Besides, the color disappeared immediately on dissolving in solvents. For the investigation of the origin of this pink, visible absorption spectra of these colored crystals were measured by rearranging the crystals side by side on a thin NaCl plate. Nujol was used as a binder. As shown in Fig. 9, the irradiated crystals of the urea adduct have an absorption maximum at 530 mμ. This region is transparent for the nonirradiated yellow crystals.

The spectrum of the irradiated thiourea adduct was completely the same as that of the irradiated urea adduct. This fact suggests that the origin of the pink has nothing to do with host molecules and may be attributed to a certain excited state of methyl α-lipoate. Barltrop, *et al.* (7) have found that if trimethylene disulfide was photolyzed at the

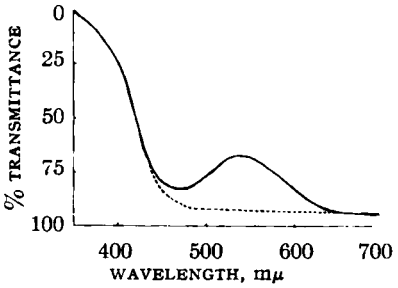


Fig. 9.—Ultraviolet spectra of crystals of methyl α-lipoate urea adduct. Key: - - - -, initial, nonirradiated crystals; ———, irradiated crystals.

temperature of liquid nitrogen a clear pale salmon color appeared. This color was attributed to free radicals of trimethylene disulfide since the colored solution faded the color of diphenylpicrylhydrazyl. As the pink observed in our experiments resembles this salmon, the origin of the pink would be attributable to free radicals of the trimethylene disulfide ring of methyl α-lipoate which are formed by the irradiation with sunlight or ultraviolet and as these radicals are restricted in the space of the crystal lattice of urea or thiourea they would exist stable without affecting further reactions.

In this way free radicals which otherwise do not exist in a stable state except at extremely low temperature can be isolated in the channel of inclusion compounds even at room temperature.

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Decarboxylation of *p*-Aminosalicylic Acid in the Solid State

By SAUL S. KORNBLUM* and BARTLEY J. SCIARRONE

The decarboxylation reaction of *p*-aminosalicylic acid in the solid state has been demonstrated to be dependent on temperature, moisture, pressure, and particle size. Typical curves are given which indicate environmental influence on the degradation reaction. A possible gross mechanism is offered which analyzes the sigmoidal-type per cent decomposition *versus* time curve obtained with dry, atmospheric, thermal degradation of *p*-aminosalicylic acid. A set of equations which describes the kinetic data is given. Using these equations, the various rate constants have been determined.

THERE HAS BEEN only a limited number of degradation studies of organic compounds in the solid state reported (1–3). The available information, with few exceptions, is sketchy and inconclusive with respect to effects of various environmental conditions. *p*-Aminosalicylic acid decarboxylates into *m*-aminophenol and carbon dioxide in solution as well as in the solid state (4). The solution kinetics of this reaction have been reported elsewhere (5–7). The decomposition reaction of *p*-aminosalicylic acid in the solid state possesses pharmaceutical interest, yet it has been interpreted only vaguely (8).

The effects of such environmental conditions as temperature, moisture, pressure, particle size, and past history of solids as they relate to the decarboxylation of solid *p*-aminosalicylic acid as well as the influence of the decomposition product are reported.

THEORY

Most real crystals are imperfect. Investigators of mechanical properties of solids have shown that no macroscopic specimen ever exhibits the theoretical strength of a perfect crystal. This has led to the recognition that some of the imperfections are dislocations (9). Observation of solids has suggested that the nucleation of a new phase may, under certain conditions, occur not homogeneously but in the neighborhood of dislocations which may be distributed uniformly throughout the crystal. The nucleation of new phases occurs when local fluctuations in the energy of the crystal are sufficient to provide the necessary activation energy. The nuclei are formed at definite localized spots where the activation is least for structure-sensitive nucleation. The number of nuclei formed in a given time thus will depend upon the number of potential nucleus-forming sites which are generally associated with some type of lattice imperfection.

Solid reactions have been classified conveniently according to the shape of the per cent decomposition *versus* time curves obtained on thermal decomposition, for which there are three main types (10) as shown in Fig. 1. For the first case, (*a*) the most rapid rate of reaction occurs at the beginning of the decomposition. This is typical of the decomposition of solids where nuclei of the new phase are rapidly formed and where there is almost instantaneous coverage of the surface with a film of the solid product. In the second case, (*b*), there is an apparent induction period due to a slow rate of production of nuclei which is followed by the rapid acceleration period and terminated by the decay period. Under favorable conditions, the rate of production of nuclei is sufficiently low for them to grow to sizes which can be examined by visual methods. For the third case, (*c*), the initial stage may be represented by a small amount of gas evolution extending over a relatively long period of time. This is followed by the acceleration of the reaction as in the second-type curve. Where melting occurs during the decomposition, the form of the curve may not correspond with any of the above types.

If decomposition occurs in the solid phase without partial melting, owing to molecular volume changes, it may be assumed that the formation of product molecules induces strain in the crystal which further cracks, forming fresh surfaces upon which decomposition can occur. A theory based on this premise was proposed by Prout and Tompkins (11) to explain their observations on the decomposition of potassium permanganate and has been extended to organic solids by Vaughan and Phillips (3). The latter workers describe the sites of nucleation as regions where decomposition is favored, that is, where the reaction occurs with a low activation energy (12). Garner (12) has reported that the surface array of product molecules has a different unit cell from that of the original substance, and this sets up strains on the crystal surface that are relieved by the formation of cracks. He states that at the mouth of these cracks, a reaction will be favored by lattice imperfections and will spread down the crevices into the crystal. Decomposition on these surfaces produces additional and further cracking. Thus a type of chain branching process develops and occasionally these reacting surfaces will interfere with one another to break the chain.

Prout and Tompkins (11) also stated that the

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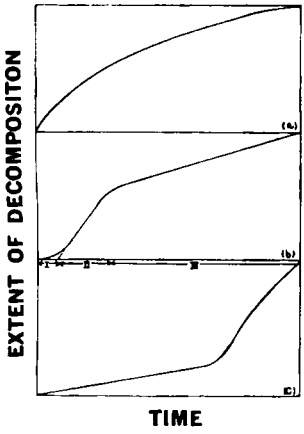


Fig. 1.—Graphic representation of three main types of decomposition – time curves in solid-state reactions; (I) induction period, (II) acceleration period (k_a), (III) decay period (k_d), which refer to case b.

rate of increase of potentially reactive molecules may be represented by

$$\frac{dn}{dt} = \frac{\alpha n}{\delta t} \qquad \text{(Eq. 1)}$$

where n is the number of potentially reactive molecules existing at the time t, δt is the average time interval between production and decomposition and α is the probability of branching. They propose that branching proceeds unhindered initially since there are many centers from which branching can originate, but as the reaction progresses considerable interference will arise. Thus when a plane of product molecules reaches a surface, either internal or external, where decomposition has already occurred the reaction will cease. If β is the probability of interference then Eq. 1 becomes

$$\frac{dn}{dt} = (\alpha - \beta)\frac{n}{\delta t} \qquad \text{(Eq. 2)}$$

EXPERIMENTAL

The powdered *p*-aminosalicylic acid of U.S.P. grade[1] used in this study was found to have an average particle size of 3.3 μ as determined by a Fisher "subsieve sizer." Whole crystals of *p*-aminosalicylic acid were prepared by recrystallization from absolute ethanol *in vacuo*. The approximate diameter of the latter was determined by means of U. S. Standard sieves, and was found to be between 40 and 80 mesh.

The powdered *p*-aminosalicylic acid used in this study proved to be stable if stored at room temperature under a dry atmosphere for a three-year period. However, during this time a slight darkening of the powder occurred, which was thought to be the result of slow oxidation.

The general procedure used in the kinetic studies was as follows. A series of 10-ml. Pyrex beakers, each containing exactly 300 mg. of *p*-aminosalicylic acid, were arranged in a desiccator previously brought to thermal equilibrium for 24 hours. The desiccator was placed in an automatically controlled oven set at the experimental temperature (70–80 ± 0.5°). At various intervals, samples were removed, dissolved in approximately 25 ml. of ethanol and titrated with standard sodium hydroxide solution using phenolphthalein as the end point indicator. An alternate spectrophotometric method of analysis was developed to determine both *p*-aminosalicylic acid and *m*-aminophenol simultaneously using the wavelengths 300 mμ for *p*-aminosalicylic acid and 230 mμ for *m*-aminophenol. The molar absorbance index (a_M) was calculated to be 6.47×10^3 L./mole/cm. for *m*-aminophenol at the wavelength 230 mμ, and 9.70×10^3 L./mole/cm. and 8.58×10^3 L./mole/cm. for *p*-aminosalicylic acid at the wavelengths 230 mμ and 300 mμ, respectively. Beer's law was obeyed with both substances over a range of 2×10^{-3} mg./ml. to 8×10^{-3} mg./ml. In all the degradation studies the reaction was followed through at least 50% decomposition.

Dry, Atmospheric Pressure Studies.—The powdered *p*-aminosalicylic acid was decomposed under atmospheric pressure, at elevated temperatures and in the absence of water vapor. The samples were stored at 70, 75, and 80°. The *p*-aminosalicylic acid powder was subjected to a degassing process using reduced pressure below 1 mm. for 24 hours, but thermal treatment showed no difference in the reaction velocity with the degassed or untreated *p*-aminosalicylic acid.

In an attempt to study the effect of particle size on the reaction, an investigation of the thermal degradation of large crystals of *p*-aminosalicylic acid (diameter not less than 0.177 mm. and not larger than 0.420 mm.) was conducted in a similar manner. The progress of the initial reaction was observed microscopically and photographed.

A study was undertaken using powdered *p*-aminosalicylic acid to determine whether or not the induction period of the degradation process was due to thermal diffusion. If thermal diffusion were the cause of any induction period, new delays in the progress of the reaction should be experienced after intermittent quenching and reheating of the powder. Thus samples were heated at 80°, quenched during the known induction period, acceleration period, and decay period by cooling to room temperature, and maintaining this temperature for at least 6 hours. The quenching periods were then followed by reheating to 80°.

The effect of carbon dioxide pressure, over the solid acid, on the decarboxylation process rate has been investigated (8) at varied pressures (20–760 mm. Hg) which demonstrated no affect on the rate

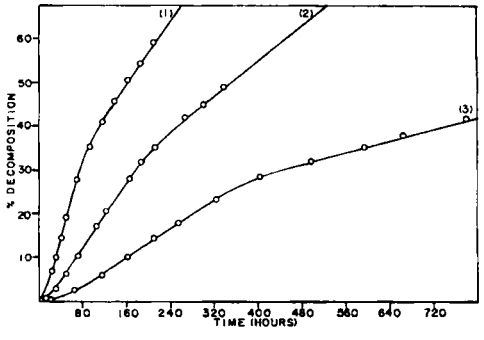


Fig. 2.—Per cent decomposition-time curves for the thermal decarboxylation reaction of *p*-aminosalicylic acid in dry, solid state at (*1*) 80°, (*2*) 75° and (*3*) 70°.

[1] The authors wish to thank Eli Lilly Co., Indianapolis, Ind., for supplying experimental quantities of *p*-aminosalicylic acid.

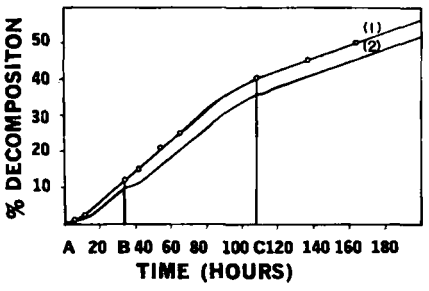


Fig. 3.—Comparison of actual data (O) with that of expected hypothetical curve were the decarboxylation of *p*-aminosalicylic acid at 80° to experience separate induction periods after quenching and subsequent heating at times *A*, *B*, and *C*.

or character of the decarboxylation process. In the present study the carbon dioxide pressure never was allowed to build up since samples were removed from the desiccator for analysis at approximately 24-hour intervals.

Dry, Reduced Pressure Study.—Since it was felt that the role of the solid product of decarboxylation, *m*-aminophenol, in the over-all reaction could be better understood if it were eliminated, the reaction was conducted at reduced pressure employing a vacuum oven. At a temperature of 80 ± 0.5° and approximately 10 mm. Hg pressure, the *m*-aminophenol readily sublimed. Under the same conditions, the *p*-aminosalicylic acid could not be detected in the sublimate. Spectrophotometric analysis of the reaction mixture demonstrated the absence of the decomposition product throughout the reaction.

Moist, Atmospheric Pressure Studies.—The effect of a constant humidity on the reaction was studied by placing samples of *p*-aminosalicylic acid over saturated solutions of appropriate inorganic salts to obtain aqueous tensions in the range between 30 and 80% absolute humidity (13). At higher humidities condensation took place and caused wetting of the powder. The desiccator containing the salt solution was allowed to equilibrate for 24 hours at the desired temperature before the samples were introduced. There appeared to be no weighable adsorption of water at 70° with an aqueous tension of 118.4 mm. Hg. If water was adsorbed by the powder, the possibility of solution kinetics would have been indicated.

Suspension Technique Study.—The utilization of a suspension technique proved useful in the solid-state decomposition study. After a screening of many vehicles, mineral oil was chosen because its inert properties eliminate interference with the reaction yet afford good heat transfer. The suspension was prepared by levigating *p*-aminosalicylic acid with mineral oil and a uniform dispersion was maintained with a power stirrer at constant speed. The reaction vessel, a Kjeldahl flask modified by the attachment of a side arm tube, allowed the withdrawal of samples for analysis without disturbing the reaction. The temperature was controlled by use of a circulating oil bath.

RESULTS

Dry, Atmospheric Pressure Studies.—The decarboxylation of *p*-aminosalicylic acid under the influence of atmospheric pressure, at elevated temperatures and in the absence of moisture is represented by the sigmoidal-type curves as given in Fig. 2. This typical per cent decomposition *versus* time curve consists of three parts that may be described as: (*a*) induction period; (*b*) acceleration period; (*c*) decay period (11). At a given temperature the induction period is found to occur only once in the thermal decarboxylation reaction. The per cent decomposition *versus* time curve represented by Fig. 3 (*1*) is the result of three consecutive heating and cooling periods at 80°. The samples were removed from the oven at the points A, B, and C and stored in a desiccator at room temperature for 6 or more hours. They were then replaced in the oven at 80° for further decomposition. If the induction period were dependent upon thermal diffusion then Fig. 3 (*2*) would be a hypothetical possibility for the per cent decomposition *versus* time curve. The existing difference in per cent decomposition at any time between Figs. 3 (*1*) and 3 (*2*) minimized the possibility of thermal diffusion since all points indicated in Fig. 3 (*1*) correspond within the limits of experimental error with those of uninterrupted runs made under the same experimental conditions. Haycock (10) describes this period as one due to the slow nuclei growth in the early stage of the reaction. The induction periods for the decarboxylation of *p*-aminosalicylic acid in a dry atmosphere decreased with increased heat intensity, and were found to be 38, 18, and 8 hours, for 70, 75, and 80°, respectively.

The acceleration period of the decarboxylation reaction for the most part can be described simply by

$$\frac{dx}{dt} = k_a = \frac{x}{t - \tau} \qquad \text{(Eq. 3)}$$

where x is the per cent decomposition, τ is the induction period, t is the time where the decomposition is x, and k_a is the rate constant for the acceleration period.

The decay period follows the second inflection point, since after this point the rate-controlling factor becomes the number of unreacted nuclei which are produced at a constant rate. The equation for the decay period is

$$\frac{dx}{dt} = k_d = \frac{x - x_{max.}}{t - t_{max.}} \qquad \text{(Eq. 4)}$$

where $x_{max.}$ is the per cent decomposition at $t_{max.}$, $t_{max.}$ is the time at which the reaction reached maximum velocity (second inflection point), and x and t are the same as in Eq. 3. Similarly, Prout and Tompkins (11) derived separate equations for both the acceleration and decay periods having similar form but expressed the rate in terms of growth of active nuclei.

The least-mean-squares equation and the above equations were used to demonstrate the validity of the rate constants calculated. The acceleration and decay periods best fit an apparent zero-order reaction equation which indicates an independence of the rate of reaction on the fraction of *p*-aminosalicylic acid remaining. A study conducted with sample sizes of 75, 150, and 300 mg. of powdered *p*-aminosalicylic acid at 80° substantiated this by rendering a k_a value of 0.456 ± 0.046 mole% $hr.^{-1}$. The second inflection point, however, oc-

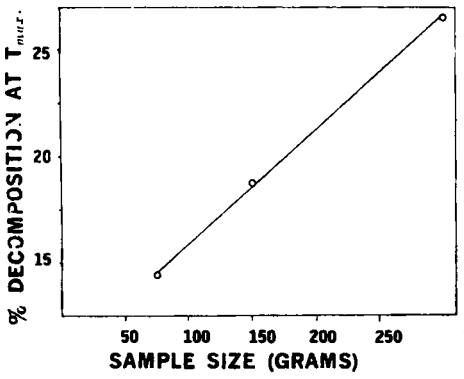


Fig. 4.—Relationship of the per cent decomposition at $t_{max.}$ *versus* the sample size for the decarboxylation of *p*-aminosalicylic at 80° under dry, atmospheric pressure.

TABLE I.—DATA OF THE DECARBOXYLATION REACTION OF DRY *p*-AMINOSALICYLIC ACID AT ATMOSPHERIC PRESSURE

T, ° C.	Induction Period (hr.)	$t_{max.}$ (hr.)	k_a (mole% hr.$^{-1}$)	k_d (mole% hr.$^{-1}$)
70	38	360	0.080 ± 0.004[a]	0.037 ± 0.002
75	18	170	0.195 ± 0.006[a]	0.094 ± 0.003
80	8	71	0.446 ± 0.011[b]	0.200 ± 0.005

[a] Average of two runs. [b] Average of three runs.

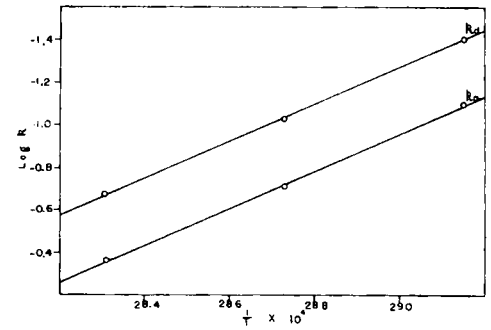


Fig. 5.—Arrhenius plot for thermal degradation at a dry, atmospheric pressure.

curred at different degrees of decomposition with all three of the sample sizes. Analysis of the data indicated a linear relationship between the per cent decomposition at the second inflection point *versus* the sample size (Fig. 4). This suggested that the β value (Eq. 2) depends on the sample size.

Table I shows the effect of temperature on the induction period, $t_{max.}$ and the values of the acceleration period rate constant (k_a) and decay period rate constant (k_d). It was possible to reproduce the per cent decomposition *versus* time curves and calculate kinetic constants within acceptable limits.

The plot of the logarithms of k_a and k_d against $1/T$ gave two straight lines (Fig. 5) with almost identical slopes, yielding activation energies of 41.0 and 40.9 kcal./mole, respectively. This showed that the reaction at each stage was the same even though the intercept changed.

The photographs[2] of *p*-aminosalicylic acid crystals point out that cracking and splintering occurs during the early stages of the solid-state reaction [Figs. 6(*a*) and 6(*b*)]. The fractures occurred at points of irregularity in the crystal. The more perfect the crystal, the greater was the amount of time needed for fracturing to be initiated. It can be seen from Fig. 6(*a*) that the cracking took place at a point of irregularity in the crystal. The fractured crystal had to be mechanically moved into the field of the microscope since fracturing apparently occurred with an explosive force. This was probably due to a release of carbon dioxide from the decarboxylation reaction. As the reaction progressed, the subsequent cracking followed more quickly, and with the approach of the acceleration period the disintegration rate markedly increased. The fracturing process of the *p*-aminosalicylic acid crystals led to a velocity rate greater than that of the powdered *p*-aminosalicylic acid during the acceleration period. The rate of degradation of the crystals is compared in Fig. 7 with that of the powdered *p*-aminosalicylic acid exposed to the same conditions. In comparing the two curves for powdered and larger crystalline *p*-aminosalicylic acid it can be seen that the induction period was longer, the acceleration period had less duration and greater velocity in the case of the larger crystalline state than the powdered form. These results may be explained

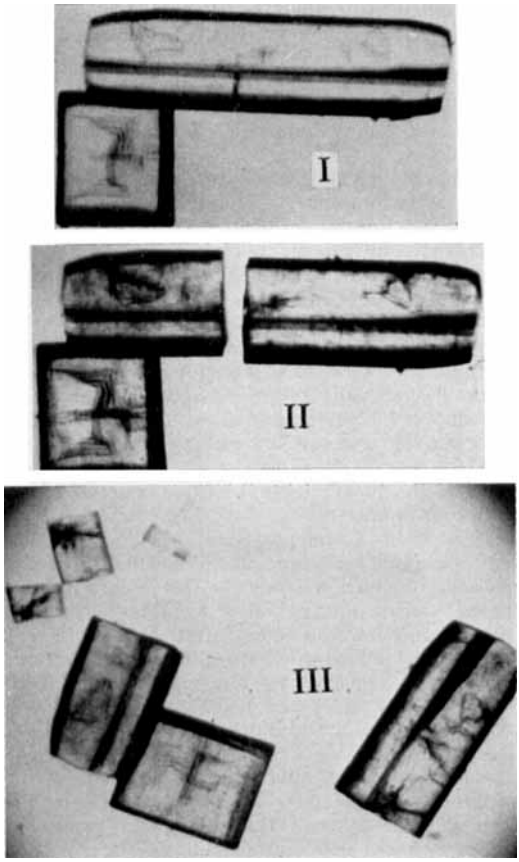


Fig. 6*a*.—Illustration of the cracking process experienced by whole crystals of *p*-aminosalicylic acid in the early stages of thermal decomposition at 80°; (I) before exposure, (II) after 7-hr. exposure, (III) after 9-hr. exposure.

[2] The assistance of Dr. A. DeMaggio is gratefully acknowledged.

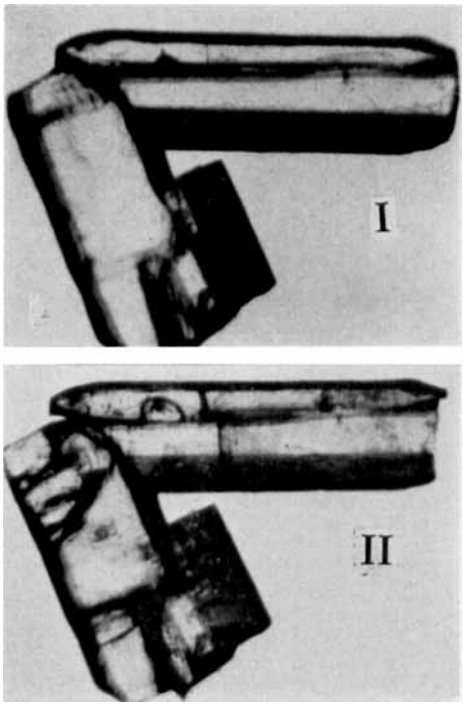


Fig. 6b.—Illustration of the cracking process experienced by whole crystals of *p*-aminosalicylic acid in the early stages of thermal decomposition at 80°; (I) before exposure, (II) after 7-hr. exposure.

by reference to the cracking process which imposed sites of reaction different from that induced by grinding. In other words, the cracking process supplies numerous new potential sites for degradation by continually cracking and fracturing, whereas the powdered *p*-aminosalicylic acid, as observed microscopically, did not appear to be further fractured. The fracturing process occurring with crystalline *p*-aminosalicylic acid was brought about either by uneven heating of the crystals or by a release of carbon dioxide generated from the decarboxylation reaction. It is felt that the apparent lack of fracturing experienced by the powdered *p*-aminosalicylic acid can be clarified on the grounds that uniform heating of the particles should be possible and also that the particles have been endowed, from past history, with numerous cracks and crevices for the release of any carbon dioxide formed.

After the second inflection point of the per cent decomposition *versus* time curve the particles, from the crystals, appeared to be of uniform size and shape, and did not appear to undergo any further change during the subsequent decay period. The duration of the acceleration period seemed to be dependent on the particle size and the physical means used in obtaining this size.

Dry Atmosphere, Reduced Pressure Study.—When the reaction was conducted under reduced pressure it was found that the induction period was maintained but without incidence of a second inflection point (Fig. 8). The rate of reaction at this reduced pressure (approximately 10 mm. Hg) increased the degradation by a multiple of 4.4. To establish the composition of the sublimate from the reaction, a "cold-finger" apparatus was utilized. The solidified vapor was shown to be *m*-aminophenol by infrared analysis and by melting point determination. This study was primarily pursued to eliminate the possibility that *p*-aminosalicylic acid also sublimed under these conditions. However, the possibility exists that *p*-aminosalicylic acid vaporizes and decomposes before it condenses to yield the same results. This point could not be resolved experimentally. Spectrophotometric analysis of the reaction mixture demonstrated the absence of decomposition product throughout the reaction. Hence, there was no accumulation of *m*-aminophenol on the surface of the *p*-aminosalicylic acid as occurs under dry, atmospheric pressure.

Moist, Atmospheric Pressure Studies.—Figures 9 and 10 represent the typical curves for the thermal decarboxylation of *p*-aminosalicylic acid in a moist atmosphere. The results of this series of experiments showed that (*a*) the induction period was greatly diminished at the lower temperature employed; (*b*) a more rapid velocity than in the dry state was evident; (*c*) once again no second point of inflection was noted. Analysis of the samples for *m*-aminophenol showed that this by-product must have been removed during the progress of the reaction by a process similar to steam distillation in the moist atmosphere.

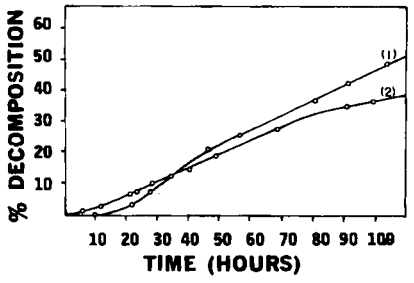


Fig. 7.—Per cent decomposition-time curves for the decarboxylation reaction of *p*-aminosalicylic acid in the dry, solid state at 80°; (*1*) whole crystals, 40/80 sieve size, (*2*) ground crystals, *ca.* 3.3 μ.

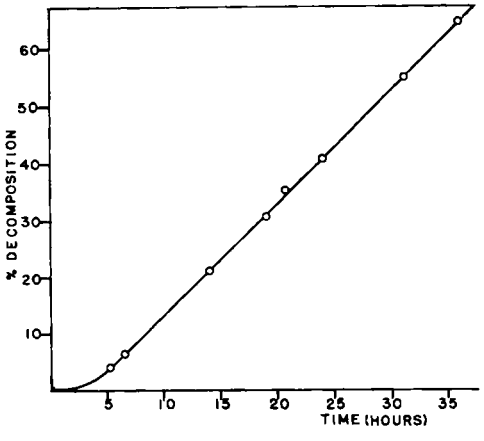


Fig. 8.—A per cent decomposition time curve for the decarboxylation of *p*-aminosalicylic acid under dry atmosphere and reduced pressure (*ca.* 10 mm.) at 80°.

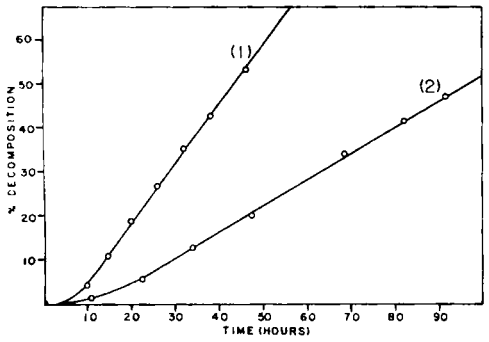


Fig. 9.—Per cent decomposition-time curves for the decarboxylation of *p*-aminosalicylic acid in a moist atmosphere at 80°; aqueous tension, (*1*) 181.0, (*2*) 8.13 mm. Hg.

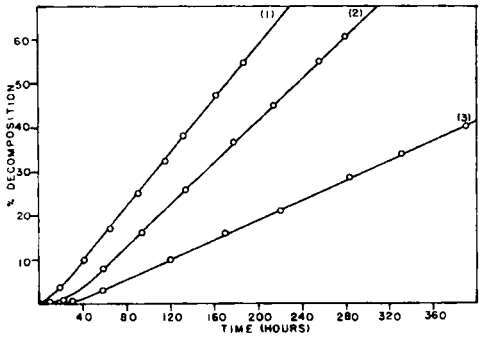


Fig. 10.—Per cent decomposition-time curves for the decarboxylation of *p*-aminosalicylic acid in a moist atmosphere at 70°; aqueous tension, (*1*) 144.0, (*2*) 118.4, (*3*) 52.3 mm. Hg.

The activation energy was reduced considerably from that obtained under dry, atmospheric conditions; however, it remained constant at all the aqueous tensions employed (Table II). The activation energy for the decarboxylation of *p*-aminosalicylic acid in aqueous solution was reported (5) as 19.4 Kcal./mole as compared to 29.5 Kcal./mole for the reaction under moist atmospheric conditions. The 10 Kcal. difference between solution kinetics and a saturated solution on the surface of a solid perhaps could be assigned to a complex of the heats of solution, dissolution, and sublimation.

A plot of the velocity constants *versus* the aqueous tension (Fig. 11) was linear, indicating that the mechanism of the reaction remained unchanged when the humidity was varied.

If one extrapolates Fig. 11 to zero pressure the velocity constant is very close to zero. Therefore, at constant temperature, if we double the vapor pressure of water the velocity constant is doubled. Zero-order solution kinetics seemed to be indicative of this relationship since the solubility of the *p*-aminosalicylic acid in the adsorbed moisture appeared to be a function of the aqueous tension over the sample. However, if we attempted to analyze,

$$\frac{d(\mathrm{PAS})}{dt} = k_1(\mathrm{PAS}) = k_0$$

where k_1 (1.986 hr.$^{-1}$) is the first-order rate constant for an aqueous soluton (5) and k_0 (0.003 hr.$^{-1}$) is the zero-order rate constant for a saturated solution on the surface, then the concentration of *p*-aminosalicylic acid in solution required to equate k_1 with reference to k_0 would be 0.0015 mole of acid. The total sample size of *p*-aminosalicylic acid (0.00196 mole) would require that approximately 80% of the solid acid be in solution for the above relationship to exist. If one considers the slow rates of dissolution and diffusion in a stagnant system, it appears presumptuous to substantiate purely solution kinetics.

Table II indicates the effect of humidity on the induction period, the *k* values and apparent activation energy.

Suspension Technique Study.—The per cent decomposition *versus* time curve obtained for the decarboxylation of *p*-aminosalicylic acid in suspension is shown in Fig. 12. It is characterized by the typical induction period followed by the reaction without incidence of a second inflection point. During the reaction a solid material collected in the neck of the reaction vessel; analysis proved it to be *m*-aminophenol. The mineral oil, therefore, provided a medium which facilitated the removal of *m*-aminophenol from the surface of the *p*-aminosalicylic acid.

TABLE II.—DATA OF THE DECARBOXYLATION REACTION OF *p*-AMINOSALICYLIC ACID UNDER MOIST, ATMOSPHERIC PRESSURE CONDITIONS

T, °C.	Aqueous Tension (mm. Hg)	Induction Period (hr.)	*k*, mole% hr.$^{-1}$[a]	Apparent Activation Energy, Kcal./mole
70	52.3	34	0.113 ± 0.005	29.3
70	118.4	26	0.245 ± 0.006	29.8
70	144.0	8	0.305 ± 0.003	29.3
80	81.3	7	0.600 ± 0.012	29.6
80	181.0	5	1.310 ± 0.023	29.5

[a] Average of two runs.

DISCUSSION

The thermal decomposition of *p*-aminosalicylic acid is characterized by the typical sigmoidal curve which has been reported for other solid-state reactions (9, 14, 15). An induction period, the duration of which was governed by experimental conditions, was experienced with all the studies. The increased induction period with larger crystals was apparently due to a limited number of dislocations prior to cracking. In time the fracturing process supplied numerous irregular chips which further disintegrated, generating new points of dislocations. After this initial period the reaction velocity exceeded that of the powdered *p*-aminosalicylic acid exposed to the same conditions. It is felt that this phenomenon was due to the abundance of nuclei formed from the explosive fracturing of the crystals. After the second inflection point of the per cent decomposition *versus* time curve the particles appeared to be of uniform size, and did not undergo further change during the subsequent decay period. The extent and velocity of the acceleration period appeared to be both dependent on the surface area of the *p*-aminosalicylic acid, and whether the active sites arose from either a thermally induced cracking process or physical grinding.

The experiments involving various environments other than dry, atmospheric pressure all produced

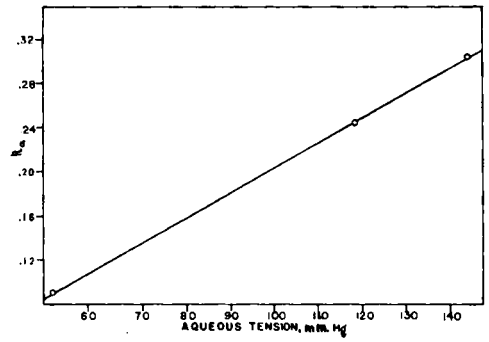


Fig. 11.—Relationship of velocity constant to the aqueous tension at 70°.

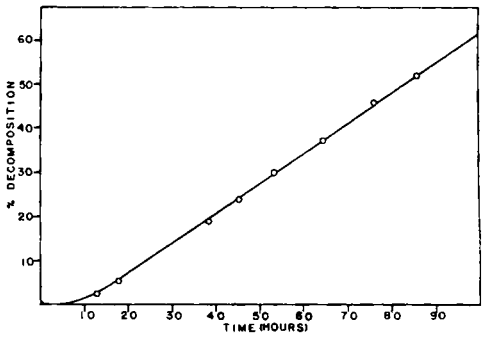


Fig. 12.—Per cent decomposition-time curve for the decarboxylation of *p*-aminosalicylic acid in a mineral oil suspension (*ca.* 0.1%) at 80°.

similar per cent decomposition *versus* time curves (Figs. 8–10, 12), which are characterized by the absence of a second inflection point. All have in common the removal of *m*-aminophenol from the reaction site. It appeared that the reduced pressure, moist atmosphere, and hot mineral oil function as transport media facilitating the removal of *m*-aminophenol from the surface of the *p*-aminosalicylic acid, which maintained the β value (Eq. 2) at a minimum. The second inflection point observed with dry, normal atmospheric conditions was brought about by an interference of the decomposition product which was eliminated under the other stated conditions. The removal of the decomposition product hastened the reaction velocity. The buildup of *m*-aminophenol on the surface of the *p*-aminosalicylic acid acted as a deterrent, decreasing the rate of reaction. Of course, decomposition must be considerable (25–30%) before the *m*-aminophenol causes a pronounced effect on the reaction rate (utilizing 300 mg. sample).

The experimental data obtained under moist atmospheric conditions were not thought sufficiently quantitative to analyze as purely solution kinetics; however, the possibility does exist. The fact that the decomposition product, *m*-aminophenol, was removed from the sample appeared to indicate that the moisture did aid in the transport of the *m*-aminophenol from the solid sample. Our findings contradict the conclusion of a previous study (8) which suggests that water functions as a catalyst. In the same study (8), it was reported that *m*-aminophenol, physically incorporated with *p*-aminosalicylic acid, also caused a catalytic effect. The grinding method utilized for introducing *m*-aminophenol possibly created new cracks in the *p*-aminosalicylic acid crystals which may account for apparent changes in the reaction rate when the *m*-aminophenol was not included.

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Assay of Glutethimide Tablets

By EDWARD SMITH

A method is presented for the isolation of glutethimide by partition chromatography and its determination by ultraviolet spectrophotometry. The acid amide, 4-ethyl-4-phenylglutaramic acid, a degradation product of glutethimide, may also be quantitatively isolated and determined. The assay is based on the absorbance of the imide in a chloroform solution at its maximum near 257.5 mμ. The identity of the imide and of the acid amide are confirmed by their ultraviolet characteristics in methanolic potassium hydroxide and by their infrared spectra.

THE N.F. XI assay procedure for glutethimide tablets (1) is a simple extraction of the imide with methanol, followed by measurement of the ultraviolet absorbance of the extract. The method provides neither for the separation of the imide from its degradation product, 4-ethyl-4-phenylglutaramic acid (acid amide), which absorbs strongly at the same wavelength, nor for other methanol soluble components which may be present in the tablets. Assay procedures have been reported which are based on the formation of a colored complex between ferric ion and the hydroxamate resulting from the reaction of the imide with alkaline hydroxylamine (2, 3). This reaction is somewhat specific, in that the imide will form a hydroxamic acid at room temperature while the acid amide will not do so. These procedures and several modifications have been tested in our laboratories and were found unsatisfactory due to the high absorbance readings of the blank, which reduce the precision and accuracy of these methods. Lactose or anions, which complex ferric ions, interfere with these methods (4). A quantitative fluorometric method for glutethimide proposed by Haycock *et al.* (4) does not provide for the separation from the acid amide which interferes.

The quantitative separation of glutethimide from its hydrolytic degradation product by ion exchange resin was recently reported in a study of the stability of the aqueous solutions of glutethimide (5). This procedure required the purification of the reagent grade resin prior to its use and did not provide for the isolation of acid amide present.

In all of the other procedures which have been reported for the determination of glutethimide, the imide is isolated before quantitation. This separation is usually done by extraction with a water immiscible solvent such as chloroform.

Many modifications of the procedure of Goldbaum *et al.* (6) for the determination of glutethimide isolated from biological samples have been published. The original method is based upon measurement of the rate of hydrolysis of glutethimide in alcoholic potassium hydroxide, determined by the ultraviolet absorption at 235 mμ. These procedures, while being suitable for low levels found in biological samples, are not practical for the assay of glutethimide tablets. Szabolcs and Vastagh (7) have determined the imide indirectly by back titrating the excess NaOH after the hydrolysis of the imide. This procedure is subject to error since anything which reacts with the alkali may interfere.

The ultraviolet and infrared absorption characteristics of some therapeutic imides, including glutethimide, have been reported by LaGrange and co-workers (8). They also review the results of the various published methods which they applied to the analysis of these imides. These procedures include the hydroxamic acid method, cobalt amine complex colorimetric procedure, hydrolysis of the imide, and the reaction of the imide with silver ion in nonaqueous media to liberate a proton which is titrated with alcoholic NaOH. The latter was reported by Gautier *et al.* (9).

Goldbaum *et al.* (6) found that glutethimide is

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This procedure has been accepted by the N.F. Committee on Revision for adoption as the official N.F. XII assay method. The chromatographic partition was modified somewhat for simplicity, in that the acid and alkaline segments are combined in a single column. Assay results from the modified method were in agreement with those reported here.

extracted quantitatively by chloroform from aqueous solution at any pH, but above pH 10 the imide tends to decompose. Acidic substances may be removed by washing the $CHCl_3$ with dilute alkali; since glutethimide is an acid too weak to form a salt, it remains in the solvent layer. This property of glutethimide permits the removal of acidic and basic interfering materials from the $CHCl_3$ extracts.

Using partition chromatography, acidic and basic substances can be separated conveniently by trapping ionically in the immobile phases of opposite basicity. Examples of the use of this technique are the N.F. XI determination of aspirin, phenacetin, and caffeine tablets (10) and a variety of pharmaceutical analyses which have been reviewed by Levine (11). With proper immobile phases to retain impurities, the pure imide can be isolated; the acid amide degradation product may also be isolated quantitatively.

METHODS

Assay.—*Preparation of Chromatographic Columns.*—Pack a pledget of fine glass wool in the base of a chromatographic tube (25 × 200-mm. test tube to which is fused a 5-cm. length of 6 or 8-mm. tubing) with the aid of a tamping rod of stainless steel, aluminum, or glass at least 12 in. long and having a disk with a diameter about 1 mm. less than that of the chromatographic tube. To 2.0 Gm. of Celite 545 or a comparable grade of infusorial earth in a 100-ml. beaker, add 2.0 ml. of approximately 4 *N* sulfuric acid and incorporate by kneading thoroughly with a flexible spatula blade until the mixture is fluffy. Transfer to a chromatographic tube, and tamp gently to compress the material to a uniform mass. In like manner, mix intimately 2.0 Gm. of Celite 545 with 2.0 ml. of approximately 1 *N* sodium bicarbonate, transfer to a second chromatographic tube, and gently compress with the tamping rod. Mount the columns so that the effluent from the acidic column flows into the bicarbonate column. Wash the columns with 50 ml. of water-saturated ether, followed by 25 ml. of water-saturated chloroform.

Procedure.—Weigh and finely powder not less than 20 glutethimide tablets. Transfer to a 100-ml. volumetric flask an accurately weighed portion of the powder equivalent to about 250 mg. of glutethimide, dilute to volume with water-saturated chloroform, and mix. Transfer 10 ml. of this solution to the top column and elute with 90 ml. of water-saturated chloroform in two equal portions, collecting the eluate in a 100-ml. volumetric flask. Dilute to volume with water-saturated chloroform and mix. Simultaneously determine the absorbance of this solution and of a solution of N.F. glutethimide reference standard in the same medium at a concentration of about 250 mcg./ml., in 1-cm. quartz cells at the maximum at about 257 mμ with a suitable spectrophotometer, using water-saturated chloroform as the blank.

TABLE I.—RESULTS OF THE ASSAY OF GLUTETHIMIDE TABLETS

Sample	No. Determin.	Av. % Recovery	S.D.	Range
Simulated A	12	100.3	0.66	99.4 to 101.4
Simulated B	12	99.6	1.27	98.3 to 102.1
		Av. % Claim		
Commercial[a]	10	100.3	0.97	98.2 to 101.6
Commercial	10	100.3	0.56	99.3 to 101.3

[a] The chloroform eluate was evaporated, then the residue was dissolved in methanol; all other determinations were made on the chloroform eluate directly.

TABLE II.—ASSAY OF SIMULATED TABLET MIXTURES BY FIVE ANALYSTS

Analyst	% Recovery Sample A	Sample B
I	101.1	99.2
	101.5	99.4
II	100.4	100.8
	101.4	101.2
III	99.8	100.4
	100.6	100.6
IV	99.4	99.3
	100.1	99.7
	100.0	99.0
	100.6	100.6
V	101.0	100.4
	101.5	100.2
	100.6	99.6
	100.3	...

Calculate the quantity, in milligrams, of $C_{13}H_{15}NO_2$ in the portion of the tablets taken by the formula $C(A_u/A_s)$ in which C is the exact concentration of the reference standard solution, in micrograms per milliliter; A_u is the absorbance of the sample solution, and A_s is the absorbance of the reference standard solution.

Identification.—Evaporate to dryness a portion of the eluate obtained in the *Assay*. The infrared absorption spectrum of a potassium bromide dispersion of the residue exhibits absorption maxima at the same wavelengths as those of a similar preparation of N.F. glutethimide reference standard.

DISCUSSION

The accuracy and precision obtained in the recovery of glutethimide are shown in Tables I and II. When known amounts of the acid amide alone and in mixtures of equal and double quantities of glutethimide were chromatographed, recovery of each present was quantitative. The acid amide was recovered from the sodium bicarbonate column by eluting it with 1% acetic acid in water-saturated chloroform. The amount present was determined by ultraviolet absorbance.

To obtain the complete spectrum of glutethimide, the chloroform eluate was evaporated and the residue dissolved in anhydrous methanol. The same quantitative results were obtained as those by the direct reading of the chloroform eluate. The latter procedure eliminated the extra steps and also the danger of decomposing the residue while evaporating the chloroform.

The ultraviolet spectra of glutethimide and of 4-ethyl-4-phenylglutaramic acid in methanol are quite similar with the principal maximum due to the

benzenoid chromophore, appearing at 257 mμ. The spectrum of the acid amide (Fig. 1) is defined more sharply with more pronounced definition of both maxima and minima.

The ultraviolet spectra of the two compounds in alkaline methanol are quite different, thus providing ready means of identifying and differentiating them. The following procedure was used to prove the identity of the separated material. About 1.25 mg. of the dried residue obtained by evaporating the eluate was transferred to a 25-ml. volumetric flask with the aid of 15 ml. of methanol. Two milliliters of 0.5 *N* methanolic KOH was added, and the solution was adjusted to volume with methanol. The ultraviolet spectrum was scanned from 220 to 350 mμ, using methanol as the blank. In alkaline methanol the ultraviolet spectrum of the acid amide is essentially the same as in neutral methanol. With the imide there is a shift in the benzenoid chromophore from sharp peaks to shoulders in the 257 and 263 region and the complete disappearance

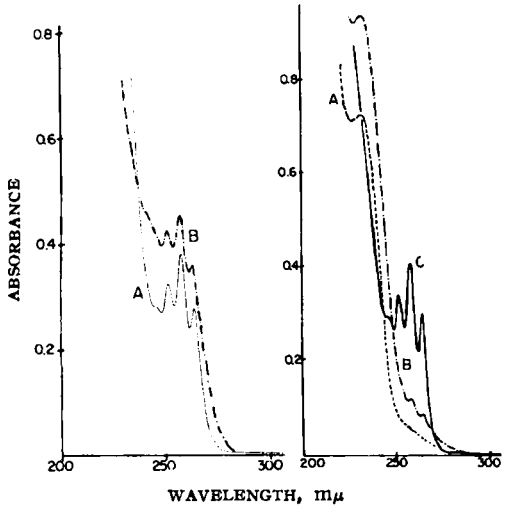


Fig. 1 (left).—Ultraviolet spectra of methanol. Key: *A*, 4-ethyl-4-phenylglutaramic acid, 50 mg./100 ml.; *B*, glutethimide, 25 mg./100 ml. Fig. 2 (right).—Ultraviolet spectra in methanolic potassium hydroxide. Key: *A*, glutarimide, 20 mg./100 ml., N/25 methanolic KOH; *B*, glutethimide, 5 mg./100 ml., N/25 methanolic KOH; *C*, 4-ethyl-4-phenylglutaramic acid, 50 mg./100 ml., N/10 methanolic KOH.

of the maximum at 253 mμ (Fig. 2). For glutethimide, the chromophore most enhanced in alkaline methanol is in the 230 to 235-mμ region. To investigate the assignment of that absorbance, unsubstituted glutarimide was prepared (12) and its spectral properties examined under the same conditions as glutethimide. In alkaline methanol a sharp peak appears in the 230 to 235-mμ region, which changes to a shoulder as the glutarimide is hydrolyzed. β-Alkyl substituted glutarimides have also been shown to exhibit this same spectral property (13, 14). Therefore, this absorbance in the 230 to 235-mμ region is due to the intact glutarimide itself and not due to a shift of the benzenoid chromophore. The absorbance of the glutethimide decreases with time as it is hydrolyzed to the acid

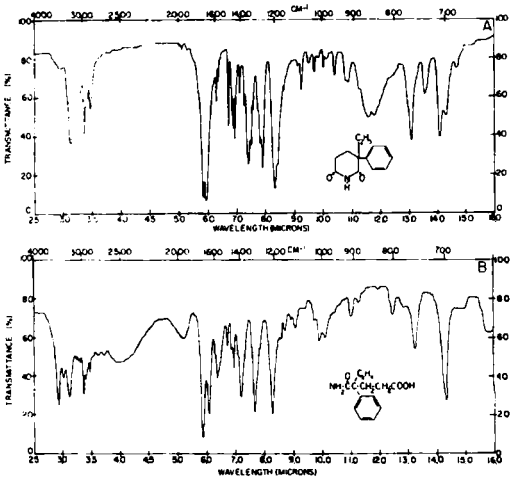


Fig. 3.—Infrared spectra. Key: *A*, glutethimide; *B*, 4-ethyl-4-phenylglutaramic acid.

amide, while the spectrum of the acid amide itself in alkaline methanol remains unchanged for at least 24 hours.

The use of acid washed Celite 545 has often caused problems due to background absorbance of impurities present. The prewashing of the Celite columns removed essentially all the background absorbance. If uncorrected, this background absorbance would cause an error of about 2%.

In the identification test, the infrared spectrum of the residue from evaporation of the eluate in a potassium bromide dispersion was compared to that of a similar preparation of N.F. glutethimide reference standard and was identical.

A similar procedure was used for the identification of any 4-ethyl-4-phenylglutaramic acid which was isolated from the eluate of the bicarbonate column. Its infrared spectrum was compared to that obtained with the acid amide prepared from the hydrolysis of glutethimide reference standard as directed in the N.F. XI identity test B for glutethimide. The infrared spectra are characteristic and suitable for the identification of both glutethimide and the acid amide as illustrated in Fig. 3. The spectra illustrated were obtained with a Perkin-Elmer model 237 spectrophotometer.

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Application of Ultrasound for Increasing Alkaloid Yield from *Datura stramonium*

By A. E. DEMAGGIO and J. A. LOTT*

A new approach is presented for the evaluation of ultrasonic energy when applied to the isolation of alkaloids from *Datura stramonium*. The method utilized made it possible to study the influence of ultrasound on the maceration process as well as during the actual extraction process. The application of ultrasound during short periods of maceration was effective in producing a greater yield of alkaloids than obtainable by conventional procedures. When applied during the process of continuous solvent extraction, ultrasound proved to be slightly more efficient in liberating the desired alkaloids from the drug. The utilization of ultrasound in commercial extraction procedures and for theoretical experimentation is discussed briefly.

A NUMBER OF investigators have demonstrated that ultrasonic energy can be utilized productively in various extraction processes. The application of ultrasound to the extraction of enzymes, nucleic acids, and other biochemically important constituents from unicellular organisms and multicellular tissues is common practice. In addition, ultrasonic energy has been employed for the extraction of oil from peanuts (1) and fish tissue (2), bitter principles from hops (3, 4), poisonous substances from animal tissue homogenates (5) and in studies of immiscible liquid-liquid extraction (6, 7). Results of these studies have supported the expectation that a greater yield of the desired materials would be obtained in a shorter time period using ultrasound than by utilizing conventional extraction procedures alone. Moreover, these selected examples also serve to illustrate the potential application of ultrasound especially for the extraction of desired materials from both plant and animal sources.

In the last decade, several interesting studies have been conducted to determine if the more efficient extractions observed when ultrasound was applied to the diverse systems indicated above could be noted in the extraction of alkaloids from medicinal plants (8–12). To determine the effects of ultrasound on the extraction of alkaloids from *Cinchona succirubra*, Schultz and Klotz (8) employed a high frequency of ultrasound (2.4 Mcy[1]) and a frequency of sound in the audible range (less than 20 Kcy[2]). An improved yield of alkaloids was obtained from samples of drug treated with the audible frequency of sound and not from those samples exposed to high frequency ultrasound. In contrast, Head, Beal, and Lauter (9) employed frequencies of 20 Kcy and 450 Kcy ultrasound to extract cinchona alkaloids and obtained an increased yield of alkaloids from ultrasonically treated samples of the drug. It should be noted that at the higher frequency of ultrasound used by Schultz and Klotz, little gaseous cavitation probably was occurring since the minimum power necessary to initiate cavitation at this frequency would be very great. The majority of ultrasonic effects, *e.g.*, disruption of cells, which would influence extraction processes, generally are attributed to the occurrence of cavitation and the accompanying energy. In view of present knowledge concerning the mechanism of ultrasonic action, it is reasonable to assume that in the absence of cavitation little or no beneficial effect of ultrasound on the extraction of alkaloids would be noted.

During the extraction of alkaloids from *Atropa belladonna*, Wray and Small (10) observed that the insonation of a maceration mixture of solvents and ground drug for 20 minutes produced the same yield of alkaloids as a conventional maceration of 8 hours when both were followed by Soxhlet extraction. Bose, Sen, and Ray (11) have reported that the maceration time for the complete extraction of alkaloids from roots of

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[1] Megacycles per second.
[2] Kilocycles per second.

Rauwolfia serpentina can be reduced from the conventional 8-hour period to 15 minutes when ultrasound at a frequency of 25 Kcy is employed. Similar results have been reported by Colian and Tomas (12), who propose that the total energy accompanying cavitation in an ultrasonic field is considerably superior to mechanical mixing in facilitating extraction.

The present study originally was initiated to determine the influence of two frequencies of ultrasound (20 Kcy and 40 Kcy) on the isolation of alkaloids from *D. stramonium*. However, early in our work the need for characterizing the effect of ultrasonic treatment during the maceration process, opposed to the effect of ultrasonic treatment during the extraction process, became evident. In none of the previously published reports has a clear distinction been made between the maceration and extraction procedures involved in the isolation of alkaloids. The maceration procedure usually consists of the soaking and swelling of the drug in a suitable solvent or mixture of solvents and is followed by the extraction procedure which consists of the physical separation of the alkaloids from the drug, commonly by percolation or continuous extraction in a Soxhlet or similar apparatus. In published reports concerned with ultrasonic extraction, ultrasound has been applied to the macerating mixture and "true" ultrasonic extraction, in the sense of repeated exposure of the drug to fresh supplies of solvent during continuous ultrasonic treatment, has not been performed previously. The construction of a special extraction apparatus made it possible for us to analyze separately ultrasonic treatment when applied during the maceration process or when applied during the extraction process. The experiments reported here were designed to determine to which phase of the isolation procedure ultrasound can be utilized most efficiently for increasing alkaloid yield or decreasing maceration or extraction time.

EXPERIMENTAL

Materials and Equipment.—Commercially available *Datura* leaf, granular (S. B. Penick); U.S.P. grade reagents and solvents; polyethylene wide mouth containers, 300 ml.; Soxhlet extractors; and a newly designed extracting apparatus (13) for ultrasonic extraction were employed. In addition, three commercial ultrasonic generators and cleaning tanks were utilized: 20 Kcy magnetostriction unit (Bendix Aviation Corp., model No. UC-4X8, 140 w.); 40 Kcy piezoelectric unit (National Ultrasonic Corp., Model No. 100, 60 w.); 40 Kcy piezoelectric unit (Circo Ultrasonic Corp., model No. 125T, 125 w.).

Procedure for Ultrasonic Maceration.—The influence of 20 Kcy and 40 Kcy ultrasonic energy when applied during the maceration of the drug was studied employing the three generators previously listed. Ten-gram samples of granular *Datura* leaf were placed in 300-ml. polyethylene containers and 80 ml. of a macerating solvent (ether, 20; U.S.P. alcohol, 12; and ammonia, 8 parts by volume) added. The recommended U.S.P. volume of macerating solvent was increased to cover the drug in the containers completely. The containers were then immersed to the neck in the water of the ultrasonic bath and the drug samples macerated with ultrasonic energy for periods of 30 minutes or 1 hour as indicated in the data. To prevent vaporization of the solvent during ultrasonic treatment, water-cooled reflux condensers were attached to the polyethylene containers, and the water in the ultrasonic bath was maintained 8–10° below the boiling point of ether (34.6°). This was accomplished by pumping the water through coils immersed in a constant temperature bath maintained at 25 ± 2°. After maceration was completed, the samples were transferred to extraction thimbles and extracted for 3 hours in a Soxhlet.

Procedure for Ultrasonic Extraction.—Ultrasonic extraction was studied employing the 20 Kcy Bendix and the 40 Kcy Circo ultrasonic generators. The 40 Kcy National ultrasonic generator was excluded from this phase of the investigation since results of the maceration studies showed it to be less efficient than its counterpart, the Circo unit. Both the National and Circo generators are piezoelectric units.

Maceration of the drug was performed in essentially the same manner indicated in the preceding section. However, in this phase of the study, ultrasound was not employed in macerating the samples. Samples of the drug were macerated by the conventional method for either a varied period of time or for a fixed period, depending on the design of the experiment. Following maceration, all samples were extracted in the newly designed extractor, while continually subjected to ultrasonic energy for various time periods.

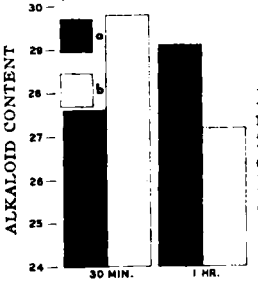


Fig. 1.—Results of 20 Kcy (Bendix) ultrasonic maceration of the drug, followed by 3 hours of conventional extraction. Key: *a*, control samples; *b*, insonated samples.

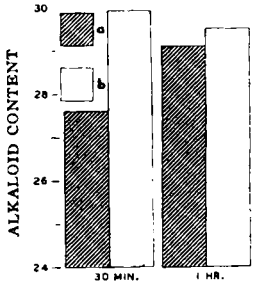


Fig. 2.—Results of 40 Kcy (National) ultrasonic maceration of the drug, followed by 3 hours of conventional extraction. Key: *a*, control samples; *b*, insonated samples.

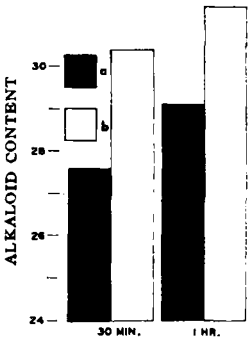


Fig. 3.—Results of 40 Kcy (Circo) ultrasonic maceration of the drug, followed by 3 hours of conventional extraction. Key: *a*, control samples; *b*, insonated samples.

Assay.—Samples were assayed for alkaloid content according to the U.S.P. XVI assay for belladonna leaf. Experiments were performed in triplicate; the assay results were averaged and expressed as milligrams of alkaloid per 10-Gm. sample of treated drug.

Results of assaying the maceration mixture for total alkaloids prior to Soxhlet extraction are not included in this report. Preliminary studies have shown that it is more satisfactory to compare results from maceration and extraction studies when similar treatments are employed—in this case, a period of continuous solvent extraction is included as a routine part of both procedures.

RESULTS AND DISCUSSION

Ultrasonic Maceration.—For the initial studies, the influence of ultrasound on the macerating mixture was observed for intervals of 30 minutes and 1 hour. Using the 20 Kcy Bendix generator, the alkaloid content of samples insonated for 30 minutes was about 9% greater than that of control or non-insonated samples (Fig. 1). When the insonation time was doubled, no increase in alkaloid yield was noted; the yield from the control samples, in this case, was slightly greater than the yield from insonated samples. A similar situation did not exist, however, in the maceration studies using the two 40 Kcy ultrasonic generators (Figs. 2 and 3).

With these 40-Kcy units there was a consistent increase in alkaloid yield when samples were macerated with ultrasound. Figure 2 shows the results obtained when samples were macerated 30 minutes and 1 hour with ultrasonic energy supplied by the National unit. A 9% increase in the yield of alkaloids was obtained when samples were exposed to ultrasound for 30 minutes, while a smaller increase (approximately 3%) was noted for samples macerated during 1 hour. Using the Circo ultrasonic generator (Fig. 3), a 10% increase in alkaloid yield was observed for samples macerated with ultrasound for 30 minutes. When samples were macerated for 1 hour with ultrasound, the increased yield of alkaloids was approximately 9% compared to control samples.

Conventional maceration without ultrasound when carried out for 1 hour was effective in causing an increase of 3% in alkaloid yield compared to samples macerated for only 30 minutes. It is interesting to note that only with the 40 Kcy Circo ultrasonic generator did samples macerated for 1 hour yield greater quantities of alkaloids than samples macerated for 30 minutes. An increase of 4% in total alkaloids was noted when samples insonated with the Circo unit for 30 minutes and 1 hour were compared. While no reason is readily apparent to account for the increase in alkaloids obtained using one ultrasonic generator and not the other two, there is some indication that after prolonged maceration, the difference in yield of alkaloids from insonated and control samples diminishes. This difference cannot be attributed to variations in the power output of the ultrasonic generators used.

From the preceding data, it is evident that the difference in the amount of alkaloids recovered between control and insonated samples is greatest during the first 30 minutes of ultrasonic maceration. Moreover, these data indicate that the 40 Kcy Circo generator is more effective than the other units employed in releasing the alkaloids from the drug during the short maceration periods used.

Short periods of ultrasonic maceration also have been effective in liberating the desired alkaloids from cinchona, belladonna, and rauwolfia. Head, Beal, and Lauter (9), employing 15 minutes of ultrasonic maceration, noted considerable increase in alkaloid yield from *C. succirubra*. A similar period of ultrasonic maceration was beneficial in extracting alkaloids from *R. serpentina* (11); while Wray and Small (10) macerated *A. belladonna* leaf with ultrasound for periods of 5 to 20 minutes and found 20-minute maceration times most effective. The similarity of results from these investigations, utilizing different ultrasonic generators and different species of medicinal plants, demonstrates that the effect of ultrasound is beneficial for increasing alkaloid yields when employed in conjunction with short periods (less than 1 hour) of maceration.

Ultrasonic Extraction.—The extraction studies to be described here differ fundamentally from previous reports concerned with ultrasonic extraction because a distinct separation of the maceration process from the actual extraction process has been achieved. For the present work, an extraction apparatus was utilized which made it possible to subject the drug sample to ultrasonic energy while, at the same time, performing continuous extraction of the liberated alkaloids. From previous data obtained during the maceration studies, it appears that a 10% increase in alkaloid yield is the maximum which can be obtained utilizing ultrasound in the manner described. The present series of experiments were undertaken to determine whether a greater yield of alkaloids could be obtained when ultrasound was applied during the extraction process rather than when applied during the maceration process.

In the first extraction experiment, the drug was extracted with ultrasound supplied by the 20 Kcy Bendix unit. To determine the interaction of maceration time and extraction time, experiments were conducted in which the drug was macerated for various time intervals (2 to 24 hours) and extracted for a fixed period of 2 hours. The 2-hour extraction period was chosen after preliminary results revealed that the maximum effect of ultrasound could be best observed during this time. A synopsis of the results is presented in Fig. 4.

A 10% increase in alkaloid yield was obtained from samples of drug macerated conventionally

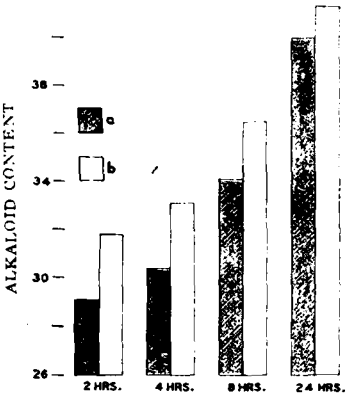


Fig. 4.—Results of 20 Kcy (Bendix) ultrasonic extraction of the drug for 2 hours, following various periods of conventional maceration. Key: *a*, control samples; *b*, insonated samples.

for 2 hours and for 4 hours and extracted with ultrasound for 2 hours. As the maceration time was increased, the difference in alkaloid content between control and insonated samples diminished; only a 3% increase was noted in samples macerated for 24 hours. These data suggest that ultrasonic extraction is most efficient when employed in conjunction with short periods of conventional maceration. However, this effect possibly could be due to further liberation of the alkaloids, such as that which occurs during maceration, rather than a direct effect of ultrasound on extraction. Therefore, a somewhat different approach was utilized in evaluating the influence of 40 Kcy ultrasonic energy.

In this case, the macerating time was fixed at 24 hours to insure complete liberation of the alkaloids, and the drug was extracted with the 40 Kcy Circo ultrasonic generator for 1 hour or for 3 hours (Fig. 5). Samples of drug extracted with ultrasound for 1 hour displayed a 15% increase in alkaloid yield when compared with control samples. This marked increase was not observed in samples extracted with ultrasound for 3 hours. In these samples little difference in yield was noted between insonated and control samples.

When a comparison is made between samples of the drug continually macerated for 24 hours and extracted for 3 hours with the 20 Kcy or 40 Kcy generator, the yield of alkaloids is greater with the 20 Kcy unit (Fig. 6). Assuming that complete liberation of the alkaloids had occurred during the 24-hour maceration period, it appears that the lower

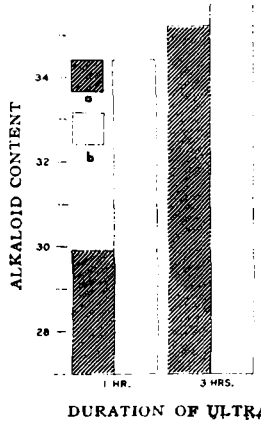


Fig. 5.—Results of 40 Kcy (Circo) ultrasonic extraction of the drug following a 24-hour conventional maceration period. Key: *a*, control samples; *b*, insonated samples.

frequency of ultrasound is more effective than the higher frequency during the extraction process.

It should be mentioned that complete exhaustion of the drug was not of primary concern during this investigation. Rather, the intent was to show the effect produced by ultrasound. This explains the relatively low yield of alkaloids obtained in certain studies. One should note also that ultrasonic action causes no detectable degradation of the active alkaloids (9, 10).

Comparisons between this study and similar but very limited work on alkaloid extraction reported in the literature are difficult to make. A great variety of ultrasonic generators, ranging from ultrasonic diathermy machines (8) to oil immersed piezoelectric crystals (9) and magnetostriction generators, have been used. However, one major similarity is evident in the increased yield of alkaloids obtained from drug samples ultrasonically treated for short periods of time (9, 10).

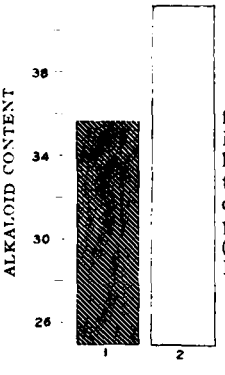


Fig. 6.—Comparative efficiency of 20 Kcy and 40 Kcy generators during 3 hours of ultrasonic extraction, following a 24-hour conventional maceration period. Key: *1*, 40-Kcy (Circo) generator; *2*, 20-Kcy (Bendix) generator.

CONCLUSIONS AND SUMMARY

As shown in the previous section, the effects of ultrasound on the liberation of alkaloids during maceration are greatest in the initial 30-minute maceration period. To explain this phenomenon, one might consider that in addition to the alkaloids present in the cell as salts of organic acids, some alkaloids may be present bound to essential cell constituents or precursors (14). The mechanism of the ultrasonic effect on maceration, then, would be to liberate the alkaloids and hasten their diffusion through the cell wall into the extracting solvent. In the absence of ultrasound, liberation of the bound alkaloids and their diffusion also occurs, but at a much slower rate. Thus, the action of ultrasonic energy would be to accelerate this rate controlling diffusional process.

If the maceration period is adequate to allow the slow diffusional process to go to completion (as in the U.S.P. overnight maceration), little difference in alkaloid yield between insonated and noninsonated samples would be observed. For this reason, it would be pointless and impractical to lengthen insonation periods if no effect of the applied ultrasound is noted during a short maceration time.

During maceration of the drug, diffusion of the alkaloids occurs as has been described. It is then necessary to separate physically the alkaloids from the drug. Ordinarily, this is accomplished during Soxhlet extraction where the residue is repeatedly

exposed to the solvent. During ultrasonic extraction, this physical separation is further enhanced by the localized stirring occurring as a consequence of cavitation. The combination of this stirring effect and the repeated washing of the drug with solvent is far superior to the simple washing procedure in Soxhlet extraction. Again, the effect of ultrasound is most noticeable during short-term extractions. For if the drug is washed often enough in the conventional procedure, all of the alkaloids eventually will be separated from the residue.

These results lead us to propose that at least two different mechanisms may be involved in the liberation of alkaloids during the isolation procedure. One of these mechanisms, occurring during the process of maceration, is stimulated or enhanced by the higher frequencies of ultrasonic energy. The other mechanism, active during the extraction process, is promoted by low frequencies of ultrasonic energy. Although the evidence supporting this hypothesis is only indirect, the experiments reported here have clearly demonstrated that it is possible to evaluate critically the effect of ultrasound on *both* phases of the procedure involved in alkaloid isolation—maceration and extraction. Furthermore, these exploratory studies indicate that the utilization of ultrasonic energy during the isolation procedure may yield valuable information concerning the exact mechanisms involved in the liberation of medicinal products from natural sources.

Ultrasonic energy definitely has utility in the extraction of alkaloid containing plants, providing that the ultrasound is of sufficient intensity and is correctly applied. Ultrasonic energy shortens considerably both the duration of the maceration and extraction process while simultaneously yielding extracts containing greater quantities of alkaloid than can be obtained by conventional procedures. Although quantitative differences in alkaloid yield have been noted, our experience has been that the maceration process lends itself most readily to the application of ultrasound. We suggest that ultrasonic treatment on a commercial scale could be utilized profitably and simply by applying ultrasound to the macerating mixture.

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Development of a Simple Automated Film-Coating Procedure

By DHIRAJ S. MODY, MORTON W. SCOTT*, and HERBERT A. LIEBERMAN

Information obtained in a series of manually controlled coating experiments was applied in developing a simple automated procedure for film-coating tablets. The equipment consists of a group of interconnected timers which signal a solenoid valve controlling the hydraulic atomization of the coating solution. The film compositions were based on mixtures of hydroxypropyl methylcellulose and ethylcellulose and were applied to tablets automatically in both 18 and 42-in. coating pans. The apparatus and coating procedure offer advantages in both research and production film-coating operations.

FILM-COATED TABLETS have gained increasing acceptance in the pharmaceutical industry; a growing number of tablet products coated in this way have reached the market. Numerous reports describing new and useful film-coating compositions have appeared in both the scientific and patent literature (1–5). The advantages of the film-coating technique include (among others) speed and convenience of operation and good control over tablet-to-tablet uniformity. A general review of the coating procedure has appeared in the reports by Wagner (6) and by Gross and Endicott (7). Recently, Lachman described a procedure for automating the film-coating operation (8). This technique was based on the use of punched-tape electronic instrumentation and may be overspecialized for general applications.

The present report describes the development of a simplified, inexpensive procedure for automatic film coating.

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EXPERIMENTAL

Materials.—The coating agents used in this study were based on mixtures of hydroxypropyl methylcellulose and ethylcellulose (9, 10) as shown in Table I.

Coating solutions were prepared by dissolving the

exposed to the solvent. During ultrasonic extraction, this physical separation is further enhanced by the localized stirring occurring as a consequence of cavitation. The combination of this stirring effect and the repeated washing of the drug with solvent is far superior to the simple washing procedure in Soxhlet extraction. Again, the effect of ultrasound is most noticeable during short-term extractions. For if the drug is washed often enough in the conventional procedure, all of the alkaloids eventually will be separated from the residue.

These results lead us to propose that at least two different mechanisms may be involved in the liberation of alkaloids during the isolation procedure. One of these mechanisms, occurring during the process of maceration, is stimulated or enhanced by the higher frequencies of ultrasonic energy. The other mechanism, active during the extraction process, is promoted by low frequencies of ultrasonic energy. Although the evidence supporting this hypothesis is only indirect, the experiments reported here have clearly demonstrated that it is possible to evaluate critically the effect of ultrasound on *both* phases of the procedure involved in alkaloid isolation—maceration and extraction. Furthermore, these exploratory studies indicate that the utilization of ultrasonic energy during the isolation procedure may yield valuable information concerning the exact mechanisms involved in the liberation of medicinal products from natural sources.

Ultrasonic energy definitely has utility in the extraction of alkaloid containing plants, providing that the ultrasound is of sufficient intensity and is correctly applied. Ultrasonic energy shortens considerably both the duration of the maceration and extraction process while simultaneously yielding extracts containing greater quantities of alkaloid than can be obtained by conventional procedures. Although quantitative differences in alkaloid yield have been noted, our experience has been that the maceration process lends itself most readily to the application of ultrasound. We suggest that ultrasonic treatment on a commercial scale could be utilized profitably and simply by applying ultrasound to the macerating mixture.

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Development of a Simple Automated Film-Coating Procedure

By DHIRAJ S. MODY, MORTON W. SCOTT*, and HERBERT A. LIEBERMAN

Information obtained in a series of manually controlled coating experiments was applied in developing a simple automated procedure for film-coating tablets. The equipment consists of a group of interconnected timers which signal a solenoid valve controlling the hydraulic atomization of the coating solution. The film compositions were based on mixtures of hydroxypropyl methylcellulose and ethylcellulose and were applied to tablets automatically in both 18 and 42-in. coating pans. The apparatus and coating procedure offer advantages in both research and production film-coating operations.

FILM-COATED TABLETS have gained increasing acceptance in the pharmaceutical industry; a growing number of tablet products coated in this way have reached the market. Numerous reports describing new and useful film-coating compositions have appeared in both the scientific and patent literature (1–5). The advantages of the film-coating technique include (among others) speed and convenience of operation and good control over tablet-to-tablet uniformity. A general review of the coating procedure has appeared in the reports by Wagner (6) and by Gross and Endicott (7). Recently, Lachman described a procedure for automating the film-coating operation (8). This technique was based on the use of punched-tape electronic instrumentation and may be overspecialized for general applications.

The present report describes the development of a simplified, inexpensive procedure for automatic film coating.

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EXPERIMENTAL

Materials.—The coating agents used in this study were based on mixtures of hydroxypropyl methylcellulose and ethylcellulose (9, 10) as shown in Table I.

Coating solutions were prepared by dissolving the

TABLE I.—FILM-COATING COMPOSITION

Ingredients	% w/v
Hydroxypropyl methylcellulose,[a] 60 HG, 50 cps.	3.75
Ethylcellulose N.F., 10 cps.	1.25
Propylene glycol U.S.P.	0.75
Titanium dioxide U.S.P.	1.98
Cab-o-sil,[b] M-5	0.02
Chloroform U.S.P.	
Isopropyl alcohol U.S.P.	
Equal parts by volume	*q.s.*

[a] Marketed as Methocel HG by the Dow Chemical Co., Midland, Mich. [b] Marketed by Cabot Corp., Boston, Mass.

film formers and glycol in a portion of the mixed solvent. A suspension of the pigment materials in a second portion of the solvent then was added. The mixtures were agitated with an Eppenbach homomixer to insure good dispersion.

Colors were included in the composition by substituting lakes and pigments for part of the titanium dioxide. Lakes of FD&C Blue No. 1, Green No. 1, Red No. 2, Red No. 3, and Yellow No. 5 were employed. Pigment materials included black and brown iron oxide and chrome green.[1] Generally, the colorant was added at a concentration of 0.2 to 0.4 w/v %.

A variety of tablet cores was used in the present experiments. Placebo tablets were formulated with spray-dried lactose and generally were compressed using $^{11}/_{32}$-in. standard concave punches. Deep concave, standard concave, square, and capsule-shaped tablets containing one or more active ingredients also were coated.

Evaluation Techniques.—Film-coated tablets were evaluated by visual inspection of film smoothness, uniformity of color, edge coverage, luster, and tablet to-tablet uniformity. The disintegration time of the film on the tablets was measured in the U.S.P. disintegration apparatus using simulated gastric fluid U.S.P. The end point was taken when the first distinct signs of film disintegration, rupture, or separation were evident.

Stability observations were obtained after storage at room temperature, 37 and 45° for various time intervals.

Hydraulic Coating Equipment.—The hydraulic coating system developed for these studies is shown schematically in Fig. 1. Basically, the system consisted of an air-driven hydraulic pump (paint sprayer type), a hand-operated spray gun, baffled coating pans, and auxiliary duct work for supply of warm air, cool air, and exhaust to the pans. Three different sized coating pans including 18, 28, and 42-in. units were used. The 18-in. pan was rotated at 30 r.p.m. The speed of the other pans was adjusted to 15 r.p.m.

The spray gun was held just within the coating pan approximately 12 in. from the surface of the (rotating) tablet bed. The air pressure to the hydraulic pump was maintained at 70 to 75 psig in all experiments.

For runs in the 18-in. pan, the batch size was approximately 30,000 tablets. Batch sizes of 75,000 and 300,000 tablets were coated in the 28 and 42-in. pans, respectively. These pan loads varied slightly with the size and shape of the tablets.

Manual Coating Procedure.—The coating procedure was based on an intermittent spraying and drying technique. The tablets were sprayed for a short interval (less than 5 seconds), then tumbled for several seconds. This "spray-on–spray-off" cycle was continued for several minutes. Cool air at ambient temperature then was admitted to the rotating pan for 5 minutes. This was followed by a 5-minute drying interval with warm air (approximately 50°). The "spray-on–spray-off" cycle then was started again and the total sequence of steps continued until the desired film weight had been applied. The run was terminated when an increase in tablet weight of 2.0% was obtained.

Coated tablets were allowed to dry completely for 8 to 12 hours in an oven at 110° F. and finished by standard polishing procedures.

Under manual operation of the equipment, the duration of the various time cycles was adjusted occasionally at the discretion of the tablet coater. For automating the film-coating operation, standardized spraying and drying cycles were established. These were accomplished by measuring the time intervals employed for each step of the process carefully in a series of successful, operator-controlled coating runs. Replicate trials in which each selected time interval was duplicated (and monitored by an observer, in addition to the tablet coater) then were conducted. The results of these experiments showed that film-coated tablets with reproducible characteristics were obtainable in separate coating runs when spraying and drying cycles were duplicated. The time cycles developed for several different tablet batch sizes are listed in Table II.

Automated Coating Procedure.—The hydraulic coating operation was automated by including a solenoid valve in the system to control the flow of the coating solution and by introducing a group of interconnected timers to control the various time cycles. The automated equipment is illustrated schematically in Fig. 2 which shows the relationship between the timer components, the solenoid valve, and the auxiliary units. The timer panel was designed to control the cool air and warm air blowers, the start and stop of the coating pan, the total coating time, and the spray-on, spray-off, and spray-repeat cycle time. The panel included stand-by switches for manual control of each function and appropriate signal lights.

The automated coating system was put in operation by closing a master power switch on the timer panel. This activated the spray-on and spray-repeat timers. The spray-on timer energized the solenoid valve for a given preset interval. The solenoid, in turn, actuated a needle control valve on the spray gun allowing flow and atomization of the coating solution. The solenoid valve was de-energized at

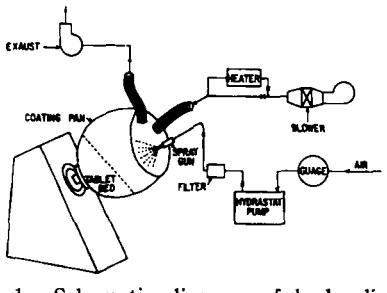


Fig. 1.—Schematic diagram of hydraulic coating system. Ten gallon hydrastat spray unit (24:1 pressure ratio), supplied by Alemite of New Jersey, Inc., Union, N. J.

[1] Lo-Micron pigments supplied by Whittaker, Clark, and Daniels, Inc., New York, N. Y.

TABLE II.—SPRAYING AND DRYING CYCLES FOR VARIOUS BATCH SIZES COATED BY MANUAL OPERATION

Batch Size, Tablets	Coating Pan, in.	Spray-On,[a] Sec.	Spray-Off,[a] Sec.	Spray-Repeat, Min.	Cool Air, Min.	Warm Air, Min.	Total Coating Time, Hr.
30,000	18	1	9	5	5	5	3 to 3.5
75,000	28	2	8	5	5	5	3.5 to 4
300,000	42	4	6	5	5	5	5.5 to 6

[a] Spray-on and spray-off operations alternated back and forth until the time period indicated in the column marked *Spray Repeat* was completed.

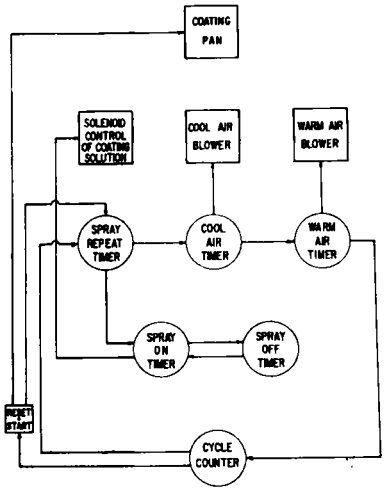


Fig. 2.—Schematic diagram of control system for automated coating operation. Solenoid valve controls the flow of the coating solution by actuating a needle valve on the spray gun (three-way solenoid valve-115 v./60c/AC and No. 22AUHPSSTC Auto-jet with 0.011-in., 40° angle nozzle). The cycle counter normally energizes the spray-repeat timer. On the last count, the counter ends the operation through the reset starter switch.

TABLE III.—TIMER PANEL SPECIFICATIONS[a]

Components	Dial Range	Dial Divisions
Spray-on timer	10 sec.	1/6 sec.
Spray-off timer	30 sec.	1 sec.
Spray-repeat timer	60 min.	1 min.
Cool air timer	60 min.	1 min.
Warm air timer	60 min.	1 min.
Cycle counter	80 counts	1 count

[a] Panel constructed by Ribble Engineering Co., Hackensack, N. J.

the end of the time period set on the spray-on timer. At this point, the spray-off timer was energized and continued to run for a preselected time interval. At the completion of this period, the spray-off timer reactivated the spray-on timer. These components alternated their operation in this manner until the time interval programmed on the spray-repeat timer was complete. The cool air timer controlling the cool air supply blower then was activated for a preset time period. At the completion of this step, the warm air timer was energized, which in turn started the flow of warm air to the pan. When the time interval set on this component was complete, the timer signalled the cycle counter, and one count was registered. The entire sequence of steps then was repeated until the total number of cycles preset on the counter had been completed. At this point the counter signalled a reset switch, and the pan and all other operations were stopped automatically.

The specifications for the timer components utilized in the automated system are listed in Table III.

Automated film-coating runs were conducted in the 18 and 42-in. coating pans. For these studies, the spray gun was held in a fixed position by suitable clamps and stand arrangement. Two spray guns were used for studies in the 42-in. pan. In these cases, the guns were positioned so that their spray patterns did not overlap. All time cycles and operating conditions reproduced those developed for manual coating as listed in Table II.[2]

RESULTS AND DISCUSSION

The film-coating solution could not be applied successfully by pouring or pneumatic spray techniques. In the latter case, the difficulties were believed to result from the premature evaporation of solvent at the nozzle. This led, in turn, to the deposition of pigment agglomerates on the tablet and "pimpled" surfaces. Application of the coating solution by pouring procedures led to mottled, uneven film coats, presumably caused by excessive film tackiness during the dry down phase. These difficulties were corrected with the hydraulic coating system.

In the present series of experiments over 1,500,000 tablets constituting 30 individual batches were coated with the hydraulic system operating under manual control. The results of these studies were used to demonstrate the flexibility of the coating solution, the reliability of the equipment, and the reproducibility obtained when duplicate time cycles were employed. In all cases, smooth and elegant film coats were obtained. Coated tablets showed complete edge coverage and high tablet-to-tablet and batch-to-batch uniformity. These qualities were evident particularly when colored film compositions were applied to white-core tablets.

No difficulties were experienced in scaling up the system for operation in the 42-in. coating pans. These experiments generally were limited, however, to tablets with a hardness of 10 Strong Cobb units or more and friability values of less than 0.2% (measured with the Roche Friabilator). At present, it is not known whether the described system has utility with softer or more friable tablets.

The application of a film thickness equal in weight to approximately 2% of the tablet core weight generally was sufficient to obtain good tablet coverage. At this thickness, the disintegration time of the film was 5 to 10 minutes. These disintegration rates did not change materially after aging the tablets at room and elevated temperatures for extended periods. No significant changes in other film characteristics were noted over these storage intervals. In two cases, however, some fading of the film color was observed. Stability results are summarized in Table IV for several representative products.

[2] To compensate for the increased flow rate obtained with the use of two spray guns with the 42-in. pan, the spray-on and spray-off cycles were changed from those established under manual operation (4 seconds on, 6 seconds off) to 2.5 seconds on and 9 seconds off.

TABLE IV.—STABILITY DATA FOR FILM-COATED TABLETS STORED AT ROOM TEMPERATURE FOR 1 YEAR

Product	Size, in.	Shape[a]	Pigment Compn.	Color		Luster		Disintegration Time of Film, Min.	
				Original	1 Yr.	Original	1 Yr.	Original	1 Yr.
A	11/32	S.C.	100 parts titanium dioxide	White	N.C.[b]	G	N.C.	5	5
B (Placebo)	11/32	S.C.	80 parts TiO_2 + 20 parts FD&C Red No. 3 Lake	Pink	S.F.	G	N.C.	5–7	5–7
C (Placebo)	11/32	S.C.	80 parts TiO_2 + 20 parts FD&C Blue No. 1 Lake	Light blue	S.F.	G	N.C.	5–7	5 7
D (Placebo)	11/32	S.C.	80 parts TiO_2 + 20 parts oxide brown	Brown	N.C.	G	N.C.	5–7	5–7
E	5/16 × 5/16	P	28 parts TiO_2 + 20 parts D&C Red No. 3 Lake + 28 parts black + 24 parts oxide brown	Reddish brown	N.C.	G	N.C.	5	5
F	10/32 × 7/8	C	40 parts TiO_2 + 60 parts FD&C Yellow No. 5 Lake	Yellow	N.C.	G	N.C.	7–10	7–10
A[c]	11/32	S.C.	100 parts titanium dioxide	White	N.C.	G	N.C.	5	5
A[d]	11/32	S.C.	100 parts titanium dioxide	White	N.C.	G	N.C.	5	5

[a] S.C., Standard concave; P, pillow-shaped; C, capsule-shaped. [b] N.C., No change; S.F., some fading; G, good. [c] Product *A* coated by automated procedure in 18-in. pan (30,000 tablets). [d] Product *A* coated by automated procedure in 42-in. pan (300,000 tablets).

Automated coating experiments were conducted in the 18 and 42-in. coating pans. Since the operating conditions required for successful coating had been previously established in an extensive number of manual runs, only limited experiments were required to develop and confirm the automated system. Six runs were undertaken and completed successfully. The results showed that tablets coated by the automatic procedure were equivalent in terms of edge coverage, luster, and disintegration time to the film-coated tablets prepared by the manual technique.

The automated film-coating system developed in this study was relatively inexpensive to assemble and simple to operate. Only four tasks were left to the tablet coater. These included (*a*) the loading of tablet cores into the pan, (*b*) the charging of coating solution to the pump, (*c*) the pressing of the start button on the timer panel, and (*d*) the unloading of finished (unwaxed) tablets. It was useful, however, to wipe the tip of the nozzle with solvent from time to time to remove any dried film which may have accumulated at the nozzle orifice. This in no way complicated the coating operation and could be completed during the routine inspections of the operation.

The capacity of the automated system is sufficient to control the coating operation in four or more individual coating pans, provided the same time cycles and film compositions are used in each. The wide range of time settings which can be programmed on the timer panel adds considerable flexibility. Although the present results are limited to the use of one basic film composition, the available flexibility suggests that the total system will have utility with other film-coating agents. Since very close control can be exerted over the spraying and drying cycles by the introduction of the automatic time controls, standardized coating conditions from batch to batch can be maintained readily.

The automated apparatus appears to have additional utility as a research tool in developing new film-coating compositions. This advantage arises because the unit can be set to give exact and reproducible coating cycles, thereby removing the influence of operator variables. For example, it was shown in one experiment that an increase of only one quarter of a second in the spray-on time resulted in overwet tablets which required immediate drying before they could be processed further. The control of the nozzle operation within such narrow time limits is virtually impossible under manual operation, but is readily achieved with the automated equipment.

SUMMARY AND CONCLUSIONS

The film composition used in the present experiments has utility in coating a broad variety of tablet shapes and sizes. The basic film composition was easily colored by the addition of suitable lakes and pigments and showed good disintegration and stability characteristics. A hydraulic atomization technique was used in applying the film coat. The introduction of timer components to control the various cycles resulted in a simple and inexpensive method of automating the coating procedure.

Batch loads of approximately 300,000 tablets were coated automatically in a 42-in. coating pan. The total coating time was 5.5 to 6 hours. For approximately two-thirds of this time (4 hours), the tablets were undergoing drying, and no additional film coats were being applied. Although these time cycles were established on the basis of manual operation, the results of the automated experiments suggested that the drying intervals could be reduced significantly. This would lead to proportional reductions in the total coating time. The automated coating equipment provides a convenient tool for exploring these opportunities, and experiments are presently planned in these directions.

The automated system offers further advantages in improving batch-to-batch uniformity and in reducing production labor charges. The system also has particular utility in research studies with new film compositions.

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Wurster Coated Aspirin I

Film-Coating Techniques

By VINCENT COLETTA and HOWARD RUBIN*

A method of coating aspirin crystals of various mesh sizes with mixtures of ethylcellulose and methylcellulose using the WARF (Wurster) air suspension apparatus is described. The results of varying the ratio of ethylcellulose to methylcellulose in the coating film have been explored. Rapidly disintegrating tablets of the coated crystals were prepared, and solubility studies were run. These showed excellent sustaining ability. Definite correlation between the solubility rate of aspirin and the ethylcellulose to methylcellulose ratio in the coating was found.

As PART OF the program of the study of dosage forms suitable for proprietary use, it was of interest to study release characteristics of crystalline drugs coated in the Wurster apparatus (1) to yield delayed release of these drugs. This paper deals with the coating of crystalline aspirin and its incorporation into rapidly disintegrating tablets. A companion paper correlates *in vitro* and *in vivo* release of certain of these preparations (2).

The Wurster apparatus was chosen because of its versatility. The coating process is not limited by particle size of the drug, the coating materials, or the solvent system; the machine has been documented as to its usefulness in applying coatings for the purpose of controlling drug release (1, 3). The 6-in. pilot model has a capacity of 0.5 to 4 Kg., and coating can be completed in 30 to 50 minutes. This chamber easily supplies quantities of material necessary for product development requirements.

The need for pH independent coatings suggested the use of combinations of ethylcellulose and methylcellulose (4), well known cellulose polymers, in different ratios as possible coating materials. It remained then to determine optimum particle size of the drug, applicable ratios of the two cellulose materials, percentage film coat, rate of application of the solubilized materials, and the particular machine settings to yield the most even continuous film coat.

EXPERIMENTAL

A typical data sheet with all operating conditions is illustrated in Table I. A soluble dye was added to the cellulose gum dispersions as a visual aid in the coating process. A large (1 gal.) Waring Blendor was used to disperse the gums. The spray run was completed in 30 minutes. By allowing the outlet temperature of the apparatus to climb to within a few degrees of the inlet temperature, there was assurance that all the solvent was evaporated off the granules. Agglomeration was prevented by adjusting air and liquid flow (5). The resultant products consisted mainly of individual crystals with a continuous coating of the polymers.

The conditions entered in Table I were standardized and used throughout the experiments. The solvent system, formulation, concentrations of plasticizer, and total cellulose polymers remained constant. In all cases, fast disintegrating tablets containing the coated aspirin—83%, cornstarch—15%, and talc—2% were prepared using $^{7}/_{16}$ in. S.C. punches. The tablets contained 325 mg. of acetylsalicylic acid. Disintegration time was less than 1 minute. *In vitro* solubility studies were run on all granules and tablets. The shells remaining after solubility studies were examined microscopically to determine degree of fracture of the coating and whether any aspirin remained in the intact shells.

Initial studies were done with 20-mesh aspirin (20–40 mesh) and 40-mesh aspirin (all pass 40 mesh). A 2.7% coating was applied to the 20-mesh material, and a 4.8% coating was applied to the 40-mesh material. The finer material required additional coating to yield approximately the same final film thickness.

Twenty-mesh aspirin crystals were selected for additional coating work; 6% coatings were applied to this material, starting with concentration of 50/50 ethylcellulose/methylcellulose with gradations to 100% ethylcellulose and 0% methylcellulose.

RESULTS AND DISCUSSION

In the first study (Table II) 20-mesh aspirin using 75% ethyl, 25% methylcellulose was the slowest, though not slow enough. It was decided to concentrate on the largest aspirin size (20 mesh) and increase the concentration of the coating material. The second series was started to determine the optimum ratio of ethylcellulose to methylcellulose.

Duplicate solubility runs indicate granulations, and tablets showed good reproducibility within the batch (2). Solubilities for duplicate coating and tablet preparations are shown in Table III. It is apparent that excellent replication was achieved in the final tablet. The accuracy of duplication from batch to batch depends only on the ability to follow through on the operating procedures of the apparatus. The Wurster apparatus is the ideal equipment for research quantities of coated drugs. The

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TABLE I.—WURSTER COATING AND GRANULATING PROCESS

Formula No.	133	*Date:*	10/17/61
Material Coated:	20–40 mesh aspirin crystals	*Quantity:*	2 Kg.
Coating:	50/50 ethylcellulose methylcellulose	*Quantity:*	120 Gm.
Coating, %:	6% based on wt. of aspirin 5.66% of total wt.		

Formula of Coating Solution:	*Quantity*
1) Methylene chloride	1800 ml.
2) Isopropyl alcohol	1800 ml.
3) Glycerin	24 ml.
4) Ethylcellulose N 10 (10 cps)	60 Gm.
5) Methylcellulose 50 Hg (50 cps)	50 Gm.
6) FD&C Green No. 3	50 mg.

Initial Coating Solution Temp.: 130° F. *Mixing Time:* 3 min.
Max. Air Flow: 96.0 c.f.m. *Recommended Air Flow:* 78.5 c.f.m.
Fluid Nozzle: 2050 *Air Nozzle:* No. 64
Atomization Pressure: 45 psig. *Coating Reservoir Pressure:* 15 psig.
Inlet Fluid Pressure: 5–9 psig. *Inlet Temp. Setting:* 125° F.
Inlet Temp. Range: 115–130° F. *Exhaust Temp. Range:* 90–110° F.
Yield: 98% *Humidity:* 45% R.H.

Remarks: Formula No. 134 tablets prepared with the coated aspirin. No agglomeration of crystals.

TABLE II.—SOLUBILITY OF ASPIRIN TABLETS

	Per Cent Dissolved (as a Function of Time— 4-hr. Recovery)			
	2.7% Coating—20 Mesh (20–40 Mesh)		4.8% Coating—40 Mesh (Pass 40 Mesh)	
	25 Ethyl 75 Methyl	75 Ethyl 25 Methyl	25 Ethyl 75 Methyl	75 Ethyl 25 Methyl
Time	134A	134B	134C	134D
1/2	42	47	77	97
1	79	65	90	98
2	94	88	100	99
3	100	94	100	99
4	100	100	100	100

use of other apparatus, *i.e.*, coating pan and hydraulic gun, is possible; but experience indicates that duplication on small batches is difficult (3).

Figures 1 and 2 clearly indicate the release rates obey first-order kinetics. The semilogarithmic solubility plots are linear. *In vitro* sustaining of acetylsalicylic acid is evident, and *in vivo* sustaining is also obtained (2).

Through the use of the Wurster apparatus and the cellulose polymers ethylcellulose and methylcellulose, it has become possible to vary the solubility release rates of aspirin crystals. The data clearly show that as the amount of ethylcellulose is increased in the ratio, the aspirin release rate is decreased. The procedure is considered very satisfactory since not only can the release be predicted but also the tablets prepared from the coated crystals give excellent duplication.

Microscopic examination of the residue after solubility determinations using stereo and polarizing microscopes indicates that most of the shells are largely intact with some fractured ones and some

TABLE III.—REPLICATION OF COATING AND TABLETING PROCEDURE

	% Dissolved as Function of Time	
	152	152A
5 min.	6	6
15 min.	13	13
30 min.	22	23
1 hr.	37	37
2 hr.	57	61
3 hr.	74	74
4 hr.	80	85

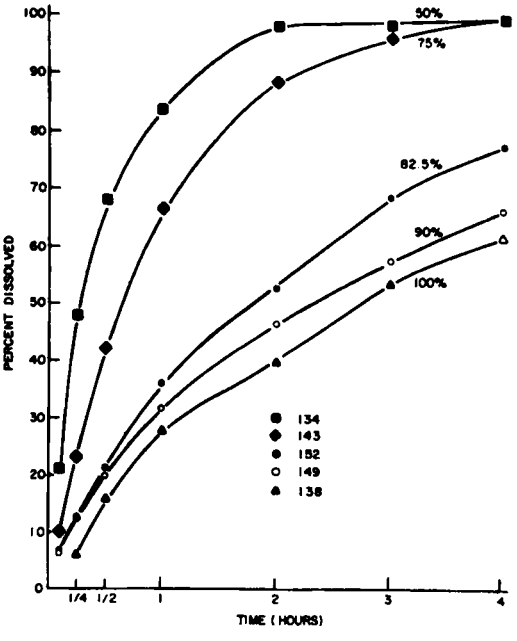


Fig. 1.—Effect of ethylcellulose content on solubility rate (6% coatings).

powdered cellulose mixture. The intact shells are free of all traces of crystalline aspirin. The variation in ratios of the two cellulose polymers with corresponding variation in solubility rate clearly

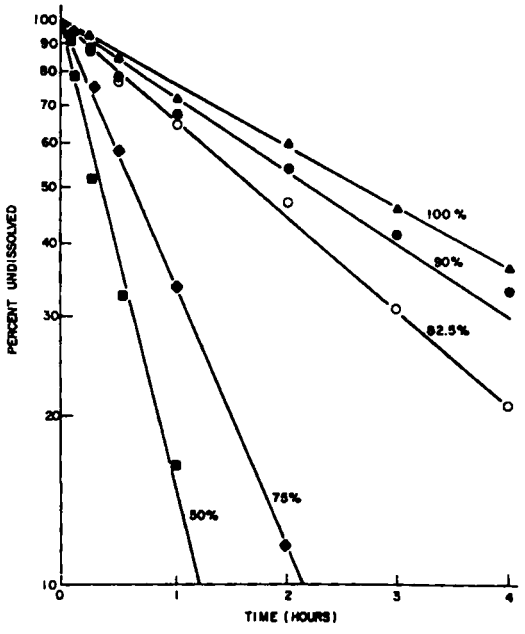


Fig. 2.—Semilog plot of per cent aspirin dissolved *vs.* time (data from Fig. 1 graph) illustrates solubility rate variations with concentration of ethylcellulose on coating.

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SUMMARY

From a purely practical pharmaceutical standpoint it is evident from the data presented that the Wurster apparatus is a valuable tool for preparing research quantities of sustained-release medications. Continuity of films and evenness of coating is possible and reproducible. The coated crystals are suitable for compression into fast disintegrating tablets that provide sustained release of aspirin. It has been shown that the solubility rate of aspirin coated with different concentrations of ethylcellulose and methylcellulose can be sustained. It has also been shown that the ratio of the two cellulose polymers affects the solubility rate, the solubility rate being inversely proportional to the concentration of ethylcellulose. This has been reflected in the solubility rate curves as shown.

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Physical Stability Testing of Pharmaceuticals

By JAMES E. TINGSTAD

The importance of physical stability of pharmaceuticals and methods of testing physical stability of tablets, capsules, suspensions, emulsions, solutions, and ointments are discussed.

Physical stability is an important problem to product formulators for three primary reasons.

Appearance.—Physicians, pharmacists, patients—all expect pharmaceutical products to look fresh, elegant, and professional no matter how long they sit on the shelf. Any slight change in physical appearance—like fading of a color—may cause these people to lose confidence in that particular product.

Uniformity.—Since most products are sold as multiple-dose packages, the formulator has to make sure that the patient receives the same amount of active ingredient in each dose. A cloudy solution or a broken emulsion means that the patient is in danger of being overdosed or underdosed.

Availability.—The formulator's ethical responsibility to the patient does not end with providing a uniform dose. If the active ingredient is not absorbed, he has failed that patient just as much as if he gave him a worthless placebo. Therefore, the formulator has to make sure that the active ingredient is just as

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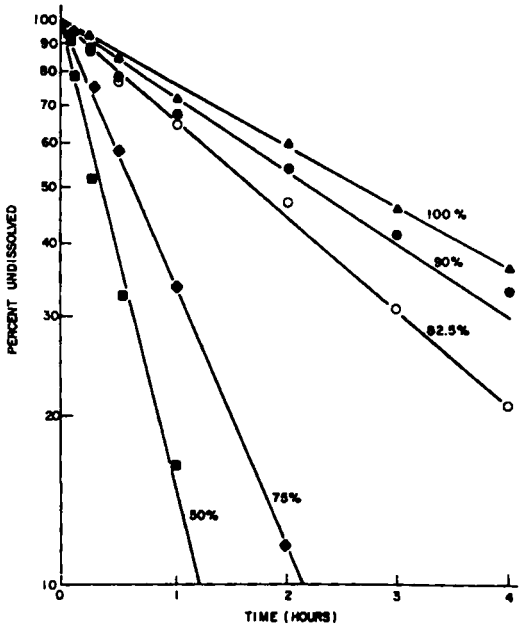


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available for absorption when the product is 1 or 2 years old as it is immediately after manufacture.

Thus it is evident that physical stability is important because it can affect the therapeutic activity of a product just as much as chemical stability.

TABLETS

A physically stable tablet should retain its original size, shape, weight, and color under normal handling and storage conditions throughout its shelf life; the *in vivo* availability of its active ingredients should not change appreciably with time.

There are two tests, among others, that are helpful in determining whether a tablet will remain intact from the time of manufacture until the patient swallows it. First, a given weight of tablets is subjected to mild, uniform, and reproducible shaking or tumbling. Visual observation then reveals if the tablets chip, crack, or split under stress. All tablets that appear to be intact after this test are then weighed to determine how much material was worn away by abrasion. The Friabilator (1) is an inexpensive satisfactory instrument for this test. This apparatus consists of a plastic cylinder with a curved shelf in it. As it rotates, the tablets are subjected to mild abrasion and then, as they drop off the shelf, to sudden shock. Results obtained using this instrument are comparative, not absolute, and to get the most from the data they should be correlated with actual stress experience.

The second test is an actual shipping test, and this should be done for all types of products. In this test the tablets are packaged in their regular shipping container and shipped by rail, air, and truck across the country and back. When they return, careful examination will often reveal that the transportation industry can do strange things to tablets. It is also a good idea to put the surviving tablets back into the Friabilator to see if subtle changes in physical stability have occurred as a result of shipping.

High speed packaging machines are very rough on tablets, and they can be utilized at an early stage to determine the strength of a tablet formulation. There are also various "drop tests" that can be used to help determine the fragility of tablets, but they should be used in addition to, and not instead of, actual shipping tests. All these tests should be performed periodically as the tablet ages because the behavior of a freshly made tablet is often quite different from that of an aged one.

The color stability of a tablet can be determined by using any appropriate colorimeter or reflectometer. It is wise also to visually compare the color of a fresh tablet with that of an aged one because fading of a color is important only when it can be detected by the naked eye. Heat, sunlight, and intense artificial light can be used to accelerate chemical deterioration of a color, but this is most useful when comparing the stability of one color with another in the same formulation. It is important to remember, however, that the system at elevated temperatures is usually different from the system at room temperature, and that it is not always correct to assume that the same things will happen, in due time, at room temperature that happen at elevated temperatures.

Tablet hardness (resistance to crushing or fracture) can be tested by using any one of the available testers (2–4). Results can vary depending on which instrument is used, so one should avoid making comparisons about physical stability when different hardness testers have been used to gather data. It is important to test the hardness of aged tablets as well as freshly made ones because many times a physician will tell a patient to cut the tablet in two and take half (whether it is scored or not). If a tablet formulation hardens excessively as it ages, a patient trying to cut an 18-month-old tablet with a paring knife may have to use such force that half the tablet crumbles while the other half flies off the table and under the refrigerator. When that happens, both the patient and his physician lose confidence in that company's ability to make good products. On the other hand, a tablet that softens excessively during storage will not hold up under normal handling conditions. Uniform hardness is especially important when the product is a chewable tablet. Finally, it should be remembered that hardness and friability are not always related; an extremely hard tablet may not pass a test in the Friabilator because of excessive chipping.

Many times it is desirable to determine the stability of a tablet to moisture. To do this, the tablets are often stored in sealed bottles in a high-humidity cabinet and their physical deterioration followed. It should be pointed out that under these conditions this test is really a measure of the resistance of the container to moisture penetration. To find out how the tablets themselves will hold up while being used under humid conditions, an additional test should be conducted. Here the tablets are stored in a container in the humidity cabinet for 1 to 4 weeks, and the container is opened three or four times a day, just as it would be in the hands of the patient. The humidity should be varied in these tests to obtain a more complete picture of the tablet's stability to moisture.

When the formulator is satisfied that a tablet formulation will reach the patient intact and elegant looking, there is still one more vitally important test to be performed before he can consider his job finished. He has to make sure that the drug will be well absorbed by the patient throughout its shelf life. This is important enough to warrant a brief theoretical discussion.

When a systemic acting drug is tested in the clinic, the proper therapeutic dose that is determined is a function of the amount of drug given and the percentage of the dose that is absorbed. To market products that are therapeutically efficient, effective, and uniform, the formulator has to make sure that the drug is well absorbed initially and that its absorption characteristics do not change appreciably throughout the product's shelf life. Changes in absorption characteristics can be caused by polymorphism, changes in particle size, changes in the dissolution rate of the active ingredient out of the tablet, and other factors.

Levy and Nelson (5) have pointed out that the nature of the formulation does affect the absorption characteristics of drugs, and evidence has been gathered at these laboratories showing that the absorption of a drug can vary considerably depending on the age of the formulation. For example, Fig. 1 shows how blood levels obtained after oral

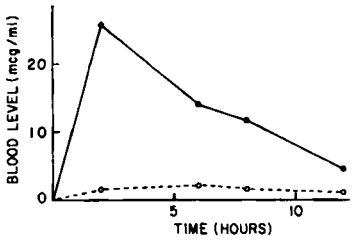


Fig. 1.—Plot showing the decrease in blood levels when an aged suspension (broken line) was administered orally to humans rather than a fresh suspension (solid line).

administration of a formulation decreased greatly as that formulation aged. Other examples could be cited, but the point to remember is this: it is unwise and dangerous to assume that just any formulation will give good drug absorption, and it is equally unwise and dangerous to assume that a well absorbed formulation will remain so throughout its shelf life.

Obviously, it is not practical to test clinically every product at regular time intervals during storage. However, there are several *in vitro* tests that can be done to help detect when changes in absorption characteristics are likely to occur. For tablets, one of the better *in vitro* tests is to determine the active ingredient's dissolution rate in artificial gastric and/or intestinal juice at 37°. The standard U.S.P. tablet disintegration apparatus can be used for these determinations (preferably without plastic disks) and ultraviolet spectroscopy can usually be used to develop a rapid assay method.

It should be emphasized at this time that dissolution rate (the rate at which the active ingredient goes into solution) is quite different from disintegration time (the time it takes for a tablet to disintegrate and the particles to pass through a standard screen). Tablets having sand as the "active" ingredient can be made so that they disintegrate very rapidly; yet blood levels of silicon dioxide would be hard to find after administering such a tablet. It has been shown (6) that disintegration time is not necessarily a good measure of dissolution rate, and it is the dissolution rate of the active ingredient that often determines the rate of absorption of that drug (5). These dissolution rate tests should be run periodically, and if no other physical changes (polymorphism, etc.) take place, an unchanging dissolution rate usually means constant *in vivo* availability. The value of these dissolution rates can be greatly enhanced if they can be correlated with *in vivo* work.

Disintegration times can be determined periodically to detect gross changes in the physical characteristics of a tablet, but this test is not sufficient unless there is a demonstrated correlation between disintegration time and dissolution rate for that particular formulation. When there is no such correlation, the dissolution rates should be determined. In the case of delayed (sustained) release formulations, release patterns are even more important and should be determined periodically during storage.

When the product is a coated tablet, the physical stability of the coat should also be investigated. Many of the above tests for plain tablets can be applied to coated tablets also.

There are two additional points concerning the physical stability testing of tablets that the formulator should keep in mind. First, it is important to recognize that elevated temperatures do not always give a true picture of what will happen, in due time, to a tablet at room temperature. For example, there are tablet formulations that swell and split at elevated temperatures; yet they remain intact indefinitely at room temperature. Also, most tablet coats will melt or soften at elevated temperatures; this seldom happens at room temperature.

Second, it is important to subject tablet formulations, especially coated ones, to freezing temperatures for at least 1 week or 2. Several times this test has revealed tablet coats that were susceptible to cracking when frozen. Subsequent shipping tests to Canada and the northern United States in the winter confirmed these observations.

GELATIN CAPSULES

It is unnecessary to spend a great deal of time discussing gelatin capsules. The capsule formulator should ascertain that: (*a*) the shells of the capsules do not soften (and stick together) or harden (and crack under slight pressure) excessively during storage, (*b*) the capsules remain intact under normal handling and shipping stress, (*c*) the color of the capsules does not change appreciably during storage, (*d*) the capsule contents, either as a powder or an oil, do not leak out of the capsule, and (*e*) the *in vitro* dissolution rate of the active ingredients does not change appreciably with time. It is especially important to test the dissolution rate of capsules at 37° rather than room temperature because gelatin capsules that dissolve in 15–30 minutes at room temperature will often dissolve in less than 2 minutes at 37°.

SUSPENSIONS

A physically stable suspension is one that can be homogeneously redispersed with moderate shaking and easily poured at any time during its shelf life, with neither the particle size distribution, the crystal form, nor the physiological availability of the suspended active ingredient changing appreciably with time.

There is not enough time to discuss thoroughly the basic physical system present in suspensions; but because of the many prevailing misconceptions about suspension systems, it is necessary to mention some of their more important theoretical aspects. Most good stable pharmaceutical suspensions are flocculated; that is, the suspended particles are physically bonded together in such a way that they form a loose, semirigid structure. This structure, in a sense, is independent of the suspension vehicle; that is, the particles hold each other up and exert no significant force on the liquid.

As a crude illustration, think of a piece of wire screen immersed in a liquid. The individual strands of wire are not supported by the liquid; rather, each strand is held in place by its neighbor. Consequently, it is the structure of the screen, and not the nature of the liquid, that determines where each strand will be in the system. Also, the wire exerts no pressure on the liquid. In the same way the particles of a flocculated suspension hold each other up, and their position in the suspension depends

primarily on the nature of their bonds with their neighbors and not upon the inherent qualities of the vehicle.

When a flocculated suspension is first made, it is well stirred and the particles are floating randomly around in the vehicle. As soon as the suspension is poured into a bottle and set on the shelf, the particles start to settle and come together. Soon (minutes to hours, depending on the system) many of the particle-particle bonds have formed and the particles continue to settle, not as individuals but as clusters of particles (agglomerates). Soon (days to weeks, depending on the system) the total structure is formed, and settling continues only because the particles are rearranging and moving closer together by forming bonds with more of their neighbors, causing the structure to compact.

Eventually, for all practical purposes, the structure becomes stable and settling stops. The sediment remains stationary like a fragile piece of wire screen sitting in the vehicle. How much volume this sediment occupies depends on the system, but in a good flocculated suspension it is usually more than half the total suspension volume, and many times it occupies all of the suspension volume. This sediment can be easily redispersed at any time by moderate shaking, and the system will then once again be completely homogeneous.

In nonflocculated suspensions, on the other hand, the particles remain as individuals, essentially unaffected by their neighbors and affected only by the suspension vehicle. These particles, being smaller and lighter than the agglomerates in a flocculated suspension, settle much more slowly. But once they have settled, they often form a rock-hard sediment on the bottom of the bottle that is extremely difficult to redisperse. These suspensions would be acceptable if one could decrease the particle size enough and increase the density and viscosity of the vehicle enough so that the particles would settle very little during the product's shelf life, but this is very hard to do in most cases. Consequently, flocculated suspensions are usually the most pharmaceutically acceptable; and, by design or by accident, most of the presently marketed suspensions are flocculated.

This discussion of suspension systems was necessary to show why some physical stability tests are valid to use while others are not. For example, Stokes law can seldom be used to examine suspension stability because it applies only to nonflocculated suspensions. And, contrary to popular belief, the many conventional viscometers are practically useless for studying physical stability of flocculated suspensions because they employ shear rates that tear the fragile structure apart, and the system thus studied has little or no relation to the actual system as it sits on the shelf. Even centrifugation (to accelerate settling) is of little value because the structure is so fragile that it breaks down under the increased force placed upon it by the centrifuge.

This can best be illustrated by imagining that a formulator has a big cardboard box full of eggs packed in a certain manner and that he wants to determine the condition of the eggs and how they are packed. To do this, he takes an axe and proceeds to open the box in a violent but effective manner. He then observes that the eggs are in

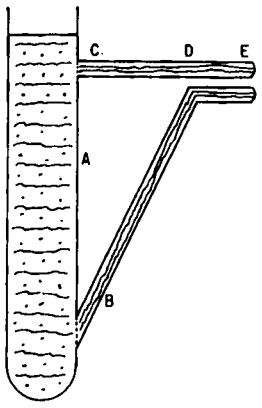


Fig. 2.—Diagram of a differential manometer. See *text* for explanation of instrument.

very poor condition and that they are packed in a very irregular and haphazard way. Poor technique leading to meaningless and erroneous conclusions? Yes, but to a certain extent this is precisely what is done to these fragile sediment structures when they are studied in the conventional manner using conventional viscometers.

How, then, should they be studied? First of all, it is necessary to determine whether the suspension under study is actually flocculated. This can be done very easily by taking advantage of the fact that the particles in a flocculated suspension support each other and exert little or no pressure on the liquid. In the apparatus shown in Fig. 2 (called a differential manometer) (7) the suspension is placed in container A and the vehicle (but not the suspended particles) is allowed to enter sidearms B and C. At point D the two sidearms are parallel and are at the same height (B appears to be lower than C only because the drawing is two-dimensional). If the suspension is flocculated, the liquid in the two sidearms will travel the same distance (*e.g.*, to point E) because of equal hydrostatic pressure (B and C are inclined slightly after point D to magnify slight differences in pressure). If the suspension is not flocculated, the weight of the suspended particles pressing down on the liquid will be greater at the bottom of container A than at the top; consequently, the hydrostatic pressure will be greater in B, and the liquid will travel further in that sidearm.

Once it has been determined that a suspension is flocculated, two simple experiments can be set up to study its physical stability. First, a measured volume of the formulation is stored in a standard size graduated cylinder and the height of the sedimented solids measured periodically by visual observation. This test does not disturb the system at all, and it is very useful for determining stability. For example, if the sediment settles rapidly to a height which is 90% of the total suspension height and then settles no further, the formulation is probably satisfactory. This technique is also useful in determining which is the best of a number of formulations. All other things being equal, the suspension with the largest sediment volume is the best formulation.

While the above test is an excellent one for observing gross characteristics of sediments, it is also desirable to be able to look inside these structures and observe the settling behavior (or rearrangement) of the agglomerates. Fortunately, there is an excellent piece of equipment available to do just

that—the Helipath attachment used with a conventional Brookfield viscometer.

This instrument consists of a slowly rotating T-bar spindle which descends slowly into the suspension as it rotates. The dial reading on the viscometer is a measure of the resistance that the spindle meets from agglomerates present at various levels in the sediment. A higher dial reading (greater apparent viscosity) means that more particles are present at that particular level in the sediment. Repeating the test at various time intervals, each time using a fresh undisturbed sample, gives an excellent picture of how the particles are rearranging (settling) within the sediment structure as the preparation sits on the shelf. It is necessary to handle these samples carefully so that the structure is not disturbed while removing the sample from the shelf. The T-bar spindle disturbs the structure slightly as it rotates, but since it descends as it turns, it continually encounters new, essentially undisturbed material.

This instrument is also very useful in comparing the stability of various formulations of the same product. Figure 3 shows the Helipath profile of two slightly different suspensions which were made at the same time. It can be seen that the agglomerates in Preparation I are settling faster than those in Preparation II (the sedimentation volume of Preparation II was greater). Preparation II proved to be the better suspension. Thus, results obtained using the Helipath, coupled with observations made on sediment volume, can reveal a great deal of useful information about suspension stability.

The important point to be remembered here is that by using the Helipath one can study the state of the sediment without first shaking the suspension to make it homogeneous. In most other viscometric tests the suspension is shaken vigorously to insure

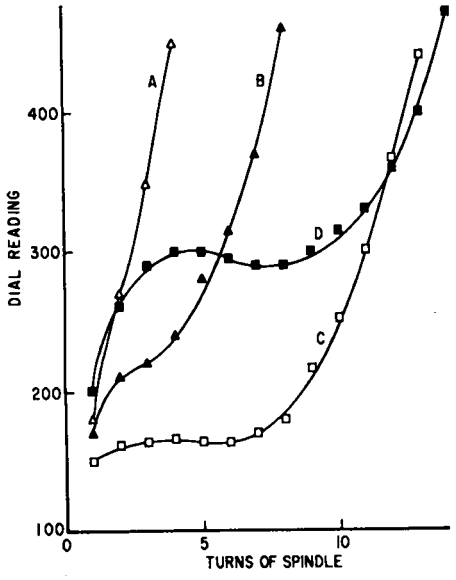


Fig. 3.—Plot illustrating data obtained using Helipath attachment to a conventional Brookfield viscometer. Curve B is for Suspension I (aged 6 months), curve A is for Suspension I (aged 9 months), curve D is for Suspension II (aged 6 months), and curve C is for Suspension II (aged 9 months).

homogeneity, then the preparation is poured into the viscometer. This, in a sense, is closely akin to opening egg cartons with axes.

These two tests are by no means the final word in suspension stability testing. There is a great deal of work that can and should be done to design still better tests. For example, it would be very helpful if the aging of a suspension could be accelerated in a way that corresponds to the actual aging that takes place on the shelf. This is being investigated in these laboratories, and preliminary results are very encouraging.

As mentioned in the definition of a stable suspension, it is also important to detect changes in particle size distribution, crystal form and, when necessary, the physiological availability of the active ingredient. The particle size distribution of the suspended drug is important because it may affect the appearance and taste of the preparation and the *in vivo* absorption of the active ingredient (*e.g.*, griseofulvin and some sulfonamides). The Coulter counter, an electronic particle counter and sizer, is an excellent tool for determining particle size distribution for particles greater than 0.5 μ. Of course, the microscope can also be used to estimate particle size distribution, but this is a tedious procedure. Changes in crystal form can be detected using X-ray diffraction or microscopic examination. If tests indicate that significant changes are taking place in crystal form, etc., it is imperative that further clinical work be done on aged formulations. The truth of this statement is clearly illustrated in Fig. 1, where blood levels dropped to practically zero when a slightly aged formulation was administered rather than a fresh one.

The following additional observations may be helpful to the suspension formulator:

Subtle chemical degradation of an ingredient can affect the physical appearance of a suspension. Recently, a sterile suspension containing a steroid ester was formulated in these laboratories. The physical appearance of the formulation, excellent at first, broke down completely within 1 month. Chemical assay for total steroid indicated good chemical stability, and considerable time and effort were spent trying to determine the cause of the trouble. Eventually, an assay was developed that differentiated between the steroid ester and its hydrolysis product, the free alcohol. It was then found that the ester was rapidly hydrolyzing to the free alcohol and that the suspending system which worked well for the ester was totally inadequate for suspending the alcohol.

Changes in crystal form can also adversely affect a suspension, either in its physical appearance or in its *in vivo* absorption. Suspensions of amorphous novobiocin acid (well absorbed) become useless in a short period of time because the drug reverts to the crystalline acid which is poorly absorbed.

It is wise to subject all suspensions to cycling temperatures; that is, to store them at elevated temperatures and then in a refrigerator, repeating this procedure several times. At elevated temperatures more of the drug will go into solution; then when the preparation is stored in the refrigerator, the excess drug in solution reprecipitates. In this way the tendency of the drug particles to grow in size can be studied.

Shipping tests should always be performed on

suspensions. In the past, several problems have been averted by observing that certain apparently good suspensions broke down during shipping. Also, the suspension should be frozen to make sure that shipping the suspension at freezing temperatures will not adversely affect it.

The pouring qualities of suspensions at refrigerated temperatures should be observed. Many patients store their fluid medications in the refrigerator, and it would prove embarrassing to market a suspension before it was discovered that it will not pour after being stored in a refrigerator.

Microscopic examination of suspension systems often gives a good picture of the state of the sediment (*e.g.*, whether the particles are interacting with each other).

Finally, when recent production lots of an established suspension product suddenly break down physically, it often helps to take a careful look at all of the ingredients to make sure that no supplier changed his manufacturing process. In the past, several such difficulties have been resolved by determining that a supplier changed his manufacturing procedure without notifying anyone. pH is another factor that should be suspected whenever things suddenly go wrong in suspension manufacture because it can have a profound effect on suspension stability.

EMULSIONS

A physically stable emulsion is one that can be homogeneously redispersed to its original state with moderate shaking and can be easily poured at any time during its shelf life.

Emulsion systems are just as complicated as suspension systems, if not more so. Again, time does not permit a thorough discussion of emulsion systems, but it is necessary to mention some of their more important theoretical aspects because the tests chosen to study physical stability should be based on certain assumptions concerning the basic physical system involved. Since most of the important pharmaceutical emulsions are oil-in-water, the discussion will be confined to this type. However, many of the remarks and tests apply to either oil-in-water or water-in-oil emulsions.

There is a good chance that most stable emulsions, especially those with a high oil content, are flocculated in much the same way as stable suspensions and that the stability of most emulsions depends on the strength of the interfacial film surrounding the dispersed oil particles. If these assumptions are correct, rate of coalescence of the internal phase is a valid measure of emulsion stability but, contrary to popular opinion, the rate of creaming is not. In these laboratories, several stable emulsions have been encountered that have a high initial creaming rate. Of course, it is best for the sake of appearance if the volume of the cream occupies as much of the emulsion volume as possible. This situation is similar to that encountered in suspensions.

When formulating an emulsion, it is important to be able to select the most stable formula, and there are two simple tests that can be used to do this. First, the various formulations are subjected to heat (50–70°) and the gross stability of the emulsions determined by visual observation or by turbidimetric measurement. The emulsion that is most stable to heat is usually the most stable at room temperature. However, this is not always the case because the systems at 60° are not the same as those at room temperature. The solubility of the emulsifiers in the two phases will be different, and the change in solubility with temperature may not be the same for both phases. Furthermore, the high temperature may chemically degrade some of the ingredients so that a distorted picture of physical stability is obtained.

The second method for estimating emulsion stability is often called the "coalescence time" test (8). In this test the emulsion system is prepared in two parts, one containing all the hydrophilic materials dissolved in water and the other containing all the hydrophobic materials dissolved in oil. Any materials that are insoluble in both phases should be dispersed in the phase that is most compatible with that material. The water phase is placed in a container, and the oil phase is carefully layered over it. Then a drop of the oil phase is introduced (*e.g.*, through a syringe attached to the bottom of the container) at the bottom of the aqueous phase; it rises through that phase and comes to rest at the interface between the two layers. After a certain length of time, the oil droplet will coalesce with the oil layer. The longer the drop takes to coalesce, the more stable the emulsion should be. This test is only roughly quantitative, but many times it can be very useful for detecting gross differences in emulsion stability at room temperature.

It was stated earlier that particle size distribution is the most valid criterion to use in determining emulsion stability. The Coulter counter is an excellent instrument for determining the particle size distribution of oil droplets larger than 0.5 μ. A good optical microscope is another useful tool, but this involves tedious work. Knoechel and Wurster (9) have refined the microscopic technique to good advantage. The Helipath attachment to the Brookfield viscometer is also useful in emulsion stability work. It gives a rapid semiquantitative picture of how the droplets (or agglomerates) are rearranging (rising) in the system.

Three additional observations on emulsion stability work may be of interest to the formulator. First, it should be recognized that temperature (low as well as high) can have a drastic effect on the behavior of emulsions. Several years ago in these laboratories an emulsion was formulated that was stable at room temperature but broke down when it was stored in a refrigerator. The reason for this was that one of the oil soluble emulsifiers precipitated out at refrigerator temperatures. This disturbed the system enough so that the emulsion broke in a few days.

Second, in preparing lots of an emulsion formulation for stability testing, it is best if at least two lots are prepared using production homogenizing equipment. Different kinds of homogenizers often produce different results, and even different sizes of the same kind of homogenizer can produce emulsions with different stability characteristics. Thus, stability results obtained solely from bench size lots may not be applicable to the finished product made in production.

Finally, following the stability of the emulsion vehicle without the oil phase being present (this can be done with suspensions also) will often yield

additional useful information about stability of the product.

SOLUTIONS

A physically stable solution retains its original clarity, color, and odor throughout its shelf life.

Solutions are relatively simple systems; the main concern of the formulator is that they retain their clarity. One should never depend on rough visual observation under ordinary light to determine clarity, especially in the case of a parenteral product; many hazy solutions pass this type of inspection. A better idea of a solution's clarity can be obtained by shining a microscope light through a diaphragm (to concentrate the beam) into the solution. Undissolved particles will scatter the light, and the solution will appear hazy. The Coulter counter can also be used to determine solution clarity, but light-scattering instruments are the most sensitive means of establishing that a solution is perfectly clear. Ordinarily, however, it is not necessary to use these more expensive instruments in this type of stability work.

It is important that a solution remain clear over a relatively wide temperature range (4–47°). At the lower temperatures one of the ingredients may precipitate because its solubility at that temperature is too low. At the higher temperatures homogeneity may be destroyed by particles flaking off glass containers or rubber closures or by chemical degradation of one of the ingredients. Further information about stability can be obtained by cycling the preparation between 4° and 47°. There are some systems that will yield a precipitate only after several weeks of this treatment, so a single 2 or 3-day cycling experiment is not always sufficient. Changes in pH will often cause precipitation of ingredients in solution, especially when these ingredients are soluble salts of insoluble acids or bases. Therefore, pH should always be followed in studying the physical stability of solutions.

When the solution is a sterile preparation, the formulator should keep in mind that foreign substances in clarifying and sterilizing filters (metals, etc.) are at times capable of causing precipitation reactions that would not ordinarily take place. Also, the filters may adsorb an ingredient and thus change the composition of the preparation. For stability work it is important to use the same type of filter that will be used in production.

OINTMENTS

A physically stable ointment retains its homogeneity and its consistency throughout its shelf life.

Hydrophobic ointments (*e.g.*, petrolatum) may be thought of as high-viscosity suspensions of active ingredients in a nonreacting vehicle. Thus, changes in particle size distribution, crystal form, etc., of the active ingredient are seldom a problem. The main physical stability problems encountered with ointments are bleeding and changes in consistency (due to aging or to changes in temperature). Bleeding occurs when fluid constituents (*e.g.*, mineral oil) separate at the top of the preparation. Visual observation is usually sufficient to detect this, but there is no known way to accelerate this process so that tendency to bleed can be predicted.

It is important to be able to define in a quantitative way the consistency of ointments because an ointment that is too soft is messy to use, and one that is too stiff is difficult to extrude out of a tube and apply. One of the instruments that is presently used to determine ointment consistency is the Penetrometer, an apparatus that allows a pointed weight to penetrate the ointment under a measurable force. The depth of penetration is a measure of the consistency of the ointment. The Helipath attachment used with a high-viscosity viscometer can also be used for this type of study.

At present the performance of another instrument, the Burrell Severs rheometer, is being investigated in these laboratories. In this instrument the ointment is loaded into a cylinder and a measured force (compressed air) extrudes a certain amount of ointment onto a receiver. The amount of ointment extruded is a measure of the consistency of the ointment. The manufacturer supplies a loading device which sucks the ointment into the cylinder, but this creates air pockets within the ointment and leads to erratic results. Much better success has been obtained in loading by melting the ointment, pouring it into the cylinder and allowing the system to cool and come to equilibrium at constant temperature. It should be recognized that remelting an ointment in this way might cause irreversible changes in the system, so it is best to obtain a sample of the ointment while it is still fluid during the original manufacturing process.

These experiments have revealed two interesting facts about ointments that should be of interest to the formulator.

First, ointments have a considerable amount of structure, and this structure takes at least 48 hours to form after the ointment has been melted. It has been found that the consistency of an ointment that has been standing 72 hours after melting is considerably more solid than that of the same ointment that has been standing for only 24 hours. It is evident from these results that rheological experiments performed on freshly made ointments may yield erroneous data. In any case, it should be determined that the structure of the ointment has reached equilibrium before any tests are performed.

Slight changes in temperature (1 or 2°) can greatly affect the consistency of an ointment. The "apparent" viscosity of one ointment formulation was twice as high at 23° as it was at 25°. Therefore, it is essential that all rheological tests on ointments be done at constant and controlled temperatures.

The physical stability problems encountered with hydrophilic creams (o/w emulsions) are similar to those of ointments and to problems discussed under *Emulsions*.

CONCLUSIONS

There are seven "rules to live by" which, if kept in mind when studying the physical stability of any system, will help the formulator to market the best possible products.

(*a*) Often the most valuable instrument in the formulator's possession is an established product whose system is similar to the one being studied. Most good physical stability tests are comparative, and using an established product as a control will greatly increase the accuracy of stability predictions.

(*b*) It is best to know as much as possible about

the basic physical systems involved in the formulations under study. A few shrewd observations early in the game will often save a great deal of trouble and effort later on.

(*c*) When using heat to accelerate a physical aging process in a heterogeneous preparation, it should be remembered that the system at elevated temperatures is different from the system at room temperature; consequently, extrapolation to room temperature stability must be done with care and caution.

(*d*) The analytical method employed to study disperse systems (suspensions, etc.) must not be too harsh because the disturbed system may have little or no relationship to the system as it sits on the shelf.

(*e*) A change in pH can often have a profound effect on physical stability; consequently, all physical stability data should include periodic measurement of pH whenever possible.

(*f*) One of the most important aspects of physical stability—physiological availability of the active ingredient—is often the most neglected. All good stability studies should include *in vitro* tests (dissolution rate, etc.) that will help detect when changes in *in vivo* absorption characteristics are likely to occur.

(*g*) Before final approval is given to the physical stability of any product, it should be taken home and used just as a patient would use it (without actually taking the medication). This "home trial" often reveals physical stability problems that never arise in the laboratory.

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Notes

Assignment of the *N*-Methyl Hydrogen NMR Peaks of Caffeine

By THOMAS G. ALEXANDER and MILLARD MAIENTHAL

The NMR spectrum of caffeine exhibits a separate peak for each of the three *N*-methyl groups. Assignment of each peak to a specific *N*-methyl group was made by comparison of the spectrum of caffeine with those of two caffeine homologs, in each of which one of the *N*-methyl groups is replaced with an *N*-ethyl group.

IN THE nuclear magnetic resonance spectrum of caffeine published by Bhacca, *et al.* (1), *N*-methyl hydrogen peaks are shown at 6.60, 6.41, and 5.99 p.p.m. However, there is no specific assignment of these to the 1, 3, and 7 positions. We undertook this study to make such assignments.

The *N*-methyl hydrogen peaks of caffeine can be selectively removed from the spectrum by (*a*) the substitution of the methyl hydrogen atoms with deuterium atoms, (*b*) the substitution of methyl groups with ethyl groups, or (*c*) the substitution of methyl groups with hydrogen atoms. The last technique might result in a significant difference in the chemical shift of the remaining *N*-methyl hydrogen peaks from the corresponding ones of caffeine, since a hydrogen atom would have considerably less inductive effect upon the rings than a methyl group. Because it was more convenient to synthesize two of caffeine's higher homologs than to prepare the partially deuterated caffeines, the second approach was used. The homologs were synthesized by treating theobromine and theophylline with ethylating agents. Samples of these compounds and of caffeine were dissolved in deuterated chloroform and their NMR spectra were obtained (Fig. 1).[1] Tetramethylsilane was used as an internal reference standard.

Caffeine—(1,3,7-Trimethylxanthine).—Eastman's white label was used.

Ethyltheobromine—(3,7-Dimethyl-1-ethylxanthine).—The sample was prepared by the method of Rodionov (2). When recrystallized from water, the product melted at 161–163°.

Ethyltheophylline—(1,3-Dimethyl-7-ethylxanthine).—The sample was prepared by a modification of Schmidt's method (3). One gram of theophylline and 2 ml. of ethyl sulfate were refluxed in 20 ml. of 5% NaOH for several hours. The solution was kept basic during the refluxing by the addition of 5% NaOH. The product, when extracted with chloroform and recrystallized from water, melted at 150–151°. (Schmidt reported a value of 154°.)

As anticipated, the spectra of each of the caffeine homologs consisted of two sharp *N*-methyl peaks, a triplet, a quartet, and a single olefinic hydrogen

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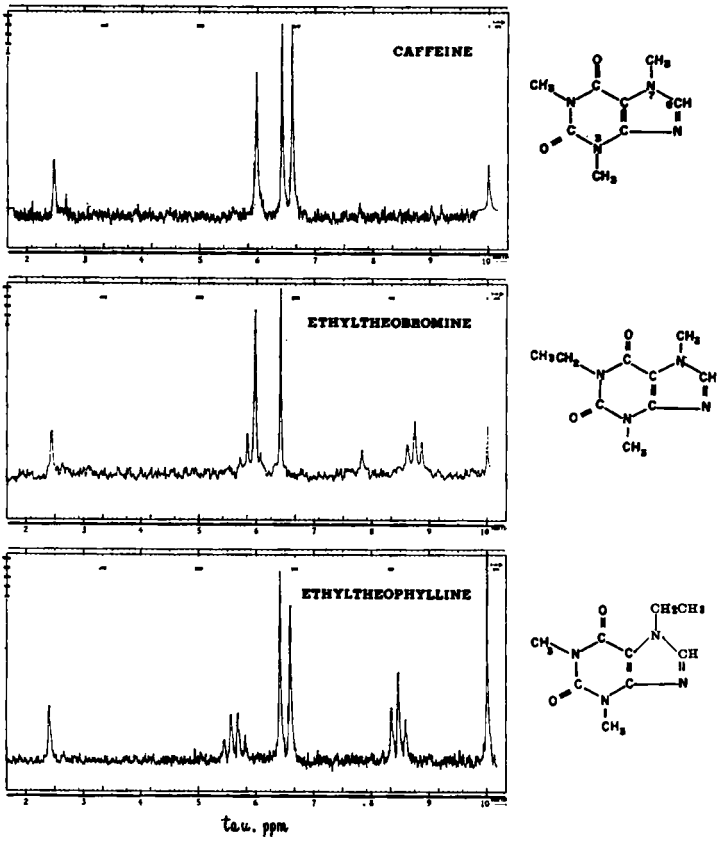


Fig. 1.—NMR spectra.

TABLE I.—ASSIGNED CHEMICAL SHIFTS IN TAU UNITS

	Caffeine			
	Reported	Observed	Ethyltheobromine	Ethyltheophylline
N-Methyl hydrogens at 1	...[a]	6.60	...	6.58
N-Methyl hydrogens at 3	...[a]	6.41	6.42	6.40
N-Methyl hydrogens at 7	...[a]	5.99	5.98	...
N-Ethyl hydrogens at 1				
Methyl triplet	...	...	8.74	...
Methylene quartet	...	...	5.89	...
N-Ethyl hydrogens at 7				
Methyl triplet	...	...	...	8.46
Methylene quartet	...	...	...	5.61
Olefinic hydrogens	2.40	2.44	2.44	2.38

[a] Unassigned *N*-methyl hydrogen peaks are shown at 5.99, 6.41, and 6.60 p.p.m. (1).

peak. The triplets and quartets result from the spin-spin interaction of the methyl and methylene hydrogens of the *N*-ethyl groups. The introduction of the methylene groups into the molecule causes very little change in the observed chemical shifts of the remaining *N*-methyl hydrogens. Thus, the technique chosen proved to be satisfactory for the interpretation of the caffeine spectrum, and it may prove valuable in similar problems of interpretation.[2]

Common to all three spectra was a sharp line between 6.40 and 6.42 p.p.m. This was assigned to position 3 since there is an *N*-methyl group at this position in each of the compounds. The other assignments are presented in Table I.

It is particularly interesting to note that the *N*-methyl hydrogens of position 1 resonate at a higher field strength than do those at positions 3 and 7. Also, the *N*-ethyl hydrogens of ethyltheobromine resonate at higher field strengths than those of ethyltheophylline. There appears to be a greater degree of magnetic shielding for those groups at position 1 than for those at position 7.

[2] The work of Graham and Rogers (4) and of Brey and Jones (5) shows that this technique would not work in the spectra interpretation of derivatives of cyclopropane and pyrazoline.

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Presence of Three New Ingredients in Spearmint Oil

By THOMAS F. BURKS* and GUNNAR GJERSTAD

By use of gas chromatography, spearmint oil N. F. was separated into 19 fractions. Co-chromatography with known reference substances conclusively demonstrated the presence of cineole, α-pinene, and linalool in the oil.

DUE TO THEIR chemical heterogeneity, volatile oils have so far defied a complete and accurate analysis. Until recently, the only available methods for proximate analyses of these oils were fractional distillation and derivative formation of variable accuracy. The relatively recent development of gas chromatography as an analytical method for volatile oils appeared to us to offer great advantages over conventional methods.

Carvone has for some time been recognized as the main ingredient of spearmint oil, the official purity rubric specifying a minimum concentration of 55% (1). Among the other known ingredients, the exact percentages of which have not been conclusively established, are pulegone (2), *d*-3-octanol (3), limonene, phellandrene, acetic and valeric acids esterified with dihydrocuminyl alcohol, and dihydrocarveol esterified with acetic acid (4). In most instances the literature gives no particular data on conformational structural formulas. The presence of pinene, linalool, and cineole has been indicated in various oils of related species from certain foreign habitats; these reports have been questioned by other workers, and we can find no substantiation hereof in American oils (4, 5).

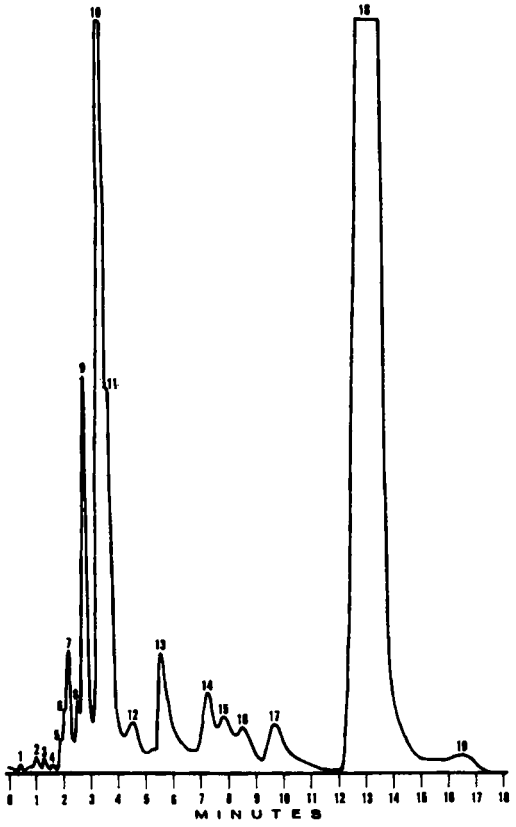


Fig. 1.—Gas chromatogram of spearmint oil N. F. on a 7.5 ft. stainless steel column of 1/4-in. diameter; Silicone Dow 710 on firebrick; helium flow 50 ml./minute; sensitivity attenuation 2 ×. Total time required for separation into 19 fractions was 18 minutes. This recording was obtained at 160°C. However, lower temperatures gave the same number of peaks.

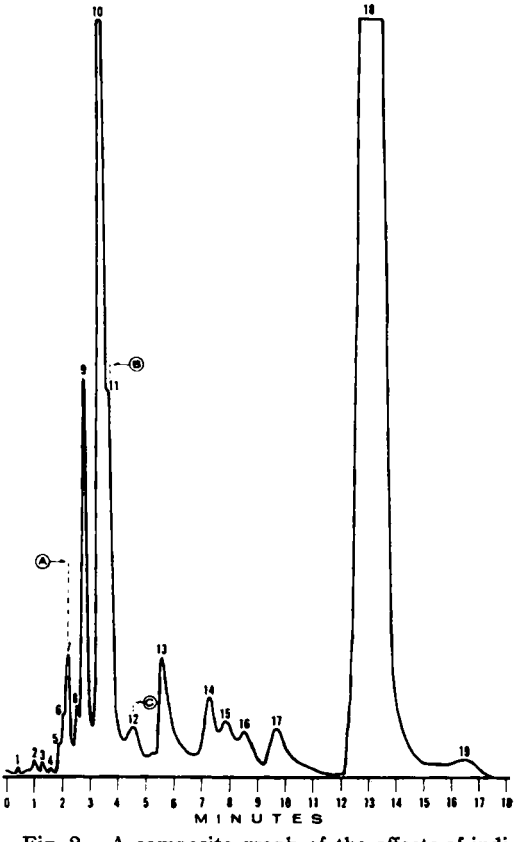


Fig. 2.—A composite graph of the effects of individual chromatograms from co-chromatography. The broken lines indicate the heights of the peaks after the reference standards were added. Key: A, pinene; B, cineole; and C, linalool.

EXPERIMENTAL

Two-microliter samples of spearmint oil N. F.[1] were chromatographed in an Aerograph model A-90-P gas chromatograph equipped with a thermal conductivity detector and a Honeywell-Brown

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This paper represents a part of a more comprehensive study of plant tranquilizers to be published elsewhere.

* Recipient of the Kilmer Prize for 1962 and the 1961 Edwin L. Newcomb Memorial Award.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

After the completion of this paper, it was noted that J. J. Broderick demonstrated the presence of cineole in spearmint oil. (Burchfield, H. P., and Storrs, E. E., "Biochemical Applications of Gas Chromatography," Academic Press, Inc., New York, N. Y., 1962, pp. 463–465.)

[1] Obtained from Magnus, Mabee & Reynard, Inc., New York, N. Y. No data on its provenience are available.

Electronik recorder of 1-mv. sensitivity. Silicone Dow 710 and diethyleneglycol succinate, both adsorbed on firebrick, were the most suitable stationary phases; the other experimental conditions are listed under Fig. 1.

Inspection of Fig. 1 reveals that the oil may be separated into a total of 19 fractions, eight representing minor and 11 major components. Due to the lack of authentic blank substances, all of these ingredients have yet to be positively identified, but work is in progress. Carvone produced the most prominent peak (No. 18) which was readily identified by use of added reference standard.

Comparison of retention volumes and co-chromatography with added pure reference standard under identical experimental conditions were employed in the identification of three compounds in the oil, the presence of which had previously been disputed. Authenticated reference substances, pure and in 2% mixtures in natural oil, were gas chromatographed individually and in various combinations. The results are presented in Fig. 2.

The addition of 2% linalool to the oil produced an increase in the size of peak 12 at a retention time of 4.3 minutes, which is identical to that of pure linalool under the stated conditions. Added cineole similarly effected an increase in peak 11, which appeared only as a "shoulder" on peak 10 in the natural oil, and clearly separated it from peak 10. The retention time of 3.7 minutes for peak 11 corresponds to that of pure cineole. Pinene, with a retention time of 2.2 minutes, caused a marked increase in peak 7.

Gas chromatography was found to be a highly versatile and valuable method for the complete analysis of complex naturally occurring volatile substances.

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Separation and Investigation of a Stable Solid Free Radical of Chlorpromazine

By F. H. MERKLE, C. A. DISCHER, and A. FELMEISTER*

A solid stable free radical of chlorpromazine was prepared and characterized. The free radical nature of this compound was verified by electron spin resonance and ultraviolet spectral studies. The molecular weight and melting point of the free radical were determined.

ALTHOUGH the pharmacological importance of chlorpromazine is well established, its metabolic fate is not fully known (1). While studies of the oxidation of chlorpromazine have been reported (2, 3) and the existence of a free radical intermediate proposed (4, 5), little is known regarding the nature of this radical in the solid state (6, 7). The preparation and characterization of this free radical intermediate was undertaken as a preliminary step to the study of its role in the electrochemical and photooxidation mechanisms of chlorpromazine.

Preparation of Free Radical Intermediate

A procedure based on that outlined by Billon (8) for the preparation of a phenazothionium salt of phenothiazine was used in the preparation and separation of solid free radical. Approximately 0.5 Gm. of chlorpromazine 5-oxide hydrochloride[1] was dissolved in 10 ml. of 70% perchloric acid. The resulting dark red solution was agitated for 10 to 15 minutes and diluted first with an equal volume of acetone and then ether. Upon cooling to about −5° a strongly red-purple, fine crystalline solid separated. This solid material will be referred to in future discussions as Compound R. The solid softened over the range of 185–195°, before melting.

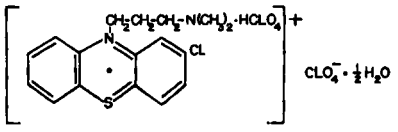
Received June 28, 1963, from the College of Pharmacy, Rutgers-The State University, Newark, N. J.
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* Present address: College of Pharmacy, Columbia University, New York, N. Y.
[1] Chlorpromazine hydrochloride and chlorpromazine 5-oxide were supplied by Smith Kline and French Laboratories, Philadelphia, Pa.

Characterization of Compound R

Schieser and Tuck (9) prepared a semiquinone free radical of chlorpromazine by dissolving chlorpromazine in concentrated sulfuric acid; electron spin resonance studies were used to confirm the presence of the free radical in the reaction mixture. Visible and ultraviolet spectra of the radical, prepared by the method of these workers, were obtained on a Beckman DK-2 ratio-recording spectrophotometer. These spectra were identical to those of Compound R, Fig. 1.

Electron spin resonance spectra indicate that Compound R is strongly paramagnetic.[2]

Elemental analysis of the free radical, Compound R, indicates that it probably exists as the hemihydrate of the diperchlorate salt. Structure I is



I

proposed. Calculated theoretical percentages of the elements in the proposed structure, based on a molecular weight of 527.5, are in good agreement with the analytical results, Table I.

[2] Electron spin resonance measurements were performed by Mrs. N. Steinberger, Columbia University, Department of Chemistry. The authors wish to thank Dr. G. Fraenkel and his staff for the use of their laboratory facilities in making these measurements.

Electronik recorder of 1-mv. sensitivity. Silicone Dow 710 and diethyleneglycol succinate, both adsorbed on firebrick, were the most suitable stationary phases; the other experimental conditions are listed under Fig. 1.

Inspection of Fig. 1 reveals that the oil may be separated into a total of 19 fractions, eight representing minor and 11 major components. Due to the lack of authentic blank substances, all of these ingredients have yet to be positively identified, but work is in progress. Carvone produced the most prominent peak (No. 18) which was readily identified by use of added reference standard.

Comparison of retention volumes and co-chromatography with added pure reference standard under identical experimental conditions were employed in the identification of three compounds in the oil, the presence of which had previously been disputed. Authenticated reference substances, pure and in 2% mixtures in natural oil, were gas chromatographed individually and in various combinations. The results are presented in Fig. 2.

The addition of 2% linalool to the oil produced an increase in the size of peak 12 at a retention time of 4.3 minutes, which is identical to that of pure linalool under the stated conditions. Added cineole similarly effected an increase in peak 11, which appeared only as a "shoulder" on peak 10 in the natural oil, and clearly separated it from peak 10. The retention time of 3.7 minutes for peak 11 corresponds to that of pure cineole. Pinene, with a retention time of 2.2 minutes, caused a marked increase in peak 7.

Gas chromatography was found to be a highly versatile and valuable method for the complete analysis of complex naturally occurring volatile substances.

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Separation and Investigation of a Stable Solid Free Radical of Chlorpromazine

By F. H. MERKLE, C. A. DISCHER, and A. FELMEISTER*

A solid stable free radical of chlorpromazine was prepared and characterized. The free radical nature of this compound was verified by electron spin resonance and ultraviolet spectral studies. The molecular weight and melting point of the free radical were determined.

ALTHOUGH the pharmacological importance of chlorpromazine is well established, its metabolic fate is not fully known (1). While studies of the oxidation of chlorpromazine have been reported (2, 3) and the existence of a free radical intermediate proposed (4, 5), little is known regarding the nature of this radical in the solid state (6, 7). The preparation and characterization of this free radical intermediate was undertaken as a preliminary step to the study of its role in the electrochemical and photooxidation mechanisms of chlorpromazine.

Preparation of Free Radical Intermediate

A procedure based on that outlined by Billon (8) for the preparation of a phenazothionium salt of phenothiazine was used in the preparation and separation of solid free radical. Approximately 0.5 Gm. of chlorpromazine 5-oxide hydrochloride[1] was dissolved in 10 ml. of 70% perchloric acid. The resulting dark red solution was agitated for 10 to 15 minutes and diluted first with an equal volume of acetone and then ether. Upon cooling to about −5° a strongly red-purple, fine crystalline solid separated. This solid material will be referred to in future discussions as Compound R. The solid softened over the range of 185–195°, before melting.

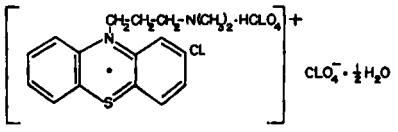
Received June 28, 1963, from the College of Pharmacy, Rutgers-The State University, Newark, N. J.
Accepted for publication November 6, 1963.
* Present address: College of Pharmacy, Columbia University, New York, N. Y.
[1] Chlorpromazine hydrochloride and chlorpromazine 5-oxide were supplied by Smith Kline and French Laboratories, Philadelphia, Pa.

Characterization of Compound R

Schieser and Tuck (9) prepared a semiquinone free radical of chlorpromazine by dissolving chlorpromazine in concentrated sulfuric acid; electron spin resonance studies were used to confirm the presence of the free radical in the reaction mixture. Visible and ultraviolet spectra of the radical, prepared by the method of these workers, were obtained on a Beckman DK-2 ratio-recording spectrophotometer. These spectra were identical to those of Compound R, Fig. 1.

Electron spin resonance spectra indicate that Compound R is strongly paramagnetic.[2]

Elemental analysis of the free radical, Compound R, indicates that it probably exists as the hemihydrate of the diperchlorate salt. Structure I is



I

proposed. Calculated theoretical percentages of the elements in the proposed structure, based on a molecular weight of 527.5, are in good agreement with the analytical results, Table I.

[2] Electron spin resonance measurements were performed by Mrs. N. Steinberger, Columbia University, Department of Chemistry. The authors wish to thank Dr. G. Fraenkel and his staff for the use of their laboratory facilities in making these measurements.

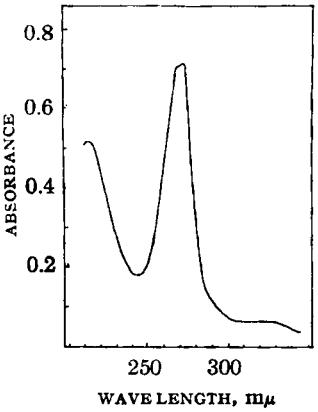


Fig. 1.—Ultraviolet spectrum of Compound R in 1 *N* sulfuric acid, immediately after solution.

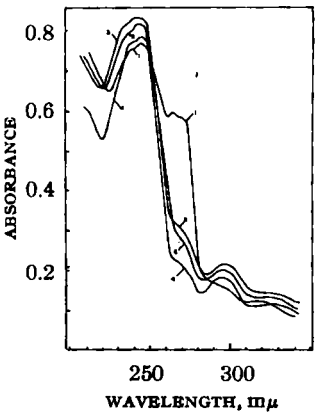


Fig. 2.—Ultraviolet spectra of an aqueous solution of Compound R. Key: Curve 1, immediately after solution; Curve 2, after 1 minute; Curve 3, after 5 minutes; Curve 4, spectrum of synthetic equimolar mixture of chlorpromazine hydrochloride and its 5-oxide.

TABLE I.—CALCULATED THEORETICAL PERCENTAGES OF THE ELEMENTS IN THE PROPOSED STRUCTURE COMPARED TO THE ANALYTICAL PERCENTAGES

Element	Per Cent Calcd.	Per Cent Found
Carbon	38.67	38.11
Hydrogen	3.98	3.77
Nitrogen	5.31	5.64
Chlorine	20.19	21.48
Sulfur	6.07	6.59
Oxygen	25.78	25.30
	100.00	100.89

The molecular weight was confirmed using a spectrophotometric technique. Solutions of chlorpromazine hydrochloride (approximately 10^{-5} *M*) were prepared in concentrated sulfuric acid. Under these conditions the chlorpromazine is converted quantitatively to the free radical, as verified by ultraviolet spectral studies. These solutions obey Beer's law at 277 mμ, with a molar absorbancy index of 4.15×10^4. Sulfuric acid solutions (9 *N*) of Compound R were then prepared. The ultraviolet spectra of these solutions of Compound R were identical to those of the free radical produced in concentrated sulfuric acid. Assuming that the molar absorbancy index of Compound R in 9 *N* acid does not differ appreciably from the molar absorbance index of the free radical produced by reaction of chlorpromazine with concentrated sulfuric acid, its molecular weight may be calculated from absorbancy measurements at 277 mμ. Three such determinations gave calculated molecular weights of 530, 519, and 547, respectively, with an average value of 532.

Reactivity of Compound R

Ultraviolet spectral studies indicate that aqueous solutions of Compound R disproportionate rapidly giving rise to equimolar mixtures of chlorpromazine and its 5-oxide. This was verified by comparison with the spectrum of a synthetic mixture of chlorpromazine hydrochloride and its 5-oxide, Fig. 2.

TABLE II.—COULOMETRICALLY DETERMINED *n* VALUES FOR THE CONTROLLED-POTENTIAL ELECTROLYSIS OF COMPOUND R

Number of Moles Taken Based on M. W. of 527.5	*n* Value Obtained
Oxidation 9.478×10^{-5}	0.98
Reduction 9.478×10^{-5}	0.94

This spontaneous disproportionation may be expressed as

2 free radicals (deep red) → 1 chlorpromazine (colorless) + 1 chlorpromazine 5-oxide (colorless) (Eq. 1)

Further evidence of the free radical nature of Compound R was obtained by controlled-potential electrolysis.[8] Samples of the free radical were oxidized and reduced electrolytically in 9 *N* sulfuric acid. The coulometrically determined *n* values (the number of equivalents per mole) are reported in Table II.

SUMMARY

On the basis of these studies it was concluded that Compound R is a free radical of chlorpromazine. The role of the free radical as an intermediate in the electrochemical and photo-oxidation of chlorpromazine will be reported shortly.

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[8] An electronic controlled-potential coulometric titrator, Model Q-2005 ORNL, was used to perform the electrolyses. Readout voltages were measured with a Non-Linear Systems 484 A digital voltmeter.

Catalytic Hydrogenation of Acetophenone

By MORRIS FREIFELDER, TAIMI ANDERSON, YEW HAY NG, and VICTOR PAPENDICK

The low pressure hydrogenation of acetophenone to 1-phenylethanol in the presence of some nickel and noble metal catalysts is described. The effects of each are noted. While palladium-on-carbon appears to be the catalyst of choice, others can be used under proper conditions. Only rhodium causes enough side reaction to discourage its use.

THE HYDROGENATION of acetophenone (I) to 1-phenylethanol (II) has been studied before. Delepine and Horeau (1) carried out the reduction[1] with Raney nickel which was promoted with alkali. They obtained II but reported no yield. Theilacker and Drossler (2) used platinum and palladium blacks from the freshly prepared corresponding oxides. A third group (3), in what was essentially a rate study, described the effect of platinum metal catalysts on the conversion of I to II.

In the reduction of I to II, competing reactions can and do take place. Other by-products are ethylbenzene (III), 1-cyclohexylethanol (IV), and ethylcyclohexane (V). Breitner and her associates (3) suggested from their work that two or more competing reactions occur concurrently or at nearly similar rates. It is our opinion that this is due to the rather high catalyst ratios that were used by this group.

Since there was a need in this laboratory for a substantial amount of II, it gave us an opportunity to reinvestigate the reaction. It was our aim to carry out the reductions at low pressure in alcoholic solution in the presence of modest ratios of commercially available catalysts and to identify the products of reaction by gas chromatography[2] and ultraviolet spectroscopy.

The results seen in the table indicate that, of those used for the reductions, 5% palladium-on-carbon[3] is indeed the best catalyst (3). They also show that the competing reactions of II to III and of II to IV do not occur concurrently with the conversion of I to II. It is likely that the rates are energy dependent and that side reaction can be easily controlled, except with 5% rhodium on alumina.[3] It is of interest that IV was still formed with the rhodium catalyst at a 3% ratio. Its propensity toward ring reduction precludes its use for the preparation of II.

Platinum oxide,[3] Raney nickel,[4] and a reduced and stabilized nickel[5] also were tried. The last named was only effective at a high ratio when promoted with sodium hydroxide (1).

EXPERIMENTAL

The following preparation is typical of those carried out with the various catalysts, except for the addition of 0.5 ml. of 20% sodium hydroxide solution in the two runs with nickel.

A solution of 30 Gm. (0.25 mole) of acetophenone in 100 ml. of ethyl alcohol was hydrogenated in the presence of 3.0 Gm. of 5% palladium-on-carbon. When one equivalent of hydrogen was absorbed, the solution was filtered from the catalyst and examined. (See Table I for results.)

TABLE I.—RESULTS

Catalyst[a]	Ratio, [b] %	Reaction Time, Hr.	Products of Reaction, % I[f]	II[h]	III[h]	IV[h]	V[h]
A	1	2	10.24	90.5	0.2	...	...
B	10	5–6	Trace	99.9	0.1	...	...
C	10	0.5 to 0.75[e]	6.28	75.9	...	16.4	2.6
	3	3.75	33.4	50.3	0.3	12.5	3.0
D	15[c]	1.5	1.09[g]	99.0	0.1	...	...
E	40[c, d]	12–15	0.2	99.3	0.5	...	...

[a] *A*, platinum oxide; *B*, 5% palladium-on-carbon; *C*, 5% rhodium on alumina; *D*, Raney nickel; *E*, G-69 nickel. [b] The ratio is based on grams of catalyst (total weight) per 100 Gm. of compound. [c] One-half milliliter of 20% sodium hydroxide solution was added. [d] When a 20% catalyst ratio was used, no uptake occurred with or without sodium hydroxide. [e] Over hydrogenation occurred; uptake was more than theoretical. [f] Determined by comparison with the ultraviolet spectrum of I. [g] After distillation, less than 1% was observed. [h] Determined by gas chromatography with available pure standards.

1-Phenylethanol.—This was used as one of the standards for gas chromatography and was prepared by hydrogenation of acetophenone in alcoholic solution at 125° and 1900 p.s.i. in the presence of barium promoted copper chromite catalyst. The product was distilled and redistilled over a column 30 cm. long packed with glass helices, b.p. 123°/55 mm. (4), reported 118°, 40 mm. and 198°, atmospheric pressure. Its ultraviolet spectrum indicated a maximum of 0.3% of acetophenone. Gas phase chromatography showed only traces of impurities.

1-Cyclohexylethanol.—Sixty grams of acetophenone was hydrogenated in the presence of 0.6 Gm. of ruthenium dioxide[5] at 100° and 1000 p.s.i. (1 hour). Distillation of the residual oil after removal of the catalyst gave a product which boiled at 188° at atmospheric pressure, n_D^{25} 1.4635 (5); yield, 88%. (Reported 189.4 to 189.8°, 761 mm., n_D^{20} 1.4677.)

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[1] These authors found that the reduction was accelerated by the addition of sodium hydroxide.

[2] Gas phase chromatography was carried out with a Barber-Colman unit, model 10, modified with a thermal conductivity detector.

[3] Available from Engelhard Industries, Newark, N. J.

[4] Supplied in a water suspension by the Harshaw Chemical Co., Cleveland, Ohio.

[5] Designated as Girdler G-69, Chemical Products Division, Chemetron Corp., Louisville, Ky.

Alkaloids of the Bark of *Teclea grandifolia*

By FRANK D. POPP and D. P. CHAKRABORTY

The bark of *Teclea grandifolia* Engl. was found to contain evoxanthine, norevoxanthine, and an unidentified alkaloid.

In 1957, GELLERT (1) reported on the constituents of the bark of *Teclea grandifolia* Engl. *Rutaceae*. In addition to identifying a number of nonalkaloidal constituents, Gellert reported the presence of about 1.2% alkaloids in the stem bark of *T. grandifolia*. However, this material was stated to be amorphous in character and was not further examined. In 1958, Paris and Stambouli (2) reported that the roots of *T. grandifolia* contained the alkaloid evoxanthine. In view of the medicinal use of *T. grandifolia* in Africa (1) and the presence of alkaloids in related genera, we now report an extension of Gellert's investigation of the stem bark of *T. grandifolia*.

The ground stem bark was extracted with naphtha to remove the nonalkaloidal constituents, and the residue was extracted continuously with 90% ethanol. The residue remaining after concentration of the ethanol extract was digested with acid and the acidic solution brought to pH 8. At this pH a precipitate was collected and chromatographed to give an alkaloid, m.p. 217–218°, in 0.04% yield. This material was homogeneous by paper chromatography (3). Its ultraviolet and infrared spectra were identical with evoxanthine; it did not depress the melting point of an authentic sample of evoxanthine.

The residual remaining after the acid digestion was extracted with methylene chloride to give after chromatography an alkaloid, m.p. 274–275°, in 0.006% yield. This alkaloid gave derivatives, tests, and spectra consistent with that of norevoxanthine (4). That it was norevoxanthine was confirmed by its conversion to evoxanthine on treatment with diazomethane.

After precipitation of the crude evoxanthine at pH 8, a chloroform extraction of the basic solution yielded after chromatography a trace of yellow solid, m.p. 185°, in less than 0.002% yield. This nitrogen containing material was homogeneous on paper chromatography, gave a positive Lebat test, had ultraviolet maxima at 208 and 278 mμ (95% ethanol) and the infrared spectra included bands at 3600 and 1620 cm.$^{-1}$ Sufficient material for further identification was not available.

While a total ethanol extract of the bark and a total naphtha extract of the bark both exhibited some activity against cell culture (KB line) (ED_{50} of 3.0×10^1 and 6.2×10^0 mcg./ml., respectively), evoxanthine itself was inactive (ED_{50} of $>1.0 \times 10^2$ mcg./ml.).[1] The total ethanol extract exhibited no activity or toxicity against the Dunning leukemia in Fisher rats.[2]

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Supported in part by Grant T-234 from the American Cancer Society and Grant RG 6475A from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

EXPERIMENTAL

Plant Material.—The tree bark (trunk) of *T. grandifolia* was supplied by the Silviculturist, Ghana. A small amount of the initial material was supplied by Dr. Gellert (1).

Extraction and Isolation.—The dried powdered bark was extracted first with naphtha and then with 90% ethanol. The ethanol extract was concentrated *in vacuo* and the concentrate digested with 8% hydrochloric acid. The acidic digest was filtered, brought to pH 8 with sodium carbonate, and a dirty green precipitate obtained. Chromatography of this precipitate over alumina (Merck, reagent chromatographic aluminum oxide) gave, with chloroform as the eluent, a solid (0.04% yield), m.p. 217–218° (from ethyl acetate). The R_f on paper chromatography using butanol:acetic acid:water (4:1:1) was 0.87 (3). This material was identical in every respect with an authentic sample of evoxanthine. The powdered residue obtained after the hydrochloric acid digestion of the ethanol extract was extracted with methylene chloride. Concentration of the methylene chloride gave a solid (0.006%), m.p. 274–275° [reported (4) for norevoxanthine, m.p. 275°]. This material appeared to be identical tc norevoxanthine in all of its reactions. That it was norevoxanthine was confirmed by reacting it with diazomethane in methanol to give evoxanthine. The norevoxanthine had an R_f of 0.79 in the above solvent system. The basic solution left after the precipitation of the crude base at pH 8 was extracted with chloroform, and the concentrated extract was chromatographed over alumina (Merck) to give in the methanol eluate a material (0.002%), m.p. 185°, with an R_f of 0.72 in the above solvent system. This material contained nitrogen and gave a positive Lebat test. The details of its spectra are included in the text.

A number of other extraction schemes were tried with the same qualitative results. Solid nitrogen containing compounds with R_f for other than 0.87, 0.79, and 0.72 were not obtained. Traces of noncrystalline materials with R_f of 0.58 and 0.69 were observed in some cases.

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[2] Results supplied by Dr. Leo Rane, University of Miami.

Synthesis of Some Dialkyl Esters of *m*-Nitrobenzenephosphonic Acid

By LINDLEY A. CATES* and TONY E. JONES

The dimethyl, di-*n*-propyl, diisopropyl, di-*n*-butyl, and diisobutyl esters of *m*-nitrobenzenephosphonic acid were synthesized for pharmacological screening. Two of the derivatives displayed some anticancer activity.

IT HAS BEEN shown that *m*-nitrobenzenephosphonic acid has *in vitro* antibacterial activity (1) and possesses the ability to immobilize *T. pallidum* (2). These cytotoxic properties, accompanied by low toxicity, prompted the synthesis of dialkyl esters of the acid for investigation as chemotherapeutic agents.

The only simple dialkyl ester of *m*-nitrobenzenephosphonic acid which has been reported is the diethyl derivative (3). The compounds reported in this paper were synthesized by the same procedure, except that a higher temperature was maintained during esterification.

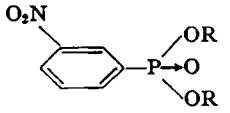
Preliminary pharmacological testing[1] of these compounds indicated that compounds II and III were toxic when tested against carcinoma 585 at a concentration of 200 mg./Kg.

EXPERIMENTAL

m-Nitrobenzenephosphonic acid was prepared according to the method of Kosolapoff (4) and converted to the dichloride by the procedure described by Freedman and Jaffe (3). These latter investigators esterified *m*-nitrobenzenephosphonic acid under conditions similar to those prescribed by Toy (5), whereby a pressure of less that 30 mm. and a temperature of not substantially over 30° were maintained during the alcohol addition. We found the use of an increased temperature during esterification more satisfactory for the synthesis of the higher homolog esters.

Preparation of Dialkyl m-Nitrobenzenephosphonates.—*m*-Nitrobenzenephosphonic acid (0.089 mole) and phosphorus pentachloride (0.187 mole) were intimately mixed in a 250-ml. three-necked flask with a glass stirring rod. The powders partially liquified with evolution of heat, and the flask was gently heated until no solid particles remained. The flask was immediately equipped with a thermometer, an outlet to a water aspirator, and an air leak. Both the air leak and outlet were protected with drying tubes. Vacuum from the water aspirator was applied (approx. 10 mm.), and the flask was gradually heated to 90° until the excess phosphorus pentachloride and most of the phosphorus oxychloride formed in the reaction were removed. Carbon tetrachloride (20 ml.) was added to the reaction mixture and the flask equipped with a dropping funnel, thermometer, and reflux condenser with an outlet to a water aspirator. Vacuum was applied (approx. 10 mm.), and the appropriate alcohol was added dropwise. Heat was applied as needed to maintain a reflux temperature for 15 minutes. Following the alcohol addition, the condenser was replaced with a water aspirator outlet and the flask heated to 90° *in vacuo* to remove the carbon tetrachloride and excess alcohol. The residual liquid was cooled and washed with 50 ml. of water, 50 ml. of 10% sodium carbonate solution, and 50 ml. of water. Vacuum distillation of the organic layers gave the pale yellow products.

TABLE I.—DIALKYL *m*-NITROBENZENEPHOSPHONATES



No.	R	B.p., °C.[a]	Yield, %[b]	Anal. % C Calcd.	C Found	H Calcd.	H Found
I	Methyl	148–152/1 mm.	49	41.6	41.6	4.4	4.7
II	*n*-Propyl	161–164/1 mm.	39	50.2	49.9	6.3	6.5
III	Isopropyl	156–160/1 mm.	58	50.2	50.1	6.3	6.6
IV	*n*-Butyl	162–167/0.6 mm.	44	53.3	52.7	7.0	7.2
V	Isobutyl	161–163/0.6 mm.	62	53.3	52.8	7.0	7.2

[a] All boiling points are uncorrected. [b] Based on analytically pure compounds achieved by triple distillation.

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* Fellow of the American Foundation for Pharmaceutical Education. Present address: College of Pharmacy, University of Houston, Houston, Tex.
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[1] By Abbott Laboratories, North Chicago, Ill., to whom the authors are indebted for these tests.

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Isolation of α-Spinasteryl-D-glucoside and α-Spinasterol from Alfalfa

By E. D. WALTER

α-Spinasteryl-D-glucoside was isolated from alfalfa and identified by infrared spectra and by identification of its products of hydrolysis. α-Spinasterol was isolated from the unsaponifiable fraction and palmitic acid from the saponifiable.

ALTHOUGH α-spinasterol was previously isolated from alfalfa by Fernholz and Moore (1) the glucoside has never been mentioned before. Blair *et al.* (2) in a study of sterols of alfalfa, stated that combined sterols were not detected at any time during its growth up to early bloom. In the process of isolation of the water-insoluble or soya-type saponin from alfalfa, an ether-soluble sterol fraction was obtained as a by-product. The same treatment that yielded β-sitosterol and β-sitosteryl-D-glucoside from coastal Bermuda grass and orchard grass (3) yielded α-spinasteryl-D-glucoside and α-spinasterol from alfalfa.

EXPERIMENTAL

One-kilogram samples of alfalfa meal (or pellets) were soaked with 10 L. of warm water for 2 hours. An extract was filtered through cloth with suction for the isolation of water-soluble saponin. The remaining hydrated meal was allowed to stand for 1 day or more in 95% alcohol (7 L.). The alcohol extract was filtered with suction, clarified with charcoal (10 Gm./L.), and concentrated to about 1.5 L. The concentrate was extracted with ether, the ether evaporated, and the residue saponified with alcoholic potassium hydroxide. Water was added and the mixture was extracted with ether; the alkali was washed out with water, and the ether extract was evaporated to near dryness.

α-Spinasteryl-D-glucoside.—About 100 ml. of skellysolve-B was added to the unsaponifiable residue. The insoluble portion was collected on a filter and washed with acetone, leaving a white powder, m.p. 290°. Yield varied from 30–55 mg./Kg. of dehydrated meal. The material recrystallized from pyridine-methanol, m.p. 290–292° (with dec.), $[\alpha]_D^{25}$ − 32.2° (in pyridine, c = 1.754, l = 0.5), showed an infrared spectrum in agreement with that presented by Obata *et al.* (4) for this glucoside isolated from sugar beet pulp.

Anal.—Calcd. for $C_{35}H_{58}O_6$: C, 73.12; H, 10.17. Found: C, 72.8; H, 10.3.

Hydrolysis of the glucoside with 2% sulfuric acid by the method of Thornton *et al.* (5) gave α-spinasterol, m.p. 167°, with an infrared spectrum in agreement with that reported by Obata *et al.* (4) and glucose which was detected by paper chromatographic comparison with known glucose.

α-Spinasteryl-D-glucoside Tetraacetate.—The glucoside (25 mg.) was dissolved in 5 ml. of pyridine and 5 ml. of acetic anhydride was added. The mixture was refluxed for 1 hour, then poured into ice water. The solid material was filtered out, washed with water, and recrystallized from alcohol, m.p. 168°, [lit. 168° (4)].

Anal.—Calcd. for $C_{43}H_{66}O_{10}$: C, 69.51; H, 8.95. Found: C, 69.1; H, 8.92.

α-Spinasterol.—The skellysolve-B-soluble part of the unsaponifiable material was put on a column of deactivated alumina. The sterol was eluted with 3% acetone in skellysolve-B, and the solvent was evaporated and recrystallized from methanol, m.p. 167°. The sterol gave a positive Lieberman-Burchard reaction. The infrared spectrum was in agreement with that of α-spinasterol (4). The yield varied from 20–70 mg./Kg. of dehydrated meal.

α-Spinasteryl Acetate.—The sterol (120 mg.) was heated on the steam bath with 10 ml. acetic anhydride for about 1 hour. The mixture was poured into ice water and the fluffy product was filtered, washed with water, dried, and recrystallized from alcohol; yield 130 mg., m.p. 181–183°, [lit. 183° (1)].

Anal.—Calcd. for $C_{31}H_{50}O_2$: C, 81.87; H, 11.09. Found: C, 81.64; H, 11.1.

α-Spinasteryl Benzoate.—The sterol (250 mg.) was dissolved in pyridine, and 0.5 ml. benzoyl chloride was added. The mixture was heated on the steam bath for 2 hours, cooled, and poured into cold dilute sodium bicarbonate solution. It was filtered after standing overnight in the cold. The residue crystallized from chloroform and absolute alcohol, m.p. 199–200°; $[\alpha]_D^{25}$ + 3.8° (in chloroform, c = 3.68, l = 0.5).

Anal.—Calcd. for $C_{36}H_{52}O_2$: C, 83.65; H, 10.15. Found: C, 83.6; H, 10.1.

Palmitic Acid.—The aqueous saponifiable fraction from the sterol separation was acidified with hydrochloric acid and extracted with ether. The acid was washed out with water, and the ether solution was clarified with charcoal. The solution was concentrated to near dryness, then dissolved in methanol. Recrystallization from methanol gave white flakes, m.p. 63°. Titration with 0.1 *N* sodium hydroxide gave an equivalent weight of 258 (palmitic acid, 256.32). The infrared spectra of this material and that of authentic palmitic acid were identical.

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Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

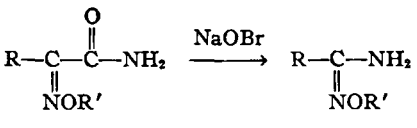
Preparation of *O*-Alkylamidoximes from α-Alkyloximinocarboxamides by the Hofmann Hypobromite Reaction

By DOMINICK A. COVIELLO

O-Alkylamidoximes were prepared by treatment of α-alkyloximinocarboxamides with sodium hypobromite. Of the five prepared, two have not been previously reported and one was reported as being an unstable oil. Structures were proven by NMR and nitrogen analysis.

Amidoximes yield *O*-alkyl ethers when treated with an alkyl halide or alkyl sulfate in the presence of base. Amidoxime hydrobromides or hydrochlorides have been treated with excess sodium ethoxide followed by an alkyl halide (1), or by shaking a sodium hydroxide solution of the amidoxime with methyl sulfate (2). Another investigator utilized alkyl halides in hydroalcoholic solutions of potassium hydroxide to *O*-alkylate amidoximes (3). Although a few aliphatic derivatives have been prepared in like manner, a recent review (4) points out that the conversion is not always successful. Attempts at *O*-alkylation of the sodium salt of ethanamidoxime with alkyl halides, for example, resulted in the formation of oils which could not be distilled without decomposition even at reduced pressure (5). On the other hand, α-oximinopropanamidoxime or α-methyloximinopropanamidoxime were alkylated with methyl sulfate in the presence of 20% sodium hydroxide solution (6) as well as hexanamidoxime which was alkylated with ethyl iodide in sodium ethoxide (7).

If one considers the conversion of a carboxamide to an amine with sodium hypobromite as a decarbonylation, it would immediately be obvious that *O*-alkylamidoximes might result from the treatment of α-alkyloximinocarboxamides with the reagent as follows:



Since *O*-alkylamidoximes have been reported as stable to hot base (8) one could expect to isolate the expected products if they were stable in base for at least 20 minutes at 70° assuming the reaction proceeded in the usual way with the amides in question.

Treatment of the *O*-benzyl ethers of five α-alkyloximinocarboxamides with sodium hypobromite produced the corresponding *O*-alkylamidoxime. Of those prepared, *O*-benzylbenzamidoxime and *O*-benzyl-2-phenethanamidoxime are known; *O*-benzylethanamidoxime has been reported as an unstable oil (5), and the *O*-benzyl ethers of propanamidoxime and hexanamidoxime are new compounds. The known compounds were characterized by comparison of melting points and NMR data, the unknown by nitrogen analysis and NMR data as summarized in Table I.

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It has been pointed out in a review (9) that as the molecular weight of the amide increases the yield of amine usually decreases in the Hofmann reaction. The results of this work are in keeping with that observation. It is felt that in the case of the reactions which produce solid *O*-alkylamidoximes, yields might be improved by a chromatographic separation.

EXPERIMENTAL

α-Alkyloximinocarboxamide intermediates have been reported previously (10).

***O*-Benzylbenzamidoxime.**—A 9.4-Gm. quantity of sodium hydroxide was dissolved in 80 ml. of water. The solution was cooled to 0° and 2.4 ml. of bromine was added. To the ice-cold solution was added 10.16 Gm. (0.04 mole) of α-benzyloximinophenylacetamide. The solution was stirred until most of the amide was in solution when heating was started with continued vigorous stirring. The reaction was accompanied by effervescence when the temperature approached 70°. Heating and stirring were continued for 20 minutes while the temperature went to 80°. The resulting oil became viscous on cooling, and standing overnight caused the oil to solidify. Two crystallizations from ethanol yielded 1 Gm. (11%) of white crystals which melted at 86 to 88.5° as compared with the reported m.p. of 90.5° (8).

***O*-Benzylphenylethanamidoxime.**—By the above procedure, 10.76 Gm. (0.04 mole) of α-benzyloximino-β-phenylpropionamide was treated with sodium hypobromite. An oil was produced which crystallized after cooling and standing overnight. Crystallization of the product from alcohol-water four times yielded 0.5 Gm. (5.2%) of buff-colored crystals melting at 52.5 to 54°; reported m.p. was 55° (11). Treatment with Norit-A yielded a white product but did not change the melting point.

***O*-Benzylethanamidoxime.**—A sodium hypobromite solution was prepared in the manner described above and 7.6 Gm. (0.04 mole) of α-benzyloximinopropionamide was added to the ice-cold solution with stirring. When solution was effected the solution was heated slowly to 75° with vigorous stirring and held at that temperature for 20 minutes, allowed to cool, and stand for 1 hour. The mixture was extracted with ether and dried over $MgSO_4$ for 24 hours. The products from three separate experiments were combined, the ether removed by distillation, and the product distilled at reduced pressure. The resulting clear oil distilled at 88–90°/0.19 mm. and weighed 8.37 Gm. (36.2%).

Anal.—Calcd. for $C_9H_{12}N_2O$; N, 17.07. Found, N, 17.00.

***O*-Benzylpropanamidoxime.**—In the manner described, 14.42 Gm. (0.07 mole) of α-benzyliminobutyramide was treated with sodium hypobromite.

TABLE I.—NMR ANALYSIS OF *O*-ALKYLAMIDOXIMES

$$R-C(=NOR')-NH_2$$

R	R'	Peak in CPS TMS Standard	Description	No. of Protons	Assignment
Methyl	Benzyl	106	Sharp singlet	3	CH_3
		275	Broad band	2	NH_2
		297	Sharp singlet	3	CH_2 (benzyl)
		438	Sharp singlet	5	Phenyl
Ethyl	Benzyl	64	Triplet	3	CH_3
		121	Quartet	2	CH_2 (ethyl)
		270	Broad band	2	NH_2
		296	Sharp singlet	2	CH_2 (benzyl)
		437	Sharp singlet	5	Phenyl
Pentyl	Benzyl	42–162	Series of three broad bands	11	Pentyl
		269	Broad band	2	NH_2
		299	Broad-sharp singlet	2	CH_2 (benzyl)
		441	Sharp singlet	5	Phenyl
Phenyl	Benzyl	276	Broad band	2	NH_2
		299	Sharp singlet	2	CH_2 (benzyl)
		440	Multiple sharp bands	10	Phenyl (2)
Benzyl	Benzyl	285	Sharp singlet	2	CH_2 (benzyl attached to carbon)
		256	Broad-sharp singlet	2	NH_2
		290	Sharp singlet	2	CH_2 (benzyl in ether linkage)
		422, 430	Two sharp singlets with some overlapping	10	Phenyl (2)

During the reaction time of 20 minutes the temperature went briefly to 92°. The mixture was cooled, allowed to stand overnight, extracted with ether: dried over $MgSO_4$, and distilled. A clear oil, 5.7 Gm. (45.7%), distilled at 81–82°/0.08 mm.

Anal.—Calcd. for $C_{10}H_{14}N_2O$; N, 15.73. Found N, 15.70.

***O*-Benzylhexanamidoxime.**—α-Benzyloximinoheptanoamide (9.2 Gm.) was reacted as described above with an appropriate amount of NaOBr. The resulting dark-red oil was distilled four times to obtain 0.5 Gm. (6%) of a yellowish oil boiling at 125–130°/0.3 mm.

Anal.—Calcd. for $C_{13}H_{20}N_2O$; N, 12.72. Found N, 12.74.

Nitrogen analyses were carried out on the Coleman nitrogen analyzer except for *O*-benzylhexanamidoxime which was analyzed by Micro-Tech Laboratories, Skokie, Ill. NMR spectra were run on the Varian A-60.

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α-Alkyloximinonitriles by Pyrolysis of α-Alkyloximinocarboxamides with Phosphorus Pentoxide

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A preparation of α-alkyloximinonitriles is described in which the corresponding α-alkyloximinocarboxamides are heated with phosphorus pentoxide *in vacuo*. Structures were proven by infrared analysis and basic hydrolysis of the nitriles to the corresponding acids.

IN A PREVIOUS COMMUNICATION (1), it was reported that attempts to cyclize β-phenethyl amides of α-alkyloximino acids to the corresponding 3,4-dihydroisoquinolines were fruitless. It was pointed out that experimental evidence indicated that the work-up of the reaction mixture apparently resulted in the hydrolysis of the amide to yield the amine moiety which could be isolated. There was no indication of the fate of the acid portion of the molecule, but it was assumed that under the conditions of the reaction, the oxime moiety decomposed thus accounting for the failure of the cyclization reaction.

In reconsidering the reaction, it was thought that it would be worthwhile to determine the fate of the acid portion of the molecule under similar conditions

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using unsubstituted α-alkyloximinocarboxamides as well as ascertain the feasibility of preparing the corresponding nitriles which would be interesting intermediates for further investigations.

Powdered α-alkyloximinocarboxamides were mixed thoroughly with a slight excess of phosphorus pentoxide and then heated with a Bunsen flame under reduced pressure. In all cases a vigorous reaction ensued during which oils were collected in reasonable yields and characterized as α-alkyloximinonitriles by infrared analysis. The characteristic nitrile peak showed up as a medium sharp band at 2235 cm.$^{-1}$ for α-benzyloximinobutyronitrile, and a medium sharp band at 2236 cm.$^{-1}$ for α-benzyloximinopropionitrile. The values are well within the reported range (2). Alkaline hydrolysis of the nitriles produced the corresponding α-alkyloximino acids.

The success of the dehydration reaction would then indicate that the failure of the Bischler-Napieralski reaction in the instance cited must be attributed to factors other than the stability of the alkyloximino group.

EXPERIMENTAL

Amides prepared according to the procedure of Woolley and co-workers (3) have been reported (4).

α-Benzyloximinobutyronitrile.—A 30.9-Gm. quantity (0.15 mole) of α-benzyloximinobutyramide was ground in a mortar and transferred to a round-bottom flask. Then 28.4 Gm. (0.2 mole) of phosphorus pentoxide was added, the flask was stoppered and manually shaken until the mixture was homogeneous. The flask was connected to a vacuum system set for downward distillation. The system was evacuated to 0.35 mm. and heating was started. Frothing and discoloration ensued as a brown oil distilled. Heating was discontinued when the temperature of the vapors fell from 129° to 50°. The reaction mixture was cooled and exhaustively extracted with benzene. The benzene extract was combined with the distillate, the benzene removed, and the residue distilled under reduced pressure. Five grams (17.3%) of an oil was collected at 102–105°/0.5 mm. The procedure followed was that outlined in the literature (5). A small amount of the oil was heated with 10% NaOH, cooled, and acidified. The solid obtained was filtered off, crystallized from alcohol-water. The melting point was 88–90°, as compared to the reported value of 86° (6). A mixed melting point showed no depression.

α-Benzyloximinopropionitrile.—In like manner, α-benzyloximinopropionamide was converted to the nitrile. In this case, in an effort to reduce mechanical losses, smaller vessels were used to run smaller amounts. Only the reaction vessel was changed for each run and in this way the yield was increased to 32%. The observed boiling point of α-benzyloximinopropionitrile was 74–76°/0.2 mm. Basic hydrolysis of the oil produced a solid melting at 85°. The melting point was consistent with that obtained when the acid was prepared in this laboratory by standard procedures (6). The melting point was not in agreement with the reported value of 73–75° (6), but nitrogen analysis of the amide did check (4). A mixed melting point showed no depression.

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Phosphorus-Nitrogen Compounds II. Some *p*-Toluidine Derivatives

By LINDLEY A. CATES and NOEL M. FERGUSON

Certain organophosphorus compounds containing substituted *p*-toluidine moieties were prepared for evaluation as cancer chemotherapeutic agents. The compounds that were synthesized include phosphoramidochloridic acids, phosphoramidates, phosphoramidothionates, a phosphorodiamidate, and a phosphorodiamidic chloride. The *S*-*p*-chlorobenzylthiuronium salt of a phosphoramidochloridic acid is also reported.

THE FIRST paper in this series (1) reported the synthesis of some carboxy- and carbethoxy-substituted aryl derivatives containing phosphorus-nitrogen bonds. This series has been extended to include some related *P–N* compounds involving substituted *p*-toluidine moieties.

Phosphoramidochloridic acids (I) constitute a relatively new class of organophosphorus compounds, of which there are few examples in the literature. Most of these compounds are arylsulfonamide derivatives (2–6), one of which was included in a group of derivatives said to possess tumor-inhibiting activities (6). All reported phosphoramidochloridic acids were prepared by treating phosphorimidic trichlorides or phosphoramidic dichlorides with formic acid (2–8). The use of an acidic reagent is not necessary in this type of synthesis, however, since the *p*-toluidine derivatives were prepared by aqueous hydrolysis of the corresponding phosphoramidic dichlorides. In either case, the acid chloride character of phosphor-

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using unsubstituted α-alkyloximinocarboxamides as well as ascertain the feasibility of preparing the corresponding nitriles which would be interesting intermediates for further investigations.

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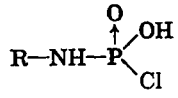
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Certain organophosphorus compounds containing substituted *p*-toluidine moieties were prepared for evaluation as cancer chemotherapeutic agents. The compounds that were synthesized include phosphoramidochloridic acids, phosphoramidates, phosphoramidothionates, a phosphorodiamidate, and a phosphorodiamidic chloride. The *S*-*p*-chlorobenzylthiuronium salt of a phosphoramidochloridic acid is also reported.

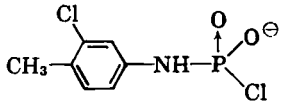
THE FIRST paper in this series (1) reported the synthesis of some carboxy- and carbethoxy-substituted aryl derivatives containing phosphorus-nitrogen bonds. This series has been extended to include some related *P–N* compounds involving substituted *p*-toluidine moieties.

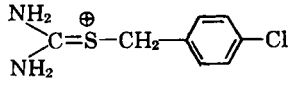
Phosphoramidochloridic acids (I) constitute a relatively new class of organophosphorus compounds, of which there are few examples in the literature. Most of these compounds are arylsulfonamide derivatives (2–6), one of which was included in a group of derivatives said to possess tumor-inhibiting activities (6). All reported phosphoramidochloridic acids were prepared by treating phosphorimidic trichlorides or phosphoramidic dichlorides with formic acid (2–8). The use of an acidic reagent is not necessary in this type of synthesis, however, since the *p*-toluidine derivatives were prepared by aqueous hydrolysis of the corresponding phosphoramidic dichlorides. In either case, the acid chloride character of phosphor-

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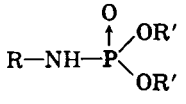


I

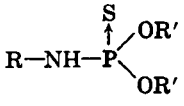




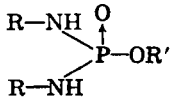
II



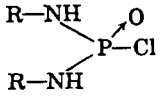
III



IV



V



VI

amidic dichlorides is sufficiently suppressed by the amido group so that the second chlorine atom is stable in neutral, or near neutral, media. Thus, the salt of a phosphoramidochloridic acid can be formed by the careful addition of dilute sodium hydroxide solution.

Investigators in the field of organophosphorus chemistry have encountered problems in the isolation and purification of some acids and esters. Phosphoramidochloridic acids possess a fair degree of water solubility and, therefore, pose similar difficulties. They can be crystallized from a dry, inert solvent to yield the anhydrous product or from an ethanol-water media to give the lower melting, hydrated form. A recent report (9) has shown that certain phosphorus-containing acids and esters can be converted to insoluble, sharp melting *S-p*-chlorobenzylthiuronium salts. Our investigations indicate that the sodium salt of (*N*-3-chloro-4-methylphenyl)phosphoramidochloridic acid is also precipitated by *S-p*-chlorobenzylthiuronium chloride to yield the pure insoluble salt II. This is the first instance of a benzylthiuronium salt of a compound containing a phosphorus-nitrogen bond and indicates another application of a new procedure for the isolation of certain organophosphorus products.

The synthetic scheme for the phosphoramidates III, phosphoramidothionates IV, and phosphorodiamidate V follows the classical preparatory methods whereby a ratio of 2 or 4 moles of amine are reacted with 1 mole of phosphorochloridate or phosphorochloridothionate in an inert solvent. The phosphorodiamidic chloride VI resulted from the refluxing of the appropriate amine with phosphorus oxychloride (2:1) in benzene over a long period of time.

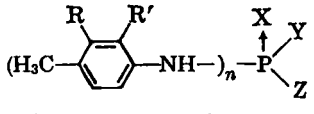
Monosubstituted *p*-aminobenzoic acids have been investigated for antibacterial properties and the chlorinated and fluorinated derivatives are among those shown to inhibit bacterial cell growth. 2-Chloro-4-aminobenzoic acid has been found to have relatively high bacteriostatic activity (10) and some antileukemic effect (11). For these reasons more attention was directed to the preparation of organophosphorus derivatives of the methyl analog of this acid. One can hypothesize that *in vivo* oxidation of the methyl group may yield the corresponding substituted *p*-aminobenzoic acid. Such a conversion may precede or follow the hydrolysis of the phosphorus-nitrogen bond by phosphamidase enzymes. Since phosphamidases are reported to occur in higher concentration in neoplastic cells than in normal cells (12), this enzymatic action is the basis for postulating a specific antimetabolic activity by the parent moiety. Also, in view of the fact that *p*-toluidine has antibacterial (13) and carcinogenic (14) properties, these derivatives may have application as antineoplastic agents.

Samples of the compounds reported in this paper have been submitted to the Cancer Chemotherapy National Service Center for preliminary evaluation.

EXPERIMENTAL

Preparation of Phosphoramidochloridic Acids.—Compounds I, II, and III were prepared by adding the appropriately substituted *p*-toluidine (0.4 mole)

TABLE I.—*p*-TOLUIDINE DERIVATIVES



No.	*n*	R	R′	X	Y	Z	M.p., °C.[a]	Formula	Analyses, % P Calcd.	P Found	Cl Calcd.	Cl Found
I	1	Cl	H	O	Cl	OH	218–220	$C_7H_8Cl_2NO_2P$	12.90	12.98	29.54	29.81
II	1	H	Cl	O	Cl	OH	88–90 dec.	$C_7H_8Cl_2NO_2P\cdot 3H_2O$	10.53	10.50		
III	1	H	CH_3	O	Cl	OH	178–179 dec.	$C_8H_{11}ClNO_2P\cdot 3H_2O$	11.32	11.33		
IV	1	Cl	H	O	OEt	OEt	85–86	$C_{11}H_{17}ClNO_2P$	11.15	11.02		
V	1	Cl	H	O	OPh	OPh	139–140	$C_{19}H_{17}ClNO_2P$	8.29	8.27		
VI	1	F	H	O	OEt	OEt	91–92	$C_{11}H_{17}FNO_2P$	11.86	12.00		
VII	1	F	H	O	OPh	OPh	115–116	$C_{19}H_{17}FNO_2P$	8.67	8.71		
VIII	1	Cl	H	S	OEt	OEt	52–54	$C_{11}H_{17}ClNO_2PS$	10.54	10.74		
IX	1	H	CH_3	S	OEt	OEt	Oil	$C_{12}H_{20}NO_2PS$	11.33	11.58		
X	2	Cl	H	O	Cl	...	154–156	$C_{14}H_{16}Cl_3N_2OP$	8.52	8.32		
XI	2	Cl	H	O	OPh	...	149	$C_{26}H_{18}Cl_3N_2O_2P$	7.35	7.36		
XII	1	Cl	H	O	Cl	$O^{\ominus}$	87–89	$C_{15}H_{17}Cl_3N_3O_2PS$	7.03	6.99		

$ClC_6H_4CH_2\overset{\oplus}{S}{=}C(NH_2)_2$

[a] All melting points are uncorrected.

dropwise with stirring to phosphorus oxychloride (0.2 mole) in 250 ml. of anhydrous ether and allowing the mixture to stand for 3 days. The suspensions were evaporated to near dryness and stirred with water for 1 hour. The residues were recrystallized following decolorization with charcoal to yield the white powdery products. Compound I was recrystallized from reagent dioxane; compounds II and III from ethanol-water.

Preparation of Phosphoramidates.—Compounds IV through VII were prepared by adding 0.1 mole of the appropriately substituted *p*-toluidine to 0.05 mole of diethyl or diphenyl phosphorochloridate in 300 ml. of reagent benzene and refluxing for 2 hours. The suspensions were evaporated to a moist mass with an air stream and gentle heat. The residues were stirred with dilute hydrochloric acid and washed with water by decantation. Recrystallization from ethanol-water following charcoal decolorization yielded the products in the form of white needles.

Preparation of Phosphoramidothionates.—Compounds VIII and IX were prepared by adding 0.2 mole of diethyl phosphorochloridothionate to 0.4 mole of 3-chloro-4-methylaniline or 2,4-dimethylaniline in 300 ml. of reagent benzene and refluxing for 2 hours. The suspensions were evaporated to a moist mass with an air stream and gentle heat. The residues were stirred with dilute hydrochloric acid and washed with water by decantation to yield red liquids. Compound VIII upon drying in a vacuum desiccator gave a red solid which was recrystallized from ethanol-water following charcoal decolorization to yield the white crystalline product having a pungent odor. Compound IX was stirred with 10% potassium hydroxide solution and dilute hydrochloric acid; each procedure was followed by water washings. The red, oily residue was dissolved in ether, decolorized with charcoal, and filtered to give a nearly colorless, clear filtrate. The filtrate was treated with anhydrous calcium sulfate, filtered and the ether removed under reduced pressure to yield the pale yellow, undistillable oil having a pungent odor.

Preparation of (N,N'-3-Chloro-4-methylphenyl)-phosphorodiamidic Chloride X.—A mixture of 3-chloro-4-methylaniline (0.1 mole) and phosphorus oxychloride (0.05 mole) in 300 ml. of reagent benzene was refluxed until negligible hydrogen chloride evolution was detected (65 hours). The reaction mixture was evaporated to dryness with an air stream and gentle heat. Recrystallization from ethanol-water following charcoal decolorization yielded the white, powdery product.

Preparation of Phenyl (N,N'-3-Chloro-4-methylphenyl)phosphorodiamidate XI.—A mixture of 3-chloro-4-methylaniline (0.2 mole) and phenyl phosphorodichloridate (0.05 mole) in 300 ml. of reagent benzene was refluxed for 2 hours. The reaction mixture upon drying in a vacuum desiccator gave a moist mass which changed to a liquid upon washing with water. Treatment with dilute hydrochloric acid yielded a yellow, waxy solid which was washed with water. Recrystallization from ethanol-water following charcoal decolorization gave the white, crystalline product.

Preparation of S-p-Chlorobenzylthiuronium Salt of (N,N' - 3 - Chloro - 4 - methylphenyl)phosphoramidochloridic Acid XII.—Five grams of compound I were suspended in water, one drop of phenolphthalein T.S. added, and 1 *N* sodium hydroxide introduced dropwise with stirring to attainment of a pale pink color and partial dissolution of the suspended material. The suspension was filtered and brought to near boiling. Ten milliliters of saturated ethanolic solution of *S-p*-chlorobenzylthiuronium chloride (9) was added to give a white viscous mass which was washed with water and dried to yield the white glassy product.

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Idoxuridine—A Preliminary Report

By LOUIS J. RAVIN, CHARLES A. SIMPSON, and ALFRED F. ZAPPALA

Data are presented to show that the principal decomposition products of idoxuridine ophthalmic solution in acidic media (pH 1.3 to 6.7) are 5-iodouracil and 2-deoxyribose. A trace of 2′-deoxyuridine was also detected. Uracil was not formed under the conditions of these experiments.

During the development of a clinically acceptable idoxuridine ophthalmic solution it was necessary to determine the type of breakdown products formed during storage at temperatures ranging from 5 to 60°. This note presents some preliminary information concerning the nature of the decomposition products formed when idoxuridine ophthalmic solution is artificially decomposed in acidic media (pH 1.3 to 6.7).

A column partition chromatographic procedure was developed (1) and subsequently used to separate the decomposition products from the idoxuridine. The individual components were then assayed by spectrophotometric methods.

Idoxuridine, some possible decomposition products, and an artificially decomposed solution of idoxuridine ophthalmic solution were chromatographed on paper using a *n*-butanol–3 *N* ammonia solvent system. A comparison of the approximate R_f values obtained are listed in Table I.

It can be seen from these data that the principal products formed when idoxuridine ophthalmic solution decomposes in acidic solution are 5-iodouracil, 2-deoxyribose, and 2′-deoxyuridine. Subsequent analysis of degraded samples by the partition column procedure substantiated that the breakdown products formed under these conditions are 5-iodouracil, 2-deoxyribose, and a trace of 2′-deoxyuridine. Figure 1 shows a plot of per cent idoxuridine *versus* per cent 5-iodouracil. The linear relationship having a slope of 0.95 indicates that for every mole of idoxuridine that decomposes, approximately 1 mole of 5-iodouracil is formed, further indicating that 5-iodouracil and 2-deoxyribose are the principal breakdown products. Uracil was not detected in the decomposed samples under the conditions of this study.

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Table I.—Comparison of Approximate R_f Values Obtained for Idoxuridine, Some Possible Decomposition Products, and An Artificially Decomposed Ophthalmic Solution

Material	R_f Values: Known Soln.	R_f Values: Artificially Decomposed Soln.
2′-Deoxyuridine	0.23	0.23 (Trace)
Uracil	0.28	...
Idoxuridine	0.31	0.31 (Large)
2-Deoxyribose	0.37	0.39 (Significant)
5-Iodouracil	0.50	0.48 (Significant)

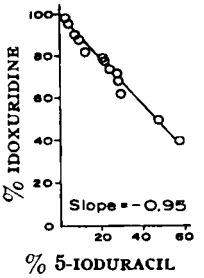


Fig. 1.—Plot showing decreasing idoxuridine and increasing 5-iodouracil concentrations for artificially decomposed idoxuridine ophthalmic solutions.

REFERENCE

(1) Smith Kline and French Laboratories, to be published.

Thin-Layer Chromatography of Iodochlorhydroxyquin

By B. P. KORZUN, S. M. BRODY, and F. TISHLER

A thin-layer chromatographic method is described for the separation of iodochlorhydroxyquin and its intermediates. The method is applicable not only to the substance but also to its pharmaceutical formulations, in which the material is suspended in ointment or cream.

The present analytical procedures (1–4) available for determining iodochlorhydroxyquin,[1] both as the substance and in formulations, do not distinguish between iodochlorhydroxyquin and its possible intermediates. The colorimetric procedure of Haskins and Luttermoser (2) and the paper chromatographic procedure of Castiglioni (3), although capable of separating 8-hydroxyquinoline from iodochlorhydroxyquin, do not separate iodochlorhydroxyquin from other halogenated hydroxyquinoline intermediates.

A semiquantitative thin-layer chromatographic procedure, using polyamide as the adsorbant, has been developed which separates iodochlorhydroxyquin from all probable intermediates, except 5,7-dichlorohydroxyquinoline. In combination with iodochlorhydroxyquin, the limit of detection is 0.25 mcg.

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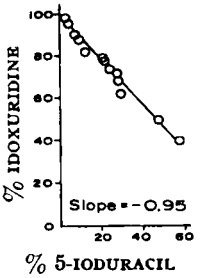


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for 8-hydroxyquinoline and 5-chloro-8-hydroxyquinoline, and it is 2 mcg. for 5-iodo and 5,7-diiodo 8-hydroxyquinoline.

EXPERIMENTAL

Preparation of Plates. The plates (20 × 20 cm.) are prepared by mixing 5 Gm. of Woelm's polyamide powder[2] and 3.5 Gm. calcium sulfate with 10 ml. distilled water. The mixture is poured into the Desaga[3] applicator and spread on five plates.[3] The approximate thickness of the coat is 250 μ. The coated plates are air-dried for 16 hours at room temperature. Prior to use, the plates are heated at 80° for 10 minutes and allowed to cool.

Preparation of Samples.—Weigh an amount of the formulation equivalent to 100 mg. of iodochlorhydroxyquin into a 250-ml. conical flask. Add about 100 ml. of acetone and heat on a steam bath for about 15 minutes, shaking frequently. Cool to room temperature, and filter into a suitable round-bottom flask. Wash the conical flask with several 5-ml. portions of acetone and filter, combining all extracts. Evaporate the acetone, and dissolve the residue in 10 ml. of dimethylformamide-methanol (1:2) with the aid of a little heat.[4] Centrifuge, if necessary, and use the clear solution for sampling (1% solution). Reference standard solutions of iodochlorhydroxyquin (1%), 5-chloro-8-hydroxyquinoline (0.025%), 8-hydroxyquinoline (0.025%), 5,7-diiodo-8-hydroxyquinoline (0.05%), and 5-iodo-8-hydroxyquinoline (0.05%) in dimethylformamide-methanol (1:2) are prepared.

Application and Development of Plates.—Five microliters of each solution, except 8-hydroxyquinoline (2 μl.) and 5-chloro-8-hydroxyquinoline (2 μl.), are applied to the plate at a point 2 cm. from the lower edge of the plate and 2 cm. apart. The plates are placed into a chamber (22.5 × 10 × 26 cm.), the bottom of which is covered with 100 ml. of methanol. The chamber is covered, and the solvent is allowed to ascend to a line drawn 15 cm. from the point of application. The time of development is approximately 1 hour. The plates are removed, air-dried, and the compounds detected by viewing either under an ultraviolet light (2660 Å.) or after spraying with Pauly reagent (5).

RESULTS AND DISCUSSION

Figure 1 shows a typical separation of the individual compounds and also a separation of a mixture. Table I lists the R_f values for the compounds. Once the contaminant is identified, a graded series of known concentrations could be run, and an approximation of the amount present can be made by comparing spot size and intensity.

[2] Available from Alupharm Chemicals, New Orleans, La.
[3] Available from Brinkmann Instruments, Long Island, N. Y.
[4] In many cases not all of the residue will dissolve due to the insolubility of many ointments in the solvent.

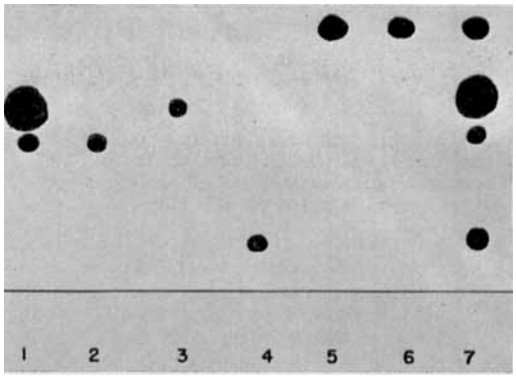
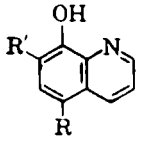


Fig. 1.—Portion of thin-layer chromatogram of (*1*) iodochlorhydroxyquin, (*2*) 5-chloro-8-hydroxyquinoline, (*3*) 5,7-dichloro-8-hydroxyquinoline, (*4*) 8-hydroxyquinoline, (*5*) 5-iodo-8-hydroxyquinoline, (*6*) 5,7-diiodo-8-hydroxyquinoline, and (*7*) mixture of all compounds, illustrating relative sizes of the spots. Line shows finish of solvent front.

TABLE I.—R_f VALUES FOR IODOCHLORHYDROXYQUIN AND INTERMEDIATES



R	R'		R_f
H	H	8-Hydroxyquinoline	0.90
Cl	H	5-Chloro-8-hydroxyquinoline	0.83
Cl	Cl	5,7-Dichloro-8-hydroxyquinoline	0.63
Cl	I	Iodochlorhydroxyquin	0.63
I	H	5-Iodo-8-hydroxyquinoline	0.50
I	I	5,7-Diiodo-8-hydroxyquinoline	0.50

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Stability of Procaine in Deuterium Oxide

By F. P. SIEGEL, F. D. HITER, S. V. SUSINA, and M. I. BLAKE

Stability of procaine in protium oxide and deuterium oxide was studied over the pH and "apparent" pH range 8 to 11. Studies were conducted at 40 and 100°.

PHARMACEUTICAL APPLICATIONS of the solvent deuterium oxide (heavy water) have not been explored. It appears from reports in the literature (1, 2) that deuterium oxide is toxic in animals only when the concentration in the body fluids reaches high levels. Since this solvent is now available in large amounts at a reasonable cost, and since deuterium oxide resembles protium oxide (ordinary water) more closely than any other solvent, it seems appropriate to study the effect of this solvent in pharmaceutical systems. Our present interest deals with the investigation of certain drugs which are labile in an aqueous medium. The stability of these drugs may be prolonged in deuterium oxide. This may prove of value, for example, in the extemporaneous preparation of ophthalmic solutions, where stability and sterility are important considerations. The effect of deuterium oxide in maintaining the sterility of such solutions is also under investigation in this laboratory.

In an earlier paper (3), the effect of deuterium oxide on the local anesthetic activity of procaine was studied. The cornea of the guinea pig was used as the test site. It was found that 0.5% solution of procaine in deuterium oxide gave the same protection as a 1.0% aqueous solution measured by the corneal reflex test.

The hydrolysis of procaine in aqueous solutions was studied by Higuchi, *et al.* (4). The rate of hydrolysis was shown to increase with hydroxide ion concentration. In the present study the rates of hydrolysis in protium and deuterium oxides are compared over the pH range 8.0 to 11.0. The effect of elevated temperature is also noted.

EXPERIMENTAL

The hydrolytic decomposition of procaine hydrochloride in protium oxide and deuterium oxide was followed by a modification of the method suggested by Higuchi, *et al.* (4). A series of ultraviolet absorption measurements was made on a solution of procaine hydrochloride undergoing hydrolysis in a constant temperature bath. Absorption measurements were made with a Beckman DU spectrophotometer model 2400 at wavelengths of 287 and 271.5 mμ. The absorbance values obtained at the first wavelength corresponding to the absorption peak of procaine were used to calculate the per cent hydrolysis. The second wavelength readings are for the hydrolytic product, *p*-aminobenzoic acid, and serve as an internal check on side reactions which may occur. These readings should be constant since the absorption properties of procaine and *p*-aminobenzoic acid are identical at this wavelength. This has been noted by Higuchi, *et al.* Degree of hydrolysis was calculated by

$$\% \text{ ester remaining} = 100 \times \frac{(k_A - k_B)}{(k_C - k_B)} \quad \text{(Eq. 1)}$$

where k_A is the absorbance of a partially hydrolyzed 0.001% procaine hydrochloride solution, k_B is the absorbance of *p*-aminobenzoate ion equivalent to a 0.001% procaine hydrochloride solution, and k_C is the absorbance of an unhydrolyzed 0.001% procaine hydrochloride solution. All readings were taken at 287 mμ.

The hydrolysis of procaine hydrochloride was studied over the pH range 8.0 to 11.0 in protium oxide and over the "apparent" pH range 8.0 to 11.0 in deuterium oxide. The hydroxide ion concentration was maintained essentially constant during the course of hydrolysis by buffering the solutions with 0.5 *M* ammonium hydroxide–ammonium chloride buffer.

The buffer solutions in deuterium oxide were prepared in the same manner as the aqueous buffers. The "apparent" pH was measured with a Beckman pH-meter (model H-2), and the solutions were adjusted to correspond to the aqueous pH values. An appropriate correction (5) to the "apparent" pH value was made to obtain pD. Analysis of the deuterium oxide buffers indicated that the deuterium oxide content was at least 99%.

A 50-ml. volumetric flask was filled to slightly below the mark with buffer solution and the flask was placed on a constant-temperature water bath set at 40 ± 0.5°. When the solution in the flask reached the temperature of the bath, 50 λ of a freshly prepared 1.0% procaine hydrochloride stock solution was added. The flask was filled to the mark with buffer solution maintained at the temperature of the bath. The flask was then stoppered and shaken thoroughly. At zero time a 3.0-ml. sample was transferred from the flask to a spectrophotometer 1-cm. cell and the absorbance was determined. This corresponded to the k_C value of Eq. 1. The progress of hydrolysis was followed by removing samples in a similar manner at varying time intervals depending on the rate of hydrolysis. These readings gave k_A values for the above expression. Exactly 500 λ of a 0.503% *p*-aminobenzoic acid stock solution was added to a 5.0-ml. volumetric flask. The flask was filled to the mark with buffer solution and mixed well. Exactly 50 λ was transferred to a second 5.0-ml. volumetric flask. The flask was filled to the mark with buffer solution and shaken thoroughly. The absorbance was obtained and this supplied the k_B in the above expression. The concentration of *p*-aminobenzoate in the final dilution is equivalent to the concentration obtainable from a 0.001% procaine hydrochloride solution upon complete hydrolysis.

Since hydrolytic decomposition is accelerated at elevated temperatures, a comparative study was conducted at 100°. Exactly 50 λ of a 1.0% procaine hydrochloride solution was added to a 50-ml. volumetric flask. The flask was filled to the mark with pH 8.0 aqueous buffer or "apparent" pH 8.0 deuterium oxide buffer solution. The solution was mixed thoroughly and placed in 5.0-ml. colorbreak ampuls. The ampuls were sealed and placed in a boiling water bath. One ampul was set aside and was used to obtain the k_C reading. Ampuls were removed from the water bath at 5-minute intervals,

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TABLE I.—COMPARISON OF RATE OF HYDROLYSIS OF PROCAINE IN PROTIUM OXIDE AND DEUTERIUM OXIDE AT 40°

Protium Oxide pH	Protium Oxide Half-life, hr.	Deuterium Oxide pD	Deuterium Oxide Half-life, hr.	$T_{1/2}$ D_2O / $T_{1/2}$ H_2O [a]
8.0	38.0	8.4[b]	115.0	3.0
8.5	13.0	8.9	38.0	2.9
9.0	6.5	9.4	13.0	2.0
9.5	4.5	9.9	9.5	2.1
10.0	3.5	10.4	6.25	1.8
11.0	2.25	11.4	2.75	1.2
8.0[c]	14[d]	8.4	7.0[d]	2.0

[a] Ratio of half-lives at "apparent" pH in D_2O and pH in H_2O. [b] pD = pH + 0.4. [c] This run at 100° C. [d] Time for this run is in minutes.

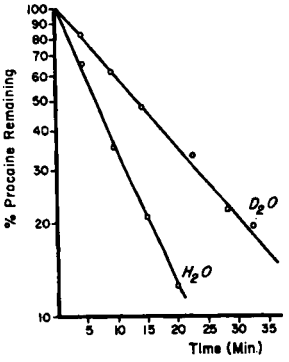


Fig. 1.—Comparison of rate of hydrolysis of procaine in protium oxide and deuterium oxide at pH and "apparent" pH 8.0 and at a 100° temperature.

plunged into an ice water bath, and the absorbance was obtained at 287 mμ. These supplied k_A readings. The k_B was obtained in the manner described for the 40° run.

The data obtained from the experiments described above are recorded in Table I. The hydrolysis rate of procaine at 100°, and pH 8 in protium oxide and "apparent" pH 8 in deuterium oxide is shown graphically in Fig. 1.

DISCUSSION

Over the pH range studied, the rate of deuteriolysis of procaine is less than the rate of hydrolysis. Over the pH and "apparent" pH range 8 to 11 and at 40° the ratio of half-lives in D_2O and H_2O is greatest at pH 8 (3.0) and decreases to a value of 1.2 at pH 11. Considerably higher ratios are obtained when the data at the equivalent pH and pD are compared. For example, when procaine in H_2O at pH 9.0 is compared with procaine in D_2O at pD 8.9, the ratio of half-lives is 5.8. However, when aqueous procaine at pH 9.0 is compared with procaine in D_2O at "apparent" pH 9.0, the ratio is 2.0, as shown in Table I. This would indicate that increased stability in D_2O is not simply a pH effect.

At 100° and pH 8 procaine is twice as stable in D_2O as in H_2O. The kinetic data for this study are shown graphically in Fig. 1. A typical first-order plot is obtained.

It was shown earlier (3) that the anesthetic activity of procaine, as observed on the cornea of the guinea pig, was greater in D_2O by a factor of 2. Since the anesthetic activity of procaine is due solely to free base which is predominant in the alkaline pH range, increased activity is, therefore, attributed to a greater stability (*in vivo*) of procaine base in deuterium oxide. Further, at the same pH and "apparent" pH, a deuterium oxide solution of procaine may contain more free base than in the corresponding aqueous solution.

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Effects of Limbic Lesions on Chlorpromazine-Pentobarbital Interaction

By MARVIN COHEN* and JOHN W. NELSON

Small lesions within the limbic system of the rat produced no consistent behavioral defects or altered responses to chlorpromazine and/or pentobarbital measured by a new behavioral scoring method.

THE LIMBIC SYSTEM is a portion of the brain located on the medial and basal walls of the cerebral hemispheres. Numerous stimulation and ablation studies (1–5) have indicated that this system is involved in the production or modification of emotional (affective) behavior. Other studies of a similar type have suggested that the psychotropic drug chlorpromazine may exert some of its behavioral effects through a mechanism involving the limbic system (6, 7). However, no portion of this system has definitely been shown to be a site for chlorpromazine. An investigation was undertaken to determine if chlorpromazine did act within the limbic system. This was done by the examination of responses to chlorpromazine after small lesions had been placed in selected areas of the limbic system.

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* Present address: IIT Research Institute, Chicago, Ill.

EXPERIMENTAL

The animals used were albino male rats of the Wistar strain and 70–100 days old. All animals were kept in groups of four and allowed free access to food and water, except during testing and operating periods. Lesions were made electrolytically with a Grass lesion maker (model LM-1). The current passed into each brain was of sufficient intensity to produce a lesion 0.5 to 1 mm. in diameter. All lesions were verified histologically by conventional paraffin embedding procedures. Sections were stained with a combination of Luxol fast blue, hematoxylin, and eosin to obtain maximum differentiation of the lesion. Sham operations were performed by anesthetizing an animal, trephining its skull, and inserting the needle electrode to the appropriate brain area without allowing electric cur-

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8.5	13.0	8.9	38.0	2.9
9.0	6.5	9.4	13.0	2.0
9.5	4.5	9.9	9.5	2.1
10.0	3.5	10.4	6.25	1.8
11.0	2.25	11.4	2.75	1.2
8.0[c]	14[d]	8.4	7.0[d]	2.0

[a] Ratio of half-lives at "apparent" pH in D_2O and pH in H_2O. [b] pD = pH + 0.4. [c] This run at 100° C. [d] Time for this run is in minutes.

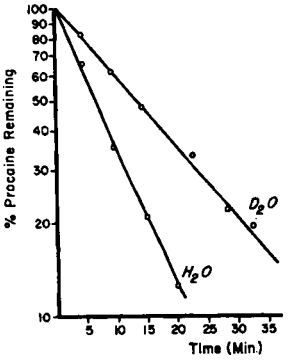


Fig. 1.—Comparison of rate of hydrolysis of procaine in protium oxide and deuterium oxide at pH and "apparent" pH 8.0 and at a 100° temperature.

plunged into an ice water bath, and the absorbance was obtained at 287 mμ. These supplied k_A readings. The k_B was obtained in the manner described for the 40° run.

The data obtained from the experiments described above are recorded in Table I. The hydrolysis rate of procaine at 100°, and pH 8 in protium oxide and "apparent" pH 8 in deuterium oxide is shown graphically in Fig. 1.

DISCUSSION

Over the pH range studied, the rate of deuteriolysis of procaine is less than the rate of hydrolysis. Over the pH and "apparent" pH range 8 to 11 and at 40° the ratio of half-lives in D_2O and H_2O is greatest at pH 8 (3.0) and decreases to a value of 1.2 at pH 11. Considerably higher ratios are obtained when the data at the equivalent pH and pD are compared. For example, when procaine in H_2O at pH 9.0 is compared with procaine in D_2O at pD 8.9, the ratio of half-lives is 5.8. However, when aqueous procaine at pH 9.0 is compared with procaine in D_2O at "apparent" pH 9.0, the ratio is 2.0, as shown in Table I. This would indicate that increased stability in D_2O is not simply a pH effect.

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Effects of Limbic Lesions on Chlorpromazine-Pentobarbital Interaction

By MARVIN COHEN* and JOHN W. NELSON

Small lesions within the limbic system of the rat produced no consistent behavioral defects or altered responses to chlorpromazine and/or pentobarbital measured by a new behavioral scoring method.

THE LIMBIC SYSTEM is a portion of the brain located on the medial and basal walls of the cerebral hemispheres. Numerous stimulation and ablation studies (1–5) have indicated that this system is involved in the production or modification of emotional (affective) behavior. Other studies of a similar type have suggested that the psychotropic drug chlorpromazine may exert some of its behavioral effects through a mechanism involving the limbic system (6, 7). However, no portion of this system has definitely been shown to be a site for chlorpromazine. An investigation was undertaken to determine if chlorpromazine did act within the limbic system. This was done by the examination of responses to chlorpromazine after small lesions had been placed in selected areas of the limbic system.

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EXPERIMENTAL

The animals used were albino male rats of the Wistar strain and 70–100 days old. All animals were kept in groups of four and allowed free access to food and water, except during testing and operating periods. Lesions were made electrolytically with a Grass lesion maker (model LM-1). The current passed into each brain was of sufficient intensity to produce a lesion 0.5 to 1 mm. in diameter. All lesions were verified histologically by conventional paraffin embedding procedures. Sections were stained with a combination of Luxol fast blue, hematoxylin, and eosin to obtain maximum differentiation of the lesion. Sham operations were performed by anesthetizing an animal, trephining its skull, and inserting the needle electrode to the appropriate brain area without allowing electric cur-

TABLE I.—RESPONSES OF ANIMALS WITH SEPTAL LESIONS

Animal No.	Response to Saline	Effect of Chlorpromazine Dose	Effect of Chlorpromazine Response, mg./Kg.	Effect of Pentobarbital Dose	Effect of Pentobarbital Response, mg./Kg.	Response to Combination
			Unoperated Animals			
13	0.5	1	1.0	17.5	10.0	7.0
31	1.0	1	2.0	17.5	7.0	7.0
47	2.0	1	3.0	17.5	10.0	10.0
14	1.5	3	4.0	5	1.5	1.0
39	2.0	3	3.0	5	2.0	4.0
55	0.5	3	0.5	5	0.5	0.5
15	2.5	5	5.0	10	1.0	6.0
45	3.0	5	5.0	10	3.0	4.0
74	1.0	5	3.0	10	2.0	6.0
16	1.0	7	3.0	15	7.0	6.0
53	0.5	7	4.0	15	3.0	7.0
76	1.5	7	5.0	15	2.0	10.0
			Operated Animals			
13[a]	0.5	1	0.5	17.5	7.0	7.0
31	0.5	1	1.0	17.5	10.0	3.0
47	2.0	1	1.0	17.5	10.0	7.0
14[a]	1.0	3	1.0	5	0.5	1.0
39	1.0	3	1.0	5	0.5	1.0
55	0.5	3	1.0	5	0.5	1.0
15[a]	1.0	5	3.0	10	3.0	6.0
45	1.0	5	5.0	10	2.0	5.0
74	1.0	5	4.0	10	2.0	6.0
16[a]	1.0	7	4.0	15	4.0	7.0
53	0.5	7	4.0	15	3.0	7.0
76	1.0	7	5.0	15	7.0	10.0

[a] Sham operation.

TABLE II.—RESPONSES OF ANIMALS WITH AMYGDALOID LESIONS

Animal No.	Response to Saline	Effect of Chlorpromazine Dose	Effect of Chlorpromazine Response, mg./Kg.	Effect of Pentobarbital Dose	Effect of Pentobarbital Response, mg./Kg.	Response to Combination
			Unoperated Animals			
75	3.0	1	2.0	17.5	10.0	7.0
73	1.0	3	4.0	5	2.5	3.0
33	1.0	3	1.0	5	1.0	2.0
46	3.0	3	3.0	5	3.0	4.0
80	0.5	5	5.0	10	2.0	6.0
21	1.0	5	5.0	10	1.0	5.0
34	1.0	5	1.0	10	3.0	4.0
78	3.0	7	4.5	15	6.0	10.0
30	1.0	7	5.0	15	3.0	5.0
36	1.0	7	5.0	15	6.0	6.0
			Operated Animals			
75[a]	1.0	1	1.0	17.5	10.0	10.0
73[a]	1.0	3	3.0	5	0.5	3.0
33	0.5	3	1.0	5	0.5	2.0
46	2.5	3	2.0	5	2.0	2.0
80[a]	1.0	5	3.0	10	1.0	4.0
21	1.0	5	3.0	10	1.0	5.0
34	1.0	5	4.0	10	3.0	5.0
78[a]	0.0	7	4.0	15	5.0	10.0
36	1.0	7	4.0	15	3.0	10.0
30	0.5	7	1.0	15	3.0	7.0

[a] Sham operation.

rent to pass. The wound was sutured, and the animal was allowed to recover.

Behavioral changes were determined by means of an original method which evaluated the responses to low (subhypnotic) doses of central depressants with a scoring system. The behavioral criteria utilized as the basis of the scoring system were loss of spontaneous motion, responses to external stimuli, and degree of ataxia. This method has been described in detail in a previous publication (8).

RESULTS

Four areas of the limbic system—the anterior (precommissural) septum, amygdaloid complex, rostral hippocampus, and medial forebrain bundle—were chosen for study. Unoperated and operated animals were tested for their responses to pentobarbital (5, 10, 15, or 17.5 mg./Kg.), chlorpromazine (1, 3, 5, or 7 mg./Kg.), and combinations of the two (3 mg./Kg. chlorpromazine + 5 mg./Kg. pentobarbital, 5 mg./Kg. chlorpromazine + 10 mg./Kg. pentobarbital, 7 mg./Kg. chlorpromazine + 15 mg./Kg. pentobarbital, or 1 mg./Kg. chlorpromazine + 17.5 mg./Kg. pentobarbital). All drugs were administered intraperitoneally. Combinations of chlorpromazine and pentobarbital were used because they gave a more sensitive indication of the behavioral effects of chlorpromazine than did the

latter administered alone, using the criteria of the behavioral method employed.

Tables I and II show the responses obtained with animals subjected to septal and amygdaloid lesions, respectively. It can be seen that small lesions in these areas produced no consistent change in behavior or in the responses to the administration of the drugs used. Similar results were obtained with the other areas investigated. The only behavioral change noted was a slight hyperexcitability; but since sham operated animals also showed this reaction, it was attributed to the operating procedure rather than to the presence of a lesion.

The results of this study indicate that investigations of behavior involving the production of lesions require a great deal of caution in the interpretation of behavioral changes that are seen. Lesion size may be one of the factors that has contributed to the many conflicting reports appearing in this field.

SUMMARY

Small lesions within the limbic system of the rat produced no consistent behavioral defects or altered responses to chlorpromazine and/or pentobarbital as measured by a new behavioral scoring method. Lesion size may be one of the factors that has contributed to the many conflicting reports appearing in this field.

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Facile Synthesis of Isoindoline and Substituted Isoindolines

By JOHN L. NEUMEYER*

The preparation of isoindoline and several derivatives bearing nuclear substituents by methods previously employed successfully only for *N*-substituted derivatives is described. The method involves the preparation and hydrogenolysis of *N*-benzyl isoindolines.

WHILE A considerable amount of work has been reported on the synthesis of *N*-substituted isoindolines and hydrogenated isoindolines by a variety of methods, difficulty was experienced by several workers (including the author) in obtaining isoindoline (VI*a*) in good yields and in a pure form. Isoindoline derivatives have been obtained by the reaction of amines with *o*-xylylene dibromide (1, 2). Bornstein (3) prepared isoindoline (VI*a*) by the hydrolytic cleavage of 2-(*p*-tolylsulfonyl)-isoindoline (prepared from *o*-xylylene dibromide and *p*-toluenesulfonamide) with phenol and hydrobromic acid in propionic acid. The reduction of *N*-substituted phthalimides with lithium aluminum hydride was a generally satisfactory method for obtaining *N*-substituted isoindolines (4, 5) and was utilized also for the preparation of tetrahydro and hexahydroisondoline (4), but failed when applied to the reduction of phthalimide. As a result, we have devised a method for the preparation of isoindoline and substituted isoindolines which was based on the lithium aluminum hydride reduction of *N*-benzylphthalimides of their corresponding isoindolines, followed by the hydrogenolysis of the benzyl moiety with palladium-on-charcoal. This route was satisfactory also for the preparation of 5-methylisoindoline (VI*b*) and 4-aminoisoindoline (VI*d*), but failed to yield 4-chloroisoindoline from the hydrogenolysis of *N*-benzyl-4-chloroisoindoline (V*c*). Treatment of V*c* with hydrogen and palladium caused the hydrogenolysis of the chlorine and the benzyl group; isoindoline (VI*a*) was the isolated product.

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* Present address: Arthur D. Little, Inc., Cambridge, Mass.

EXPERIMENTAL[1]

N-Benzylphthalimide.—This was prepared from potassium phthalimide (0.5 mole) and benzyl chloride (0.5 mole) in *N*,*N*-dimethylformamide in 75% yield by the method of Billman and Cash (6), crude, m.p. 111–112°. The compound was used in subsequent reactions without further purification. [Lit. (6) m.p. 115–116°.]

N-Benzyl-4-methylphthalimide (IIb).—This was obtained from potassium 4-methylphthalimide (I*b*) and benzyl chloride; yield: 72%, m.p. 122.5 to 123.5°.

Anal.—Calcd. for $C_{16}H_{13}NO_2$: C, 73.99; H, 5.77; N, 5.57. Found: C, 73.87; H, 5.82; N, 5.63.

N-Benzyl-3-chlorophthalimide (IIc).—This was obtained from potassium 3-chlorophthalimide (I*c*) and benzyl chloride; yield: 98%, m.p. 137 to 138.5° (recrystallized from *n*-butanol).

Anal.—Calcd. for $C_{15}H_{10}NO_2Cl$: C, 66.30; H, 3.71. Found: C, 66.29; H, 3.71.

N-Benzyl-3-nitrophthalimide (IId).—A mixture of 19.3 Gm. (0.1 mole) of 3-nitrophthalic anhydride and 10.7 Gm. (0.1 mole) of benzylamine was fused at 200–250° and the mixture allowed to cool. Upon recrystallization from isopropyl alcohol, 23 Gm. (83%) of yellow flakes, m.p. 140–141°, were obtained.

Anal.—Calcd. for $C_{15}H_{10}N_2O_4$: C, 63.83; H, 3.57; N, 9.93. Found: C, 63.68; H, 3.74; N, 9.90.

N-Benzyl-3-aminophthalimide (IIe).—A solution of 25 Gm. (0.089 mole) of *N*-benzyl-3-nitrophthalimide in 290 ml. of benzene was hydrogenated in a Parr hydrogenator at room temperature using 0.5 Gm. of platinum oxide until the hydrogen uptake

[1] Both the melting points and the boiling points were uncorrected. All melting points were obtained in a Thomas Hoover silicone oil filled capillary melting point apparatus. The assistance of Mr. R. C. Pharo with a number of the experiments reported herein, of Mr. J. E. Zarembo and his staff in carrying out the analyses, and of Mr. H. Adelman in the interpretation of infrared spectra is gratefully acknowledged.

latter administered alone, using the criteria of the behavioral method employed.

Tables I and II show the responses obtained with animals subjected to septal and amygdaloid lesions, respectively. It can be seen that small lesions in these areas produced no consistent change in behavior or in the responses to the administration of the drugs used. Similar results were obtained with the other areas investigated. The only behavioral change noted was a slight hyperexcitability; but since sham operated animals also showed this reaction, it was attributed to the operating procedure rather than to the presence of a lesion.

The results of this study indicate that investigations of behavior involving the production of lesions require a great deal of caution in the interpretation of behavioral changes that are seen. Lesion size may be one of the factors that has contributed to the many conflicting reports appearing in this field.

SUMMARY

Small lesions within the limbic system of the rat produced no consistent behavioral defects or altered responses to chlorpromazine and/or pentobarbital as measured by a new behavioral scoring method. Lesion size may be one of the factors that has contributed to the many conflicting reports appearing in this field.

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Facile Synthesis of Isoindoline and Substituted Isoindolines

By JOHN L. NEUMEYER*

The preparation of isoindoline and several derivatives bearing nuclear substituents by methods previously employed successfully only for *N*-substituted derivatives is described. The method involves the preparation and hydrogenolysis of *N*-benzyl isoindolines.

WHILE A considerable amount of work has been reported on the synthesis of *N*-substituted isoindolines and hydrogenated isoindolines by a variety of methods, difficulty was experienced by several workers (including the author) in obtaining isoindoline (VI*a*) in good yields and in a pure form. Isoindoline derivatives have been obtained by the reaction of amines with *o*-xylylene dibromide (1, 2). Bornstein (3) prepared isoindoline (VI*a*) by the hydrolytic cleavage of 2-(*p*-tolylsulfonyl)-isoindoline (prepared from *o*-xylylene dibromide and *p*-toluenesulfonamide) with phenol and hydrobromic acid in propionic acid. The reduction of *N*-substituted phthalimides with lithium aluminum hydride was a generally satisfactory method for obtaining *N*-substituted isoindolines (4, 5) and was utilized also for the preparation of tetrahydro and hexahydroisondoline (4), but failed when applied to the reduction of phthalimide. As a result, we have devised a method for the preparation of isoindoline and substituted isoindolines which was based on the lithium aluminum hydride reduction of *N*-benzylphthalimides of their corresponding isoindolines, followed by the hydrogenolysis of the benzyl moiety with palladium-on-charcoal. This route was satisfactory also for the preparation of 5-methylisoindoline (VI*b*) and 4-aminoisoindoline (VI*d*), but failed to yield 4-chloroisoindoline from the hydrogenolysis of *N*-benzyl-4-chloroisoindoline (V*c*). Treatment of V*c* with hydrogen and palladium caused the hydrogenolysis of the chlorine and the benzyl group; isoindoline (VI*a*) was the isolated product.

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EXPERIMENTAL[1]

N-Benzylphthalimide.—This was prepared from potassium phthalimide (0.5 mole) and benzyl chloride (0.5 mole) in *N*,*N*-dimethylformamide in 75% yield by the method of Billman and Cash (6), crude, m.p. 111–112°. The compound was used in subsequent reactions without further purification. [Lit. (6) m.p. 115–116°.]

N-Benzyl-4-methylphthalimide (IIb).—This was obtained from potassium 4-methylphthalimide (I*b*) and benzyl chloride; yield: 72%, m.p. 122.5 to 123.5°.

Anal.—Calcd. for $C_{16}H_{13}NO_2$: C, 73.99; H, 5.77; N, 5.57. Found: C, 73.87; H, 5.82; N, 5.63.

N-Benzyl-3-chlorophthalimide (IIc).—This was obtained from potassium 3-chlorophthalimide (I*c*) and benzyl chloride; yield: 98%, m.p. 137 to 138.5° (recrystallized from *n*-butanol).

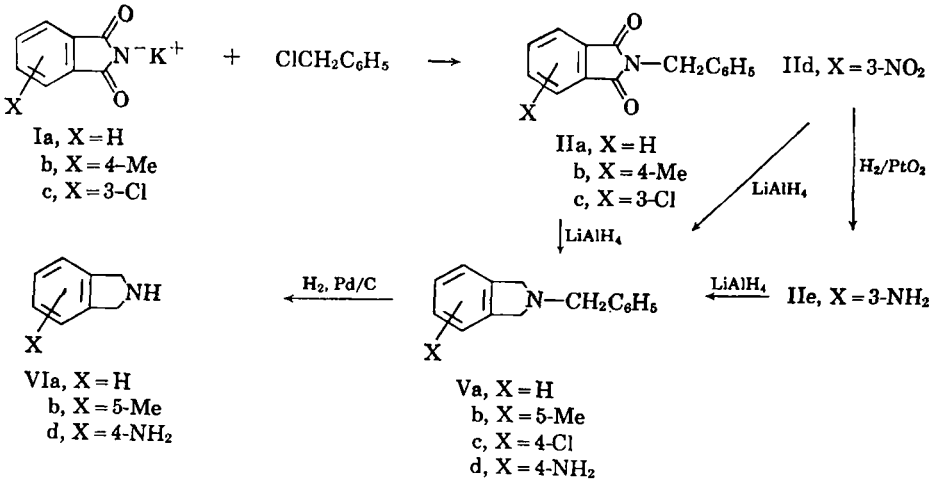
Anal.—Calcd. for $C_{15}H_{10}NO_2Cl$: C, 66.30; H, 3.71. Found: C, 66.29; H, 3.71.

N-Benzyl-3-nitrophthalimide (IId).—A mixture of 19.3 Gm. (0.1 mole) of 3-nitrophthalic anhydride and 10.7 Gm. (0.1 mole) of benzylamine was fused at 200–250° and the mixture allowed to cool. Upon recrystallization from isopropyl alcohol, 23 Gm. (83%) of yellow flakes, m.p. 140–141°, were obtained.

Anal.—Calcd. for $C_{15}H_{10}N_2O_4$: C, 63.83; H, 3.57; N, 9.93. Found: C, 63.68; H, 3.74; N, 9.90.

N-Benzyl-3-aminophthalimide (IIe).—A solution of 25 Gm. (0.089 mole) of *N*-benzyl-3-nitrophthalimide in 290 ml. of benzene was hydrogenated in a Parr hydrogenator at room temperature using 0.5 Gm. of platinum oxide until the hydrogen uptake

[1] Both the melting points and the boiling points were uncorrected. All melting points were obtained in a Thomas Hoover silicone oil filled capillary melting point apparatus. The assistance of Mr. R. C. Pharo with a number of the experiments reported herein, of Mr. J. E. Zarembo and his staff in carrying out the analyses, and of Mr. H. Adelman in the interpretation of infrared spectra is gratefully acknowledged.



had ceased (30 minutes). The catalyst and benzene were removed yielding 19.0 Gm. (85%) of yellow needles, m.p. 148.5 to 149.5°.

Anal.—Calcd. for $C_{15}H_{12}N_2O_2$: N, 11.11. Found: N, 11.35.

N-(Benzyl)isoindoline (Va).—The procedure for the reduction of the imide to the isoindoline was similar to the method previously described (4). To 1000 ml. of ether (dried over sodium) in a 2-L. flask fitted with a stirrer, Soxhlet extractor, and a thermometer was added 23 Gm. (0.6 mole) of lithium aluminum hydride. The thimble of the Soxhlet extractor was charged with 70 Gm. (0.3 mole) of *N*-benzylphthalimide and continuously extracted for 24 hours. With stirring, 100 ml. of 10% sodium sulfate was slowly added to the ether mixture while the reaction flask was cooled in an ice bath. The precipitate was filtered off and washed two times with 200 ml. of ether; the combined ether extracts were dried over magnesium sulfate, the ether was eliminated, and the residue distilled, yielding 37.5 Gm. (60%) of an oil, b.p. 180° (0.35 mm.), n_D^{25} 1.5897. This oil solidified on standing (m.p. 36–37°) and darkened when exposed to light and air. [Lit. (2) m.p. 41° recrystallized from alcohol.]

N-(Benzyl)-4-chloroisoindoline (Vc).—This was obtained from II*c* in 66% yield; b.p. 144° (0.5 mm.), n_D^{25} 1.5945. The compound darkens on standing.

Anal.—Calcd. for $C_{15}H_{14}ClN$: C, 73.91; H, 5.79; N, 5.75. Found: C, 73.69; H, 5.47; N, 5.62.

N-(Benzyl-5-methylisoindoline (Vb).—This was obtained from II*b*, b. p. 126–130° (0.04 mm.), m.p. 47–49°. The analytical sample, m.p. 57–58°, was recrystallized from ethanol-water.

Anal.—Calcd. for $C_{16}H_{17}N$: C, 86.05; H, 7.68; N, 6.27. Found: C, 85.67; H, 7.53; N, 6.31.

N-(Benzyl)-4-aminoisoindoline (Vd).—This was obtained from II*e* in 45% yield; b.p. 152–157° (0.001 mm.), m.p. 64–65°. The compound was characterized as the phenylurea derivative, prepared from phenyl isocyanate in ether, m.p. 178–179°.

Anal.—Calcd. for $C_{22}H_{21}N_3O$: C, 76.94; H, 6.16; N, 12.24. Found: C, 77.25; H, 6.37; N, 12.06.

The reduction of *N*-benzyl-3-nitrophthalimide (II*d*) (0.07 mole) with lithium aluminum hydride (0.21 mole) by the aforementioned procedure also yielded 27% of V*d*.

Isoindoline (VIa).—Hydrogenolysis of 9.5 Gm. (0.043 mole) *N*-(benzyl)isoindoline (V*a*) in 100 ml. of ethanol was carried out by shaking the solution in a Parr apparatus for 16 hours with 2 Gm. of 5% palladium-on-carbon under an initial pressure of 50 p.s.i. hydrogen at 50°. Distillation of the product gave 3.8 Gm. (75%) of VI*a* b.p. 105° (20 mm.), n_D^{25} 1.5650, a clear oil which solidifies on standing in the refrigerator, m.p. 17°. [Lit. (2) b.p. 115° (30 mm.), n_D^{25} 1.5698, m.p. 16.0 to 16.5°.] The *p*-toluenesulfonyl derivative was prepared by treating 0.5 Gm. of isoindoline (VI*a*) with 0.6 Gm. of *p*-toluenesulfonyl chloride in 3 ml. of pyridine. The mixture was warmed for 30 minutes, allowed to cool, and poured over ice. The yellow crystals which precipitated were filtered and recrystallized from ethanol, m.p. 175 to 175.5°. [Lit. (3) 175–176° prepared from *o*-xylylene dibromide and *p*-toluenesulfonamide.]

5-Methylisoindoline (VIb).—This was prepared by the hydrogenolysis of V*b* in the aforementioned manner in 76% yield, b.p. 111° (14 mm.), m.p. 43–44°.

Anal.—Calcd. for $C_9H_{11}N$: C, 81.16; H, 8.38; N, 10.52. Found: C, 80.91; H, 8.05; N, 10.30.

This compound was further characterized by preparing the phenylurea derivative, m.p. 160–161°.

Anal.—Calcd. for $C_{16}H_{16}N_2O$: C, 76.16; H, 6.39; N, 11.10. Found: C, 76.41; H, 6.33; N, 11.21.

4-Aminoisoindoline (VId).—This was obtained by the hydrogenolysis of V*d* in the aforementioned manner (80% yield); b.p. 137–140° (0.1 mm.), m.p. 86–87°.

Anal.—Calcd. for $C_8H_{10}N_2$: C, 71.61; H, 7.51; N, 20.88. Found : C, 71.35; H, 7.60; N, 20.61.

The dihydrochloride of VI*d* was prepared from methanol and hydrogen chloride and recrystallized from methanol-ether, m.p. 283–284°.

Anal.—Calcd. for $C_8H_{10}N_2 \cdot 2HCl$: C, 46.35; H, 5.84; N, 13.53. Found: C, 46.24; H, 5.80; N, 13.61.

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Determination of Trace Elements in Drugs by Neutron Activation Analysis

By M. M. TUCKERMAN*, L. C. BATE, and G. W. LEDDICOTTE

Results of determinations of trace elements in drugs by neutron activation analysis are presented for more than 150 samples including inorganics, salicylates, steroids, vitamins, antibiotics, and chlorinated compounds. Trace elements found include aluminum, antimony, arsenic, bromine, chlorine, copper, gallium, manganese, mercury, scandium, sodium, and thorium.

MONOGRAPHS setting standards for drugs frequently have limit tests for "heavy metals." The tests are more accurately described as "substances colored by sulfide ions under the conditions specified for preparing the sample as compared visually with a lead standard." The sensitivity of the test varies with the metallic ions present and the acidity of the solution. The visual comparison suffers from the differences in color of the various metal sulfides.

Since the test normally sets limits in the parts-per-million range, about 1 Gm. of material must be destroyed. Organic materials obtained from good manufacturing processes almost invariably give no discernible color when tested. The considerations of the cost involved in performing the assay on the more expensive synthetic drugs, and of the possible amounts of impurities that could be introduced by milligram-level doses, have led to a re-examination of the rationale for this test by the Subcommittee on Heavy Metals of the Board of Revision of the United States Pharmacopeia.

A preliminary general investigation of trace elements in drugs was undertaken to collect data for recommendations for tests for trace contaminants. Nondestructive neutron activation analysis was chosen as a rapid method which provided adequate sensitivity for all toxic elements and all common contaminants (except lead) if 100-mg. samples were irradiated in the neutron flux available in the air-cooled portion of the Oak Ridge graphite-moderated reactor (1).

Neutron activation is based on the work of Fermi (2) as further developed by Curie (3), Livingston (4), Hevesy (5), Seaborg (6), and Clark and Overman (7). A comparison of the limits of neutron activation with other methods of analysis has been made in a review by Meinke (8). The physical, chemical, and nuclear limitations of the method have been recognized in a review by Smales (9).

Neutron activation analysis is a method for quantitative and qualitative determinations of the elements in a sample using the technique of nuclear transformation. When a sample is irradiated by neutrons produced in a nuclear reactor or other source, some of the atoms in the sample interact with the neutrons and are converted into radioactive isotopes of the same element (10).

The product produced by irradiation of a stable isotope with neutrons is usually radioactive and produces gamma rays. The energies of the emitted gamma rays and the relative numbers emitted at each energy is the gamma spectrum, a characteristic of each isotope which can be used for qualitative identification (11).

Quantitative measurement is made either by comparison of the sample with a standard (12) or by mathematical calculation based upon a knowledge of the irradiating neutron flux and the geometry and efficiency of the gamma-ray detector (13).

For most samples, the recorded gamma spectrum is complex, consisting of the sum of the spectra from the isotopes present. These can be identified by the process of "spectrum stripping" in which the spectral contribution from an isotope present in the sample is electronically subtracted from the recorded spectrum of the sample (14). This process can be repeated, subject only to the statistical nature of emission of radiation, until all isotopes present have been identified and quantitatively determined. Although results presented in this paper were obtained through the process of spectrum stripping by manual operations, a computer-controlled process has been shown to be feasible (15).

EXPERIMENTAL

Solid samples in Bakelite-capped glass vials were placed in a polyethylene secondary container and were irradiated for appropriate periods of time from a few hours to 1 week in the air-cooled hole of the X-10 graphite-moderated reactor at Oak Ridge National Laboratory. Assays for aluminum were done by irradiation of the sample in polyethylene primary and secondary containers for 1 minute in the pneumatic tube of the Oak Ridge "swimming pool" reactor. After irradiation, samples were weighed and placed in clean containers for counting. Counting was done with a single 3 × 3 in. thallium-activated sodium iodide crystal optically coupled to a 3-in. Dumont photomultiplier tube. This assembly was contained in a graded lead–copper–cadmium shield about 3 ft. in diameter. The geometry of the counter was adjusted and polyethylene absorbers for beta-radiation were used as needed to make proper use of the equipment. The gamma-ray spectrum was obtained by sorting the pulses from the phototube, using a Radiation Instruments Development Laboratory 200-channel analyzer. These spectra were analyzed by manual spectrum stripping using liquid standards of known concentrations irradiated simultaneously with the sample.

RESULTS AND DISCUSSION

The results of the assays are shown in Table I. These results represent a very small sampling compared with the total number of pharmaceutical batches produced. No general conclusions, therefore, should be drawn from the data.

Quite unexpected, however, was the widespread

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TABLE I.—TRACE ELEMENTS FOUND IN DRUG SAMPLES[a]

Substance	No. of Samples	Elements Detected											
		Al	Sb	As	Br	Cl	Cu	Ga	Mn	Hg	K	Sc	Na	Th
Acetophenetidin	5				2		3		2				2	
Acetophenetidin	4												5	
Acetylsalicylic acid	5				3				1				2	
Acetylsalicylic acid	5								3				3	
Aluminum hydroxide	3												8	
Amodiaquine hydrochloride	3				4		4						5	
Ascorbic acid	10			2			2		2	3			4	
Benzoic acid	5								2				4	
Bismuth subcarbonate	3				4		3		3				4	
Bismuth subcarbonate	1												6	
Calcium carbonate	5				4		4						5	
Calcium-*d*-pantothenate	4				5		4	3	5				5	
Calcium gluconate	4				6								4	
Cherry juice	1						3		3		7			
Chloramphenicol	4				4								4	
Chloramphenicol palmitate	4			2	4				3				4	
Chlormezanone	3								4				4	
Chloroquine phosphate	3			3	4		4		3				5	
Cortisone acetate	3				6									
Diphenhydramine hydrochloride	4				6								4	
Diphenylhydantoin	4						4						5	
Ferrous gluconate	3				5		4		5				5	
Hydrocortisone	3				5									
Hydrocortisone acetate	3				4								4	
Magnesium stearate	4		2										6	
Magnesium trisilicate	4											3	8	
Methocel	5					6							6	
Methyl salicylate	5				2	2	1						2	
Niacin	5	4					3		2	3		1	4	2
Methyl salicylate	5				3		2						3	
Niacinamide	5									3			5	
Phenol	3												3	
Phenylephrine hydrochloride	3		3		4								4	
Prednisolone	3				6									
Predisolone acetate	3				5								5	
Prednisone	3				6									
Riboflavin	3			2	3	7			2	3			4	
Talc	2								6			3	7	
Talc	2											3	6	
Tannic acid	4						4		3		6		5	
Contamination from ascorbic acid							6		4				6	

[a] Key: Average of samples in which element was detected. Blank, not detected; 1, less than 0.01 p.p.m.; 2, 0.010 to 0.099 p.p.m.; 3, 0.10 to 0.00 p.p.m.; 4, 1.0 to 9.9 p.p.m.; 5, 10 to 99 p.p.m.; 6, 100 to 990 p.p.m.; 7, 1000 to 9,900 p.p.m.; 8, 10,000 to 49,000 p.p.m.

occurrence of bromine, which may indicate the presence of brominated intermediates, bromine in raw materials, or bromine in the plant atmosphere. Wide differences in sodium content for groups of samples of acetophenetidin, acetylsalicylic acid, methyl salicylate, and talc clearly indicate the origin of samples from different manufacturers. The presence of copper in a grossly discolored portion of a sample of ascorbic acid emphasize the need for care in the manufacture of this substance. The occurrence of arsenic, antimony, and mercury in some samples, albeit in quantities which would not be detected by the U.S.P. heavy metals test, shows the possibility of contamination and the need for retention of the U.S.P. test.

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Synthesis of Some Amides and Amines Containing the 1,4-Benzodioxan Nucleus as Potential Adrenolytic Agents

By MARTIN SCHREIBMAN, CLIFTON E. MILLER, WILLIAM H. SHELVER, and JAMES P. VACIK

Ten substituted 1,4-benzodioxan carboxamides and five substituted aminomethyl-1,4-benzodioxans have been synthesized for evaluation as potential adrenolytic agents.

NUMEROUS COMPOUNDS containing the 1,4-benzodioxan nucleus have been synthesized and pharmacological evaluation of a number of these compounds has revealed a variety of pharmacological activities (1). Most of the active adrenolytic agents contain a mono- or disubstituted aminomethyl group in the 2 position of the benzodioxan nucleus. The substituents on the nitrogen and position of the nitrogen have been shown to affect the activity of the benzodioxans as adrenergic blocking agents (2–4). Very few examples of 2-arylaminomethyl-1,4-benzodioxans have been reported. Therefore, the synthesis of some 2-arylaminomethyl-1,4-benzodioxans was undertaken to explore the effect of this type of substitution upon pharmacological activity. In addition to the variation in the substituent attached to the nitrogen, this series will produce a decrease in the basicity of the compounds as compared to previously prepared aliphatic derivatives of the benzodioxan nucleus. Because of the potent adrenolytic activity of several bis-benzodioxan derivatives (5, 6) some compounds of this type were also synthesized.

The preparation of these derivatives was first attempted by the reaction of 2-chloromethyl-1,4-benzodioxan with the appropriate amine, a procedure commonly used for the synthesis of this type of compound. Because this route required large amounts of 2-chloromethyl-1,4-benzodioxan, a modified synthesis was initially developed for the chloro compound. This material had been reported previously to have been prepared from the reaction of 2-hydroxymethyl-1,4-benzodioxan with thionyl chloride and an organic base in the presence of an inert solvent (7). A more convenient route for the preparation of 2-chloromethyl-1,4-benzodioxan involved the reaction of excess thionyl chloride with the alcohol in the absence of an acid acceptor. The product obtained in this manner was identical in all respects to the product obtained utilizing the older procedure. The reaction of aniline with 2-chloromethyl-1,4-benzodioxan produced the desired amine in moderate yield. However, when the latter reaction was carried out using *p*-phenylenediamine no desirable product was isolated. A variety of reaction conditions and isolation procedures was attempted with no success.

An alternate procedure for the synthesis of substituted aminomethyl-1,4-benzodioxans has been reported by Koo (8). In this method the appropriate amine is reacted with 1,4-benzodioxan-2-carbonyl chloride to produce the amide which upon reduction with lithium aluminum hydride produced the desired amine. This method proved to be more successful and resulted in the synthesis of the compounds shown in Tables I and II. Attempts to reduce several of the compounds failed to result in the isolation of identifiable products. Since certain benzodioxancarboxamides have been reported to show an effect on the central nervous system (9), the intermediate carboxamides in this study are also of pharmacological interest. The compounds synthesized have been submitted for a general pharmacological screening and the results obtained will be the subject of a subsequent communication.

The general procedure for the synthesis is given below and the specific properties and analytical data are cited in Tables I and II.

2-Chloromethyl-1,4-benzodioxan.—Twenty-five grams (0.15 mole) of 2-hydroxymethyl-1,4-benzodioxan (10) in a 500-ml., two-neck flask equipped with magnetic stirrer, dropping funnel, and reflux condenser was reacted with 36 Gm. (0.30 mole) of thionyl chloride at room temperature. The reaction mixture was refluxed for 30 minutes and excess thionyl chloride removed by distillation at atmospheric pressure. The crude product was distilled and the 2-chloromethyl-1,4-benzodioxan collected at 110–112° (5 mm.), n_D^{25} 1.5510, yield 83% [reported b.p. 132° (14 mm.) (10)]. The product thus obtained had an infrared spectrum compatible with its structure, was light yellow, and was pure enough for subsequent reactions. The yellow color may be removed by washing the thionyl chloride stripped product with water and drying prior to vacuum distillation. A preparation was carried out with a fourfold quantity of reactants and gave an over-all yield of 77%.

2-Phenylaminomethyl-1,4-benzodioxan.—To a 100-ml. three-neck flask was added 10 Gm. (0.054 mole) of 2-chloromethyl-1,4-benzodioxan and 25 ml. (0.27 mole) of aniline. The mixture was refluxed under nitrogen for 2 hours, and the excess aniline removed by rendering the mixture basic with aqueous sodium hydroxide and steam distilling. The residue remaining was extracted twice with ether. The ethereal extracts were combined, dried over anhydrous sodium sulfate, and evaporated to yield a brownish colored oil. Aqueous hydrochloric acid (5 *M*) was added to the oil, producing a solid material. The solid material was recrystallized twice from propyl alcohol and was identical with the material formed by the alternate procedure.

1,4-Benzodioxan-2-carboxamide.—Koo's method (8) was used to obtain the 1,4-benzodioxan-2-carbonyl chloride used in the following reaction. 1,4-Benzodioxan-2-carbonyl chloride in 50 ml. of benzene was added dropwise with stirring over a period of 1 hour to a solution of the appropriate amine in 150 ml. of boiling benzene. The mixture was stirred and refluxed for an additional 2 hours. A 2:1 molar ratio of the appropriate amine to acid chloride was used when the amine had one reactive amino group. When the amine had two reactive

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TABLE I.—AMIDES OF 1,4-BENZODIOXAN-2-CARBOXYLIC ACID

Amine Used	M.p.,[b] °C.	Molecular Formula	Analysis[a] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Yield, %	Recrystallization Solvent
Aniline	116–118	$C_{15}H_{13}NO_3$	70.57	70.76	5.19	5.09	78	Ligroin
1-Phenylpiperazine	138–139	$C_{19}H_{20}N_2O_3$	70.35	70.62	6.22	6.35	73	Methylcyclohexane
2-Amino-4-methylpyridine	109	$C_{15}H_{14}N_2O_3$	66.65	66.57	5.22	5.33	67	Ethanol and Water
p-Dimethylaminoaniline	127–128	$C_{17}H_{18}N_2O_3$	68.44	68.23	6.08	5.90	62	Methylcyclohexane
p-Phenylenediamine	271–275 dec.	$C_{24}H_{20}N_2O_6$	66.66	66.41	4.66	4.61	63	Dioxane and Water
trans-1,4-Aminomethylcyclohexane	209–210	$C_{26}H_{30}N_2O_6$	66.93	67.02	6.48	6.30	63	Ethanol
cis-1,4-Aminomethylcyclohexane	124–125	$C_{26}H_{30}N_2O_6$	66.93	66.80	6.48	6.36	77	Ethanol and Water
Diphenylamine	127–128	$C_{21}H_{17}NO_3$	76.12	76.09	5.17	5.28	89	Ethanol and Water
Phenothiazine	138–140	$C_{21}H_{15}NO_3S$	69.91	69.91	4.18	4.29	Quant.	Ethanol and Water
p-Nitroaniline	179	$C_{15}H_{12}N_2O_5$	60.05	60.15	4.03	4.09	Quant.	Benzene

[a] Elemental analysis was performed by Alfred Bernhardt Microanalytical Laboratories, 433 Mülheim (Ruhr), Höhenweg 17, Germany. [b] Melting points reported here are uncorrected.

TABLE II.—(1,4-BENZODIOXAN-2-YLMETHYL) AMINES

Amine Used	M.p.[b] of Salt, °C.	Molecular Formula	Analysis[a] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Yield, %	Recrystallization Solvent
Aniline	191–192	$C_{15}H_{16}ClNO_2$	65.10	64.85	5.82	5.90	60	Isopropanol
1-Phenylpiperazine	240–244	$C_{19}H_{24}Cl_2N_2O_2$	59.53	59.42	6.31	6.31	37	Ether and Ethanol
p-Dimethylaminoaniline	195–199	$C_{17}H_{21}ClN_2O_2$	57.15	57.29	6.21	6.38	43	Ether and Ethanol
p-Phenylenediamine	234–236	$C_{24}H_{26}Cl_2N_2O_4$	60.38	60.23	5.49	5.38	64	Ether, Ethanol, and HCl
1,4-Aminomethylcyclohexane	261–265	$C_{26}H_{36}Cl_2N_2O_4$	61.05	61.37	7.10	6.97	40	Ether and Ethanol

[a] Elemental analysis was performed by Alfred Bernhardt Microanalytical Laboratories, 433 Mülheim (Ruhr), Höhenweg 17, Germany. [b] Melting points reported here are uncorrected.

amino groups, a one to one ratio of the amine to acid chloride was employed. Here, the addition of the last half of the amine was delayed until the reaction had been refluxed for 1 hour, and it was added to the reaction mixture over a period of 1.5 hours; the mixture was refluxed for 2 additional hours. In either of the above cases, after standing overnight, the resultant mixture was treated with cold water to dissolve the precipitated amine salt. The benzene layer was washed with 10% aqueous potassium hydroxide, with 2.5 *N* aqueous hydrochloric acid, and finally twice with water. It was dried with anhydrous sodium sulfate and evaporated. If the amine had a second nonreactive nitrogen or the amide formed was only slightly soluble in cold benzene, modified techniques for obtaining the product were employed. If the amide was only slightly soluble in benzene, cold water was added and the product removed by filtration. If the amine had a second nonreactive nitrogen, cool water was added and the benzene layer separated. The benzene was washed once with potassium hydroxide solution and twice with water. In this latter technique most of the amine was separated from the amide in the benzene layer which was dried over anhydrous sodium sulfate and evaporated leaving the product which was purified by recrystallization from the appropriate solvent. The solvents of recrystallization, analysis and physical properties are listed in Table I.

Substituted Aminomethyl-1,4-benzodioxans.—2-Carboxamides were added in a 1:4 molar ratio to lithium aluminum hydride suspended in anhydrous ether and the reaction mixture was stirred and refluxed for a period of 8 hours. Ethyl acetate (20 ml.) was added dropwise to decompose the lithium aluminum hydride and 200 ml. of 20% potassium sodium tartrate in water was added to the reaction mixture. The ether layer was separated and the water layer extracted twice with ether. The ethereal extracts were combined, washed twice with water, and dried over anhydrous sodium sulfate. Evaporation of the ether yielded an oil or solid material which was dissolved in absolute ether to which was added hydrogen chloride to form the appropriate amine salt. When the compound reduced had two carboxamide groups, a 1:8 ratio of amide to lithium aluminum hydride was employed. The properties of the compounds prepared are listed in Table II.

SUMMARY

A number of previously unreported substituted aminomethyl-1,4-benzodioxans have been synthesized. A general method for the synthesis of 2-aminomethyl-1,4-benzodioxans containing an aryl or heteroaryl substituent on the nitrogen has been developed.

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"Aggregation" Phenomenon and Some Ephedrine Isomers

By HAROLD H. WOLF, MARTIN A. SCHUSTER*, and GEORGE LANCIAULT

Experiments have shown that three ephedrine isomers, D(−) ephedrine, L(+) ephedrine, and D(−) pseudoephedrine, are significantly more lethal to grouped *versus* isolated mice. The magnitude of this effect is considerably less than that observed with racemic amphetamine. A fourth isomer, L(+) pseudoephedrine, did not demonstrate this phenomenon.

OUR INTEREST in the pharmacology of a series of ephedrine isomers was first stimulated by the findings of LaPidus, *et al.* (1), who reported that D(−) pseudoephedrine blocked the pressor activity of D(−) ephedrine. It appeared worthwhile to initiate a study into the central nervous system activity of these compounds and to compare critically their activity with that of racemic amphetamine as well as with that of two additional ephedrine isomers which were available—L(+) ephedrine and L(+) pseudoephedrine.

As a starting point for these studies, a series of experiments was designed to evaluate the incidence of lethality to these agents observed in an isolated *versus* aggregated environment; the object of this work was to establish the presence or absence of the well-known "aggregation" phenomenon (2–7) with these isomers. The results obtained constitute the basis for this note.

EXPERIMENTAL

Novice, adult, male, albino mice of a random bred Swiss strain (Maxfield Animal Supply, Cincinnati, Ohio) were used as experimental animals. These mice, which ranged in weight from 17 to 23 Gm., were housed in groups of 16 to 22 animals each in metal cages 24 × 23 × 18 cm. They were maintained on Purina laboratory chow and had free access to food and water except during the experimental test periods.

All experiments were conducted in both an aggregated and isolated environment. Aggregation was defined as placing three mice in a metal cage 7 × 7 × 7.5 cm. with a wire mesh bottom; isolation consisted of placing a solitary mouse in a similar cage. The room temperature was maintained at approximately 25° throughout the study.

Aqueous solutions of all drugs were administered intraperitoneally in a constant volume of 1 ml. per 100 Gm. body weight. With the exception of L(+) ephedrine, which was obtained as the hydrochloride salt, the free base of the ephedrine isomers was dissolved in a few drops of 1:20 hydrochloric acid and the volume of the resulting solution adjusted with distilled water. Amphetamine[1] was administered as the sulfate salt. The pH of the drug solutions was approximately 5. In all instances drug dosage was calculated as free base.

To determine the dose lethal to 50% of animals (LD_{50}) for each drug, groups of nine animals were randomly selected, injected with various doses of the agent under investigation, and placed in either an aggregated or isolated environment. The incidence of lethality and general behavior of the animals were recorded every 15 minutes for a period of 3 hours. Dead animals in the group situation were replaced with noninjected animals to maintain a constant degree of aggregation. This procedure was repeated until a minimum of three points were established in the range between 0 and 100% lethality in both environmental situations. The results obtained were then plotted on logarithmic probability paper, and a regression line was fitted to the plotted points by eye. From this plot of the data, the LD_{50}, 95% fiducial limits, and the lethality potency ratio, (LPR = LD_{50} isolation/LD_{50} aggregation) were calculated by the method of Litchfield and Wilcoxon (8).

RESULTS AND DISCUSSION

The LD_{50}'s and LPR's obtained for the agents under investigation are shown in Table I. It is evident from these results that, except for L(+) pseudoephedrine, all drugs were significantly more lethal to grouped *versus* isolated mice. Thus an "aggregation" phenomenon was demonstrated with D(−) ephedrine, L(+) ephedrine, D(−) pseudoephedrine, and racemic amphetamine. The findings pertaining to D(−) ephedrine are in agreement with those reported by Chance (3), who also found this agent to be considerably more lethal in animals subjected to aggregation. To our knowledge, the central nervous system activity of the remaining ephedrine isomers has never been critically investigated.

Although three of the four ephedrine isomers tested demonstrated the "aggregation" phenomenon, an examination of the LPR's reveals that the magnitude of these responses was significantly less than that observed with amphetamine. Furthermore, no statistical difference was apparent among the LPR's of those ephedrine isomers which were shown to be more lethal in an aggregated environment.

In addition to the quantitative differences in

TABLE I.—LETHALITY OF SELECTED STIMULANTS IN AGGREGATED AND ISOLATED MICE[a]

Drug	LD_{50}[b] Aggregated	LD_{50}[b] Isolated	LPR[c]
D(−) Ephedrine	177 (138–209)	270 (229–319)	1.53 (1.13–2.06)
L(+) Ephedrine	255 (234–278)	323 (299–349)	1.31 (1.19–1.43)
D(−) Pseudoephedrine	248 (214–288)	312 (292–324)	1.26 (1.07–1.49)
L(+) Pseudoephedrine	220 (194–248)	224 (193–260)	1.02 (0.76–1.38)
(±) Amphetamine	12.2 (9.5–15.5)	104 (97.1–110)	8.5 (6.6–11.1)

[a] Three-hour observation period. [b] mg./Kg. Values in parentheses are 95% fiducial limits. [c] Lethality potency ratio = LD_{50} isolation/LD_{50} aggregation. Values in parentheses are 95% fiducial limits.

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The authors thank Dr. Jules LaPidus for supplying the L(+) ephedrine and D(−) pseudoephedrine.
*National Science Foundation Undergraduate Research Participant, 1963.
[1] Marketed as Benzedrine Sulfate by Smith Kline and French.

lethality described above, the agents investigated manifest some striking qualitative differences in toxicity. Although the design of the test cages did not permit continuous observation, it was possible to make intermittent examinations of general behavior. On the basis of these observations it was apparent that the toxicity of racemic amphetamine and D(−) ephedrine could be qualitatively differentiated from that of the other ephedrine isomers employed. For example, pilo erection was commonly observed in mice which had been treated with either racemic amphetamine or D(−) ephedrine and then placed in either isolation or aggregation. In contrast, little pilo erection was observed in animals subjected to similar environmental conditions after having been treated with L(+) ephedrine, D(−) pseudoephedrine, or L(+) pseudoephedrine.

Animals injected with lethal doses of either racemic amphetamine or D(−) ephedrine and then placed in an isolated environment demonstrated tremors, loss of righting reflex, clonic and tonic convulsions, and acute respiratory failure leading to death. These animals invariably died within 1 hour after drug administration, usually in the throes of a convulsion. On the other hand, there appeared to be two distinct causes of death in mice treated with lethal doses of either of the above agents and then placed in a crowded environment. Those animals that died within the first hour succumbed to convulsions (as described above), whereas exhaustion due to prolonged hyperactivity or repeated episodes of clonic convulsions was the apparent cause of death in animals that expired 1 or more hours after drug administration. These mice lost their righting reflex, appeared comatose, and experienced progressive respiratory difficulty until apnea occurred and death ensued.

In contrast to the above, the pattern of toxicity observed in mice treated with L(+) ephedrine, D(−) pseudoephedrine, or L(+) pseudoephedrine was essentially the same for both environmental situations. Death was caused by acute respiratory failure preceded by clonic or tonic convulsions, and always occurred within 1 hour after drug administration.

An explanation for the failure of L(+) pseudoephedrine to demonstrate an "aggregation" phenomenon is not readily available. However, a number of preliminary experiments conducted in our laboratory, *e.g.*, determination of the behavioral changes accompanying drug administration, low frequency electroshock seizure threshold studies, and examination of drug-induced alterations in levels of central nervous system activity measured by chemoshock techniques indicate that several major quantitative and qualitative differences exist among these four ephedrine isomers. It is anticipated that further investigation into the exact nature of central stimulation induced by these agents will help elucidate the findings reported herein.

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Colorimetric Microdetermination of Plasma Free Fatty Acids

By DONALD C. KVAM, J. G. SCHMIDT, D. A. RIGGILO, and D. G. GALLO

The application of a colorimetric method to the determination of free fatty acids in plasma is described. The method is simple, sensitive, and yields results comparable to those obtained with the titrimetric procedure.

THE MEASUREMENT of unesterified fatty acid levels in biological samples is usually accomplished by microtitration of the acidity present in suitably prepared extracts (1). Such microtitration techniques require special equipment and even with care and experience may give variable results. Ayers reported a colorimetric method for the estimation of fatty acids based upon the finding that copper or cobalt salts of monocarboxylic acids dissolve in chloroform to give colored solutions (2). Iwayama subsequently developed an essentially new procedure, also based upon the solubility of the copper salts of C_{10}–C_{22} monocarboxylic acids in chloroform, but its sensitivity was not great enough to be useful for the determination of plasma free fatty acids (3). The sensitivity of the latter procedure was greatly increased by Duncombe, who measured the concentration of copper in the chloroform solution with diethyldithiocarbamate (4). We have adapted this colorimetric method to the determination of plasma free fatty acids following their extraction by the technique of Dole and Meinertz (1). The resulting procedure is simple, very sensitive and reproducible, and uses commonly available laboratory equipment.

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EXPERIMENTAL

Reagents.—The following reagents were used.

Extraction Solution.—Mix 1 *N* H_2SO_4, hexane, and isopropyl alcohol in the volume ratio 20:200:780.

Cupric Nitrate Reagent.—Mix 450 ml. of 1 *M* triethanolamine, 50 ml. 1 *N* acetic acid, and 500 ml. 5% cupric nitrate solution.

Sodium Diethyldithiocarbamate, 0.1%.—Dissolve 100 mg. $(C_2H_5)_2NCS_2Na \cdot 3H_2O$ in 100 ml. normal butanol (prepare fresh daily).

Stock Standard Fatty Acid Solution.—Dissolve 56.8 mg. stearic acid in 100 ml. of hexane.

Working Standard Fatty Acid Solution.—Dilute 10 ml. of stock standard to 100 ml. with hexane. This solution contains 0.2 μmoles/ml.

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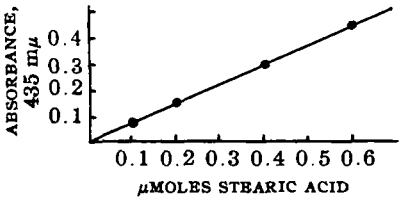


Fig. 1.—Typical standard curve.

Procedure.—The extraction of the plasma free fatty acids is accomplished as described by Dole and Meinertz (1), except that hexane rather than heptane is used as the hydrocarbon solvent due to its lower boiling point.[1] Transfer 0.25 ml. plasma to a 20 × 150 mm. screw-cap culture tube. Screw caps having Teflon liners should be used throughout. Add 0.75 ml. of distilled water and 5.0 ml. of the extraction solution. Shake the mixture well and allow to stand 5 minutes. Add 2.0 ml. of distilled water and 3.0 ml. of hexane, shake well, and allow the two phases to separate.[2] Transfer 4.0 ml. of the upper layer to a clean 20 × 150 mm. screw-cap culture tube and evaporate the solvent using gentle heat and a stream of nitrogen. Dissolve the residue in 5.0 ml. of chloroform and add 2.5 ml. of the cupric nitrate reagent. Shake well and centrifuge for 5 minutes at 2000 r.p.m. Carefully remove the upper aqueous layer by suction and transfer 2.0 ml. of the chloroform phase to a clean 10 × 75 mm. cell. Care must be taken at this point to avoid carrying over any of the aqueous phase, which contains a large amount of copper. Add 0.1 ml. of 0.1% sodium diethyldithiocarbamate, mix well, and determine the absorption of the solution at 435 mμ using a reagent blank which has been carried through the entire procedure. Maximum color develops immediately and is stable for over 2 hours. Deviations in the plasma and reagent volumes described are permissible, providing the ratios between reagents are not altered.

Standard Curve.—Transfer 0.5, 1.0, 2.0, and 3.0 ml. aliquots (equivalent to 0.4, 0.8, 1.6, and 2.4 μmoles free fatty acid per ml. plasma) of the working standard fatty acid solution to clean 20 × 150 mm. screw-cap culture tubes. Add hexane to a total volume of 3.0 ml., 3.0 ml. of distilled water, and 5.0 ml. of extraction solution. Shake well and allow to stand 5 minutes. Transfer 4.0 ml. of the upper layer to clean 20 × 150 mm. screw-cap culture tubes and carry through the remaining procedures as described above.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical standard curve obtained by absorbance *versus* μmoles stearic acid. Although we have observed only small variations in extinction, the standard curve should be checked daily for best results.

As reported by Ayers (2), the copper salts of monocarboxylic acids shorter than C-8 and of di- or tricarboxylic acids are not soluble in chloroform and do not interfere with the procedure. The copper salts of phospholipids are somewhat soluble and will interfere when present in large amounts. Synthetic *l*-dipalmitoyl lecithin and cephalin, obtained from California Corporation for Biochemical Research, were found to have, respectively, approximately 25 and 50% of the molar absorption of stearic acid. For this reason an extraction technique which discriminates against the phospholipids is required. When excessive amounts of phospholipids are present in the sample, the double extraction procedure of Dole and Meinertz (1) may be necessary.

TABLE I.—THE EFFECT OF EPINEPHRINE INJECTION ON PLASMA FREE FATTY ACIDS IN RATS

Epinephrine Dose,[a] mg./Kg./i.p.	No. Rats	Plasma Free Fatty Acids, μmoles/ml.	% Increase
Control	25	0.43 ± 0.03[b]	
0.02	10	0.57 ± 0.05	33
0.10	15	0.74 ± 0.05	72
0.50	10	0.92 ± 0.10	114

[a] Expressed as the base. [b] Standard error of the mean.

The results obtained with the colorimetric procedure are comparable to those obtained by the titrimetric method. In one study the free fatty acid concentration of five different samples of rat plasma was determined by both methods. The group mean was 0.61 μmoles/ml., with a standard deviation of 0.11 as determined colorimetrically, while the results of the titrimetric analysis gave values of 0.63 ± 0.06 μmoles.

Due to the extreme insolubility of fatty acids in aqueous media, it has been difficult to carry out recovery studies. However, the addition of stearic acid dissolved in a small amount of isopropanol to plasma has yielded recovery values averaging 95%.

The colorimetric method as described has been applied to the determination of plasma free fatty acids in both dogs and rats. It reflects accurately the changes produced by manipulations known to decrease or increase the level of plasma free fatty acids. For example, it is well accepted that epinephrine has the ability to cause large increases in these levels (5). Table I presents the results obtained 15 minutes following the intraperitoneal injection of various quantities of *l*-epinephrine bitartrate in saline into rats of the Sprague-Dawley strain. The control free fatty acid level as well as the increases in plasma free fatty acids in response to graded doses of *l*-epinephrine are similar to the results obtained by Maickel *et al.* in the same strain using the microtitration procedure (6). Similar results have been obtained with dogs and cats. The convenience and high sensitivity of this colorimetric procedure make it an extremely useful technique for the study of changes in plasma free fatty acids under a variety of experimental conditions.

[1] When five samples of a pooled rat plasma were extracted with heptane as the hydrocarbon solvent and the fatty acids determined by the above procedure, a value of 0.31 ± 0.016 μmoles/ml. was obtained. This compares favorably with the value of 0.32 ± 0.007 μmoles/ml. obtained with the same plasma when hexane was utilized in the extraction mixture.

[2] To aid in transferring the hexane phase, an additional 0.5 to 1.0 ml. of hexane may be added carefully after the phases have separated following shaking. This has no effect on the final result, provided the same volume is added to all tubes.

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Structure of Lomatin, A New Coumarin

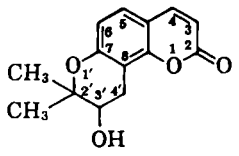
Sir:

Examination of the coumarin content of the petroleum ether extract of *Lomatium nuttallii* (A. Gray) has revealed that the principal component is a compound, m.p. 187–188°, $[\alpha]_D^{25°}$ + 74.8° (EtOH), corresponding to the formula $C_{14}H_{14}O_4$. We have named this substance *lomatin*, and our structural studies indicate that it is (+)-3′-hydroxy-3′,4′-dihydroseselin (I). Infrared spectra[1] (Nujol mull) show principal absorption frequencies at 3500 (free OH), 1700 (C=O), 1600 and 1487 (aromatic C=C), 1281 and 1075 cm.$^{-1}$ (OH deformation and C—O stretching). Ultraviolet spectra show $\lambda_{max.}^{EtOH}$ 246 mμ (log ε 3.78), 257 (3.56), and 329 (4.11); $\lambda_{min.}^{EtOH}$ 242 mμ (log ε 3.49), 252 (3.55), and 264.5 (3.30).

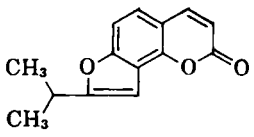
The nuclear magnetic resonance (NMR) spectrum of I showed absorption at 1.37 and 1.41 p.p.m. (*gem*-dimethyl) and at 2.6 p.p.m. (hydroxyl). Absorptions were observed at 2.93 and 3.11 p.p.m. (J_{ab} = 5 cps.) for the 4′-protons and at 3.89 p.p.m. (J_{ab} = 5 cps.) for the 3′-proton. Peaks at 6.16 and 7.60 p.p.m. (J_{ab} = 10 cps) are assigned to protons at positions 3 and 4, respectively, whereas protons at positions 6 and 5 are found to absorb at 6.77 and 7.22 p.p.m. (J_{ab} = 8 cps.), respectively. The hydroxyl proton absorption at 2.6 p.p.m. (concentration and temperature dependent) was eliminated by exchange with D_2O, confirming the infrared OH stretching at 3500 cm.$^{-1}$. Formation of an acetate, m.p. 141°, combined with negative tests for phenol, conclusively showed the presence of an alcoholic hydroxyl group. Acid catalyzed dehydration yielded dihydroöroselone (II), confirming the suspected coumarin character of the nucleus. Furthermore, it revealed the presence of a ring system attached through an ethereal oxygen at position 7 to position 8.

Possible structures of the furanocoumarin type, based on II as a dehydration product, would be III, IV, and V. Of these, III is the structure of columbianetin, unequivocally established previously (1). Structure IV would be a hemiketal ruled out by the insensitivity of lomatin to acid and by nonconformity to the NMR spectrum. Structure V likewise does not conform to the NMR spectrum but, in addition, is eliminated on the basis of the alkaline hydrolytic data on the acetate of I because esters derived from coumarins having a hydroxyl group on the benzylic carbon at position 8 of the coumarin nucleus are unusually sensitive to alkali, having half-lives of only a few minutes at best. Such esters are subject to a rapid S_N1 type of acyloxyl expulsion triggered by the neighboring phenoxide anion when the coumarin lactone ring is opened initially by base (2, 3). No such rapid hydrolysis was observed in the case of the acetate of I, inasmuch as the hydrolytic data showed a half-life of 28 minutes for this ester. The remaining structural possibilities were VI and I, assuming that a ring contraction could occur under the conditions of dehydration to yield II. Structure VI is eliminated by the hydrolytic arguments against V, although the NMR conceivably could be applicable. Thus, I was the most likely candidate, and this assignment was confirmed in two ways. The first was by marshaling the hydrolytic data for comparable compounds. A comparison with hydrolytic data reported by Willette and Soine (3) on 3′-acetoxy-4′-methoxy-3′,4′-dihydroseselin (VII) with that of the acetate of I shows that the data are compatible with I. Compound VII has been shown to have a half-life of 8.5 minutes under similar conditions of alkaline hydrolysis which showed a half-life of 28 minutes for the acetate of I. Rabinovitch and Schramm (4) have shown that the relative rates of basic hydrolysis of 2-ethoxyethanol-1-acetate to ethyl acetate at 25° is 2.41. Portoghese and Malspeis (5) have, likewise, shown similar relative rates of basic hydrolysis for 2-methoxyethyl and ethyl esters of (±) - α - (2 - piperidyl) - phenylacetic acid. The ratio of rates of hydrolysis of the acetate of I and VII (3.29) is in fair agreement with the above authors. Further evidence for a secondary alcohol function in I was obtained by comparing the NMR spectra of I and its acetate. It is well known (6) that a methine proton attached to a carbon bearing a secondary hydroxyl group usually exhibits absorption in the NMR at a frequency about 60 to 70 cps. higher than when

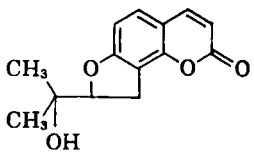
[1] The authors have previously but erroneously (3) reported that the absorption frequencies of (±)-I were 3636 (free OH), 1720 (C=O), 1295 and 1090 cm.$^{-1}$ (OH deformation and C—O stretching). The corrected values, identical with those of (+)-I, are given in this communication and were obtained with a Perkin-Elmer 521 grating infrared spectrophotometer.



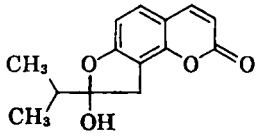
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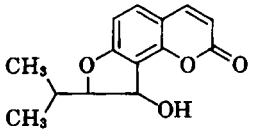
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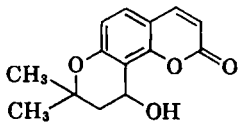
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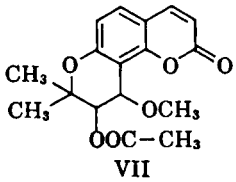
IV



V



VI



VII

the hydroxyl is acetylated. The frequency shift in the case of I and its acetate was 70 cps., in excellent agreement with its assigned character.

Finally, direct comparison of the NMR spectra of racemic I and its acetate[2] with their natural counterparts revealed that they were superimposable.

This represents the first report to our knowledge of the natural existence of I, although it may be pointed out that the structurally undefined hydroxy-containing coumarin recently reported by Hata *et al.* (7) from the roots of *Angelica formosana* Boiss., m.p. 187–188°, with the same empirical formula may well be identical with lomatin. The reported data, however, are not sufficient to make a firm identification. Finally, it should be noted that racemic I also has been reported by Mustafa *et al.* (8), in 1961, through controlled hydrolysis of visnadin with alcoholic sulfuric acid, followed by aluminum isopropoxide reduction.

[2] Previously prepared in these laboratories (3). It may be noted that the present work actually validates the well-accepted, but not necessarily proven, structure of racemic I.

(1) Willette, R. E., and Soine, T. O., THIS JOURNAL, **53**, 275(1964).
(2) Smith, E., *et al.*, *J. Am. Chem. Soc.*, **79**, 3534(1957).
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Correlation of *In Vivo* Metabolism Rate and Physical Properties of Sulfonamides

Sir:

It has been shown (1–3) that the post-equilibration phases of the disposition of several sulfonamides can be described mathematically in terms of two competitive first-order processes: one is excretion of apparent unchanged drug, and the other is metabolism. By measuring the appearance of apparent unchanged drug and metabolite in the urine, apparent first-order rate constants can be assigned to these two processes (1–3). In a series of antibacterial sulfonamides, for which the major metabolic fate is acetylation, attempts were made to correlate the rate constant for the metabolic process (k_m) with the lipid/water partition ratio, assuming the rate limiting step to be diffusion to the site of acetylation (3). However, such correlation apparently did not exist.

In addition to the sulfonamides already referred to (3), rate constants for *in vivo* acetylation (k_m) have been calculated for six additional sulfonamides using data available in the literature (5). The partition ratios of these sulfonamides in the system chloroform/pH 7.4 phosphate buffer range from 0.03 to 3.13 (6). To minimize possible variations in experimental technique, single literature sources were used for ionization constants (5) and extent of plasma protein binding (6). Assuming plasma to be at pH 7.4, the proportion of the sulfonamide which is both free from protein binding and nonionized may be calculated (Table I).

Considering the current theories of drug diffusion across cell membranes (4), it appears that for a molecule to cross such a membrane it must be (*a*) free of plasma protein binding, (*b*) nonionized, and (*c*) sufficiently lipophilic. If the lipophilicity of the nonionized molecule is of sufficient magnitude, however, it is conceivable that this property is no longer rate limiting in the diffusion process; this rate is limited instead by the proportion of the molecule in plasma which is both free of protein binding and in the nonionized form.

The data presented in Table I were subjected to statistical analysis (7) to obtain the rank order correlation coefficients for the relationship between k_m and "per cent free," "per cent nonionized," and "per cent free and nonionized," respectively. A significant correlation existed between k_m and per cent free (correlation coefficient 0.73; $p < 0.01$) and between k_m and per cent nonionized (correlation coefficient 0.63; $p < 0.05$). Most strikingly, a highly significant correlation was found between k_m and "per cent free and nonionized" (correlation coefficient 0.85; $p <$ 0.001). Therefore, it appears that in the series of sulfonamides studied, rate of metabolism *in vivo* can be correlated with ionization constant and extent of plasma protein binding.

TABLE I.—PHYSICAL PROPERTIES AND RATE CONSTANTS FOR METABOLISM OF SULFONAMIDES

Compd.	pKa	% Plasma Binding[a]	% Free	% Nonionized at pH 7.4	% Free and Nonionized at pH 7.4	k_m, Hr.$^{-1}$
Sulfanilamide	10.5	13	87	99.92	86.93	0.043
Sulfacetamide	5.3	19	81	0.79	0.64	0.016
Sulfaphenylpyrazole	5.8	99	1	2.45	0.03	0.0012
Sulfamethoxypyridazine	7.0	92	8	28.47	2.28	0.0078
2-Sulfapyrimidine	6.4	47	53	9.09	4.82	0.013
2-Sulfa-5-methoxypyrimidine	6.5	90	10	11.19	1.12	0.0053
2-Sulfadimethylpyrimidine	7.4	81	9	50.0	9.50	0.06
4-Sulfadimethoxypyrimidine	5.9	99	1	3.07	0.03	0.0017
Sulfaethylthiadiazole	5.1	97	3	0.50	0.02	0.0020
Sulfathiazole	7.0	78	22	28.47	6.26	0.0302
Sulfisomidine	7.4	90	10	50.00	5.00	0.0097

[a] The source of binding data (6) gave extent of binding at three plasma concentrations; the average of the values quoted for the two lower concentrations was used.

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Some Possible Applications of Aqueous Thermometric Titration to Pharmaceutical Analysis

Sir:

Recent work in thermometric titration has indicated that there is a wide range of systems to which this powerful analytical method should be applicable (1). The enthalpy change of a reaction, in addition to having a free energy component, has a contribution from the entropy change, *viz.*, $\Delta H = \Delta F + T\Delta S$. Thus, thermometric titration (being an enthalpy method) will often work where free energy methods, such as potentiometric titration, fail. Since almost every reaction has associated with it a measurable enthalpy change, thermometric titration may prove extremely useful for pharmaceutical analysis.

Briefly, a thermometric titration involves adding a concentrated titrant to a dilute titrate in an "adiabatic" titration cell and measuring the temperature change as a function of the volume of titrant added. A quantitative measure of the amount of titrate is obtained either from the volume of titrant necessary to reach the end point (a change in slope in the temperature–volume plot) or from the amount of heat absorbed or evolved during the reaction (1). (See Curve A in Fig. 1.)

Well-defined thermograms for titrations in aqueous solution can be obtained with several substances whose official or other assay procedures require nonaqueous titration with perchloric acid in glacial acetic acid. While such nonaqueous titrations are quite useful, the authors feel that, in general, aqueous thermometric titration is more convenient. The main disadvantage of thermometric titration is that while it can be performed without electronic instrumentation, such a procedure is tedious and time-consuming. On the other hand, the rapid instrumental procedure requires only simple equipment.

The thermograms shown in Figs. 1 and 2 were obtained by adding titrant at a rate of 0.6 ml./minute from a Menisco-matic buret (American Instrument Co., Silver Spring, Md.) into 100 ml. of titrate contained in a Dewar flask equipped with a stirrer. The temperature was measured with a 2000-ohm bead-in-glass-probe thermistor in a bridge circuit similar to that described by Jordan (2). The output of the bridge was recorded with a 1.25 or 5-mv. recorder. For these exploratory titrations, no particular precautions were taken to assure that the titrant and titrate were initially at the same temperature.

Figure 1 shows three thermograms for systems which exhibit at best poorly defined aqueous potentiometric titration curves. Curves A and C in Fig. 1 have reasonably well-defined end points. The official assay procedures (3) for both chlorpheniramine maleate and nicotinamide require acetous titration with perchloric acid and visual end point detection. In aqueous solution, neither titration gives a discernible potentiometric end point. An advantage of the thermometric method over the U.S.P. method for chlorpheniramine maleate assay is that while in the latter both the pyridine nitrogen of the chlorpheniramine and the bimaleate ion are titrated *together*, in the thermometric method the one end point observed reflects only the chlorpheniramine concentration. While the curvature in the thermogram for the titration of the theophylline in aminophylline with NaOH (Curve B) makes the detection of the end

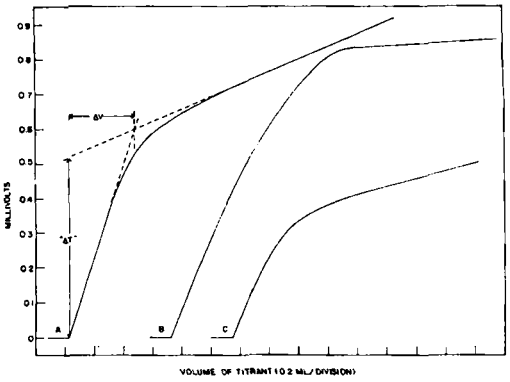


Fig. 1.—Thermograms for titrations in aqueous solution. Key: A, 100 ml. 0.01 *M* chlorpheniramine maleate *vs.* 2 *M* HCl; B, 100 ml. 0.01 *M* aminophylline *vs.* 2 *M* NaOH; C, 100 ml. 0.01 *M* nicotinamide *vs.* 2 *M* HCl. All concentrations are approximate.

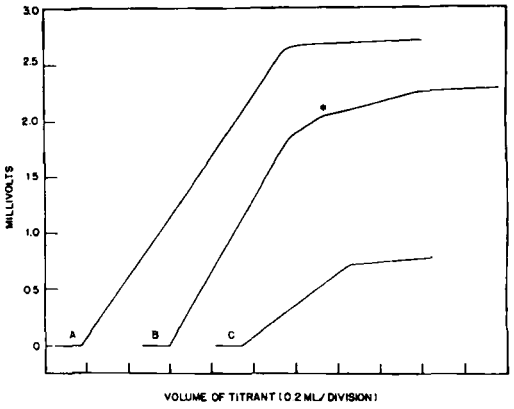


Fig. 2.—Thermograms for titrations in aqueous solution. Key: A, 100 ml. 0.005 *M* aminophylline *vs.* 1 *M* $AgNO_3$; B, 100 ml. 0.01 *M* chlorpheniramine maleate *vs.* 2 *M* NaOH; C, 100 ml. 0.01 *M* chlorpromazine hydrochloride *vs.* 2 *M* NaOH. All concentrations are approximate.

point difficult, the measurement of the temperature rise is quite easy. The corresponding aqueous potentiometric titration curve exhibits a poorly defined end point (4).[1] Medwick and Schiesswohl (5) have described a nonaqueous potentiometric assay of ethylenediamine and theophylline in aminophylline using acetous perchloric acid and an acetic acid–acetic anhydride solvent. No theophylline end point was observed in aqueous thermometric titration with acid.

Figure 2 shows thermograms for three titrations which give fair-to-good potentiometric end points in aqueous solution. In each case, however, the thermometric end point is at least as easy to identify as the potentiometric end point. Curve A represents the titration of theophylline in aminophylline with silver ions. In comparison with the titration of chlorpheniramine maleate with acid (Fig. 1, Curve A), the titration with alkali (Curve B) exhibits two true end points, the first corresponding to the neutralization of the bimaleate ion and the second to the neutralization of the chlorpheniramine ion. The false "end point" indicated by the asterisk is due to the delay of the precipitation of the chlorpheniramine base after the start of the second neutralization. The U.S.P. procedure (6) for chlorpromazine hydrochloride assay calls for acetous potentiometric titration with perchloric acid in the presence of mercuric acetate. No end point is apparent in the direct aqueous thermometric titration of chlorpromazine hydrochloride with acid since neither the phenothiazine nitrogen nor the chloride ion is sufficiently basic. The aqueous titration with base shown in Fig. 2 (Curve C) represents the neutralization of the protonated quaternary nitrogen of the chlorpromazine ion.

The precision obtainable even from titrations without rigid titrant–titrate temperature control is indicated by the following data. End point determinations of five replicate titrations of a solution of chlorpheniramine maleate with 2 M HCl gave an average value of 0.01073 M with an average deviation of ± 0.00005 M. The temperature rise was constant to $\pm 1.1\%$. For nine replicate titrations of ~ 0.005 M aminophylline with 1 M $AgNO_3$, the end-point-determination result was a theophylline concentration of 0.00951 $\pm$ 0.00006 M with the temperature rise constant to $\pm 0.7\%$.

In cases where the end points are fairly sharp, we have been able to run titrations with an automatic end-point-determination device (7). The details of thermometric assay procedures of the type reported here for pure materials and for substances in various dosage forms are now being investigated.

[1] The curve obtained by Bartilucci and Discher (4) using a smooth platinum indicating electrode is slightly more definitive than a potentiometric titration curve that the authors have obtained using a glass electrode.

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JOURNAL OF Pharmaceutical Sciences

September 1964 volume 53, number 9

Review Article

Oximes Antagonistic to Inhibitors of Cholinesterase

Part I

By ROBERT I. ELLIN and J. HENRY WILLS

ORGANOPHOSPHORUS INHIBITORS OF CHOLINESTERASE

Diisopropyl phosphorofluoridate (DFP) and isopropyl methylphosphonofluoridate (sarin) are common prototypes of compounds possessing the general formula $R_1R_2P(O)X$, where R_1 and R_2 are either alcoholic, phenolic, mercaptic, amidic, alkyl, or aryl groups and X may be fluoride, chloride, cyanide, carboxyl, phosphate, pyrophosphate, phenoxy, thiol, or thiophenoxy radicals. These compounds are powerful inhibitors of enzymes which possess carboxylic esterase activity, *i.e.*, true cholinesterase, pseudocholinesterase, ali-esterase, chymotrypsin, trypsin, thrombin, and acetylesterase. However, one should not assume that all esterases are inhibited by organophosphorus compounds; for example, diethyl-*p*-nitrophenylphosphate inhibits only one of the two enzymes in serum capable of hydrolyzing various *p*-nitrophenylesters (1, 2).

CHOLINESTERASES

The existence of an enzyme or enzymes capable of hydrolyzing acetylcholine is well established (3, 4). The true cholinesterase of Mendel and Rudney (5) is found predominantly in nervous tissue and erythrocytes of most species, whereas pseudocholinesterase predominates in the blood plasma (6, 7). Ali-esterase, also present in blood, does not hydrolyze choline esters. The pseudo form does not play a significant part in the neurohumoral transmission of cholinergic impulses, although it can hydrolyze acetylcholine in addition to other esters. If acetylcholine, as suggested by Burn (8), is involved in the spontaneous rhythmic activity of such tissues as heart muscle and cilia, pseudocholinesterase may play an important nonsynaptic role in these tissues or may supplement the action of true cholinesterase wherever high concentrations of acetylcholine occur. Dixon and Webb (9) prefer to use the terms "acetylcholinesterase" and "cholinesterase" for true and pseudocholinesterase. As these names are used interchangeably in the literature, we shall not in this report adhere to a single nomenclature but shall attempt to be as lucid as possible when referring to the individual enzymes.

How biochemical catalysts work is a question germane to the understanding of all biochemical processes. In applying this statement to acetylcholinesterase, one would hope to establish a mechanism for its catalytic action. Many enzymes have been highly purified; in fact, the sequence of amino acids in ribonuclease has been worked out by Hirs *et al.* (10). However, one must modify this optimistic attitude by stating that the stereo-structure of enzymes is presently unknown and that no enzymatic process is completely understood. Considerable progress had been achieved with the esterase enzymes in experiments designed to identify the active site or sites responsible for the catalytic action and en-

Received from the Physiology Division, Directorate of Medical Research, U. S. Army Chemical Research and Development Laboratories, Edgewood Arsenal, Md.

EDITOR'S NOTE: Additional considerations of oximes antagonistic to inhibitors of cholinesterase will be discussed in the concluding portion of this review, Part II, which will appear in the October issue of THIS JOURNAL.

zyme specificity (11). In many instances, the so-called catalytic entities consist of nonproteinoid cofactors, such as pyridoxal phosphate, diphosphopyridine nucleotide (DPN), metal ions, etc. Enzymatic processes are known to be centered around these cofactors, which are relatively simple molecules when compared with the enzyme. One may logically initiate studies by noting the influence of these cofactors on enzymatic reactions. Unfortunately, the problem of elucidating the mechanism of esterase action is much more difficult because the esterases are completely protein in nature and there is no cofactor.

Acetylcholinesterase has not yet been isolated in a completely pure state. Purified enzyme from the electric eel has an activity corresponding to 7×10^3 μmoles of acetylcholine hydrolyzed per minute per milligram of protein at 25° and pH 7.0 (12). The equivalent weight of the purified form has been estimated to be from 2×10^6 to 1.2×10^7 (13, 14). Much of the work on the mechanism of esterase inhibition has been carried out with pure compounds of lower molecular weight, such as crystalline chymotrypsin and trypsin. Classical experiments have shown that, when chymotrypsin is inhibited with DFP, TEPP, or paraoxon, 1 mole of inhibitor reacts with 1 mole of protein and liberates 1 mole of acid. One molecule of phosphorus, with its attached alkyl or alkoxy groups and oxygen atom, is bound to one molecule of enzyme (15–22). A similar conclusion was reached for acetylcholinesterase by calculating the equivalent weight from the amount of phosphorus bound to an enzyme preparation after complete inactivation with DFP and comparing this value with independently derived equivalent weights (17). The inhibition results from a stoichiometric, nonreversible, covalent reaction. The bimolecular rate constant for the reaction of sarin with eel cholinesterase was shown to be as high as 6.3×10^7 L. mole^{-1} minute^{-1} at 25° at pH 7.4 (10). Kinetic data showed also that reactions with the various inhibitors occur at different rates. The phosphorus was so tightly bound to both chymotrypsin and pseudocholinesterase that it could not be removed by treatment with trichloroacetic acid.

A mole-for-mole reaction of esterase with inhibitor indicates that there is one active site per mole of enzyme. A rather ingenious method for determining the concentration of active centers in enzyme preparations was presented by Cohen and Warringa (23). They treated crude ox red cell cholinesterase with butyrylcholine, a strong competitive inhibitor of the active center. The enzyme was then saturated with excess unlabeled DFP. Excess DFP and the reversibly combined butyrylcholine were then removed by dialysis. The activity of the resultant enzyme was determined and found to be 100% of the original activity. The preparation was then treated with radioactive DFP; the reaction product was dialyzed. The amount of radioactive phosphorus bound to the inactivated enzyme was then determined (24) and was used to calculate that there can be only one active center per equivalent of the enzyme.

MECHANISMS OF ACTION OF CHOLINESTERASES

We would do well to examine the fine structures of the active surfaces of cholinesterases before examining the mechanism of ester hydrolysis. Since Adams and Whittaker (25) suggested the presence of an anionic site on the surface of the enzyme, a number of reports on this general subject have been published by Wilson and by Bergmann (26–34). Their conclusions indicate that acetylcholinesterase contains two principal subsites: (*a*) an anionic site which binds and orients the cationic portion of the substrate and (*b*) an esteratic site, HG:, containing an acidic hydrogen atom and a basic group represented by a pair of electrons (Fig. 1*a*). The anionic site is largely concerned with specificity, while the esteratic site is supposedly responsible for the catalytic activity. A theory has been proposed that pseudocholinesterase has one anionic site for each esteratic site, while acetylcholinesterase has

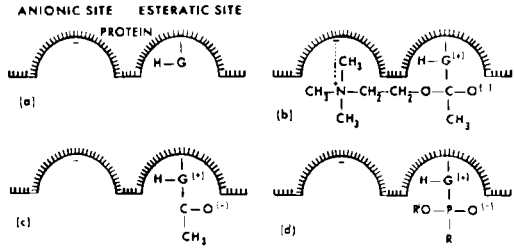


Fig. 1.—(*a*) Diagram of the active sites on the cholinesterase molecule; (*b*) diagram of the interaction between cholinesterase and acetylcholine; (*c*) diagram of acetylated cholinesterase; and (*d*) diagram of phosphorylated cholinesterase (70).

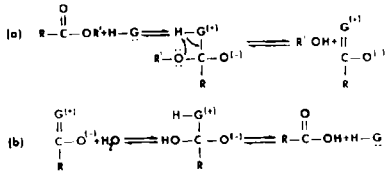


Fig. 2.—Mechanism of acetylcholine hydrolysis (R = CH_3; R′ = $[(CH_3)_3N—CH_2—CH_2]^+$) by cholinesterase (:G—H) (33).

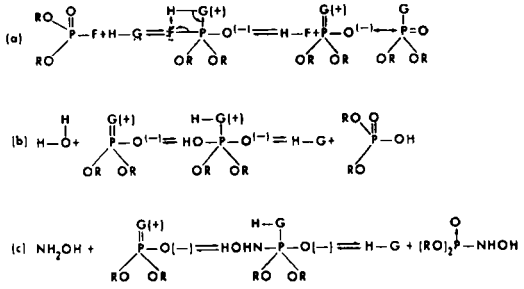


Fig. 3.—(*a*) Reaction of DFP (R = isopropyl) with cholinesterase; (*b*) regeneration of inhibited enzyme by water; and (*c*) regeneration of inhibited enzyme by hydroxylamine (48).

two anionic sites (35). Papers by Friess (36, 37) tend to confirm the two-site theory and in addition suggest that the esteratic site binds to a negative group of the ester rather than to the positive carbonyl carbon. The last concept is not widely accepted.

A diagram representing the interaction of acetylcholine with cholinesterase is shown in Fig. 1*b*. The combination of acetylcholine with the subsite of the enzyme is the rate-controlling step of the reaction. Both groups of the esteratic site are required for catalytic activity, but only the basic group is required for binding. Hydrolysis then occurs at the esteratic site, resulting in the unstable acetylated enzyme shown in Fig. 1*c* after diffusion away of the choline liberated in the reaction. Acetic acid promptly splits from this site, restoring the enzyme to its original state (Fig. 1*a*). As illustrated in Fig. 2, an incipient hydrogen bond sets off an electronic cycle which results in the splitting off of choline (if acetylcholine is the substrate), with the subsequent formation of an acyl enzyme. The acyl enzyme complex reacts with water within a few microseconds to produce regenerated enzyme and acid. The reaction with water apparently involves exchange of oxygen between carboxylate ions and water (30).

MECHANISMS OF ACTION OF CHOLINESTERASE INHIBITORS

Organophosphorus compounds also phosphorylate the enzyme at the esteratic site (Fig. 1*d*). The mechanism of inhibition of acetylcholinesterase parallels the reaction of the enzyme with its normal substrate. When DFP reacts with acetylcholinesterase, a reversible enzyme-inhibitor complex involving a covalent bond between the basic group of the esteratic site and the electrophylic phosphorus atom of DFP is formed. A transfer of a proton from the acid group to the halide atom follows, resulting in the splitting out of a halogen acid and the formation of phosphorylated enzyme (Fig. 3*a*). The reaction of the latter with water is now rate determining. Reactivation of cholinesterase now occurs rather slowly (Fig. 3*b*). The rate of hydrolysis would depend also on the nature of the alkyl group or groups within the phosphorylating agent.

Studies carried out with DFP-inhibited chymotrypsin, trypsin, acetylcholinesterase, and aliesterase (24, 38, 39) indicate that in each enzyme the phosphoryl group is attached to a hydroxy group of serine. Examination of the pH profile of acetylcholine hydrolysis, catalyzed by cholinesterase, indicates the existence of a basic group at an esteratic site which has a pKa value of about 6.5 (40, 41). Wagner-Jauregg and Hackley (42) pointed out that histidine is the only naturally occurring amino acid which (*a*) has a pKa value within this region and (*b*) can react with DFP in aqueous solution at neutral pH. Photochemical oxidation (43) of chymotrypsin in the presence of methylene blue led to the loss of one histidine residue and a corresponding loss of enzyme activity. There are, however, two amino groups in cystidyl diglycine which have pKa's of 6.36 and 6.96. Porter *et al.* (44) suggest that the serine molecule at the esteratic site is present in the form of a cyclic anhydride, a Δ2-oxazoline. The structure of the latter would be basic enough to react with DFP at a pKa value of 5. Bergmann (40) also does not believe that the serine molecule is representative of the esteratic site because it does not contain a group responsible for the measured pKa value and does not provide an explanation for the spontaneous hydrolysis of the intermediate acyl enzymes. Evidence, however, does favor the roles of the imidazole group of histidine and the hydroxyl group of serine as coparticipants in the active center.

Many researchers believe that acylation of the imidazole ring of histidine occurs first; they hold that a secondary reaction takes place during subsequent hydrolysis to produce phosphorylated serine. Peptides thus far isolated from inhibited enzyme indicate that histidine is a good distance from the serine in the peptide chain. However, it is reasonable to suppose that the peptide chains are flexible and that these two amino acids can approach one another. The hypothesis concerning migration of a phosphoryl group fits the results of work on the "aging" phenomenon, in which resistance to reactivation develops following initial inactivation of the enzyme (17, 45). A group in Holland (46) feels, however, that aging is not caused by transfer of the phosphoryl group, but rather by changes in structure of that group on the inhibited enzyme. They were able to show with C^{14}- and P^{32}-labeled DFP that in-

hibited pseudocholinesterase is spontaneously converted from a reactivatable diisopropyl phosphorylated enzyme into a nonreactivatable monoisopropyl enzyme. In both cases, the phosphorus was bound to a hydroxyl group in serine.

DEVELOPMENT OF OXIMES AS ANTIDOTES TO ORGANOPHOSPHATES

The value of oximes for treating organophosphate poisoning was suggested initially by experiments involving hydroxylamine. Hestrin (47) in 1949 found that acetylcholinesterase catalyzed the formation of acetohydroxamic acid when the enzyme was incubated with hydroxylamine and acetate. An analysis of this reaction suggests that the latter is bound to the enzyme, the resulting product then being attacked by hydroxylamine to release intact enzyme and hydroxamate. Wilson and his collaborators (27, 33, 48) subsequently demonstrated that cholinesterase inhibited by tetraethyl pyrophosphate (TEPP) can be reactivated rapidly by hydroxylamine and more slowly by choline. Reactivation with hydroxylamine is similar in mechanism to the reaction of the acetylated enzyme with water. The nitrogen atom reacts with the electrophylic center of the inhibited enzyme to produce a phosphorylated hydroxamate and reactivation of the enzyme (Fig. 3*c*).

Jandorf (49) showed that hydroxylamine reacts with sarin in a stoichiometric manner at neutral pH and room temperature. The mechanism indicated a mole-for-mole reaction between the two, followed, in excess hydroxylamine, by additional decomposition. Mole ratios between sarin and hydroxylamine greater than three increase markedly the rate of destruction of sarin above that by hydrolysis. This reaction is not the same as the reaction with acyl esters. Instead of the ester linkage's splitting, the P-F bond splits and 1 mole each of nitrogen and of ammonia are liberated (Fig. 4). Choline is, at best, a weak nucleophilic agent. Its activity is attributed to the electrostatic attraction of its quaternary ammonium center to the anionic site of the enzyme. Yet choline is almost completely inactive toward DFP-inhibited enzyme. It then appears that the anionic site is functional in TEPP-inhibited enzyme but not in that inhibited by DFP. Similarly, hydroxylamine partially reactivates diethyl phosphorylated chymotrypsin (50) but reactivates DFP-inhibited acetylcholinesterase somewhat less. A rational explanation for these differences in molecular behavior may be found in steric shielding of the anionic site by the large isopropyl radicals of DFP.

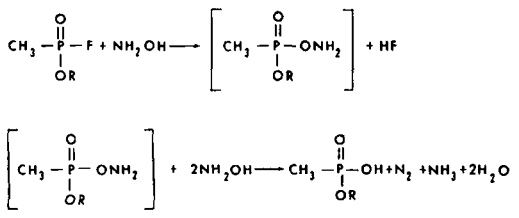


Fig. 4.—Reaction of hydroxylamine with sarin (R = isopropyl) (49).

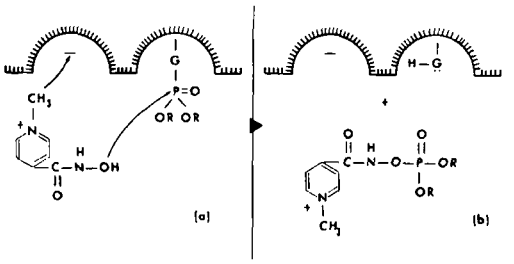


Fig. 5.—Diagrammatic illustration of the reactivation of DFP-inhibited enzyme by isonicotinic acid methiodide (52).

In a search for a more potent nucleophylic compound than hydroxylamine, nicotinehydroxamic acid (51) and the corresponding methiodide (the latter containing both a reacting nucleus and a suitably positioned cationic center) were prepared. Subsequent tests showed that these compounds were better reactivators than hydroxylamine. Isonicotinehydroxamic acid, picolinehydroxamic acid, and their corresponding methiodides were also prepared. Kinetic studies were consistent with the idea that the hydroxamate ions were the reactivating species (52). Reactivation of cholinesterase by picolinehydroxamic acid methiodide probably takes place *via* a nucleophilic attack on the phosphorus atom of the inhibited enzyme, followed by subsequent regeneration of the enzyme in a bimolecular displacement. Here, the hydroxamate may be phosphorylated by the mechanism proposed in Fig. 5. In all but one instance (53), quaternary hydroxamic acids were better than their tertiary analogs as reactivators of TEPP-inhibited enzyme and poorer for DFP-inhibited enzyme. Picolinehydroxamic acid was the exception in that it was more active than its quaternary derivative in reactivating cholinesterase inhibited by either TEPP or DFP. Wilson (53) explains this phenomenon as due, in part, to the low basicity of the zwitterion, which decreases the affinity of the hydroxamate for the phosphorus atom in the inhibited enzyme.

At the same time, Wagner-Jauregg (54), seeking compounds of low toxicity which would react

rapidly with sarin, synthesized a number of hydroxamates. A study of the chemical reaction between a hydroxamic acid and sarin showed that the first step is a reaction between the organophosphorus compound and the hydroxamate in which a phosphorylated hydroxamate and HF are formed. The phosphorylated hydroxamate undergoes a rearrangement in which hydrolyzed organophosphorus compound and isocyanate are formed. The isocyanate then reacts with another molecule of the hydroxamic acid to form *N*-phenylcarbamyl benzhydroxamate. Hurd and Bauer (55) had reported a similar mechanism when they reacted benzhydroxamic acid with benzenesulfonylchloride.

Wilson and Meislich (51) showed that DFP-inhibited cholinesterase could be reactivated *in vitro* by hydroxamates; however, there was a question whether hydroxamates would be effective on sarin-inhibited enzyme; although there are many similarities between the *in vitro* reactions of DFP and sarin, there are significant differences between their biochemical reactivities. Nicotinehydroxamic acid methiodide proved to be effective on sarin-inhibited enzyme also.

Hydroxamic acids having pKa values of 7.5 and higher accelerated the decomposition of DFP and sarin. Hackley *et al.* (56) determined the pKa's of 15 hydroxamic acids and reported that those with values between 7.8 and 9.3 reacted rapidly with sarin. Stolberg and Mosher (57) studied the effects of substituents in the *ortho* position of hydroxamates, using the rationale that the reaction rates of certain *ortho*-substituted phenyl derivatives were more than 100 times greater than would normally be expected. They found, however, that a vicinal hydroxamic group did not produce a marked increase in the rate of reaction of benzhydroxamic acids with sarin. *Cis*-hexahydrophthalohydroxamic acid, with a pKa of 9.75, was the most active of the series. Their

TABLE I.—REACTIVATION OF INHIBITED CHOLINESTERASE BY VARIOUS ISONITROSO COMPOUNDS (59)

Agent	Concn.	Reactivation (%) of Cholinesterase Inhibited by TEPP[a]	Sarin[b]	DFP[c]
Diisonitrosoacetone (DINA)	0.01	78	100	32
Monoisonitrosoacetone (MINA)	0.01	97	100	15
Isonitrosoacetophenone (INAP)	0.01	83	65	45
Isonitrosoacetylacetone (INAA)	0.01	24	33	4
Diacetylmonoxime (DAM)	0.01	11	5	0

[a] Inhibitor, 10^{-6} *M* for 10 minutes at 25°. [b] Inhibitor, 10^{-7} *M* for 10 minutes at 25°. [c] Inhibitor, 10^{-6} *M* for 10 minutes at 25°.

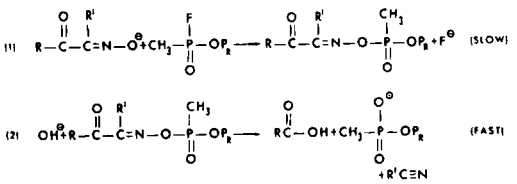


Fig. 6.—Reaction of MINA (R = CH_3; R' = H) with sarin (60).

measurements also indicated that the reactive form was the hydroxamate species, and that the increased activity was dependent only on the ionization constant of the hydroxamic acid. The reaction of sarin with hydroxamic acids followed a linear free energy Brönsted type of relationship when the reaction rates were plotted against the ionization constants. Steinberg *et al.* (58) indicated that in a series of hydroxamates an optimal ionization constant can be predicted for reactivation of inhibited enzyme.

With modified concepts of nucleophilicity and acidity in mind, a series of isonitroso compounds were prepared and tested (59) for their ability to reactivate inhibited cholinesterase; diisonitrosoacetone (DINA), monoisonitrosoacetone (MINA), isonitrosoacetophenone (INAP), isonitrosoacetylacetone (INAA), and diacetylmonoxime (DAM) were a few of the compounds investigated. The extents to which several of these isonitroso compounds reactivate acetylcholinesterase inhibited by TEPP, DFP, or sarin during a 10-minute reactivation period are given in Table I.

MECHANISMS OF ACTION OF OXIMES

Green and Saville (60) reacted monoisonitrosoacetone (MINA) with sarin and reported that 1 mole of oxime reacted for each mole of sarin decomposed. The reaction was accompanied by the liberation of 3 moles of acid—an aliphatic acid, hydrogen fluoride, and a phosphorus acid (Fig. 6). Second-order rate constants, determined from the rate of acid liberation, indicate that the rate-determining step is the initial reaction between the oxime and sarin.

A large variety of oximes, with pKa's ranging from 5 to 12, were subsequently examined. Working on the hypothesis that the anionic site survives inhibition of acetylcholinesterase by organophosphorus compounds, Wilson and Ginsburg (61) decided to combine a good reactivating group, such as an oxime, with a suitably placed quaternary nitrogen atom. Pyridine-2-aldoxime methiodide (Fig. 7*a*), better known as 2-PAM, P-2-AM, or 2-formyl, *N*-methylpyridinium oxime iodide (2FMPOI) resulted from these efforts.

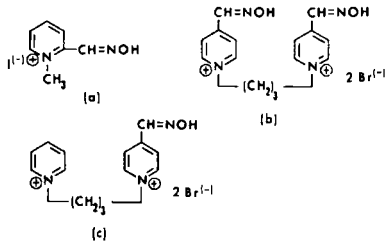


Fig. 7.—Structures of pyridinium aldoximes used in the therapy of nerve gas poisoning: (*a*) pyridine-2-aldoxime methiodide, 2-PAM or 2FMPOI; (*b*) 1,1′-trimethylene bis(4-formylpyridinium bromide) dioxime, TMB-4 or B4FPBOBr$_2$; (*c*) 1,1′-trimethylene bispyridinium-4-formyloxime dibromide or 4FPPOBr$_2$.

2-PAM was effective in reactivating enzymes inhibited by either TEPP or DEP; *i.e.*, this quaternary salt was 50,000 times faster than picolinehydroxamic acid and almost 10^6 times as active as NH_2OH when tested against TEPP-inhibited enzyme. At about the same time, Davies and Green (62) using a somewhat different train of reasoning (63), also found that 2-PAM *in vitro* is an effective reactivator of cholinesterase inhibited by organophosphorus compounds. Childs, Davies, *et al.* (59) reported work on a series of oximes, the most potent in reactivating cholinesterase inhibited by TEPP, DFP, or sarin being 2-PAM.

A and *B* configurations of the quaternary pyridinium aldoximes were prepared (64) and their physical properties listed. The *A* series was made by treating pyridine-2-carboxaldehyde methiodide (PCAM) with hydroxylamine at −5° while the *B* series was prepared by refluxing pyridine-2-aldoxime (P2A) with methyl iodide. The *A* form (colorless) was stable at low temperature but reverted to the *B* (yellow) form on standing at room temperature. The following facts led Wilson and Ginsburg to the postulation that the *B* series was of the anti configuration: (*a*) furan-2-aldehyde and pyrrol-2-aldehyde, when warmed with hydroxylamine, yield the anti configuration, while the syn configuration forms in the cold (65–68), (*b*) members of the *B* series have the higher acidities and therefore are anti because this configuration brings the hydroxyl group closer to the positive center and to the ring (69), and (*c*) the *A* series, having lower melting points and greater solubilities, corresponds to the syn series of benzene and pyrrole oximes.

Wilson (70) in 1959 advanced an explanation for the action of 2-PAM on the basis of molecular complementarity. From a series of experiments he determined that the esteratic site of cholinesterase is occupied by the organophosphorus moiety and that the anionic site is still functional. He then determined that: (*a*) an ideal reactivator should be bound to the organophosphorus-inhibited enzyme in such a way that a nucleophylic grouping, such as the oxygen in the anti configuration of 2-PAM, should fall one bond length from the phosphorus atom, (*b*) that the anti configuration of the 4-PAM molecule would also be satisfactorily oriented, and (*c*) that 3-PAM would not have the proper spatial requirements and would be devoid of enzyme reactivating activity. Experiments confirmed the inactivity of 3-PAM. The 2-PAM anti was 40 times more active than the 4-PAM anti, and the syn configuration of 2-PAM was inactive in reactivating inhibited enzyme. This report appeared to foreshadow the time when one could tailor therapeutic compounds on the basis of attainable physicochemical data.

Poziomek *et al.* (71) reinvestigated the reported configuration of syn and anti forms of the oximes and phenylhydrazones derived from pyridine-2-carboxaldehyde methiodide (PCAM) and found that there are differences of at least 29 mμ between the wavelengths of maximum absorption for the *A* and *B* series. Previous studies with isomeric benzaldehyde oximes (72, 73), furfuraldehyde oximes (74), and picolinaldehyde phenylhydrazones (75) had shown that there are only small differences between the absorption maxima. In addition, the group reported that PCAM forms hydroxaminohydroxymethyl, hydrazinehydroxymethyl, and hydroxyphenylhydrazomethyl derivatives with hydroxylamine, hydrazine, and phenylhydrazine, respectively; these various findings led to the proposal of carbinolamine structures—rather than oxime ones—for the *A* series. These results would cloud the configuration previously assigned to the syn configuration of 2-PAM and leave in doubt the configuration of anti-2-PAM. Data from our laboratories (76) suggest that the previously mentioned *A*-series 2-PAM is not a pure compound but a mixture of a carbinolamine and a geometric isomer (x-isomer) of 2-PAM. The *B* series, or the reputed *trans* 2-PAM, degrades in alkaline solution *via* a bimolecular (E2) elimination mechanism in which a β-hydrogen is removed and simultaneously a multiple covalent bond is formed. This type of mechanism is reported to possess stereospecificity (77–79) such that groups in the *trans* position are eliminated more rapidly than similar groups in the *cis* position. The rate of degradation of the x-isomer of 2-PAM in alkaline solution was about 75 times as rapid as that of the *B*-series 2-PAM. The x-isomer, then, should be the

trans configuration; consequently, the *B*-series 2-PAM would be the syn isomer. These results reverse present concepts concerning the configurations of the *A* and *B* series of 2-PAM. In addition, the rate of reactivation by syn-4-PAM of eel acetylcholinesterase inhibited by sarin was found to be nearly three times that by the corresponding anti-isomer (80). The molecular complementarity theory of Wilson predicts that anti-4-PAM would be the more active reactivator.

Irrespective of oxime configuration or structural requirements, the oxime with the lower pKa of a geometrically isomeric pair was shown to be the more efficient reactivator of inhibited acetylcholinesterase (81). There may be an inherent fallacy in generalizing the conclusion, however. It is generally accepted that the dissociated species of the oxime is responsible for reactivation of inhibited enzyme. In experiments performed at or near physiological pH, the oxime with the lower pKa will contain a greater fraction of the ionized form than that with the higher pKa value. The latter would not appear, then, to be as effective a reactivator as the former. On the other hand, a weakly nucleophilic oxime, even though completely dissociated, may be ineffective as a reactivator.

In vivo experiments also proved that 2-PAM was an effective antidote, especially when administered with atropine, against many anticholinesterase agents. Kewitz pointed out early that 2-PAM, being a quaternary salt, would be poorly soluble in lipids, so that its physiological actions would tend to be limited to those on peripheral effectors (81) and not to extend to the central nervous system (82). Wilson subsequently proposed (83) a quaternary pyridinium aldoxime in which the methyl group of 2-PAM would be replaced by an alkyl group of high molecular weight to increase its lipid solubility. Pyridine-2-aldoxime dodecyliodide (2-PAD) was synthesized and found to be 40,000 times more soluble in chloroform than 2-PAM and 200 times less soluble in water. The addition of 2-PAD to a mixture of 2-PAM and atropine, used as an antagonist to sarin in the mouse, raised the LD_{50} dose of sarin to nearly double that in mice given only the mixture of 2-PAM and atropine as antagonist. However, 2-PAD was far less effective than 2-PAM in reactivating a fly-brain cholinesterase preparation inhibited by DFP (84).

Another attempt to enhance the activity of the pyridinium aldoxime molecule was carried out by Poziomek *et al.* (85). They thought that, because compounds possessing two quaternary nitrogen atoms are more effective reversible inhibitors of acetylcholinesterase than those having a single charged group, a bis-quaternary oximino compound should form a stronger association complex with inhibited enzyme and thereby enhance the reactivation process. A series of 1,1′-polymethylene-bis-(4-formylpyridinium bromide) dioximes was prepared (Fig. 7*b*), in which the number of methylene groups between the pyridine rings varied from 2 to 10. Some of these compounds were more rapid reactivators of sarin-inhibited eel cholinesterase than 2-PAM. The most effective in treating poisoned animals was 1,1′-trimethylene-bis-(4-formylpyridinium bromide) dioxime, abbreviated TMB-4. Hobbiger *et al.* (86) independently and simultaneously, reached a similar conclusion. They prepared a number of monoximes and dioximes of bis-pyridinium ions (Fig. 7*b,c*) and studied the affinities of these compounds for cholinesterase, their interactions with TEPP, and their abilities to reactivate TEPP-inhibited cholinesterase. They reported that every compound in this series is a more potent reactivator than 2-PAM, that TMB-4 has the greatest therapeutic potential and indicated that, should TMB-4 be applicable to species other than mice, 2-PAM would become obsolete as an antidote for TEPP poisoning. Recently Loomis (87) synthesized a series of bis-quaternary bis-aldoximes and a tris-quaternary tris-oxime containing the benzene ring in the chain connecting the quaternary nitrogens. The bis-oximes with the lowest *in vitro* activities had the highest toxicities *in vivo*. The tris-oxime was a better reactivator of sarin-inhibited acetylcholinesterase than 2-PAM but was 26 times as toxic.

The reaction mechanism for the reactivation of sarin-inhibited enzyme by 2-PAM is postulated as a nucleophilic substitution by oximate ions on the phosphorus atom, giving rise to the product *O*-(isopropyl methylphosphono)-1-methyl-2-formylpyridinium iodide oxime, $(CH_3)_2-CH-O-P(O)(CH_3)-O-N=CH-C-(CH)_4-N^+-CH_3$.

Hackley and Owens (88) unsuccessfully attempted to prepare and isolate phosphorylated 2-PAM. However, the phosphorylated 4-PAM was synthesized. The compound decomposes at room temperature, but may be stored in a desiccator at 0° for several months with little decomposition. It was a potent inhibitor of eel acetylcholinesterase and quite toxic to mice, with an i.v. LD_{50} of 0.2 mg./Kg. If phosphorylated 2-PAM (P-2-PAM) is formed when inhibited enzyme is reactivated, one would expect

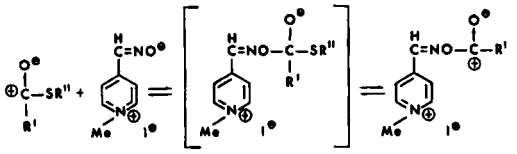


Fig. 8.—Reaction of a thioester with 4-PAM (90).

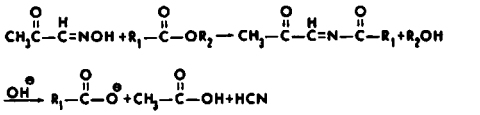


Fig. 9.—Reaction of MINA with an acylating agent in the body (99).

it also to be toxic. One supposes, because of the low toxicity of 2-PAM even in the presence of sarin (89), that P-2-PAM, upon forming in the body, is rapidly hydrolyzed to the corresponding nontoxic phosphorus-containing acid and some salt of intact oxime.

O'Neil *et al.* (90) proposed that part of the therapeutic effect of oximes may be due to their interference with acetylcholine synthesis. Their suggested mechanism (Fig. 8) is that oximes compete with choline for acetyl CoA (a thioester) and thereby prevent acetylation of choline. A mechanism which decreases the production of acetylcholine would have, of course, effects resembling to some extent those of reactivation of acetylcholinesterase. This type of reaction may be unimportant practically (91); however, we believe that interference with production of acetylcholine may be a useful therapeutic procedure in organophosphorus poisoning if sufficiently active, nontoxic compounds with this action can be found.

A study was recently conducted on the ability of pyridine-2-aldoxime to form chelates with metal ions (92), the rationale being that these compounds could be of value in treating poisoning by organophosphorus anticholinesterases because the hydrolysis of DFP is known (93, 94) to be accelerated by the presence of the cupric chelate of α,α'-dipyridyl, a compound related structurally to P2A. Courtney *et al.* (95) described a number of metal chelates which catalyze hydrolyses of DFP and sarin. A number of stable chelates of P2A are being tested for their physiological activities in antagonizing poisoning by organophosphorus compounds.

DISTRIBUTION AND METABOLISM OF OXIMES

As would be expected, the oximes have definite toxic actions. Hydroxylamine reacts with many substances normally found in the body and is appreciably toxic. Riemann (96) reported that the feeding of hydroxylamine hydrochloride to rats resulted in a considerable enlargement of the spleen and a marked reduction in the size of the thyroid gland. Hydroxylamine affects a number of enzyme systems; for example, it inhibits tryptophan pyrrolase, both *in vivo* and *in vitro* (97), glutamic-oxalacetic transaminase in liver and brain, and glutamic-pyruvic transaminase in liver.

Although many studies have been performed on the reaction *in vitro* of hydroxamic acids with organophosphorus compounds, relatively little work has been done *in vivo*. Reports concerning the distribution and fate of hydroxamates in animals or humans have not been published. Epstein and Freeman (98) studied the prophylactic and therapeutic efficacies of a number of selected hydroxamic acid derivatives in protecting against sarin in mice and stated that the large doses required for protective action would limit the usefulness of these compounds in humans. Of the aliphatic isonitroso derivatives, DINA was the most toxic, followed by MINA and DAM, successively.

The breakdown of DINA is accompanied by the production of 2 moles of hydrogen cyanide (HCN). The breakdown of MINA (Fig. 9) gives rise to 1 mole of HCN, but there is no production of HCN during the biotransformation of DAM (99, 100). It was felt that sufficient amounts of HCN could build up from doses of DINA and MINA to account for their toxicity; however, the acute toxicity of DAM would be due to the compound itself. Sodium thiosulfate and sodium nitrite were subsequently shown to protect rats against poisoning by MINA. A small increase in urinary excretion of thiocyanate has been observed after administration of large doses of 2-PAM to rats (101), but the amount of cyanide formed in the body during metabolism of this oxime was considered to be too small to contribute to the compound's toxicity.

In experiments with MINA in dogs and rabbits Askew *et al.* (99) showed that there is an inverse relationship between the concentrations of cyanide in plasma and in whole blood; that is, samplings showed a steady decrease in concentration of oxime accompanied by a corresponding rise in the concentration of cyanide in plasma. Maximum concentrations of MINA and DAM in blood were found within 10 minutes after i.p. injections of the oximes. Rutland (102), in studying the disappearance of MINA and DAM from the blood of male albino rats

after i.p. injections of the oximes, found that maximum concentrations of oxime in blood were reached within an average of 12 minutes. Dultz *et al.* (100) showed that in rats DAM enters rapidly into most tissues, but somewhat slower into skeletal muscle and fat. Studies were also performed on the disappearance of DAM in the presence of tissue slices. Incubation of DAM with slices of rat liver resulted in a 25% reduction of the concentration of this oxime in the reaction vessel during the first hour; brain and kidney produced only a 5% decrease in oxime concentration, while other tissues caused even smaller decreases.

Jager *et al.* (103) studied the distribution, destruction, and elimination of DAM in humans. They found that the time required for the concentration of DAM in serum to fall 50% from its initial level after i.v. injection of the oxime was more than 7 hours. Dultz (100) reported times of 4.5 hours for dogs and approximately 2 hours for rats. The rather low rate of elimination of DAM from plasma could be significant in determining the potential usefulness of this oxime as a prophylactic agent against poisoning by organophosphorus anticholinesterases. After 6 hours, less than 10% of the DAM injected was found in the urine. DAM was found within 1 hour after its injection in the cerebrospinal fluid, indicating that the so-called blood-brain barrier is relatively permeable to this oxime.

If a choice of a therapeutic agent for poisoning by sarin, DFP, etc., had to be made today, the most likely candidate would be some salt of 2-PAM. Though other compounds may be found at some future date to be of greater value, this oxime is effective and, in addition, there is a comparatively large amount of information about its toxicity, pharmacology, stability, and metabolism. Table II gives recoveries of oxime from pooled rabbit tissues and fluids after 50 mg./Kg. of 2-PAM was injected intravenously during a 1-hour period. The greatest concentration of 2-PAM was found in the kidney.

In a study undertaken to determine the distribution of 2-PAM within experimental animals, 2-PAM was synthesized with a C^{14} label in the methyl group (112). Though only 64% of the labeled compound injected was recovered, the distribution of the label among the tissues of injected mice was measured. The liver, duodenum, jejunum, ileum, kidney, caecum, colon, and blood contained about 20% of the activity after 1 hour; the urine contained 44%. Only trace amounts of the label were found in the brain. A labeled metabolite identified tentatively as *N*-methyl-2-pyridone was an important excretory product. Ten minutes after i.v. injection of the labeled oxime into a cat, the blood was estimated to contain only about 5% of the label; after 2.5 hours, only 0.3% remained in the blood.

TABLE II.—CONCENTRATION OF 2-PAM IN RABBIT SERUM AND TISSUES AFTER INTRAVENOUS INJECTION OF 50 mg./Kg. (103)

Tissue	Concn.[a] Animal No. 1	Animal No. 2
Serum	1.5	0.7
Spleen	2.0	1.5
Liver	2.9	1.3
Kidney	10.3	4.5
Brain	0.6	0.1[b]

[a] Concentration expressed as milligrams of PAM per 100 ml. of serum or per 100 Gm. (wet weight) of tissue. [b] Less than 0.6 mg. PAM cannot be measured accurately with the technique employed for tissues.

Two good analytical methods are available for determining 2-PAM in biological material. The method of Blom (104), later modified by Csaky (105), can be applied to any oxime. After deproteinization of a sample, the supernatant is hydrolyzed with acid on a boiling water bath. The resulting hydroxylamine is oxidized by iodine to nitrous acid, which is used to diazotize sulfanilic acid. The resulting diazonium salt is coupled with *N*-(1-naphthyl) ethylenediamine dihydrochloride to produce a violet color. The method is applicable to most experiments, but there were several pitfalls. One must be certain that the pH of the acid solution used in the hydrolysis is effective in producing quantitative splitting. We found that various conditions of equilibrium tend to cause incomplete reactions.

A much simpler method may be used. In acid solution, 2-PAM exhibits an absorption peak at 292 mμ; in basic solution, 2-PAM shows a significant bathochromic shift at 335 mμ, which is attributed to the oximate anion (106). This phenomenon is quite advantageous, for at this wavelength the likelihood of interference from most biological materials is greatly reduced. The method is about 100 times more sensitive than the colorimetric assay and has been adapted for the analysis of 2-PAM in biological tissues and fluids by several authors (107–109). Creasey and Green (107) report reproducible determination of 2-PAM in skeletal muscle, blood, liver, kidney, urine, and feces of the rat.

When 2-PAM is incubated aerobically with rat liver, it is completely metabolized. Unlike DAM, 2-PAM is removed from the human body primarily by renal excretion. The half-life in man of 2-PAM, given in an i.v. dose of 15 mg./Kg., was found to be 0.8 hours (103). Approximately 80% of the oxime was excreted within 6 hours, most of the unchanged oxime being found

in the urine within the first 30 minutes. No appreciable binding of 2-PAM to human serum protein was observed, and the oxime did not enter the erythrocytes. The excretion of 2-PAM in urine is, therefore, intrinsically rapid. Studies of the prophylactic activity of the oxime *in vivo* (110, 110*a*) indicated the need for appreciable concentrations in the blood. The persistence of effective concentrations of oxime in the blood after administration of a dose of a salt of 2-PAM should be prolonged by preventing rapid renal elimination of the oxime. Berglund *et al.* (109) demonstrated that the renal elimination of 2-PAM is markedly reduced in dogs which receive an intravenous infusion of sodium bicarbonate.

Sundwall (111) investigated the rate of absorption of 2-PAM in human volunteers after intramuscular or oral administration. After intramuscular injection into 10 subjects (in a dosage of 30 mg./Kg.), a maximum concentration in plasma averaging about 15 mcg. per ml. was found after approximately 20 minutes. A progressive fall in the concentration of oxime ensued. After 90 minutes the concentration was about 9 mcg. per ml. A plot of individual results indicated that there were two rates of absorption from intramuscular injections: one fairly rapid, with the plasma concentration rising to about 20 mcg. per ml. after 5 minutes, and a slower one, with the plasma concentration rising to only 10 mcg. per ml. after 20 minutes. After oral administration, a slow rise in plasma concentration occurred, reaching a maximum in about 2.5 hours. Twenty-three per cent of the oral dose was excreted within 4 hours. Later Sundwall (111) designed experiments to determine the effectiveness of various concentrations of P2S (pyridine-2-aldoxime methylmethanesulfonate) in antagonizing the neuromuscular block produced by organophosphorus cholinesterase inhibitors. Anesthetized cats were given approximately one LD_{50} of inhibitor and then an intramuscular injection of oxime. Plasma levels of P2S above 4 mcg. per ml. counteracted neuromuscular block, bradycardia, hypotension, and respiratory failure.

Recently Barkman (113) determined plasma levels of P2S in human volunteers following the self-administration of 1.5 ml. of a 50% concentration of oxime (a dose of about 10 mg./Kg.) contained in an automatic injector. Within 6 minutes, plasma concentrations of over 4 mcg./ml. were reached; they were maintained for about 90 minutes. After a dose of 20 mg./Kg., the plasma concentration of oxime was maintained above 4 mcg./ml. for about 170 minutes.

One significant and controversial point still exists: 2-PAM is essentially an ionic compound and thus quite polar. It would not be expected to cross the blood brain barrier. Jager *et al.* (103) were not able to demonstrate 2-PAM in cerebrospinal fluid 70 minutes after it had been administered intravenously. Recently Clark and Roth (114) reported the synthesis of 2-PAM with a C^{14} atom in the carbonyl position of the side chain. Autoradiography of the rat brain following i.v. injection of 20 mg./Kg. of labeled 2-PAM showed (115) that unmetabolized 2-PAM entered all anatomical areas of the brain in concentrations shown previously to be adequate for reactivation of inhibited acetylcholinesterase. For example, 1.2 mcg. of oxime per gram of wet tissue was found in cerebral cortex 10 minutes after injection of the oxime. The conclusion of Roth's group that a quaternary compound can pass the blood-brain barrier readily is contrary to the generally accepted view that quaternary amines enter the CNS much more slowly than the corresponding tertiary amines.

The question of whether 2-PAM penetrates brain is one on which there are conflicting opinions and inconsistent evidence. Although therapeutic doses of 2-PAM will reactivate *in vitro* cholinesterase of brain inhibited by organophosphorus compounds and will reactivate *in vivo* cholinesterases of muscle, blood, and parotid gland, they do not reactivate *in vivo* cholinesterase of brain (81, 82, 102, 116–120). Kewitz (81), and Kewitz and Nachmansohn (82), and Rosenberg (121) found that use of repeated or enlarged doses of 2-PAM is capable of reactivating cholinesterase in brain, but Rutland (102) found that an i.p. dose of 100 mg./Kg. of 2-PAM reactivated only 4% of the cholinesterase of the brain of the rat after intoxication *in vivo* by sarin.

Sakai *et al.* (122) reported that 2-PAM is unable to overcome the paralysis of respiratory centers induced by parathion or paraoxon. On the other hand, Wislicki (123) found that 2-PAM, injected i.v. into the anesthetized (sodium pentobarbital) cat, promptly slows the rate of repetition of phrenic volleys, with the appearance of a relative inspiratory apneusis, and Brown *et al.* (124) reported rapid improvement in ventilation in animals poisoned with tabun or sarin and then treated first with atropine and later with 2-PAM. They believe that the improvement is too abrupt to be explained solely by reactivation of cholinesterase. Longo *et al.*

(125) found that, although i.v. injection of 2-PAM has no effect on the EEG of the rabbit, administration of 2-PAM before i.v. injection of sarin doubles or triples the dose of Sarin required to establish a "grand mal" pattern; prophylaxis with 2-PAM was said to abolish entirely the desynchronization of the EEG pattern induced by sarin prior to the appearance of the "grand mal" pattern. Brown (126) reported that P2S, even when injected intracisternally, had no effect on the respiratory paralysis and cardiovascular actions of intracisternal injections of sarin. Edery reported (127), however, that cerebral intraventricular injections of oximes, including 2-PAM, do have some effectiveness in overcoming the toxic effects from cerebral intraventricular injections of ethyl pyrophosphate. Tong and Way (128) found that cerebral intraventricular injection of 2-PAM is capable of modifying the lethal effects of injections of paraoxon, TEPP, or DFP following i.p. injections of atropine and 2-PAM, protection against the lethal actions of paraoxon and TEPP being especially marked.

In addition to the evidences of some central action of 2-PAM cited previously, this oxime has been noted to induce rapid recovery of consciousness in patients poisoned by parathion (129–132) and to have an anticonvulsant action in the same type of patient (130, 132). One is forced to conclude, therefore, that 2-PAM has some ability to penetrate the blood-brain barrier despite its quaternary character and to induce effects dependent upon the presence of the oxime within the brain. The work of Firemark and his collaborators (115) seems to show that significant crossing of the blood-brain barrier by 2-PAM can occur within 10 minutes in the rat.

Studies of the metabolism of 2-PAM are incomplete and are presently proceeding within a number of laboratories. *In vitro* studies indicate that 2-PAM forms the following products in aqueous solutions at various pH values: 2-cyanopyridine methiodide, pyridine-2-carboxaldehyde methiodide, pyridine-2-carboxylic acid methiodide, 2-carbamidopyridine methiodide, and *N*-methyl-α-pyridone (133–135). A graphic representation of the reactions involved in the degradation of 2-PAM is shown in Fig. 10. The products of decomposition do not interfere with the ultraviolet analysis of 2-PAM in basic media and are readily separated and identified by paper chromatographic techniques (136).

Kramer (137) analyzed human urine following oral administration of 2-PAM. His report describes the isolation of both the intact drug and a fluorescent metabolite. The latter is described as highly polar and having both a carboxyl function and a weakly acidic group. A number of procedures used to characterize the metabolite indicated that it was a derivative of *N*-methyl-picolinic acid.

Since the liver had been implicated as the major biotransformation site of several oximes, Way *et al.* (138–140) studied the metabolism of C^{14} radiolabeled 2-PAM by perfusing the oxime through isolated rat liver. They eventually separated two radioactive fractions: one had ultraviolet spectral characteristics similar to those of 2-PAM, and the other was thought to be an *O*-conjugated form of *N*-methyl-α-pyridone. Later, Way (141) described the isolation and characterization of a second metabolite, methyl-2-cyanopyridinium ion, by procedures involving adsorption on charcoal, paper electrophoresis, ion-exchange chromatography, paper chromatography, and ultraviolet analysis.

Kalser (112) found at least six chromatographically separable metabolites in the urines of mice as early as 20 minutes after injection of 2-PAM. She indicated that one metabolite was

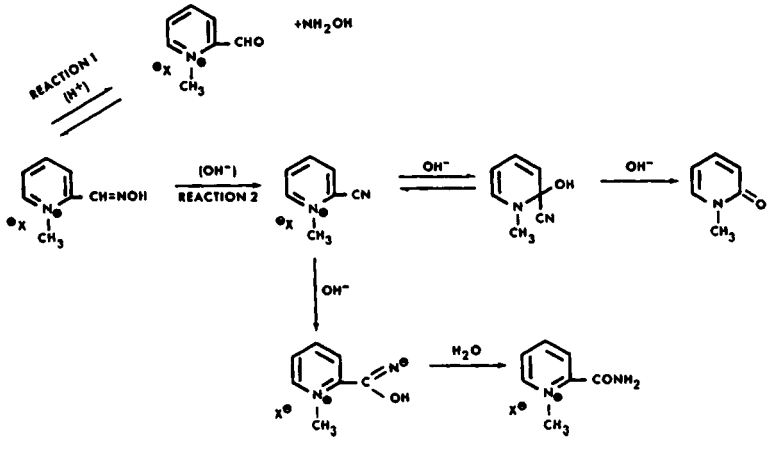


Fig. 10.—Mechanism of the decomposition of 2-PAM in aqueous solution (133–135).

probably *N*-methyl-α-pyridone. Firemark *et al.* (115) reported that rat urine contained as many as 11 distinct chromatographic bands. The largest band was identified as unmetabolized 2-PAM. No attempt was made to identify the other products.

Since 2-PAM is known to produce cyanide ion *in vitro* at physiological pH, experiments were carried out by Enander *et al.* (101, 142, 143) with the methanesulfonate salt (P2S) to determine whether formation of cyanide contributes to the toxicity of the oxime. The increase in excretion of thiocyanate, the product of detoxication of cyanide in the body, in the urines of humans given P2S orally was statistically insignificant. Another compound, metabolite *X*, that is easily converted to cyanide but is not identical with thiocyanate, was found also. The same investigations subsequently detected 2-cyanopyridinium ion (PCNM) in the urines of rats and humans given P2S by mouth. The amount in rat urine during the first day corresponds to about 8% of the oxime administered, opposed to 0.3% in the urines of human volunteers. Thiocyanate and a compound with properties similar to those of metabolite *X* were subsequently found in the urines of rats injected with PCNM. P2S appears, therefore, to be converted to PCNM, which then is degraded to metabolite *X* and thiocyanate.

This group of investigators (143) also found that 80 to 90% of C^{14}-labeled 2-PAM injected into rats was excreted in the urine within 24 hours. A radiochromatograph of the urine demonstrated the presence of five compounds. The major component was unchanged oxime. A second component was identified as PCNM. A third component had an R_f value identical with that of *N*-methyl-pyridinium-carboxylic acid. Another peak had the same R_f value as metabolite *X*. About 5% of the injected radioactivity was recovered as *N*-methyl-α-pyridone picrate from the urines of rats; the same metabolite was also demonstrated in mice.

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Essential Oils and Their Constituents XX

Detection and Estimation of Menthofuran in *Mentha arvensis* and Other Mint Species by Coupled Gas-Liquid–Thin-Layer Chromatography

By ISHWAR C. NIGAM* and LEO LEVI

The occurrence of menthofuran in *Mentha arvensis* is reported for the first time. From 0.01 to 0.04 per cent of the heterocycle was determined by means of coupled gas-liquid–thin-layer chromatography in genuine essential oils obtained from plants grown in Argentina, Brazil, Formosa, India, Japan, and South Africa. Its presence at such low concentrations could not be detected by conventional chemical tests, *e.g.*, Flückiger's reaction, or by modern instrumental techniques, including ultraviolet and infrared spectrophotometry applied to analytical specimens directly. The classical distinction between *Mentha piperita* (peppermint) and *M. arvensis* (mint), ostensibly based on the presence or absence of menthofuran, is therefore no longer justifiable. Other mint species, including spearmints (*Mentha cardiaca* Gerard ex Baker cultivar Scotch or Highland spearmint, *Mentha spicata* L. cultivar common or native American spearmint, and *Mentha viridis*), pennyroyal (*Mentha pulegium* L. and *Hedeoma pulegioides* (L.) Pers.), bergamot mint (*Mentha citrata* Ehrh.), *Mentha sylvestris* L., and *Mentha rotundifolia*, contained trace amounts of menthofuran also. The experimental data suggest a reappraisal of presently acknowledged concepts regarding both biogenesis and chemotaxonomy of the genus *Mentha*.

ALMOST ONE-HUNDRED years ago Flückiger observed that peppermint oil gave a characteristic color reaction when heated in glacial acetic–nitric acid solution (1). In 1890, Polenske suggested that the phenomenon was due to the presence of a volatile, nonnitrogenous constituent in the essential oil (2). Thirty years later the compound, menthofuran, was isolated by Carles as an "ether-oxide" from the flower oil of Italian peppermint (3). Its constitution was established soon afterwards by Wienhaus and Dewein (4). In 1936, Treibs synthesized the heterocycle and demonstrated that its properties were identical with those of the natural isolate (5).

As a criterion of identity for *M. piperita*, Flückiger's test came to be applied widely and with only slight modifications. It is generally carried out as follows. Three drops of the essential oil are mixed in a dry test tube with 5 ml. of a solution of 1 vol. of nitric acid in 300 vol. of glacial acetic acid, and the tube is placed in a beaker of boiling water. Within 5 minutes the liquid develops a blue color, which on continued heating deepens and shows a copper fluorescence, then fades, leaving a golden yellow solution (6). With *M. arvensis*, this sequence of color changes is not observed, the test solution attaining merely a light yellowish tinge which undergoes no marked change during the 5-minute heating period.

Offering a valuable criterion for distinguishing readily two closely allied and commercially most important products of the genus *Mentha*, the reaction was soon considered and still is an official specification (7, 8). As a spot test requiring but 1 drop of material, it was later utilized by Zolotovich for the examination of *M. arvensis* as well as *M. piperita* oils and many of their hybrids (9).

Studies carried out recently in our laboratory on products of different geographical origins confirmed the specificity generally ascribed to the reaction (10). Yet, not always were experimental data entirely satisfactory, and the investigation was continued. It is the purpose of this paper to report the results that we obtained using coupled gas-liquid–thin-layer chromatography as the method of analysis.

EXPERIMENTAL

Apparatus and analytical procedures were previously described (11).

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* National Research Council of Canada Postdoctoral Fellow, 1962–1963.

Materials

Essential Oils.—Oil of mint (*M. arvensis*) was provided through the courtesy of Dr. Juan-Carlos Tuja, Cultivos Industriales, S.A., Buenos Aires, Argentina, Drs. M. T. Magalhães and O. R. Gottlieb, Instituto de Química Agrícola, Rio de Janeiro, Brazil; Dr. T. S. Chou, Bureau of Commodity Inspection and Quarantine, Republic of China, Taipei, Taiwan; Dr. I. C. Chopra, Regional Research Laboratory, Jammu-Tawi, Kashmir State, India;

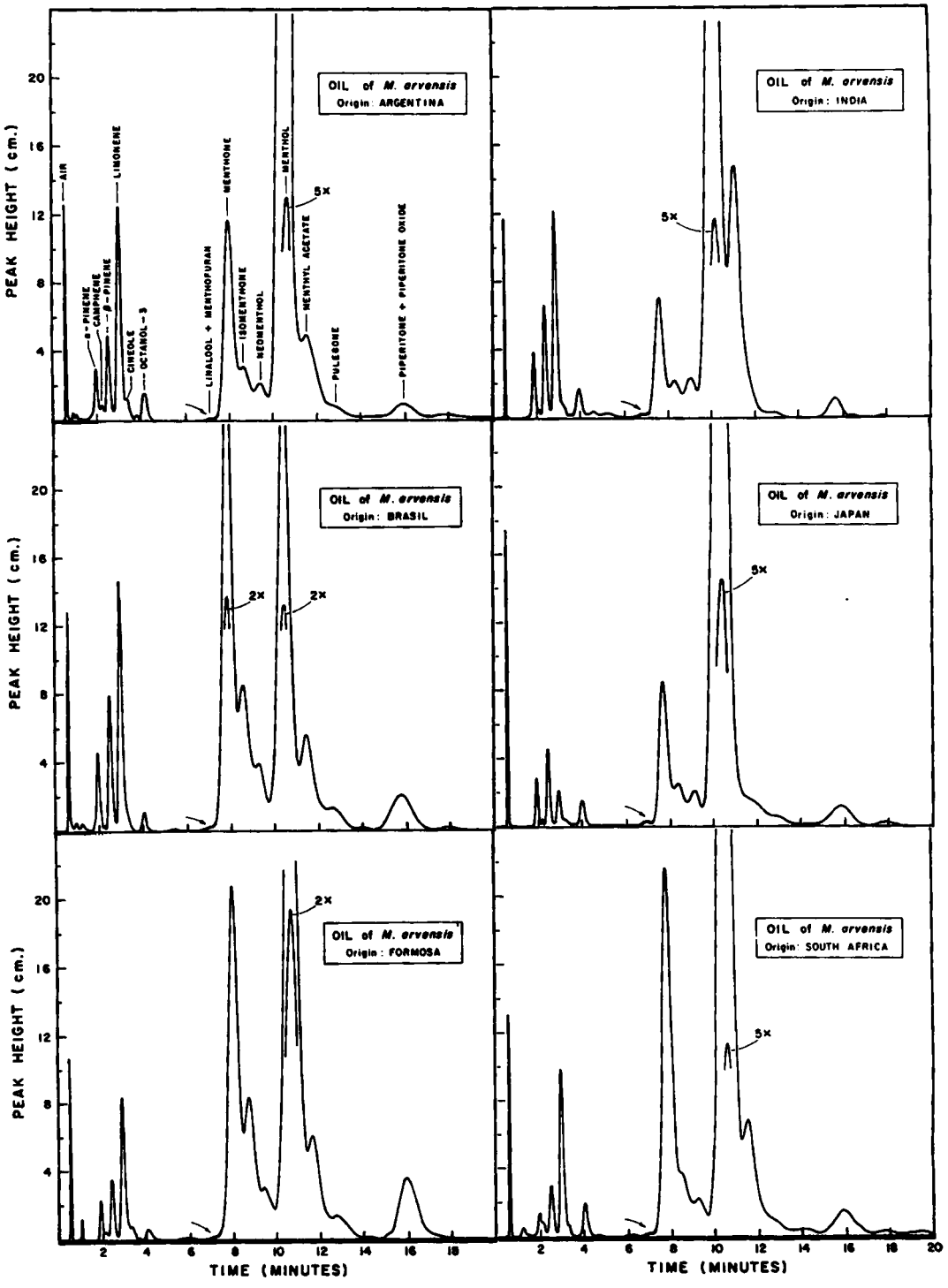


Fig. 1.—Gas chromatograms of *M. arvensis* oils. Analytical peaks reproduced in heavy lines. Column—SAIB (20%) on Chromosorb W (acid-washed), 60–80 mesh; temperature, 170° C.; helium, 75 ml./minute; sample volumes, 2 μl.

Dr. Teikichi Hiraizumi, Takasago Perfumery Co., Ltd., Tokyo; Dr. Sumio Shimizu, Laboratory of Agricultural Chemistry, Shinshu University, Ina-Nagano Ken; Dr. Nagamori Ikeda, Laboratory of Plant Breeding, Okayama University, Tsushima, Okayama City, Japan; Dr. D. H. S. Horn, Council for Scientific and Industrial Research, National Chemical Research Laboratory, Pretoria, South Africa; and Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

Oil of spearmint (*M. cardiaca, M. spicata, M. viridis*) was supplied through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich., and Dr. C. Scholtens, N. V. Chemische Fabriek "Flebo," Hoogezand, The Netherlands.

Oil of pennyroyal (*M. pulegium* L. and *Hedeoma pulegioides* (L.) Pers.) was provided through the courtesy of Dr. E. Guenther, Fritzsche Brothers, Inc., New York, N. Y.; Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.; Dr. J. M. Quigg, Compagnie Parento, Ltd., Toronto, Ontario, Canada; Destilaciones Bordas Chinchurreta, S.A., Seville, Spain.

Oil of bergamot mint (*M. citrata* Ehrh.) and oil of *M. sylvestris* L. was supplied through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

Oil of *M. rotundifolia* was provided through the courtesy of Dr. Sumio Shimizu, Laboratory of Agricultural Chemistry, Shinshu University, Ina-Nagano Ken, Japan.

Essential Oil Constituents.—Menthofuran (ex *M. piperita*, freshly distilled for use as reference standard); $\epsilon^{EtOH}_{max.\ 219\ m\mu}$ = 6,750. *d*-Pulegone (ex *M. pulegium* L.) was provided through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

Isolation of Menthofuran Fraction from Two Grams of Brazilian Oil of *M. arvensis*

Sample aliquots of 50 μl. each were subjected to gas chromatography (column: SAIB (20%) on Chromosorb W; temperature, 170° C.; helium: 75 ml./minute) (12) and effluents emerging just prior to menthone (retention time ~6.5 to 7.5 minutes) trapped in carbon tetrachloride. Following evaporation of solvent, the residue obtained was examined by infrared and ultraviolet spectroscopy, thin-layer chromatography, and Flückiger's classical color test.

Coupled Gas-Liquid–Thin-Layer Chromatograms of Essential Oils

Samples ranging from 25–100 μl. were injected into the column and fractions emerging within 6.5 to 7.5 minutes (see Fig. 1) deposited directly on chromatoplates mounted at the vapor exit as previously reported (11). *n*-Hexane was applied for the development of condensates thus spotted, and a 5% solution of vanillin in concentrated sulfuric acid served as spray reagent.

Identification and Estimation of Menthofuran in Analytical Specimens

To 10-Gm. samples of an authenticated Japanese oil of *M. arvensis* (Flückiger test negative) were added accurately weighed amounts of menthofuran and preparations containing from 0.10 to 1.00% of the heterocycle made up as reference standards. They served as controls for compound identification and the appraisal of product recovery. Following deposition of 0.5 to 1-μl. aliquots of these mixtures on chromatoplates, development in *n*-hexane for about 45 minutes, and treatment with the 5% vanillin-sulfuric acid reagent, characteristic deep orange-red spots were readily observed. On prolonged standing, they developed a bluish-purple hue.

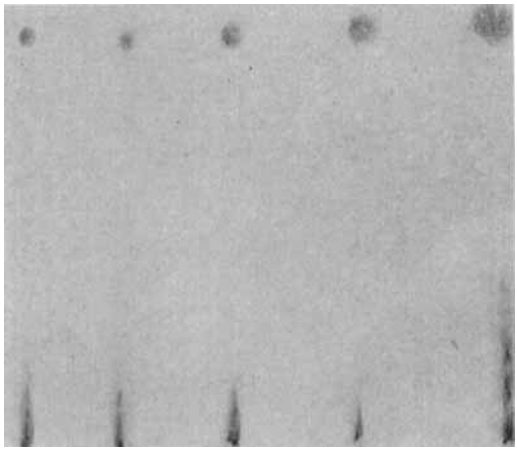
RESULTS AND DISCUSSION

Characterization of Menthofuran Fraction Obtained from Brazilian *M. arvensis*

Infrared analysis of the isolate, prepared in accordance with the procedure described and spread as a thin film between two salt plates, showed weak yet distinct bands characteristic of menthofuran at 1680, 1640, 1565, and 735 cm.$^{-1}$ Marked absorptions throughout the 1200 and 920 cm.$^{-1}$ regions indicated admixture with menthone and linalool, respectively. A Perkin-Elmer double beam, model 221 spectrophotometer equipped with prism grating interchange and automatic gain control was used to make the measurements. It was operated at fivefold scale expansion and flushed with dry nitrogen during the experiment. Although the analytical specimen was of heterogeneous composition, purification by rechromatography was not attempted for fear of recovering insufficient material to carry out further tests.

The crude isolate was therefore subjected to direct ultraviolet analysis and its U.V. characteristics compared with those of the essential oil from which it had been obtained. Taken up in 25 ml. of ethyl alcohol it exhibited peak absorbance (ϵ = 2.1) at 220 mμ, indicative of the presence of approximately 0.03% of menthofuran in the essential oil.

Similar examination of a number of *M. arvensis* oils showed invariably the occurrence of strong maxima or shoulders throughout the 220–240 mμ region; the specimen which had served as starting material for preparation of the menthofuran concentrate displayed a maximum at 231 mμ ($E^{1\%}_{1\ cm.}$ = 43.9). Hence the presence of trace amounts of



1 *M. arvensis* | *2* *M. spicata* | *3* *M. cardiaca* | *4* Menthofuran | *5* Isolate from *M. arvensis*

Fig. 2.—CGT-Chromatograms of analytical peaks from *1, M. arvensis; 2, M. spicata; 3, M. cardiaca;* and TL-Chromatograms of *4*, menthofuran standard, and *5*, menthofuran isolate from *M. arvensis* as described under *Experimental.*

menthofuran in these oils escaped recognition by direct ultraviolet analysis. Thus, the addition of as much as 1% of the heterocycle to genuine preparations could not be detected with any degree of reliability. It is because of such relatively intense absorption by nonmenthofuran constituents throughout the analytical wavelength range that *M. piperita* oils fail, likewise, to submit to menthofuran determinations by direct ultraviolet spectrophotometry.

Following recovery of the concentrate from its ethanolic solution, a 1-μl. aliquot was examined along with a 1-μl. aliquot of reference standard (0.1% menthofuran) by thin-layer chromatography using *n*-hexane as developing solvent. Deep orange-red colorations of identical R_f values were observed following treatment of the chromatoplate with the spray reagent (see Fig. 2).

The remainder of the analytical sample was used for observing Flückiger's reaction. The characteristic sequence of colorations was seen clearly and a strong positive test obtained within 1 minute. Thus, each of the analyses had supplied ample evidence for the presence of menthofuran in *M. arvensis*, and its occurrence in this species can now be considered ascertained beyond doubt.

The most convenient and perhaps also the most convincing of the criteria of identity established were those assembled by thin-layer chromatography carried out under the experimental conditions specified. Of a great many terpene compounds examined similarly in our laboratory, none showed the migration characteristics plus color response typical of menthofuran. Pertinent chemical and chemotaxonomic applications of the technique were reported (13), and further data illustrative of its scope for the analysis of essential oils and their constituents are to be published elsewhere.

TABLE I.—TLC AND CGTC OF ESSENTIAL OILS OF THE GENUS *Mentha*

Essential Oil	TLC Sample Vol., 2 μl.	CGTC Sample Vol., 25–100 μl.	Menthofuran, %
Mint			
M. arvensis L.			
Argentina	−	+	0.01
Brazil APPA-MA-1	−	+	0.02
APPA-MA-4	−	+	0.01
Formosa	−	+	0.03
India	−	+	0.04
Specimen obtained from Japanese mint plants raised and processed in State of Kashmir			
Japan "Sanbi"	−	+	0.04
"Hakubi"	−	+	0.02
South Africa	−	+	0.03
Strain of imported Japanese mint			
Spearmint			
M. cardiaca Gerard ex Baker cultivar Scotch or Highland	−	+	0.02
M. spicata cultivar common or native American	−	+	0.01
M. viridis	−	+	0.02
Pennyroyal[a]			
M. pulegium L.			
Spanish	+	+	0.30
Moroccan	+	+	0.25
Hedeoma pulegioides (L.) Pers.	+	+	0.05
Oregon race	−	+	0.02
M. sylvestris L.[a]	−	+	0.05
M. citrata Ehrh.[a]	+	+	0.07
M. rotundifolia[a]			
French strain, Main constituent neo-neo-isopulegol	+	+	0.06
Italian strain, Main constituent piperitenone oxide	−	+	0.02

[a] Specimens stored prior to analysis under nitrogen and at 5°C. for periods up to 3 years. Menthofuran contents of freshly distilled oils probably higher than reported.

Coupled Gas-Liquid–Thin-Layer Chromatograms

The gas chromatograms of six *M. arvensis* oils of different geographical provenance are shown in Fig. 1.

Qualitatively their profiles are similar. Yet quantitative correlations of peaks observed for biochemically related constituents assures classification of these oils according to geographical origin (10).

In each of the chromatograms a minor peak or small shoulder precedes the emergence of menthone. Shown in heavy lines it illustrates the effluent examined by coupled gas-liquid–thin-layer chromatography as described under *Experimental*. Codeposition of linalool and menthone as well as other constituents emerging during collection of the effluent does not interfere with the analyses because of the selectivity of the solvent system and spray reagent applied. In each instance color intensities observed were compared with those produced by accurately measured aliquots of menthofuran (0.1%)–*M. arvensis* calibration standards examined under the same experimental conditions. The presence of 0.01 to 0.04% of menthofuran as a genuine constituent of *M. arvensis* was thus established (see Table I).

Correlation of these results with those reported in previous investigations (10, 11, 14, 15) illustrates the occurrence of a given constituent at vastly different concentrations in various species belonging to a given genus. Such findings will make imperative revisions of our present concepts concerning the biogenesis of essential oils and their constituents and aid in the development of more reliable methods of plant classifications by physicochemical techniques. Because of its insensitivity, Flückiger's test, as this study has shown, may still be considered of distinct *practical* value to the industry. Yet its fundamental *biogenetic* significance and *chemotaxonomic* importance, as implied in the literature throughout the years, no longer exist.

One of the *M. arvensis* oils assayed, a commercial sample obtained from England, gave a positive menthofuran test (0.14%) when 2-μl. aliquots were spotted for direct thin-layer chromatography. Since adulteration of oils of *M. arvensis* with oils of *M. piperita* would be unprofitable, the observation is probably indicative of genuine compositional variations to be encountered. Quite likely, menthofuran concentrations up to 0.2% are still true criteria of *M. arvensis* authenticity.

For a time it was thought that contamination of *M. arvensis* with wild growing plants might account for the experimental observations. Accordingly, a Brazilian oil from such a species,[1] grown experimentally in the region of Colorado (Paraná State), was subjected to similar analyses. It contained 0.12% of menthofuran. (Pulegone content ~50%.) Comparable data were obtained for an American *M. arvensis* oil[2] produced from plants grown experimentally in Wisconsin.

It would thus appear that the presence of menthofuran in *M. arvensis* cannot be ascribed to contamination with wild growing mints. Since, furthermore, and for the purpose of clarifying this point, essential oils of different geographical origins were analyzed—some of them produced from authenticated strains cultivated under carefully controlled conditions—the possibility of the presence of menthofuran *via* admixture should also be ruled out. Contamination with peppermint can likewise be discounted, for *M. piperita* is not grown in Brazil, Formosa, and Japan. Only experimental plantations exist at this time in Argentina and India. Finally, and equally important is the fact that commercially exploited *M. arvensis* fields in the various producing countries are carefully supervised at all times to insure that only essential oils of highest quality and uniformity are reaching the world's markets. Menthofuran is, evidently, a genuine constituent of the species itself.

Sensitivity of Analytical Techniques

Examination of samples by the glacial acetic–nitric acid reaction proceeded as expected for *M. arvensis* oils. Faintly yellowish solutions formed without intermediate colorations being observed. Similar analyses of menthofuran–*M. arvensis* calibration standards proved that positive tests were obtained only at menthofuran concentrations equal to or greater than 0.4% by weight. Thin-layer chromatography yielded distinctly positive results when applied to 1-μl. aliquots of menthofuran–*M. arvensis* reference standards containing as little as 0.1% of menthofuran by weight. *M. piperita* oils examined similarly gave strongly positive tests with less than 0.5 μl. sample aliquots necessary for analysis. Having at our disposal reliable calibration standards, we screened conveniently menthofuran

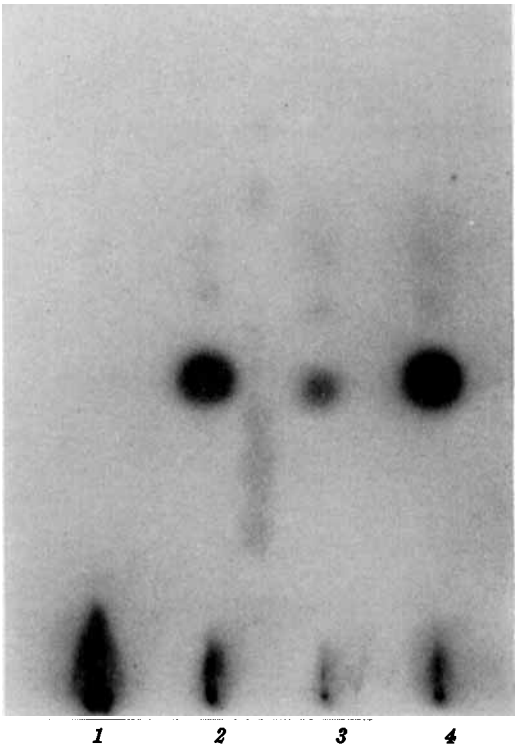


Fig. 3.—Thin-layer chromatograms of *1*, *M. arvensis* (presence of menthofuran not detected); *2*, *M. piperita* (Italo-Mitcham)—menthofuran 6%; *3*, *M. piperita* (English-Mitcham)—menthofuran 2.5%; and *4*, *M. piperita* (U.S.A.-Yakima)—menthofuran 9%. Sample volume—0.5 μl.

[1] Courtesy of Dr. M. T. Magalhães, Instituto de Química Agrícola, Rio de Janeiro, Brazil.
[2] Courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

TABLE II.—SENSITIVITY OF VARIOUS TECHNIQUES

Method of Analysis	Limit of Sensitivity
Ultraviolet spectroscopy	>1%
Flückiger reaction	0.4%[a]
Differential infrared spectroscopy	0.25%
Thin-layer chromatography	0.05% (0.5 mcg./μl.)
CGTC	0.01%[b]

[a] Sensitivity in solution slightly better than that reported by Zolotovich (0.5%) for spot test carried out on filter paper with but 1 drop of oil and reagent, respectively (9). [b] Subject to further refinement through increase of sample volumes used for analysis. Such refinement does not apply to thin-layer chromatography which as a rule processes larger complex samples less efficiently than smaller ones. Thus, none of the *M. arvensis* oils listed in Table I gave positive menthofuran tests when 10-μl. aliquots were spotted for analysis. The feature is a decided advantage of CGTC over TLC (see Table I) and outweighs the disadvantage of material losses which might occur during gas chromatography.

contents of peppermint oils available in our collection, as shown in Fig. 3.

An infrared method for the estimation of menthofuran in *M. piperita* oils was reported by Naves. Based on absorption intensities observed at 735 cm.$^{-1}$ it permitted detection of 1% of the heterocycle in genuine products. *M. arvensis* oils examined comparably failed to show the analytical band (16). We applied infrared spectroscopy likewise during the course of this study and confirmed Naves' results. None of the essential oils displayed infrared absorptions suggesting the presence of menthofuran. Only when using differential techniques (17, 18) and operating the instrument at fivefold scale expansion was it possible to detect the addition of 0.25% of menthofuran to authenticated preparations. With menthofuran concentrations in *M. arvensis* below this level direct infrared procedures would presently appear to be inadequate methods for such analyses.

The sensitivity of the various techniques applied is summarized in Table II.

Examination of Essential Oils from Other Mint Species

CGTC-analyses based on the experimental techniques described were also carried out on oil of spearmint (*M. cardiaca, M. spicata, M. viridis*), oil of pennyroyal (*M. pulegium* and *Hedeoma pulegioides*), oil of bergamot mint (*M. citrata*), oil of *M. sylvestris*, and oil of *M. rotundifolia*. The presence of menthofuran in each of these species was thus likewise established for the first time (see Table I).

BIOCHEMICAL SIGNIFICANCE OF EXPERIMENTAL RESULTS

The specificity of the metabolic pathways governing formation of carvone derivatives in spearmints and of menthone derivatives in peppermints was first pointed out by Kremers (19). Later, Reitsema substantiated and expanded these observations on the basis of a series of profound and extensive studies, grouping mint oils into the spearmints, peppermints, and lemon mints. Spearmints were characterized by the presence of 2-oxygenated *p*-menthanes, (carvone group), peppermints (including *M. arvensis* and *M. pulegium*) by the presence of 3-oxygenated *p*-menthanes (menthone group), and lemon mints by the presence of acyclic compounds. The coexistence of both 2- and 3-oxygenated *p*-menthanes in a given species was questioned and formation of one or the other of the three types considered exclusive (20). Subsequent workers acknowledged and endorsed this classification (21, 22). Recently, however, the occurrence of trace amounts of 3-oxygenated terpenoids, menthone and isomenthone, in spearmints was observed in this laboratory (14). The present study demonstrates the existence of a cyclic constituent, menthofuran, in *M. citrata*. Furthermore, the occurrence of pulegone, a possible precursor to menthofuran formation, was likewise established in this species by the CGTC technique. Small amounts of the cyclic ketone occurred also in *M. sylvestris* and *M. rotundifolia*. Its presence in *M. cardiaca, M. spicata, M. viridis*, and *M. arvensis* was previously reported (10, 14).

These observations appear to confirm that similar stereospecific biochemical processes are taking place in most, if not all, species of the genus *Mentha*, but at very different rates (23). A critical biogenetic scheme correlating the experimental data reported with others now assembled will make the subject of a forthcoming publication (24).

This study has shown how a delicate constituent not readily identified by direct application of classical chemical as well as modern instrumental methods of analysis may be detected and estimated in the various species of a given genus by means of the CGTC technique. In this regard it serves as a further example of the value of this method for the more thorough exploration of specific compositional regions comprising a gas chromatogram, for the isolation and determination of a minor constituent in a complex natural material, and hence for the establishment of new, sensitive criteria of product authenticity.

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Methanesulfonate Esters of Cholane[1] Alcohols

By FREDERIC C. CHANG

Some methanesulfonate esters of cholane alcohols derived from desoxycholic acid have been prepared and submitted for screening as antitumor agents. Infrared spectra of the dimesylates have distinctive patterns in the 870 to 1000 cm.$^{-1}$ region, each of which is shown to be virtually superimposable on a composite spectrum of a pair of corresponding mono-mesylates. Antitumor screening test results indicate that the 3α,12α-, and 12α,24-compounds are inactive and harmless to test animals, but the 3α,24-isomer is quite toxic.

THE DISCOVERY that 1,4-butanediol dimesylate[2] possesses significant antitumor activity has aroused interest in the methanesulfonyloxy derivatives of other types of compounds (1, 2). Access to a number of cholanediols made available during the course of other steroidal research (3–5) led us to prepare and submit for antitumor screening their dimesylate derivatives.

Syntheses of 3α,12α-dimesyloxycholane (I), 3α,24 - dimesyloxycholane (II), 12α,24 - dimesyloxycholane (III) by conventional methanesulfonyl chloride treatment in pyridine solution of the corresponding dihydroxycholanes derivable from desoxycholic acid—3α,12α-cholanediol (IV), 3α,24-cholanediol (V), and 12α,24-cholanediol (VI), respectively—are relatively straightforward. However, the 24-mesyloxy group can undergo further reaction with chloride ion formed in the reaction, as was encountered previously in the preparation of tosylates (3), and it was found necessary to avoid excessive reaction times in the mesylation of II and III. Use of thin-layer chromatography (TLC) facilitates following the course of reaction.

As predictable from knowledge of relative acylation rates of the hydroxyl groups in bile acids (6), the axial 12α-hydroxyl, additionally hindered by the C-17 side chain and possibly by the A-ring (7), is least accessible and reacts much more slowly than hydroxyl at either 3α or 24. Thus, IV or VI in the presence of 1.5 mole equivalents of mesyl chloride after 30 minutes at room temperature are converted into 3α,12α-cholanediol 3-mesylate (VII) and 12α,24-cholanediol 24-mesylate (VIII), respectively, without trace of dimesylation, as shown by TLC.

The primary C-24 hydroxyl group probably reacts faster than the secondary 3α in the mesylation reaction, as is true in the successful selective tosylation of 3α,24-cholanediol to the 24-monotosylate (3), but under the conditions of the experiments reported here mesylation at both 3α and 24 positions proceeded too rapidly to demonstrate relative rates. In fact, when V was treated with an equimolar amount of methanesulfonyl chloride even at 0° and followed by TLC, the first spot taken immediately after mixing (zero time), when developed, showed the production of two new spots, presumably corresponding to the two possible mono-mesylates. At 2-minute reaction time, an additional spot, with R_f value of 3α,24-cholanediol dimesylate had appeared. Spots taken at 20 minutes and longer show that the reaction had stopped and no further change in concentration of the three products or of the starting diol is seen, when apparently all the methanesulfonyl chloride has been consumed. Attempts to separate the mixture by either crystallization or column chromatography have failed.[3]

Infrared spectra of the three dimesylates all have a strong sharp band near 1170 cm.$^{-1}$ and bands near 1350 cm.$^{-1}$ (Fig. 2a), which are characteristic of sulfonate esters and are attributed to $-SO_2$ stretching vibrations (8). In addition, each spectrum has a distinctive absorption pattern in the 870 to 1000 cm.$^{-1}$ region, which can be used for differentiation and easy identification of the three isomers. Study reveals that these patterns are distinctive because of the marked individuality of the isolated mesyloxyl groups in the 3α, 12α, and 24 positions in the 870 to 1000 cm.$^{-1}$ region. These characteristic absorption bands differ among themselves considerably, but each is remarkably constant in shape and intensity at least for the compounds in the cholane and cholanic acid series.[4] Thus, in this infrared region the spectra of the dimesylates

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I am indebted to Prof. Philip C. Merker for guidance and consultation on pharmacological aspects of this work. Terry Richardson and Jo Thedford have contributed valuable technical assistance.

[1] All cholane derivatives mentioned in this paper are 5β-cholanes.

[2] Marketed as Myleran by Burroughs Wellcome & Co., Tuckahoe, N. Y.

[3] Failure by the latter method is not surprising, as R_f values by TLC of the two mono-mesylates and the dimesylate differ by little. By comparison, in the same developing system the two corresponding mono-tosylates (3) had quite different R_f values.

[4] However, mesyloxy compounds containing neighboring groups with possible interactions have not been studied.

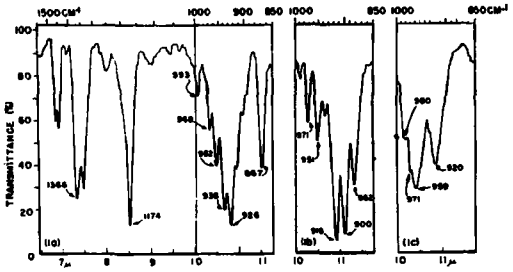


Fig. 1.—Partial infrared spectra of cholanol mesylates. (*a*) 3α-Cholanol mesylate; (*b*) 12α-cholanol mesylate; (*c*) 24-cholanol mesylate (all 4% in CCl_4).

are each virtually superimposable on a composite spectrum of a mixture consisting of the corresponding cholanol mesylates in equal proportions. Figure 1 shows partial spectra of the three cholanol mesylates: 3α-mesyloxycholane (IX), 12α-mesyloxycholane (X), and 24-mesyloxycholane (XI). Figure 2 gives spectra of the three dimesylates I, II, and III, each compared with the corresponding mixture in appropriate concentration.

The mono-mesylates VII and VIII, mentioned above, and a number of other cholane derivatives,[5] were recognized as mesylates and correctly assigned to substituent position through their distinctive infrared bands in this region.

Screening Results.—Compounds I and III in screening tests conducted by the Cancer Chemotherapy National Service Center on Sarcoma 180 in mice and lymphocytic leukemia in rats, were devoid of antitumor activity and quite harmless to test animals at daily doses of 125 mg./Kg., over a period of seven days. In contrast, compound II proved to be extremely toxic even in 0.50 mg./Kg. doses and could be administered only at low dosages. This degree of toxicity is unusual among steroids; furthermore, endocrine evaluation data, obtained from CCNSC, suggests that the 3α,24-compound may have thymolytic and antisplenic activity without marked antiflammatory effect. These results and the marked difference in biological activity between the isomeric dimesylates invites further investigations which we are pursuing.

EXPERIMENTAL

Microanalyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.; Weiler and Strauss, Oxford, England; and Alfred Bernhardt Mikroanalytisches Laboratorium, Mulheim, Germany. Melting points were taken on an electrical micro hot-stage and are uncorrected. These mesylate melting points were found to be dependent on the rate and length of heating; samples were placed on the hot-stage at one degree below the melting point, with the block temperature increasing about 0.33 degree per minute. Optical rotations were determined in 2% chloroform solution at about 25°. Infrared spectra were recorded on a Perkin-Elmer Infracord spectrophotometer (model No. 137).

Mesylation Procedure.[6]—The cholane alcohol, dried by azeotropic distillation of a benzene solution and dissolved in 7 volumes of freshly distilled pyridine, is cooled to nearly 0° in an ice bath, and while the reaction flask is twirled in the ice bath, an appropriate quantity (see below) of methanesulfonyl chloride (Eastman Kodak Co. No. 5388) is added through a pipet. After five minutes, the flask is removed from the bath and allowed to stand at room

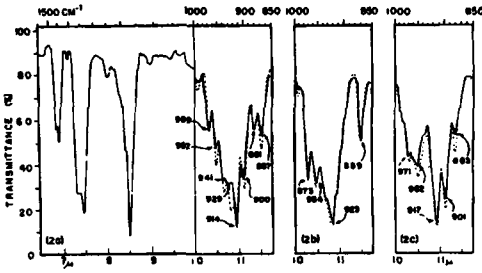


Fig. 2.—Partial infrared spectra of cholanediol dimesylates (——) and corresponding mixtures (- - -). (*a*) 3α,12α-Cholanediol dimesylate (2.5% in CCl_4); and 3α-cholanol mesylate-12α-cholanol mesylate (1:1 mixture of 4% solutions in CCl_4). (*b*) 3α,24-Cholanediol dimesylate (2.5% in $CHCl_3$); and 3α-cholanol mesylate-24-cholanol mesylate (1:1 mixture of 4% solutions in $CHCl_3$). (*c*) 12α,24-Cholanediol dimesylate (2.5% in CCl_4); and 12α-cholanol mesylate-24-cholanol mesylate (1:1 mixture of 4% solutions in CCl_4).

temperature. Crystals of pyridinium chloride separate as the reaction proceeds. The reaction can be followed readily by thin-layer chromatography (see below). When the reaction is complete, the flask is returned to the ice bath and ice chips are added, slowly to avoid undue temperature rise. In a few instances, the product crystallized at this point. Generally, processing requires transfer to a separatory funnel with ether, washing successively with cold dilute hydrochloric acid, neutralizing with dilute sodium bicarbonate solution and finally with water. After separation and treatment of the ethereal layer with a desiccant, and removal of the ether, the product is crystallized with a suitable solvent (see below).

Thin-layer Chromatography.—Plates were coated with silica gel G (Merck, Darmstad) using DeSaga equipment, developed in ligroin–ethyl acetate[7] (1:1), and sprayed with vanillin–sulfuric acid–ethanol (9).

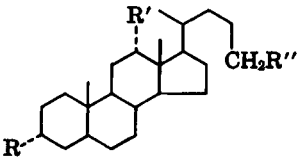
3α,12α-Cholanediol Dimesylate (I).—This was prepared from 3α,12α-cholanediol (4). Methanesulfonyl chloride used, 6 mole equivalents; reaction time, 3 hours. Crystallized from *i*-propyl ether as clear, dense prisms, m.p. 117.2 to 118.2°, $[\alpha]_D$ +

[5] Methyl 12α-mesyloxy-3-cholenate, methyl 12α-mesyloxy cholanate, methyl 3α,12α-dimesyloxycholanate, 3β-chloro-5-cholene-24-ol mesylate, 3β-chloro-11-cholene-24-ol mesylate [all described in (5)].

[6] Procedure used in the preparation of all mesylate esters described in this paper. Variations were only in proportion of methanesulfonyl chloride used, and in reaction time; these are stated under the individual compound descriptions. In general, yields obtained were nearly quantitative.

[7] Ligroin marketed as Skellysolve B by Skelly Oil Co., m.p. 63–70°, purified by sulfuric acid treatment and distillation.

TABLE I.—MESYLATES[a] OF CHOLANE ALCOHOLS



	Mesylate	R	R'	R''
I	3α,12α-Cholanediol Dimesylate	OMs[b]	OMs	H
II	3α,24-Cholanediol Dimesylate	OMs	H	OMs
III	12α,24-Cholanediol Dimesylate	H	OMs	OMs
VII	3α,12α-Cholanediol 3-Mesylate	OMs	OH	H
VIII	12α,24-Cholanediol 24-Mesylate	H	OH	OMs
IX	3α-Cholanol Mesylate	OMs	H	H
X	12α-Cholanol Mesylate	H	OMs	H
XI	24-Cholanol Mesylate	H	H	OMs

[a] Mesylate = Methanesulfonate. [b] OMs = Methanesulfonyloxy group.

70.0°, $\nu_{max.}$ (CCl_4) 1339, 1174, 969, 952, 941, 929 (sh), 914, 900, 881, 867 cm.$^{-1}$.

Anal.—Calcd. for $C_{26}H_{46}O_6S_2$ (518.76): C, 60.21; H, 8.94. Found: C, 60.56; H, 8.99.

3α-24-Cholanediol Dimesylate (II).—This compound was prepared from 3α,24-cholanediol (3, 10). Three mole equivalents of methanesulfonyl chloride were used. The reaction time was 1 hour. Needles of II crystallized from ligroin, m.p. 138.9 to 141.2°, $[\alpha]_D$ + 33.5°, $\nu_{max.}$ ($CHCl_3$) 1351, 1171, 973, 954, 923, 869 cm.$^{-1}$.

Anal.—Calcd. for $C_{26}H_{46}O_6S_2$ (518.76): C, 60.21; H, 8.94. Found: C, 60.32; H, 9.22.

12α,24-Cholanediol Dimesylate (III).—This was prepared from 12α,24-cholanediol (11).[8] Six mole equivalents of methanesulfonyl chloride were used. The reaction time was 3 hours. Crystallized from ligroin, III separated as clear, thin rectangular plates, m.p. 102.1 to 103.5°, $[\alpha]_D$ + 49.4°, $\nu_{max.}$ (CCl_4) 1350, 1179, 961, 952, 917, 901, 883 cm.$^{-1}$.

Anal.—Calcd. for $C_{26}H_{46}O_6S_2$ (518.76): C, 60.21; H, 8.94. Found: C, 60.02; H, 8.69.

III is quite stable at room temperature and can be dissolved by reflux in ligroin providing the material is stirred. Overheating, even at steam bath temperatures, causes quick decomposition of the compound.

3α,12α-Cholanediol 3-Mesylate (VII).—This was prepared from 3α,12α-cholanediol (4). The amount of methanesulfonyl chloride used was 1.5 mole equivalents and the reaction time was 1 hour. VII crystallized from ligroin as thin regular hexagons, m.p. 117.2 to 119.0°, $[\alpha]_D$ + 51.3°, $\nu_{max.}$ (CS_2) 3636, 1033 (12α-OH); 1361, 1174, 968, 954, 933, 917, 870 cm.$^{-1}$.

Anal.—Calcd. for $C_{25}H_{44}O_4S$ (440.60): C, 68.15; H, 10.07. Found: C, 67.96; H, 9.99.

12α,24-Cholanediol 24-Mesylate (VIII).—This was prepared from 12α,24-cholanediol (11). The amount of methanesulfonyl chloride used was 1.5 mole equivalents, and the reaction time was 1 hour. VIII crystallized from ligroin in bundles of needles, m.p. 97.4 to 98.6°, $[\alpha]_D$ + 35.8°, $\nu_{max.}$ (CS_2) 3663, 1036 (12α-OH); 1366, 1179, 982, 971, 960, 923 cm.$^{-1}$.

Anal.—Calcd. for $C_{25}H_{44}O_4S$ (440.60): C, 68.15; H, 10.07. Found: C, 67.87; H, 9.86.

3α-Cholanol Mesylate (IX).—This was prepared from 3α-cholanol (3). The amount of methanesulfonyl chloride used was 1.5 mole equivalents and the reaction time was 1 hour. IX crystallized from *i*-propyl ether as clear prisms, m.p. 114.5 to 115.5°, $[\alpha]_D$ + 44.1°, $\nu_{max.}$ (CCl_4) 1366, 1174, 993, 968, 956, 936, 926, 867 cm.$^{-1}$.

Anal.—Calcd. for $C_{25}H_{44}O_3S$ (424.60): C, 70.71; H, 10.45. Found: C, 70.57; H, 10.26.

12α-Cholanol Mesylate (X) (5).—This was prepared from 12α-cholanol (4). The amount of methanesulfonyl chloride used was 5 mole equivalents and the reaction time was 3 hours. X crystallized from methanol as thin plates, m.p. 99.3 to 99.8°, dec., $[\alpha]_D$ + 64.7°, $\nu_{max.}$ (CCl_4) 1344, 1174, 971, 951, 916, 900, 882 cm.$^{-1}$.

Anal.—Calcd. for $C_{25}H_{44}O_3S$ (424.60): C, 70.71; H, 10.45. Found: C, 70.92; H, 10.32.

24-Cholanol Mesylate (XI).—This was prepared from 24-cholanol (3, 12). The amount of methanesulfonyl chloride used was 1.5 mole equivalents, and the reaction time was 1 hour. XI crystallized from ligroin in micro needles, m.p. 89.5 to 91.2°, $[\alpha]_D$ + 26.6°, $\nu_{max.}$ (CCl_4) 1370, 1348, 1181, 980 (sh), 959, 920 cm.$^{-1}$.

Anal.—Calcd. for $C_{25}H_{44}O_3S$ (424.60): C, 70.71; H, 10.45. Found: C, 70.88; H, 10.65.

[8] Our preparation crystallized from benzene in thick elongated hexagons, m.p. 134.9 to 135.9°, $[\alpha]_D$ + 39.1° (chf.).

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Dielectric Constant Correlations with Solubility and Solubility Parameters

By WILLIAM G. GORMAN and GARY D. HALL

Correlations and lack of correlations of dielectric constants (DEC's) with solubility and solubility parameters are discussed. By restricting various solvent systems to solvents exhibiting similar bonding characteristics, such as by using members of homologous chemical series or by using solvent blends, essentially linear relationships were obtained for plots of log mole fraction of solute *versus* the DEC of the solvent or solvent system. Relationships of this type were demonstrated for (*a*) a nonpolar liquid in a series of polar polyethylene glycols, (*b*) a nonpolar liquid in a series of semipolar polypropylene glycols, (*c*) nonpolar liquids in blends of polar and semipolar solvents, (*d*) polar liquids in blends of nonpolar and semipolar solvents, and (*e*) a solid organic solute in blends of polar solvents. A ternary phase diagram for the system methyl salicylate/isopropanol/water is presented and resolved into a straight line by this procedure. This approach is presented as a means for the quantitative evaluation of solubility in homologous solvent series and solvent blends.

THE CONCEPT of solubility parameters as proposed by Hildebrand (1, 2) appears to have been accepted as one of the basic theories in the area of solubility. However, limitations such as the lack of readily available thermodynamic data, difficulty in readily determining such data, and the limited areas of applicability have prompted continued efforts to secure other solubility relationships.

Paruta *et al.* (3) have reported their observations on correlations of dielectric constants with solubility parameters. From a comparison of a number of solvents, they have proposed that a linear relationship exists between these two values which can be expressed by

$$\delta = 0.22\,\epsilon + 7.5 \qquad \text{(Eq. 1)}$$

where δ is the solubility parameter, and ϵ is the dielectric constant (DEC). They also have indicated that the best correlation between solubility parameters and dielectric constants was obtained with those solvents which associate primarily through hydrogen bonding, whereas Hildebrand (4) has carefully noted that justification for the theory of solubility parameters rests upon the London dispersion forces and that its use for polar substances is somewhat questionable.

EXPERIMENTAL

If we assume, as claimed by Paruta *et al.*, that DEC's are directly related to solubility parameters, correlations of solubility with the DEC's would be expected to follow a pattern similar to those presented by Hildebrand. For example, Hildebrand (5) has shown that plots of log mole fraction of solute *versus* the square of the difference between the solubility parameters of the solute and of the solvent result in a linear relationship. Assuming that a direct relationship exists between DEC's and solubility parameters, substitution of DEC values for the solubility parameters in these plots would be expected to produce similar type plots. A graph of this type (Fig. 1) was prepared for the solubility of iodine in various nonpolar solvents utilizing data presented by Hildebrand (5) and dielectric constant values as reported in the literature (6, 7). The log mole fraction of solute was plotted *versus* the square of the difference between the DEC's of solute and solvent as well as against the square of the difference between the solubility parameters of solute and solvent in order that a direct comparison could be made of the two relationships. The excellent correlation between solubility and solubility parameters may be observed readily, compared to the rather poor correlation with dielectric constants.

Hildebrand (8) has also shown good correlation for the solubility of gases in various solvents by plotting log mole fraction of solute *versus* the solubility parameter of the solvent. A plot of this type was prepared (Fig. 2) for the solubility of dichlorodifluoromethane[1] in several solvents of varying polarity, again substituting dielectric constants for solubility parameters. The solubility data were obtained from (9, 10), while DEC values consisted of both those reported in the literature (6) and determined experimentally. All experimental DEC values were determined with a Sargent model V oscillometer (11) using the purest commercially available solvents which were dried over calcium chloride prior to use. Although a vague linear trend was apparent, the solubility correlation was again rather poor. This is believed due to a wide variation in the chemical nature of the solvents employed with a resultant variation in their molecular associations. It was noted, however, that those solvents of similar chemical types (*e.g.*, *n*-alkyl alcohols, polyethylene glycols, and polypropylene glycols) showed closer trends toward linearity. This suggested the possibility of demonstrating linear correlations between solubility and dielectric constants by restricting the solvents to similar chemical types, such as homologous series in which similar molecular associations would be contemplated.

This concept is illustrated in Fig. 3, where solu-

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[1] Marketed as Freon 12 by E. I. du Pont de Nemours & Company, Wilmington, Del.

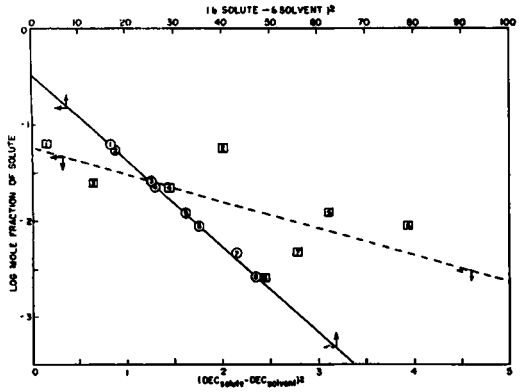


Fig. 1.—Correlation of the solubility of iodine with O, solubility parameters (δ) and □, dielectric constants (DEC's) of various solvents. Key: *1*, $CHBr_3$; *2*, CS_2; *3*, $CHCl_3$; *4*, $TiCl_4$; *5*, CCl_4; *6*, c-C_6H_{12}; *7*, $SiCl_4$; *8*, CCl_2F-$CClF_2$.

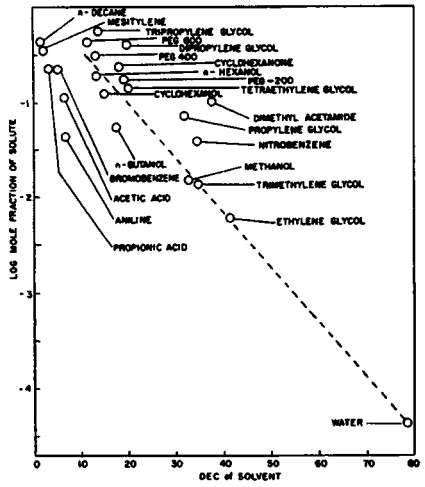


Fig. 2.—Correlations of the solubility of dichlorodifluoromethane with the dielectric constants (DEC's) of various solvents.

bility (10) of solutes dichlorotetrafluoroethane[2] and trichloromonofluoromethane[3] expressed as log mole fractions, were plotted *versus* the experimentally determined DEC's of two homologous series—polypropylene glycols and polyethylene glycols, respectively. In general, good agreement was observed in each case. In addition to showing solubility trends, this graph indicates the probable solubility of these solutes in other glycols of the series. For example, the solubility of dichlorotetrafluoroethane in polypropylene glycol 250 could be estimated from the graph.

If the above assumption is valid, even better agreement might be expected by further restriction of the molecular associations between solute and solvent. It was felt that this might be accomplished through the use of solvent blends. Previous investigations, such as the correlation of solubility with solubility parameters as reported by Chertkoff and Martin (12) and correlations with dielectric constants as reported by Paruta *et al.* (3) and Moore (13, 14) have been based on solvent blends which qualitatively restrict the molecular associations in the systems.

In order to investigate systems of this type for possible correlations of solubility with the DEC of the solvent system, use was made of liquid ternary phase systems. These systems were selected as they present solubility data covering the full range of miscibility for the mixtures. Figure 4 illustrates such a diagram for the system methyl salicylate/isopropanol/water prepared using experimental data. The log mole fractions of methyl salicylate, calculated from the weight values in Fig. 4, were plotted in Fig. 5 against the dielectric constants of the corresponding water/isopropanol mixtures. Also included in Fig. 5 are the systems chloroform/acetone/water and carbon tetrachloride/ethanol/glycerol utilizing the solubility data of Bonner (15) and McDonald *et al.* (16), respectively. For each system the DEC's of the solvent blends were determined experimentally to compensate for deviation from the theoretical linear values. Linear relationships were realized over most of the solubility range. The possibility of wide correlation in such plots is illustrated, for example, by the curve for carbon tetrachloride/glycerol/ethanol which covers a solute concentration range of 3 to 97% by weight of carbon tetrachloride.

The solubilities of polar solutes in nonpolar-semi-

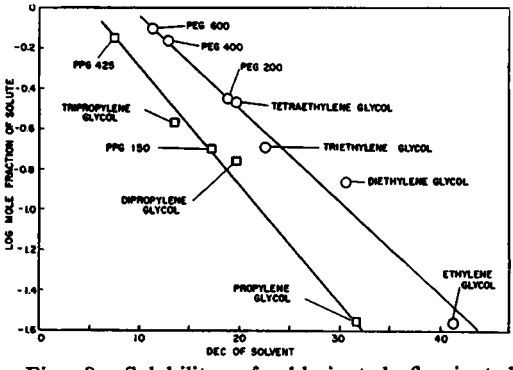


Fig. 3.—Solubility of chlorinated fluorinated hydrocarbons in homologous series of polyethylene glycols. Key: O, trichloromonofluoromethane; □, dichlorotetrafluoroethane.

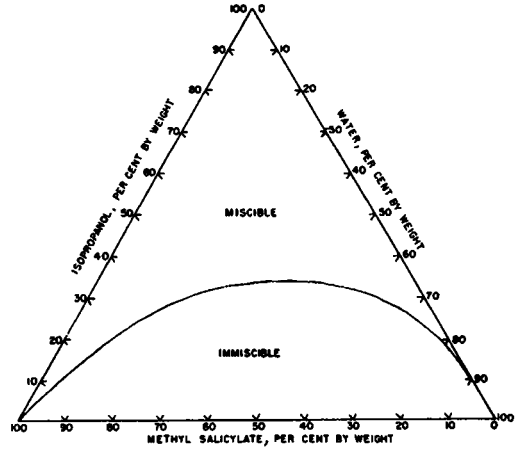


Fig. 4.—Ternary phase diagram of the system methyl salicylate/isopropanol/water.

[2] Marketed as Freon 114 by E. I. du Pont de Nemours & Company, Wilmington, Del.
[3] Marketed as Freon 11 by E. I. du Pont de Nemours & Company, Wilmington, Del.

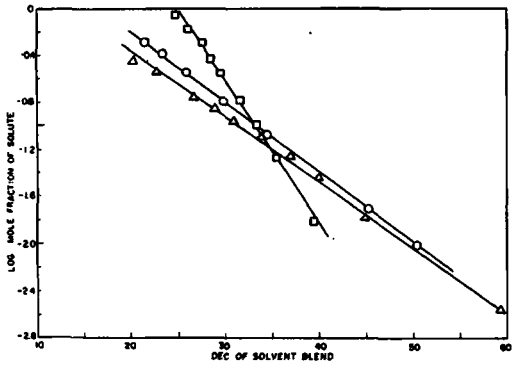


Fig. 5.—Solubility of various nonpolar solutes in polar solvent blends. Key: □, carbon tetrachloride in ethanol-glycerol; O, methyl salicylate in isopropanol-water; △, chloroform in acetone-water.

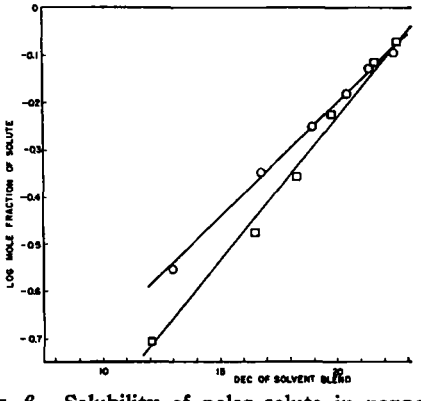


Fig. 6.—Solubility of polar solute in nonpolar/semipolar solvent blends. Key: O, water in ethanol-dichlorodifluoromethane; □, water in hexylene glycol-carbon tetrachloride.

polar solvent blends are presented in Fig. 6. The solubility of water in mixtures of dichlorodifluoromethane/ethanol (10) and of carbon tetrachloride/hexylene glycol (17) was expressed again in terms of log mole fraction of solute *versus* DEC of the solvent blend. DEC values of the carbon tetrachloride/hexylene glycol mixtures were determined experimentally, while the values of dichlorodifluoromethane/ethanol mixtures were estimated because of their high volatility at room temperature. A straight line relationship was obtained over most of the miscibility range.

In view of the success of the above correlations for liquid systems, possible extension of the concept to include the solubility of solids was considered. Figure 7 illustrates the solubility of secobarbital in water/ethanol, water/glycerol, and water/propylene glycol mixtures based on the solubility data presented by Udani and Autian (18). Essentially linear relationships were again obtained for each blend. It will be noted, however, that the extent of solubility not only varies with the dielectric constant of each solvent mixture, but also varies from one solvent mixture to another, although the bonding characteristics of each blend are somewhat similar. This suggests that the solubility of a given solute is not only affected by the DEC of the solvent system, but also varies with the particular solvent system employed. Therefore, although the desired solubility of a solute is achieved in a solvent blend at a given DEC, it does not indicate that the same degree of solubility can be expected for a different solvent blend even at the same DEC. In their study on the solubility of benzoic acid in mixed solvents, Chertkoff and Martin (12) also had similar variations when using solubility parameters.

SUMMARY AND CONCLUSIONS

Although a general linear relationship was reported to exist between dielectric constants (DEC's) and solubility parameters, serious discrepancies were noted when such correlations were extended to solubility data. However, by limiting the solvent system or systems to those exhibiting similar bonding characteristics, such as found in a chemical series, essentially linear relationships were demonstrated by plots of log mole fraction of solute *versus* the dielectric constant of the solvent system. Similar solvent restrictions by the use of solvent blends were investigated. Ternary phase diagrams of such systems were resolved into straight lines when plotted in this manner. Extension of this procedure to include solid solutes was also presented. A typical pharmaceutical solubility system consisting of secobarbital in a series of solvent blends was investigated. Although generally linear relationships were obtained, a common line for all the solvent blends was not observed, indicating that solubility varies with the solvent system employed as well as with its DEC.

It is felt that the above correlations are not strictly linear, but are essentially so over a large enough portion of the curve to make the relationship useful. Deviations from linearity may appear, for example, when extremely high concentrations of solute are present. For this reason the best approximations of solubility are obtained by interpolation between determined points rather than by extrapolation.

The solubility of polar solutes in less polar solvents (Fig. 6) resulted in lines with positive slopes while negative slopes were obtained for the solubility of nonpolar solutes in solvents of higher polarity (Figs. 5 and 7). It is, therefore, probable that a solute with polarity intermediate to that of the solvents would result in a curve which peaks at a certain DEC value. Systems such as those studied by Chertkoff and Martin may be in this category.

In the preparation of solubility graphs such as de-

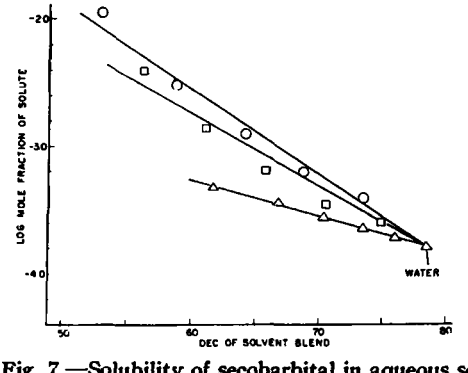


Fig. 7.—Solubility of secobarbital in aqueous solvent blends. Key: O, secobarbital in ethanol-water; □, secobarbital in propylene glycol-water; △, secobarbital in glycerol-water.

scribed in this paper, the solute mole fractions can be calculated readily and the DEC's of the solvent systems can be determined experimentally, accurately and quickly. The resulting graphs may be used for estimating the quantitative solubility of a solute in a solvent of a homologous series, for estimating the quantitative solubility of a solute in a solvent blend, for estimating the composition of a solvent blend required to dissolve a given amount of solute, for evaluating the efficiency of various solvents as coupling agents or ingredients of solvent blends, and for predicting the probable solvency properties of related solvents.

Thus, the use of dielectric constants presents an intriguing approach for the determination and correlation of solubility phenomenon.

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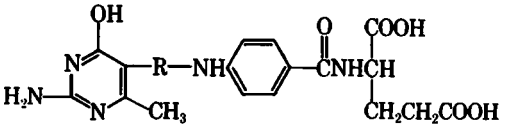
Analogs of Tetrahydrofolic Acid XI

Synthesis and Enzymic Evaluation of *p*-{[*N*-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)carbamoyl methyl]amino}benzoyl-L-glutamic Acid

By B. R. BAKER and KRISHNA SACHDEV

Condensation of ethyl α-acetamidoacetoacetate (XII) with guanidine carbonate afforded 5-acetamido-2-amino-4-hydroxy-6-methylpyrimidine (X). Acid hydrolysis of X followed by reaction with bromoacetyl bromide gave 2-amino-5-(bromoacetamido)-4-hydroxy-6-methylpyrimidine (XI). Reaction of XI with aniline or *p*-aminobenzoyl-L-glutamic acid in dimethylsulfoxide resulted in formation of 2-amino-5-(anilinoacetamido)-4-hydroxy-6-methylpyrimidine (XIII) and the title compound (III), respectively. III was a poor inhibitor of both dihydrofolic reductase and 5,10-methylene-tetrahydrofolate dehydrogenase, showing inhibition of about the order obtained with *p*-aminobenzoyl-L-glutamic acid.

A PYRIMIDYL ANALOG (I) (2–4) of tetrahydrofolic acid has been found to bind to folic reductase five times better than the substrate folic acid (4), to bind to 5,10-methylene-tetrahydrofolate dehydrogenase about one-thirtieth as well as the substrate (1), and to bind to thymidylate synthetase one-sixth as well as the substrate (5); *dl*-5,10-methylene-tetrahydrofolate was the substrate for the latter two enzymes. In contrast, the analog (II) is only one-eightieth as effective as I as an inhibitor of folic reductase (1), but II binds somewhat better to 5,10-methylene-tetrahydrofolate dehydrogenase than does I (1). It was concluded that the basic *p*-amino group of



I, R = $-CH_2CH_2CH_2-$
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III, R = $-NHCOCH_2-$

the *p*-aminobenzoyl-L-glutamate moiety was probably one of the essential groups for binding to folic reductase, but not to 5,10-methylene-tetrahydrofolate dehydrogenase. It was therefore of interest to synthesize III for enzymic evaluation; III has a different bridge between the pyrimidyl and aminobenzoyl moieties, but still retains the basicity of the latter moiety.

A key intermediate in the synthesis of III (see Scheme I) was 2,5-diamino-4-hydroxy-6-methylpyrimidine (IX), a compound described only once in the literature (6). Bromination of 2-amino-4-

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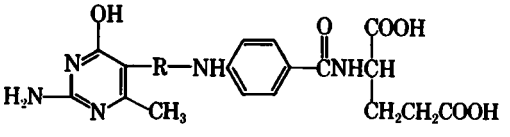
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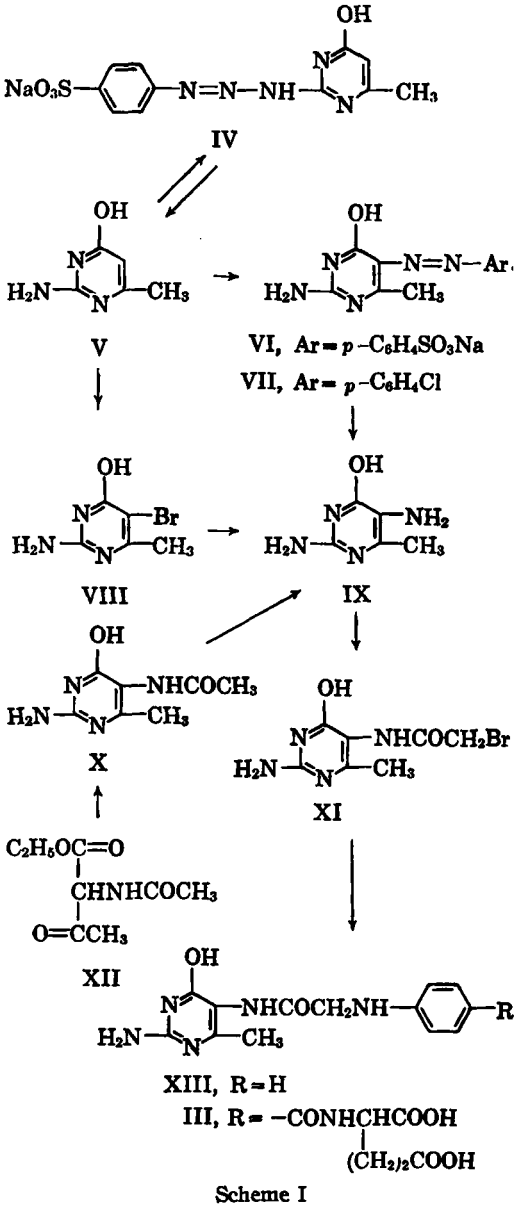


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Scheme I

hydroxy-6-methylpyrimidine (V) according to Jaeger (6) gave a 98% yield of the 5-bromopyrimidine (VIII) as the hydrobromide salt. Reaction of VIII with concentrated ammonia water at 200° as previously described (6) gave only traces of the desired 5-aminopyrimidine (IX).

Todd, *et al.* (7), have introduced the 5-amino group in certain pyrimidines by coupling with a diazonium salt followed by reduction of the resultant azo derivative; although they made a number of 5-aminopyrimidines in this way, a number of other pyrimidines were merely surveyed to see if coupling with a diazonium salt to form an azo compound took place—as evidenced by color development. In the latter category was 2-amino-4-hydroxy-6-methylpyrimidine (V) which they described gave a color with *p*-chlorophenyl diazonium chloride attributed to formation of the azo dye (VII). We have investigated this reaction in more detail using the more stable diazonium salt from sulfanilic acid; coupling to V in sodium carbonate solution occurred with formation of an insoluble sodium salt, presumably with structure VI. When the insoluble azo dye was reduced in warm water with sodium hydrosulfite, the product did not agree in properties with the 5-aminopyrimidine (IX) prepared earlier from the 5-bromopyrimidine (VIII); the ultraviolet and infrared spectra indicated that a mixture of the starting material (V) and the desired 5-aminopyrimidine (IX) was obtained. That such was the case was readily verified by thin-layer chromatography by comparison of this mixture with IX (obtained from VIII) and V.

At first glance it would appear that V must have been carried along with the insoluble azo compound (VI). That such was not the case was shown by thin-layer chromatography; although two spots were present in the dye, one moved slower than V and one moved faster than V. It is clear that one spot must be the desired 5-azopyrimidine (VI); it is probable that the second spot was an azoaminopyrimidine (IV), which on reduction would regenerate V. Attempts to rearrange this supposed *N*-azo derivative (IV) to a 5-azo derivative (VI) in dilute acetic acid, according to the general procedure of Rosenhaus and Unger (8) were unsuccessful. It is clearly possible that other azo dyes mentioned by Todd, *et al.* (7), may also be mixtures of *N*-azo and 5-azo aminopyrimidines.

A simple and successful route to the 5-aminopyrimidine (IX) *via* X was finally found. Condensation of ethyl α-acetamidoacetoacetate (XII) (9) with guanidine carbonate in boiling ethanol gave the 5-acetamidopyrimidine (X) in 30–40% yield. Since this range of yields was obtained within 2 hours and did not increase with increasing reaction time, ethanolic cleavage of the keto ester (XII) was probably a competitive reaction (10). When the guanidine carbonate reaction with XII was run under noncleavage conditions by using *tert*-butyl alcohol (10) as the solvent, the yield of 5-acetamidopyrimidine (X) was increased to 72%.

Hydrolysis of the 5-acetamidopyrimidine (X) with boiling 6 *N* hydrochloric acid gave the desired 5-aminopyrimidine (IX) hydrochloride in 83% yield; the free base (IX) obtained from the hydrochloride was uniform on thin-layer chromatography and was identical with IX prepared from the 5-bromopyrimidine (VIII). When the 5-aminopyrimidine (IX) hydrochloride was re-

acted with excess bromoacetyl bromide in aqueous sodium bicarbonate suspension, the 5-(bromoacetamido)pyrimidine (XI) was obtained in 69% yield; although XI had no melting point, it was uniform on thin-layer chromatography and was clearly distinguishable from the starting material (IX) and had an infrared spectrum similar to X.

Displacement of bromide from the 5-(bromoacetamido)pyrimidine (XI) with excess aniline in dimethylsulfoxide at ambient temperature proceeded smoothly to the 5-(anilinoacetamido)-pyrimidine (XIII) in 73% yield. A similar reaction between XI and *p*-aminobenzoyl-L-glutamic acid to give the tetrahydrofolate analog (III) proceeded less effectively; partly due to the difficulty in isolation and purification of the product the yield was only 13%.

When the 5-(anilinoacetamido)pyrimidine (XIII) was assayed as an inhibitor of the dihydrofolic reductase from pigeon liver, no inhibition was observed up to a concentration of 0.5 m*M;* in contrast, 0.5 m*M* 2-amino-5-(3-anilinopropyl)-4-hydroxy-6-methylpyrimidine (1) gave 40% inhibition of this dihydrofolic reductase when 6 μM dihydrofolate and 9 μM reduced triphosphopyridine-nucleotide (TPNH) were employed (1). Similarly, III was a poor inhibitor of this dihydrofolic reductase, only 20% inhibition being observed at a concentration of 6 m*M;* this result contrasts with the 50% inhibition of this enzyme shown by 0.10 m*M* concentration of I.

When III was assayed as an inhibitor of the 5,10-methylene-tetrahydrofolate dehydrogenase from pigeon liver in the presence of 0.03 m*M dl*-5,10-methylene-tetrahydrofolate and 0.10 m*M* TPN (1), 50% inhibition was obtained at a concentration of 3.2 m*M;* this compares not too favorably with 1.1 m*M* of I and 0.47 m*M* of II required for 50% inhibition of this enzyme (1). In fact, III showed about the same order of inhibition of the two enzymes as *p*-aminobenzoyl-L-glutamic acid, indicating that the pyrimidyl moiety of III made no contribution toward the binding of the compound to either enzyme.

At this time an exact explanation of why III is not so good an inhibitor of dihydrofolic reductase or 5,10-methylene-tetrahydrofolate dehydrogenase as I or II cannot be given. Since III should have all the necessary binding groups for both of these enzymes, it is possible that this alternate side chain in III (compared to I) could cause some steric interference with enzyme binding in the region of —NHCO— group, or the —NHCO— group of the bridge could have an unfavorable influence on the pK's or tautomerism of the groups in the pyrimidine moiety that are necessary for binding. Whether III would be a better inhibitor than I and II of some of the other enzymes in folic cofactor area (2) remains to be determined.

EXPERIMENTAL

Melting points were determined in capillary tubes with a Mel-temp block; those melting points below 230° are corrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer model 137B spectrophotometer. Ultraviolet and visible spectra were determined with a Perkin-Elmer model 202 spectrophotometer. Thin-layer chromatography (TLC) was performed on silica gel on glass plates with methanol as the developing solvent; spots were detected with iodine vapor. Concentration of all solutions was performed by spin-evaporation *in vacuo* at a bath temperature of 50–70°.

5 - Acetamido - 2 - amino - 4 - hydroxy - 6-methylpyrimidine (X).—Ethyl α-acetamidoacetoacetate (XII) was prepared by the reaction of phenyl diazonium chloride with ethyl acetoacetate followed by reductive acetylation as described by Pfister, *et al.* (9), in 76% over-all yield for the two steps. The crude, waxlike, but crystalline XII was employed for condensation with guanidine since better over-all yields were obtained; losses in recrystallization of the low melting XII were high.

A mixture of 0.93 Gm. (5 mmoles) of XII, 12 ml. of *tert*-butanol and 0.45 Gm. of guanidine carbonate was refluxed with magnetic stirring for 10 hours during which time the product separated. The mixture was evaporated and the residue triturated with 20 ml. of cold water. The product was collected on a filter and washed with ice-water; yield, 0.67 Gm. (72%), m.p. 305–308° dec. Recrystallization of an identical sample, prepared in ethanol as the reaction solvent, from ethanol gave white crystals of a hydrate, m.p. 308–310° dec.; $\lambda_{max.}^{EtOH}$ 227, 291 mμ; $\nu_{max.}^{KBr}$ 3300, 3100 (NH); 1700 (amide C=O); 1670–1630 (broad); 1580, 1530, 1510 cm.$^{-1}$ (NH, pyrimidine). The compound traveled as a single spot on TLC.

Anal.—Calcd. for $C_7H_{10}N_2O_2 \cdot H_2O$: C, 42.0; H, 6.05; N, 28.0. Found: C, 42.3; H, 5.91; N, 27.7, 28.0.

2,5 - Diamino - 4 - hydroxy - 6 - methylpyrimidine (IX) Hydrochloride.—A solution of 2.0 Gm. of X in 20 ml. of 6 *N* hydrochloric acid was refluxed for 30 minutes, then evaporated to dryness. The residue was triturated with methanol; yield, 1.6 Gm. (83%) of hydrochloride, m.p. 255–260° dec.; $\nu_{max.}^{KBr}$ 3330 (NH); 1705 (C=NH$^+$); 1680, 1540, 1480, 1460 cm.$^{-1}$ (NH, pyrimidine). This material was suitable for conversion to XI.

The free base (IX) crystallized when a concentrated aqueous solution of the hydrochloride was neutralized with sodium bicarbonate. The base (IX) is quite water soluble and had m.p. 270–275° dec.; $\lambda_{max.}^{EtOH}$ 245, 305 mμ; $\nu_{max.}^{KBr}$ 3400, 3300, 3200, 3100 (NH); 1680, 1640, 1580, 1550, 1530, 1500 cm.$^{-1}$ (NH, pyrimidine). This compound gave a single spot on TLC that moved slower than X, but was the same as IX prepared in traces from 2-amino-5-bromo-4-hydroxy-6-methylpyrimidine as described by Jaeger (6). Jaeger (6) recorded a m.p. of 275°, the yield being a "small amount."

2 - Amino - 5 - (bromoacetamido) - 4 - hydroxy-6-methylpyrimidine (XI).—To a stirred suspension of 1.41 Gm. (8 mmoles) of IX hydrochloride in

50 ml. of water containing 3.8 Gm. of sodium bicarbonate was added 3.03 Gm. (16 mmoles) of bromoacetyl bromide in one portion. The mixture was vigorously stirred for 7 hours at ambient temperature during which time the nature of the suspended material changed. The product was collected on a filter and washed with water, then ethanol; yield, 1.28 Gm. (60%) of white solid which partially melts about 255° but does not completely melt below 320°. This material was suitable for further reactions.

To the aqueous filtrate was added 1.00 Gm. of sodium bicarbonate and 1.3 Gm. of bromoacetyl bromide. After being stirred for 4 hours, the solution deposited an additional 0.10 Gm. (9%) of product identical with the first crop. A similar preparation was purified for analysis by two recrystallizations from absolute ethanol: white crystals, m.p. above 300°, that gave a strong halogen test; $\lambda_{max.}^{EtOH}$ 227, 291 mμ; $\nu_{max.}^{KBr}$ 3400, 3300–3000 (NH); 1690 (amide C=O); 1670–1630, 1580, 1540, 1500 cm.$^{-1}$ (NH, pyrimidine). This compound gave a single spot on TLC which moved faster than X and IX.

Anal.—Calcd. for $C_7H_9BrN_4O_2 \cdot {}^1/_3EtOH$: C, 33.3; H, 4.02; N, 20.3. Found: C, 33.2, 33.3; H, 3.92, 3.80; N, 20.0.

2 - Amino - 5 - (anilinoacetamido) - 4 - hydroxy-6-methylpyrimidine (XIII).—To a solution of 522 mg. (2 mmoles) of XI in 5 ml. of dimethylsulfoxide was added 0.37 ml. (4 mmoles) of aniline. After standing at ambient temperature in a stoppered flask for 2 days, the solution was diluted with 20 ml. of cold water. After 20 minutes in an ice bath, the mixture was filtered and the product washed with water; yield, 400 mg. (73%), m.p. 200–205° dec., that was soluble in 0.1 *N* hydrochloric acid, in contrast to XI. Two recrystallizations from aqueous ethanol gave white crystals of a hydrate, m.p. 210° dec.; $\nu_{max.}^{KBr}$ 3540, 3300, 3280 (NH, OH); 1720 (amide C=O); 1680–1580, 1530, 1490 (NH, C=C, pyrimidine); 763, 690 cm.$^{-1}$ (C_6H_5-). This compound could not be freed completely from solvated water. Analyses varied depending upon the drying conditions.

Anal.—Calcd. for $C_{13}H_{15}N_5O_2 \cdot 1{}^1/_2H_2O$: C, 52.6; H, 6.20; N, 23.5. Found: C, 52.7; 52.9; H, 6.12, 5.98; N, 23.1, 23.1.

Further drying at 100° in high vacuum over P_2O_5 gave the following analytical results:

Anal.—Calcd. for $C_{13}H_{15}N_5O_2 \cdot {}^1/_2H_2O$: C, 54.8; H, 5.75; N, 24.5. Found: C, 54.8; H, 6.04; N, 24.5.

p - {[N - (2 - Amino - 4 - hydroxy - 6 - methyl-5 - pyrimidyl)carbamoylmethyl]amino} benzoyl-L-glutamic Acid (III).—A solution of 0.783 Gm. (3 mmoles) of XI and 1.20 Gm. (4.5 mmoles) of *p*-aminobenzoyl-L-glutamic acid in 10 ml. of dimethylsulfoxide was allowed to stand in a stoppered flask at ambient temperature for 3 days. The solution was diluted with 40 ml. of water and adjusted to pH 3.8 with 1% aqueous sodium bicarbonate. The finely divided precipitate was collected by centrifugation and washed with water. The crude product was dissolved in the minimum of 1% aqueous sodium bicarbonate. After clarification by filtration, the solution was acidified with glacial acetic acid. The product was again collected by centrifugation and washed with water; yield, 0.175 Gm. (13%). Recrystallization from methanol gave a white powder that had no melting point and did not give a resolved infrared spectrum. Solvate was removed only after drying for 24 hours at 100° in high vacuum over P_2O_5. The analytically pure material then gave a negligible Bratton-Marshall test for diazotizable amine (4) and had $\lambda_{max.}^{pH\ 1}$ 280 mμ (ε 25,200), $\lambda_{max.}^{pH\ 8.4}$ 288 mμ (ε 24,400), $\lambda_{max.}^{pH\ 13}$ 282 mμ (ε 25,700).

Anal.—Calcd. for $C_{19}H_{22}N_6O_7$: C, 51.1; H, 4.93; N, 18.8; O, 25.1. Found: C, 50.9; H, 4.90; N, 19.0; O, 25.2.

No better yields were obtained when the dry *N,N*-dimethylformamide was employed as the solvent. When the reaction was run in dimethylsulfoxide at 80–90° for 5 hours, the isolated product (11%) was considerably darkened and had about 7% of Bratton-Marshall positive material after short treatment with hot 3 *N* hydrochloric acid (1).

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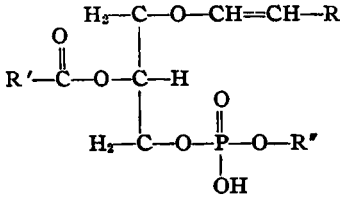
Plasmalogens II

Studies on Formation of Cyclic Acetals from Alkenyl Glycerol Ethers

By CLAUDE PIANTADOSI, MICHAEL F. FROSOLONO*, CARL E. ANDERSON and ALLEN F. HIRSCH

The 1-alkenyl glycerol ethers were converted to the corresponding cyclic glycerol acetals. The conditions necessary for their cyclization were studied, and no cleavage of the alkenyl linkage was observed. The compounds isolated from the cyclization reaction were identical with the synthetic cyclic glycerol acetals prepared by a different route.

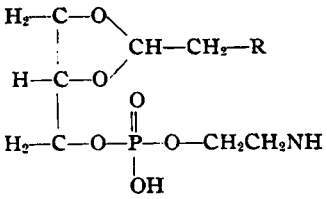
AT THE PRESENT TIME, naturally occurring plasmalogens are thought to be derivatives of glycerylphosphoryl choline, ethanolamine or serine, containing a fatty acid residue and an enol ether linkage as typified by I, first proposed by Klenk and Debuch (1), where R and R′ are long chain alkyl groups and R″ represents ethanolamine, choline, or serine.



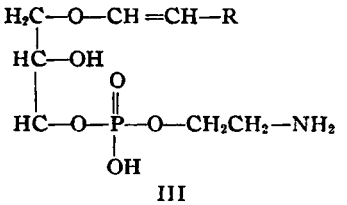
I

Evidence has recently been presented by Davenport and Dawson (2) that the cyclic acetal, II, isolated by Feulgen and Bersin (3) and Thannhauser, *et al.* (4), is an artifact formed by the acid hydrolysis of ethanolamine lysoplasmalogen, III; however, Landowne and Bergmann (5) have presented evidence for the occurrence of the cyclic form II in the sea anemone *A. elegantissima*, although Rapport and Alonzo (6) in a study of lipid extracts from the tissues of 11 marine invertebrates stated that their study did not produce proof either for or against an acetal structure.

Since the synthesis of 3-(1-alkenyloxy)-1,2-

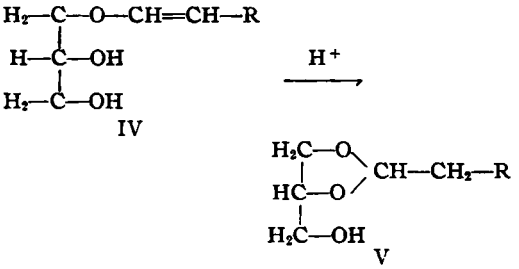


II



III

propanediols, IV, has been accomplished by this laboratory (7, 8), it was of interest to study the conditions necessary for their cyclization to the corresponding cyclic glycerol acetals, V.



IV V

EXPERIMENTAL[1]

Cyclization of 3-(1-Octenyloxy)-1,2-propanediol (Table I).—The cyclization of 3-(1-octenyloxy)-1,2-propanediol to 2-heptyl-4-hydroxymethyl-1,3-dioxolane is given as an example of the method used in the cyclization experiments. The other experiments were analogous, varying mainly in the choice of the cyclizing agent, reaction temperature, and time. To 5.0 ml. of 3-(1-octenyloxy)-1,2-propanediol dissolved in 10 ml. chloroform-isobutanol (1:1) mixture was added 10 ml. of aqueous 10% (w/v) trichloroacetic acid. The mixture was heated at 37° for 1 hour with vigorous stirring. The reaction mixture was allowed to stand at room temperature (25°) for approximately 17 hours. At the end of this time, sufficient *N* sodium hydroxide was added to neutralize the acid. The organic layer was extracted with diethyl ether, washed with water, and dried over anhydrous potassium carbonate. The solvent was removed *in vacuo* and the remaining oil distilled

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* U. S. Public Health Service Training Grant No. 5TI-GM-404-03.

[1] All boiling points and melting points are uncorrected. Analyses were determined by G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England, and by Spang Microanalytical Laboratory, Ann Arbor, Mich. All infrared spectra were automatically recorded with a Perkin-Elmer infracord spectrophotometer, housed in an air-conditioned room. The instrument was calibrated by means of known absorption bands. The liquid samples were scanned in a sodium chloride mull cell. No solvent was used.

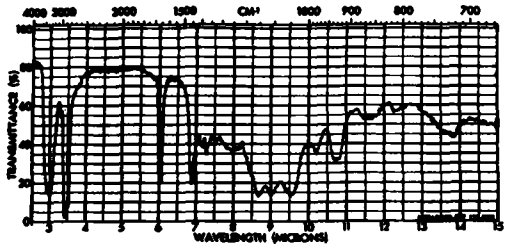


Fig. 1A.—3-(1-Octenyloxy)-1,2-propanediol.

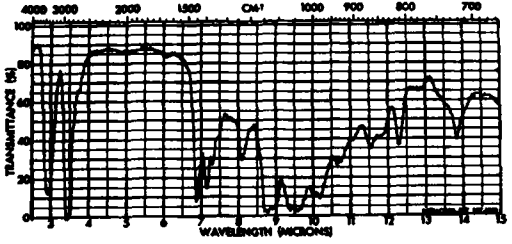


Fig. 1B.—Product isolated after treatment of 3-(1-octenyloxy)-1,2-propanediol with 10% TCA.

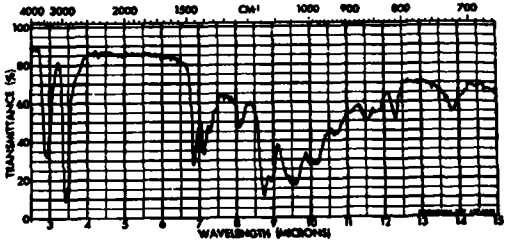


Fig. 1C.—Synthetic 2-heptyl-4-hydroxymethyl-1,3-dioxolane.

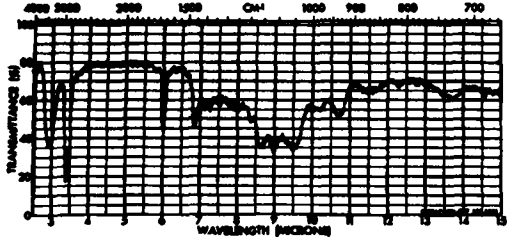


Fig. 2A.—3-(1-Decenyloxy)-1,2-propanediol.

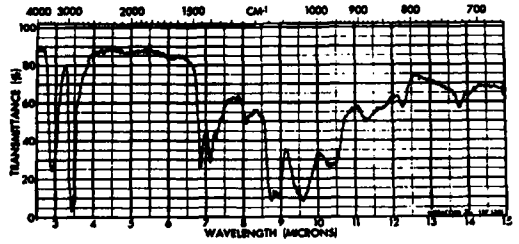


Fig. 2B.—Product isolated after treatment of 3-(1-decenyloxy)-1,2-propanediol with glacial acetic acid.

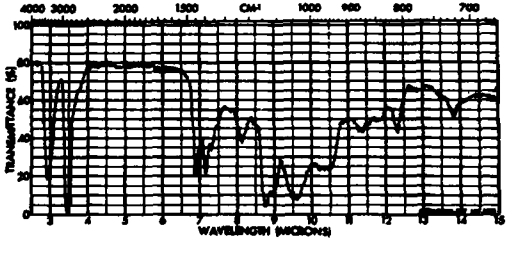


Fig. 2C.—Synthetic 2-nonyl-4-hydroxymethyl-1,3-dioxolane.

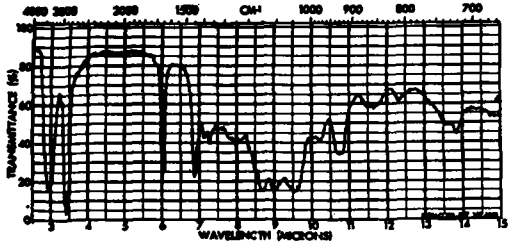


Fig. 3A.—3-(1-Dodecenyloxy)-1,2-propanediol.

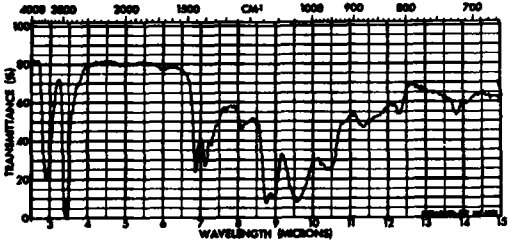


Fig. 3B.—Product isolated after treatment of 3-(1-dodecenyloxy)-1,2-propanediol with glacial acetic acid.

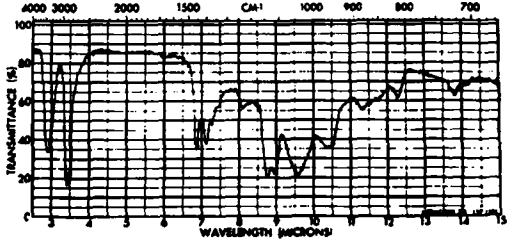


Fig. 3C.—Synthetic 2-undecyl-4-hydroxymethyl-1,3-dioxolane.

TABLE I.—PHYSICAL CONSTANTS OF GLYCEROL ENOL ETHERS, ISOLATED CYCLIC GLYCEROL ACETALS, AND SYNTHETIC CYCLIC GLYCEROL ACETALS

Glycerol Enol Ether				Cyclization Conditions[d]				Compound Isolated after Cyclization			Synthetic Cyclic Glycerol Acetal			
	b.p. °C./mm.	nD	(°C.)	Acid	Solvent	Temp., °C.	Time, hr.	b.p. °C./mm.	nD	(°C.)		b.p. °C./mm.	nD	(°C.)
3-(1-Octenyloxy)-1,2-propanediol[a]	120/0.02	1.4657	(20.0)	A	CI	37	1.0	80/0.01	1.4514	(20.0)[f]	2-heptyl-4-hydroxymethyl-1,3-dioxolane[i]	80/0.01	1.4531	(20.0)
				B	None	65	0.5	80/0.01	1.4514	(20.0)				
				A	CI	37[e]	1.0	80/0.01	1.4514	(20.0)				
				B	None	60[e]	1.0	80/0.01	1.4514	(20.0)				
3-(1-Decenyloxy)-1,2-propanediol[b]	130/0.05	1.4667	(20.0)	A	CI	37	1.0	95/0.02	1.4526	(25.6)[g]	2-nonyl-4-hydroxymethyl-1,3-dioxolane[j]	95/0.02	1.4560	(20.0)
				C	CI	37	1.0	95/0.02	1.4538	(25.5)				
				D	CI	37	1.0	95/0.02	1.4540	(25.0)				
				B	None	37	1.0	95/0.02	1.4539	(25.6)				
				B	None	50	1.0	95/0.02	1.4541	(25.0)				
				B	None	37	0.5	95/0.02	1.4538	(25.5)				
3-(1-Dodecenyloxy)-1,2-propanediol[c]	165/0.05	1.4684	(20.0)	B	None	60	1.0	135/0.25	1.4570	(20.0)[h]	2-undecyl-4-hydroxymethyl-1,3-dioxolane[k]	134/0.24	1.4575	(23.0)

[a] *Anal.*—Calcd. for $C_{11}H_{22}O_3$: C, 65.31; H, 10.96. Found: C, 65.19; H, 11.15. [b] *Anal.*—Calcd. for $C_{13}H_{26}O_3$: C, 67.78; H, 11.38. Found: C, 67.94; H, 11.28. [c] *Anal.*—Calcd. for $C_{15}H_{30}O_3$: C, 69.72; H, 11.70. Found: C, 69.70; H, 11.74. [d] Acid (cyclization agents): A, 10% w/v trichloroacetic acid; B, glacial acetic acid; C, 10% w/v trichloroacetic acid with 1.40 Gm. $HgCl_2$; D, 90% v/v glacial acetic acid. Solvents: CI, chloroform-isobutanol. [e] Compound isolated at end of 1 hour without allowing reaction mixture to stand for 17 hours. [f] *Anal.*—Calcd. for $C_{11}H_{22}O_3$: C, 65.31; H, 10.96. Found: C, 65.05; H, 11.08. [g] *Anal.*—Calcd. for $C_{13}H_{26}O_3$: C, 67.78; H, 11.38. Found: C, 68.23; H, 11.21. [h] *Anal.*—Calcd. for $C_{15}H_{30}O_3$: C, 69.72; H, 11.70. Found: C, 69.54; H, 11.63. [i] *Anal.*—Calcd. for $C_{11}H_{22}O_3$: C, 65.31; H, 10.96. Found: C, 65.46; H, 11.00. [j] *Anal.*—Calcd. for $C_{13}H_{26}O_3$: C, 67.78; H, 11.38. Found: C, 67.85; H, 11.44. [k] *Anal.*—Calcd. for $C_{15}H_{30}O_3$: C, 69.72; H, 11.70. Found: C, 69.90; H, 11.68.

through a 5-in. Vigreux column to yield one product. The structures assigned were supported by their infrared spectra (Figs. 1–3). The 2,4-dinitrophenylhydrazone of the aldehyde was prepared from the isolated product, m.p. 106°. Reported m.p. 106° (9). The 2,4-dinitrophenylhydrazones of the aldehydes obtained from the isolated cyclization products of 3-(1-decenyloxy)-1,2-propanediol and 3-(1-dodecenyloxy)-1,2-propanediol were also prepared and the melting points were, respectively, 104° and 106°, which agreed with the iterature values (9).

Preparation of 2-Alkyl-4-hydroxymethyl-1,3-dioxolanes.—The 2-alkyl-4-hydroxymethyl-1,3-dioxolanes used as reference compounds were synthesized by a completely independent procedure described in the literature (10).

The 3-(1-alkenyloxy)-1,2-propanediols (Figs. 1A, 2A, 3A) show a characteristic absorption in the infrared region at 1653 $cm.^{-1}$ attributed to the —O—CH=CH— group (11). As expected, this absorption is absent in the infrared pattern of the cyclic glycerol acetals, thus the use of the infrared spectrophotometer enabled one to demonstrate cyclization very readily. The infrared patterns (Figs. 1B, 2B, 3B) of the material resulting from the cyclization of the alkenyl glycerol ethers showed no absorbance in the 1650 $cm.^{-1}$ region and were in all respects identical to the infrared patterns of the appropriate reference compounds. The refractive indices, boiling points, and elemental compositions of the materials isolated from the cyclization procedure agreed favorably with the reference compounds (Table I).

DISCUSSION

The results presented here support the conclusions reached by Davenport and Dawson in their work with ethanolamine lysoplasmalogen. In the experiment conducted with 10% aqueous trichloroacetic acid with 1.40 Gm. mercuric chloride as the cyclization agent, and 3-(1-decenyloxy)-1,2-propanediol (Table I), only one product was formed, 2-nonyl-4-hydroxymethyl-1,3-dioxolane, and no free aldehyde could be detected. Davenport and Dawson, however, state that the free aldehyde is generated from ethanolamine lysoplasmalogen in the presence of the mercuric ion, although a small amount of the cyclic form remains. This may be due to the fact that the experiments reported in the present work were based on the synthetic, 3-(1-alkenyloxy)-1,2-propanediols, as compared with the ethanolamine lysoplasmalogen utilized by the above authors.

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Interaction of Preservatives with Macromolecules V

Binding of Chlorobutanol, Benzyl Alcohol, and Phenylethyl Alcohol by Nonionic Agents

By C. K. BAHAL and H. B. KOSTENBAUDER

Possible interaction of chlorobutanol and two aromatic alcohols, benzyl and phenylethyl, with polysorbate 80, polyvinylpyrrolidone, and methylcellulose was investigated by equilibrium dialysis studies. All these common alcoholic preservatives were found to exhibit reversible association with polysorbate 80. Only chlorobutanol was bound by polyvinylpyrrolidone, and none of the alcohols interacted with methylcellulose.

QUANTITATIVE DATA have previously been presented for the extent of association occurring between a number of common preservatives and nonionic macromolecules such as polyethylene glycols, polyvinylpyrrolidone, methylcellulose, and polysorbate 80. Data are available for the binding of preservatives such as phenols (1–6), carboxylic acids (1, 7, 8), and quaternary ammonium compounds (9). This communication is concerned with the presentation of data for additional classes of common preservatives, chlorobutanol, and the aromatic alcohols. Although there is microbiological evidence to indicate that chlorobutanol is inactivated in the presence of polysorbate 20 and it has been observed that chlorobutanol interacts with macromolecules, there has been no previous investigation of the magnitude of the interaction.

Deeb and Boenigk (10) found that a 0.5 per cent aqueous solution of chlorobutanol was a more effective antibacterial than a 0.75 per cent solution of chlorobutanol in 3 per cent polysorbate 20. They also observed that the solubility of chlorobutanol was greatly increased in the presence of polysorbate 20. Inactivation of chlorobutanol and phenylethyl alcohol in presence of polysorbate 60 has been reported by Barr and Tice (11).

Although there have been no reports of inactivation of benzyl alcohol in the presence of macromolecules, this preservative was included for comparison.

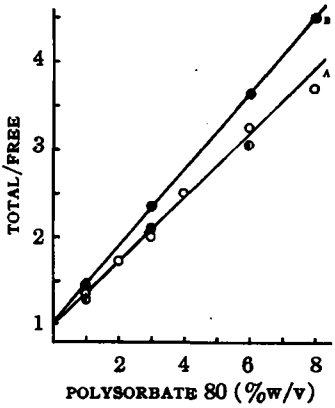


Fig. 1.—Binding of chlorobutanol by polysorbate 80. Key: A, at 30°—total chlorobutanol in solution: ○, 2.87 × 10^{-2} *M*; △, 4.37 × 10^{-2} *M*; ◐, 5.78 × 10^{-2} *M*. B, at 45°—total chlorobutanol in solution: ●, 2.91 × 10^{-2} *M*.

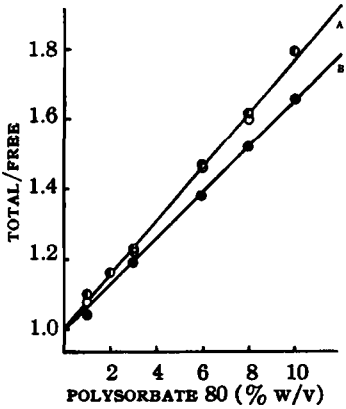


Fig. 2.—Binding of benzyl alcohol by polysorbate 80. Key: A, at 30°—total benzyl alcohol in solution: ○, 9.31 × 10^{-2} *M*; ◐, 19.43 × 10^{-2} *M*. B, at 45°—total benzyl alcohol in solution: ●, 19.43 × 10^{-2} *M*.

EXPERIMENTAL

Materials.—Chlorobutanol, anhydrous, U.S.P.; benzyl alcohol, reagent grade; phenylethyl alcohol, N.F.; polysorbate 80[1]; polyvinylpyrrolidone[2]; methylcellulose, 10 cps.[3]; and nylon[4] and cellophane[5] membranes were used.

Dialysis Procedure.—The technique used in the present studies was essentially the same as that employed by Miyawaki, *et al.* (4), with the exception that the films of nylon and cellophane were employed with plastic dialysis cells instead of as dialysis bags. Nylon membranes, which have been shown to be impermeable to polysorbate 80 (3), were selected for use in studies involving polysorbate 80, while cellophane was used for polyvinylpyrrolidone and methylcellulose studies.

Each dialysis cell consisted of two Plexiglas[6]

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[1] Polysorbate 80 is polyoxyethylene 20 sorbitan monooleate. Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

[2] Plasdone C, Antara Chemicals, New York, N. Y.

[3] Methocel, 10 cps., Dow Chemical Co., Midland, Mich.

[4] Plaskon Nylon, 0.0005 in., Allied Chemical Corp., New York, N. Y.

[5] Visking Cellulose Casing, Visking Corp., Chicago, Ill.

[6] Rohm & Haas Co., Philadelphia, Pa.

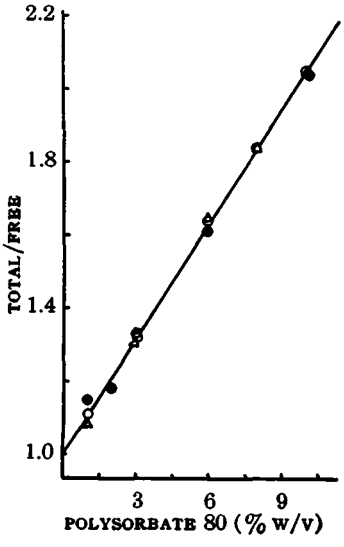


Fig. 3.—Binding of phenylethyl alcohol by polysorbate 80. Key: at 30°—total phenylethyl alcohol in solution: ●, 3.93 × 10^{-2} *M*; ○, 7.62 × 10^{-2} *M*. At 45°—total phenylethyl alcohol in solution: △, 3.93 × 10^{-2} *M*.

blocks, 6.3 × 6.3 × 2.6 cm., each half with a cavity having a capacity of 20 ml. Threaded Plexiglas plugs provided access to the cell cavities. To assemble the cells, a semipermeable membrane was clamped between the two symmetrical halves, solutions were pipeted into each cavity as required and the stoppers, fitted with polyvinyl chloride washers, were screwed in tightly. The cells were then rotated at 9 r.p.m. in a constant temperature water bath maintained at the required temperature ±0.2° until equilibrium was established. Using nylon membranes, equilibrium was established at 30° in 6 days for chlorobutanol, 96 hours for phenylethyl alcohol, and 48 hours for benzyl alcohol. Changes in volume of the two cell compartments, found only with polyvinylpyrrolidone and methylcellulose, were recorded and necessary corrections were made for the macromolecule concentration.

Assay Methods.—Benzyl alcohol and phenylethyl alcohol were assayed with a Beckman DU spectrophotometer at wavelengths of 256 mμ and 258 mμ, respectively. Chlorobutanol was determined by alkaline hydrolysis followed by determination of the chloride ion by potentiometric titration with 0.01 *N* silver nitrate solution, using a platinum-tungsten electrode pair (12).

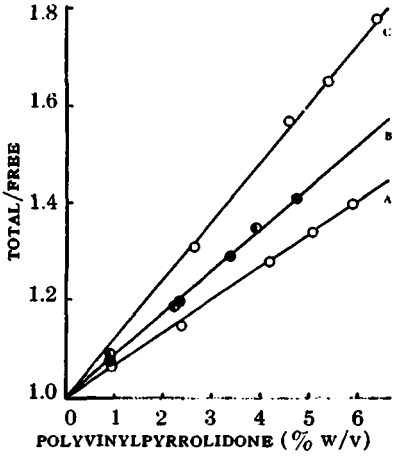


Fig. 4.—Binding of chlorobutanol by polyvinylpyrrolidone. Key: A, at 15°—total chlorobutanol in solution: ○, 2.85 × 10^{-2} *M*. B, at 30°—total chlorobutanol in solution: ●, 2.87 × 10^{-2} *M*; ◐, 5.77 × 10^{-2} *M*. C, at 45°—total chlorobutanol in solution: ○, 2.88 × 10^{-2} *M*.

RESULTS

Polysorbate 80.—Data for interaction of preservatives with polysorbate 80 are plotted in Figs. 1–3. The ratio of total/free preservative was essentially constant over the range of preservative concentrations utilized. With 3 per cent polysorbate 80 at 30°, 50% chlorobutanol, 18% benzyl alcohol, and 25% phenylethyl alcohol were bound. With an increase in temperature from 30 to 45°, the magnitude of interaction increased for chlorobutanol, decreased for benzyl alcohol, and remained unchanged for phenylethyl alcohol.

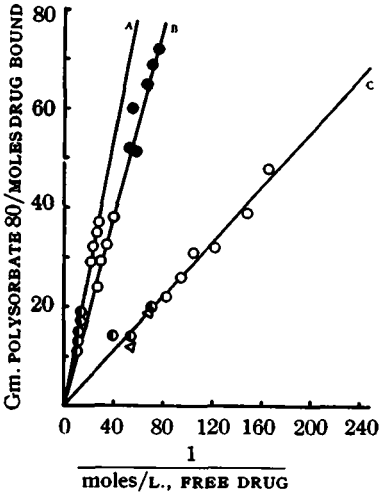


Fig. 5.—Langmuir-type plots for binding of preservatives by polysorbate 80 at 30°. Key: A, total benzyl alcohol in solution: ○, 9.31 × 10^{-2} *M*; ◐, 19.43 × 10^{-2} *M*. B, total phenylethyl alcohol in solution: ●, 3.93 × 10^{-2} *M*; ○. 7.62 × 10^{-2} *M*. C, total chlorobutanol in solution: ○, 2.87 × 10^{-2} *M*; △, 4.37 × 10^{-2} *M*; ◐, 5.78 × 10^{-2} *M*.

Polyvinylpyrrolidone.—The results of dialysis studies with polyvinylpyrrolidone are plotted in Fig. 4. Of the preservatives studied, only chlorobutanol interacted with polyvinylpyrrolidone and the extent of binding increased with an increase in temperature.

Methylcellulose.—None of the preservatives studied showed any interaction with methylcellulose.

DISCUSSION

Nature of Interaction.—Langmuir-type plots for chlorobutanol, benzyl alcohol, and phenylethyl alcohol (such as Fig. 5), when extrapolated to infinitely high free preservative concentration, show a line passing through the origin rather than an intercept corresponding to a limiting binding capacity. Such plots suggest a binding mechanism similar to partitioning into a separate phase, rather than adsorption on a micellar surface or to specific

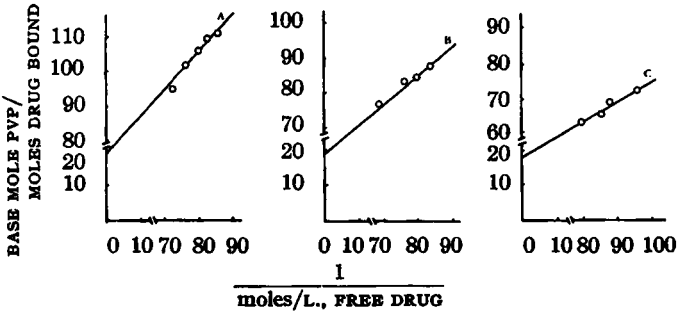


Fig. 6.—Langmuir-type plots for binding of chlorobutanol by polyvinylpyrrolidone (PVP). Key: A, at 15°—total chlorobutanol in solution: 2.85×10^{-2} *M*; B, at 30°—total chlorobutanol in solution: 2.87×10^{-2} *M*; C, at 45°—total chlorobutanol in solution: 2.88×10^{-2} *M*. [Drawn by method of least squares (17).]

TABLE I.—INTERACTION OF CHLOROBUTANOL WITH POLYVINYLPYRROLIDONE

Temp., °C.	Binding Constant *K*, L. mole^{-1}	Enthalpy Change (ΔH) cal. mole^{-1}	Free Energy Change (ΔG) cal. mole^{-1}	Entropy Change (ΔS_M) cal. deg.$^{-1}$ mole^{-1}
15	17.5	4078	−1639	27.8
30	22.9	4078	−1887	27.7
45	35.4	4078	−2255	27.9

sites on the macromolecule. This behavior is in direct contrast to the interaction of *p*-hydroxybenzoic acid esters with polysorbate 80 (3).

Langmuir-type plots for interaction of chlorobutanol with polyvinylpyrrolidone, Fig. 6, indicate a limiting binding capacity (0.05 sites per base mole of the polymer). The binding sites did not change with an increase in temperature. Thermodynamic functions calculated from data for binding of chlorobutanol by polyvinylpyrrolidone are presented in Table I.

The positive (unfavorable) enthalpy and the large positive unitary entropy term for interaction of chlorobutanol with polyvinylpyrrolidone are similar to values observed for binding of nonpolar molecules such as benzene or nitrobenzene to polyvinylpyrrolidone (13). Molyneux and Frank (13) and others (14, 15) have attributed interactions of this type mainly to the large unitary entropy change accompanying the disruption of the "iceberg" structure of water molecules around hydrocarbon groups in aqueous solution (hydrophobic bonding).

Temperature dependency of interactions with polysorbate 80 are not readily interpreted. Isooctane-water partition coefficients for chlorobutanol, benzyl alcohol, and phenylethyl alcohol all show an increase in the ratio, concentration in organic phase/concentration in aqueous phase, with an increase in temperature from 30 to 45° (16). If the interaction with polysorbate 80 were simply an oil/water partitioning phenomenon, one might expect that the binding to surfactant would vary with temperature in the same way as the oil/water partition coefficient varies. Undoubtedly, the interaction with the micellar surfactant is considerably more complex.

SUMMARY

When chlorobutanol is used as a preservative in the presence of polysorbate 80 or polyvinylpyrrolidone and when benzyl alcohol and phenylethyl alcohol are used in presence of polysorbate 80, it is advisable to use concentrations of these preservatives slightly in excess of the concentrations normally employed in aqueous solutions. Data presented in this work can be used to calculate the actual concentration required according to the procedure previously described (5). Methylcellulose did not interact with any of the preservatives used in this study and polyvinylpyrrolidone showed no tendency to bind benzyl alcohol or phenylethyl alcohol.

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Antiradiation Compounds VI

Metal Chelates and Complexes of 2-Mercaptoethylamine and 2-Mercaptoethylguanidine

By WILLIAM O. FOYE and JAMES MICKLES

To provide stable derivatives of 2-mercaptoethylamine (MEA) and 2-mercaptoethylguanidine (MEG) and to test one of the current hypotheses of the mechanism of radiation protection in animals (that metal binding is an important reaction in impeding cellular oxidations), metal complexes of MEA and MEG have been prepared. Reaction of the trithiocarbonate zwitterions of MEA and MEG with metal salts provided straight chain (2:1) metal complexes, and direct reaction of MEA and MEG with metal salts in acid solution provided cyclic (1:1) chelates. Structures of these compounds have been elaborated. One of the compounds, the Zn(II) complex of MEG has shown appreciable radiation protection in mice.

THE ABILITY to bind metal ions has been considered a possible mechanism for the protective action of the mercaptoamine and other chelating antiradiation agents in animals (1–4). Metal ion chelation or complexation may operate either for scavenging ions of copper or iron to prevent or interrupt cellular oxidations (5) initiated by radiation, for stabilization of the cuprous state of cuprous-containing enzymes (6), or for protection of the metal constituents of enzymes from free radical or ionic attack through transient chelation by the protective compound. In the event of such possibilities as these, administration of a preformed chelate to the animal would be expected to lower the radiation protective effect of the agent. Conversely, if the agent acts in the form of a metal chelate, or possibly is transported as a metal chelate, use of the preformed chelate should give equal or better protective ability than use of the agent alone.

Comparison of the radioprotective abilities of compounds capable of metal-ion chelation with those of the metal chelates themselves has been made in several instances. Alexander (7), for instance, showed that the copper chelates provided less protection to mice than the agents themselves, using diethyldithiocarbamate, dithiooxamide, and oxine. Rixon and Whitfield (8) found that disodium EDTA gave some protection to rats, whereas the copper complex of EDTA afforded little or no protection. An example where radiation protection was provided by metal complexes was reported by Smirnova (9) with the use of polyamine iron complexes.

Other evidence that metal-ion chelation may be of importance in radiation protection of animal cells also exists. Corradi (10) has claimed that such chelating agents as diethyldithiocarbamate, EDTA, *N*-nitroso-*N*-phenylhydroxylamine, and nitrilotriacetate are more active than MEA when given in equivalent fractions of the toxic dose. Protective action by metal ions themselves has been reported by Butler and Robins (11), who found that both ferrous and ferric ions protected trypsin against irradiation by electrons, possibly through complexation with the enzyme. A quantitative correlation between metal-binding ability and radiation protection has been observed by Jones (2), who found a constant ratio between the stability constant of the copper chelate and the protective effect of such agents as MEA, cysteine, histamine, EDTA, ethylenediamine, salicylic acid, and glycine. Knoblock and Purdy (12) have also shown a correlation between the instability constant of the copper complex and the radiation protective capacity in a series of mercaptoamines.

To compare the radioprotective abilities of 2-mercaptoethylamine (MEA) and 2-mercaptoethylguanidine (MEG) with the protective effects of their metal complexes, several reactions that might lead to metal complexes were investigated. Straight-chain metal sulfide complexes of MEA (I) and MEG (II) were obtained from the reaction of the trithiocarbonate zwitterions of MEA (13) and MEG (14) in dimethylformamide with aqueous solutions of divalent metal sulfates. The decomposition of trithiocarbonate-metal complexes to the metal alkyl sulfides appears to be a general reaction and takes place quantitatively (15). The metals employed were Zn (II), Cu (II), and Fe (II).

Cyclic metal complexes, or chelates, of MEA and MEG were obtained from the reactions of the ligands directly with metal salts. A 1:1

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$$^{+}NH_3CH_2CH_2S\overset{\overset{S}{\|}}{C}S^{-} + MSO_4 \rightarrow {}^{+}NH_2CH_2CH_2SMSCH_2CH_2NH_3{}^{+}SO_4{}^{-} + CS_2$$

I

$$NH_2CNHCH_2CH_2S\overset{\overset{S}{\|}}{C}S^{-} + MSO_4 \rightarrow NH_2\overset{\overset{NH_2^+}{\|}}{C}NHCH_2CH_2SMSCH_2CH_2NH\overset{\overset{NH_2^+}{\|}}{C}NH_2\ SO_4{}^{-} + CS_2$$

II

zinc (II) chelate of MEA was prepared using a procedure of Greth and Reese (16), which employs zinc oxide and carbon disulfide in aqueous alkali. Apparently an intermediate carbon disulfide complex decomposed to give the metal chelate. Copper (II) chelates (1:1) of MEA and MEG were obtained from aqueous solution by direct reaction of the sulfate with the ligand at pH 8.0 and allowing the pH to drop. In the case of MEG, it was necessary to keep the pH from dropping below 3.0 in order to obtain a crystalline product of good degree of purity. A similar reaction between ferrous sulfate and MEG, however, gave less drop in pH, and the product was identical with the open complex obtained from Fe (II) and the trithiocarbonate zwitterion of MEG. The chelates (III and IV) were distinguished from the complexes by elemental analyses, a much greater drop in pH during chelate formation, a difference in ratio between ligand and metal in the chelates (1:1) and the complexes (2:1). The complexes, in general, were more highly colored.

Aqueous solubility of both the complexes and chelates was quite limited, with generally less than 0.01 Gm. of substance soluble in 100 ml. of water. Since the antiradiation tests discussed below are generally carried out using 1–3% solutions administered by i.p. injection, these compounds must have been given in suspension.

Analytical evidence for the complexes and chelates prepared is recorded in Table I. The agreement between found and theoretical values was, in general, good for compounds of this nature where exact duplication of the product on successive runs is often difficult. In the majority of cases, agreement of four or five elements with theoretical values was obtained. The compounds were analyzed at two different commercial laboratories, and values for carbon and hydrogen generally concurred. Reproducible values for nitrogen and sulfur were more difficult to obtain, and were not obtained for the complexes of MEG. In the case of the Fe (II) MEG complex prepared directly from MEG rather than from the MEG trithiocarbonate, however, acceptable values for nitrogen and sulfur were obtained (see *Experimental*).

For the Fe (II) complex of MEA, carbon, hydrogen, nitrogen, and metal analyses indicated the presence of four sulfur atoms instead of the three required by the general formula. Formulation of the product as either the bis-bisulfate of the Fe (II) complex or the sulfate of the Fe (III) complex provided theoretical values agreeing for these four elements but not for sulfur. Although the color of the product would suggest that it is the Fe (III) rather than the Fe (II) complex, no indication of Fe (III) was obtained with thiocyanate ion.

Antiradiation Properties.—Antiradiation testing of some of the compounds described was carried out at the Walter Reed Army Institute of Research under the direction of Dr. D. P. Jacobus. Tests were made in mice using lethal doses of X-radiation as previously mentioned (17), 30-day survivial being taken as evidence of protection. The MEG–zinc complex (II) was tested at a dosage level of 50–150 mg./

$$HCl\cdot NH_2CH_2CH_2SH + MSO_4 \rightarrow \begin{array}{l} CH_2\text{——}CH_2 \\ | \quad\quad | \\ NH_2 \quad S \\ \searrow \swarrow \\ M^{+}\ Cl^{-} \end{array}$$

III

$$HBr\cdot NH_2CH_2CH_2S\overset{\overset{NH_2^+Br^-}{\|}}{C}NH_2 \xrightarrow[NH_4OH]{} NH_2\overset{\overset{NH_2^+Br^-}{\|}}{C}NHCH_2CH_2SH + MSO_4 \rightarrow \begin{array}{l} CH_2\text{——}CH_2 \\ | \quad\quad\quad | \\ S \quad\quad NH \\ \quad\quad\quad \| \\ \quad\quad\quad C \\ \quad\quad\quad | \\ \quad\quad\quad NH_2 \\ \searrow \swarrow \\ M^{+}\ Br^{-} \end{array}$$

IV

TABLE I.—PHYSICAL PROPERTIES OF METAL COMPLEXES AND CHELATES

A. COMPLEXES OF MEA: $(^{+}NH_3CH_2CH_2S)_2M\ SO_4^{=}$

M	Yield, %	Color	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found	Sulfur, % Calcd.	Sulfur, % Found	Metal, % Calcd.	Metal, % Found
Zn(II)	81	White	$C_4H_{14}N_2O_4S_2Zn$	15.22	15.22	4.47	4.67	8.87	9.11	30.46	30.07	20.70	19.65
Cu(II)	74	Brown	$C_4H_{14}N_2O_4S_2Cu$	15.31	15.21	4.50	4.05	8.92	8.46	30.65	30.62	20.24	20.24
Fe(II)	73	Tan	$C_4H_{18}N_2O_8S_4Fe$[a]	11.91	12.08	3.75	3.89	6.95	7.03	31.80	26.65	13.84	15.35

B. COMPLEXES OF MEG: $(NH_2{}^{+}CNHCH_2CH_2S)_2\ M\ SO_4^{=} \cdot n\ H_2O$

M	Yield, %	Color	n	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Metal, % Calcd.	Metal, % Found	Water, %[b] Calcd.	Water, %[b] Found
Zn(II)	71	White	1	$C_6H_{20}N_4O_6S_2Zn$	17.25	17.01	4.82	4.79	15.65	14.43	4.31	4.05
Cu(II)	50	Red-brown	1	$C_6H_{20}N_4O_6S_2Cu$	17.32	18.92	4.85	4.75	15.27	14.66	4.33	4.52
Fe(II)	84	Green	2	$C_6H_{22}N_4O_8S_2Fe$	16.90	16.77	5.17	4.85	13.10	13.94	8.45	6.69

C. CHELATES OF MEA AND MEG

Chelate	Yield, %	Color	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found	Sulfur, % Calcd.	Sulfur, % Found	Metal, % Calcd.	Metal, % Found
Zn(II)MEA	71	White	C_2H_6NOSZn[c]	15.25	14.47	3.84	3.63	8.89	10.30	20.36	22.97	41.50	45.75
Cu(II)MEA	92	Yellow	$C_2H_6NSClCu$	13.72	13.44	3.45	4.53	8.00	8.79	18.31	19.66	36.28	36.24
Cu(II)MEG	59	Yellow	$C_3H_9N_3SBrCu$	13.77	13.57	3.08	3.81	16.06	14.80	12.25	12.87	24.28	24.79

[a] Formulated as bis-bisulfate. [b] Determined by weight loss on vacuum drying at 100°. [c] The anion is hydroxyl ion.

Kg. and was described as giving good protection, but somewhat less than that afforded by MEG itself. The MEG–copper complex (II) gave only slight protection, as did the zinc chelate of MEA (III). The latter two compounds were appreciably more toxic than the MEG–zinc complex, however, and could be tested only at dosage levels under 50 mg./Kg.

EXPERIMENTAL

Analyses for carbon, hydrogen, nitrogen, and sulfur were determined by both Weiler and Strauss, Oxford, England, and Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. Metal analyses were done by ashing at 900°. Melting points were taken on a Fisher-Johns block.

Complexes of 2-Mercaptoethylamine.—The following procedure is representative. An aqueous solution (20 ml.) of 4.32 Gm. (0.015 mole) of zinc sulfate heptahydrate or 3.74 Gm. (0.015 mole) of cupric sulfate pentahydrate or 4.18 Gm. (0.015 mole) of ferrous sulfate heptahydrate was added dropwise with stirring to a solution of 4.39 Gm. (0.03 mole) of the zwitterion of 2-aminoethyltrithiocarbonic acid (13) in 150 ml. of dimethylformamide. A precipitate immediately formed. Stirring was continued for an hour after the addition was complete, and the product was isolated and washed with benzene followed by ether. The product was placed under vacuum to remove the excess solvent and was then air dried. The complexes decomposed on heating above 200°.

Complexes of 2-Mercaptoethylguanidine.—The following procedure is representative. An aqueous solution (15 ml.) of 2.16 Gm. (0.0075 mole) of zinc sulfate heptahydrate or 1.87 Gm. (0.0075 mole) of cupric sulfate pentahydrate or 2.09 Gm. (0.0075 mole) of ferrous sulfate heptahydrate was added dropwise with stirring to a solution of 2.93 Gm. (0.015 mole) of the zwitterion of 2-guanidoethyltrithiocarbonic acid (14) in 100 ml. of dimethylformamide. A precipitate formed immediately. Stirring was continued for an hour after the addition was complete, and the product was isolated and washed with benzene followed by ether. The product was placed under vacuum to remove the excess solvent and was then air dried. The complexes decomposed above 200°.

Zinc Chelate of 2-Mercaptoethylamine.—2-Mercaptoethylamine hydrochloride (11.3 Gm., 0.1 mole) (Chemicals Procurement Labs.) was dissolved in 65 ml. of water and neutralized by the addition of an aqueous solution of sodium hydroxide (4 Gm., 0.1 mole). To this solution was added 8.1 Gm. (0.1 mole) of zinc oxide, and the resulting suspension was stirred while the temperature was maintained at 40°. Carbon disulfide (10 ml., 0.15 mole) was added dropwise with stirring. The temperature was maintained at 40°, and stirring was continued for 3 hours. During this period the color of the suspension turned from yellow to white. A white solid was isolated and washed with water followed by ethanol, and the product was dried in a vacuum desiccator. The yield was 11.1 Gm. (71.4%) of a white product which decomposed above 210°.

Cupric Chelate of 2-Mercaptoethylamine.—2-Mercaptoethylamine hydrochloride (3.42 Gm., 0.03 mole) was dissolved in 35 ml. of water and the pH of the resulting solution was adjusted to 8.0 with dilute ammonium hydroxide. Ten milliliters of a solution containing 3.74 Gm. (0.015 mole) of cupric sulfate pentahydrate was added dropwise with stirring, and at a pH of 4.5, a yellow precipitate appeared. After the addition was complete, the pH had decreased to 2.3. Stirring was continued for an hour after the addition was complete, and the yellow product was isolated and washed with water followed by ethanol. The yield was 2.4 Gm. (92.2%), and the product decomposed above 200°.

Cupric Chelate of 2-Mercaptoethylguanidine.—*S*-β-Aminoethylisothiouronium bromide hydrobromide (8.52 Gm., 0.03 mole) (Matheson, Coleman and Bell) was dissolved in 35 ml. of water and the pH of the resulting solution was adjusted to 8.0 with dilute ammonium hydroxide. Ten milliliters of a solution containing 3.74 Gm. (0.015 mole) of cupric sulfate pentahydrate was added dropwise with stirring, and at a pH of 6.5, a yellow precipitation appeared. The pH was allowed to decrease to 3.0 and was maintained at this value by the alternate dropwise addition of dilute ammonium hydroxide and the cupric sulfate solution. Stirring was continued for 1

hour after the addition was complete, and the yellow product was isolated and washed with water followed by ethanol. The yield was 2.3 Gm. (58.7%), and the product decomposed above 110°.

Ferrous Complex of 2-Mercaptoethylguanidine.—*S*-β-Aminoethylisothiouronium bromide hydrobromide (8.52 Gm., 0.03 mole) was dissolved in 35 ml. of water and the pH was adjusted to 8.0 using dilute ammonium hydroxide. A solution of 4.18 Gm. (0.015 mole) of ferrous sulfate heptahydrate in 15 ml. of water was added dropwise with stirring and a dark green precipitate formed immediately. After the addition was complete, the stirring was continued for 1 hour and the pH of the solution had decreased to a value of 6.6. The green product was isolated and washed with water followed by ethanol. The yield was 1.1 Gm. (17.3%) of a dark green product which started to decompose at 225°.

Anal.—Calcd. for $C_6H_{22}FeO_6S_3$: C, 16.90; H, 5.17; Fe, 13.10; N, 19.71; S, 22.56. Found: C, 17.06; H, 5.16; Fe, 11.99; N, 19.00; S, 22.24.

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Investigation of Egyptian Basil Essential Oils by Simple Chromatographic Method

By L. HOERHAMMER, A. E. HAMIDI*, and G. RICHTER

Two kinds of basil oil are produced in Egypt under the so-called white and red basil oils. The present study reveals the occurrence of terpineol, linalool, cineole, citral, eugenol, esterified terpineol, geraniol, linalool, and citronellol with acetic and formic acids (geranyl and/or citronellyl acetate, linalyl and/or terpinyl acetate and citronellyl formate) a sesquiterpene alcohol (nerolidol?) and unidentified terpenes in both types of oil. The red type contains in addition methyl chavicol and cinnamic acid ester. The white type contains methyl cinnamate and (safrol ?). A simple economic method for the oil investigation is described.

THE GENUS *Ocimum* includes, according to Hegi (1), 50–60 species in Africa, Asia, and America. Guenther (2) and Gildemeister and Hoffmann (3) state that there is a great possibility of cross pollination in the species of *O. basilicum* L., hence the occurrence of a larger number of varieties and physiological forms is possible. They claim that it seems appropriate to classify the basil oil types according to their chemical composition and geographical sources rather than ascribing them to definite plant varieties. They mention main types of *Ocimum* plants according to the chemical compound dominating in the oil.

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Since no previous work has been reported on the analysis of the Egyptian basil volatile oils, this study was undertaken to reveal their constituents. This study is also made to verify, as far as possible with future studies, the appropriate chemotaxonomical order of the Egyptian basil plants. Further aim of the study is to find out a simple economic method for the oil investigation which could be applied easily in agricultural experimental stations occupied with the production of essential oils, where expensive and delicate apparatus cannot always be afforded easily.

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hour after the addition was complete, and the yellow product was isolated and washed with water followed by ethanol. The yield was 2.3 Gm. (58.7%), and the product decomposed above 110°.

Ferrous Complex of 2-Mercaptoethylguanidine.—*S*-β-Aminoethylisothiouronium bromide hydrobromide (8.52 Gm., 0.03 mole) was dissolved in 35 ml. of water and the pH was adjusted to 8.0 using dilute ammonium hydroxide. A solution of 4.18 Gm. (0.015 mole) of ferrous sulfate heptahydrate in 15 ml. of water was added dropwise with stirring and a dark green precipitate formed immediately. After the addition was complete, the stirring was continued for 1 hour and the pH of the solution had decreased to a value of 6.6. The green product was isolated and washed with water followed by ethanol. The yield was 1.1 Gm. (17.3%) of a dark green product which started to decompose at 225°.

Anal.—Calcd. for $C_6H_{22}FeO_6S_3$: C, 16.90; H, 5.17; Fe, 13.10; N, 19.71; S, 22.56. Found: C, 17.06; H, 5.16; Fe, 11.99; N, 19.00; S, 22.24.

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Investigation of Egyptian Basil Essential Oils by Simple Chromatographic Method

By L. HOERHAMMER, A. E. HAMIDI*, and G. RICHTER

Two kinds of basil oil are produced in Egypt under the so-called white and red basil oils. The present study reveals the occurrence of terpineol, linalool, cineole, citral, eugenol, esterified terpineol, geraniol, linalool, and citronellol with acetic and formic acids (geranyl and/or citronellyl acetate, linalyl and/or terpinyl acetate and citronellyl formate) a sesquiterpene alcohol (nerolidol?) and unidentified terpenes in both types of oil. The red type contains in addition methyl chavicol and cinnamic acid ester. The white type contains methyl cinnamate and (safrol ?). A simple economic method for the oil investigation is described.

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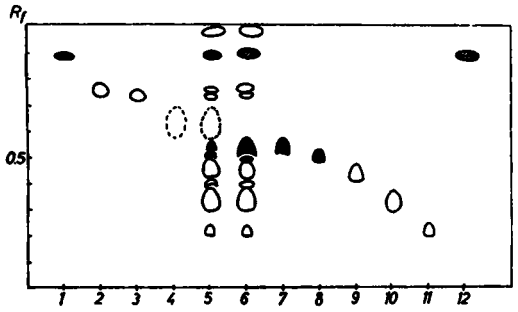


Fig. 1.—Thin-layer chromatogram of Egyptian white and red basil essential oils. Key: 1, safrol; 2, citronellyl formate; 3, geranyl acetate; 4, methyl cinnamate; 5, white basil oil; 6, red basil oil; 7, eugenol; 8, citral; 9, cineole; 10, linalool; 11, terpineol; 12, methyl chavicol.

EXPERIMENTAL

White Basil Oil

The plant from which the oil was obtained was named by the royal botanic gardens, Kew, U.K., as *O. basilicum* L. var. *basilicum*. It is an erect plant with much branching, slightly pubescent, and attaining a height of 80 cm. or more. The racemes are dense with white flowers, hence the name white basil. It grows in the whole Nile valley giving two to three cuts during the year.

Examination of the Oil.—The freshly cut herb (branches and flowering shoots) was steam distilled by the Medi inal and Aromatic Plants Research Division, Ministry of Agriculture, at Barrage north of Cairo. The oil was yellowish having a sweet odor resembling methyl cinnamate. It had the following physicochemical constants: specific gravity (20°), 0.9647; optical rotation, −4.80; refractive index (20°), 1.5078; acid number, 1.4; and ester number, 156.8.

Chromatographic Analysis

An informatory thin-layer chromatogram[1] of the oil (Fig. 1) was prepared to gain a preliminary knowledge of the possible occurrence of the oil constituents, by applying a simple method described by Hoerhammer and co-workers (6). The detection of the spots was carried out by the following reagents after Hoerhammer and co-workers (7): osmium tetraoxide, antimony trichloride, and anisaldehyde. According to the results of this chromatogram, the oil was fractionated on a column using 0.2–0.5 mm. activated silica gel as adsorbant. The column was 1-cm. i.d. and 28 cm. long. A small pledget of cotton was placed in the bottom of the column and the elution was carried out by a mixture of benzene and ethyl acetate (95:5). The fractions 1–31 of 0.5 ml. each and 32–96 of 3 ml. each were col'ected and thin-layer chromatographed (Figs. 2–4). These chromatograms show the distribution of the oil constituents isolated on the column and hav ng R_f values corresponding to authentic test references.

Verification of the Occurrence of the Oil Constituents

Terpineol.—The amount of terpineol in the oil seemed to be small. Therefore a larger column of 3-cm. i.d. and 28 cm. long was used as described above. The oil amount used was 5 ml. The first 61 fractions of 5 ml. each, fraction 62 of 30 ml., and fractions 63–64 of 200 ml. were collected. In the last fraction, which contained mainly linalool, terpineol which had an R_f value on thin-layer chromatogram as that of an authentic terpineol sample, could be detected. Since geraniol has on a thin-layer chromatogram an R_f value like terpineol, to verify the absence of geraniol in the oil, a paper chromatogram was carried out as described by Hoerhammer and co-workers (8). Pure geraniol and the obtained terpineol were tested. There were two different R_f values indicating the absence of geraniol in the oil. The occurrence of terpineol in the oil was also ascertained by obtaining its nitrosochloride derivative (9) in the form of white needle crystals which were identical to crystals obtained by the same manner from pure terpineol. We could not determine the melting point of these few crystals obtained from basil oil.

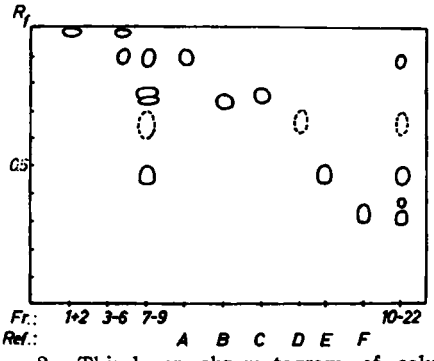


Fig. 2.—Thin-layer chromatogram of column fractionated Egyptian white basil essential oil. Key: Fr., fractions, Ref., test references; A, safrol; B, geranyl acetate; C, citronellyl formate; D, methyl cinnamate; E, cineole; F, linalool.

Linalool.—The fractions 59–66 (Fig. 4) were combined and the solvent evaporated under reduced pressure. An authentic sample of linalool produced the same R_f value as the isolated linalool on the thin-layer chromatogram. The oxidation of the collected fractions with Beckman oxidation reagent (10) at 16° into citral and examining the oxidation product by thin-layer chromatography, resulted in an R_f value and spot color the same as pure linalool treated the same way. The occurrence of linalool was also proved by treating these fractions with 2,4-dinitrophenylhydrazine according to Vogel (11). For comparison, pure linalool was treated the same.

Cineole and Cinnamic Acid Ester.—The fractions 23–29 (Fig. 3) were combined and the solvent evaporated as usual. The oily residue was fractionated on a column as described previously for the oil fractionation. Chloroform was used as eluent. Forty-four fractions of 3 ml. each were collected. The fractions 31–33 on standing for a few hours produced white needle crystals of pleasant odor. These crystals had on thin-layer chromatogram an R_f value and melting point (36.5°) identical to an authentic sample of methyl cinnamate. The oily residue was treated with 2 *N* NaOH for 1 hr. A eucalyptus odor was noticed indicating the presence of cineole. Its occurrence was also proved by means of paper chromatography against pure cineole (7).

The aqueous saponifiable solution was rendered

[1] All thin-layer chromatograms were eluted by a mixture of benzene and ethyl acetate (95:5 v/v) unless otherwise stated, and silica gel G Merck was used as adsorbant.

acidic by means of sulfuric acid. A white precipitate was formed which, after recrystallization, had the odor and melting point (135°) of pure cinnamic acid (see under *The Esters*).

Citral.—This compound was detected by thin-layer chromatography against pure citral. The reproduction of the spots was achieved by means of 2,4-dinitrophenylhydrazine (concentrated solution in 2 *N* HCl). Its occurrence was also proved as follows: 0.5 ml. of the oil was treated with 2,4-dinitrophenylhydrazine to obtain the citral hydrazones, whose crystallization was not possible. The solution was then shaken with petroleum ether (b.p. 30–68°). The petroleum ether extract was dried over anhydrous sodium sulfate and thin-layer chromatographed against the hydrazones of authentic citral. A mixture of benzine (b.p. 50–70°) and ethyl acetate (95:5 v/v) was used for elution. The R_f values and spots color were identical. This system was found applicable to separate the two types of citral hydrazones.

The Esters and Their Corresponding Acids and Alcohols.—The fractions 7–9 (Fig. 2) were combined, reduced to a small volume, and column-fractionated as outlined above. For elution, a mixture of benzene and *n*-heptane (19:1 v/v) was used. Fifty-four fractions of 1 ml. each were collected and thin-layer chromatographed. There were at least three spots indicating the presence of three ester types and a fourth spot with a higher R_f value (Fig. 2). The presence of the corresponding acids was verified through their hydroxamic acids by treating 0.5 ml. of the oil with hydroxylamine as described by Hais (12). Authentic samples of formic, acetic, and cinnamic acids were treated in the same way. These hydroxamic acids were paper chromatographed applying a system described by Hais (13) and using filter paper S.S. 2043b,gl., washed. For the reproduction of the spots, an

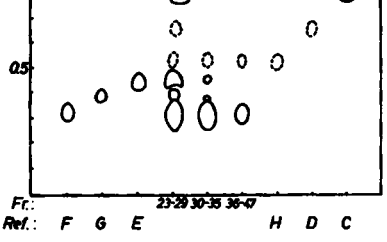


Fig. 3.—Thin-layer chromatogram of column fractionated Egyptian white basil essential oil. Key: Fr., fractions; Ref., test references; F, linalool; G, geraniol; E, cineole; H, citral; D, methyl cinnamate; C, citronellyl formate.

ethanolic solution of 0.1% ferric chloride was used. The spots were identical both in R_f values and color indicating the presence of the three acids respectively.

A portion of these concentrated fractions was thin-layer chromatographed against the available authentic samples of geranyl, citronellyl, terpinyl, and linalyl acetates, citronellyl formate, and methyl cinnamate. Miller and Kirchner (14) described several systems for the separation of terpene alcohols and esters on silicic acid glass plates. Our trials with most of these systems using silica gel were unsuccessful. Therefore a mixture of *n*-heptane, *n*-hexane, and diisobutylketone (1:1:5) was used for elution. The spots were developed by spraying with antimony trichloride. There were (*a*) a lower spot corresponding to methyl cinnamate, (*b*) above it, another spot corresponding to geranyl and/or citronellyl acetate, (*c*) a third higher spot corresponding to linaryl and/or terpinyl acetate, and (*d*) a fourth farther higher spot corresponding to citronellyl formate. It was not possible by this system to differentiate between geranyl and citronellyl acetate on one hand, and linalyl and terpinyl acetate on the other hand. The verification of the corresponding alcohols was carried out as follows. The ester fractions were saponified as usual. The liberated alcohols were paper chromatographed (8) against authentic samples of geraniol, terpineol, citronellol, and linalool. The obtained R_f values were identical, respectively. It is noteworthy to state that the reproduction of a methyl cinnamate spot can also be achieved by spraying the plates with 2,4-dinitrophenylhydrazine, where a stable yellow color is produced. Methyl chavicol could not be detected in these fractions.

Eugenol.—The isolation of this compound on the column was unsuccessful. It was obtained by shaking 0.5 ml. of the oil with 3% aqueous solution of sodium hydroxide, extracting the phenolic substances with ether, drying the ether extract over anhydrous sodium sulfate and thin-layer chromatographed against authentic eugenol. The spots reproduced by anisaldehyde reagent were identical in their R_f values and color.

Safrol.—In the ester fractions 7–9 (Fig. 2) a fourth compound with higher R_f value could be observed; presumably a phenol ether. It had the same R_f value on the thin-layer chromatogram and spot color when sprayed with anisaldehyde and exposed to osmium tetraoxide as an authentic sample of safrol.

Sesquiterpene Alcohol (Nerolidol?).—The fractions 23–29 (Fig. 3) contained a compound located on the thin-layer chromatogram between linalool and cineole. It had an R_f value as an authentic sample of nerolidol. When exposed to osmium tetraoxide it showed an unsaturated property presumably a sesquiterpene alcohol.

Terpenes.—The fractions 1–6 (Fig. 2) contained the terpenes. These were not investigated further because of lack of test references.

Red Basil Oil

The plant from which the oil was produced was named by the royal botanic gardens, Kew, U.K., as *O. basilicum* L. var. *purpurascens* Benth The morphological feature of the plant does not differ greatly from the white type except that the flowers are violet to red, hence the name red basil.

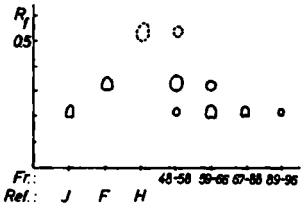


Fig. 4.—Thin-layer chromatogram of column fractionated Egyptian white basil essential oil. Key: Fr., fractions; Ref., test references; J, terpineol; F, linalool; H, citral.

Examination of the Oil.—The red basil oil which was produced by the same method as the white basil oil, had a strong spicy odor and its color was reddish. It had the following physicochemical constants: specific gravity (20°), 0.9111; optical rotation, −10.75°; refractive index (20°), 1.4830; acid number, 6.5; and ester number, 14.5.

Chromatographic Analysis

The oil was investigated the same way as the white basil oil. It had the same chemical composition (Fig. 1) except for the following:

Methyl Chavicol.—This could be detected by thin-layer chromatography against authentic methyl chavicol.

Cinnamic Acid Ester.—Cinnamic acid could be detected by its hydroxamic acid as described in the white basil oil investigation. The isolation of the ester on the column or on the chromatoplates was unsuccessful.

Safrol.—Could not be detected.

Eugenol.—Could be easily isolated on the column.

CONCLUSION AND DISCUSSION

The investigation of the two Egyptian basil oils undertaken in this study, revealed the occurrence of terpineol, linalool, cineole, eugenol, esterified geraniol, citronellol, linalool, and terpineol with acetic and formic acids (geranyl and/or citronellyl acetate, linalyl and/or terpinyl acetate, citronellyl formate), and a sesquiterpene alcohol (nerolidol?) in both types of oils. In addition, the white type contained methyl cinnamate and (safrol?). The red type contained methyl chavicol and traces of cinnamic acid ester. The presence of terpineol, citral, and safrol has not been reported before in the *O. basilicum* oils. Therefore the basil oil types outlined by Guenther (2) and Gildemeister and Hoffmann (3) cannot be applied in arranging these two oil types in any of the reported types. This viewpoint is supported by the occurrence of citral in other *Ocimum* species, *e.g.*, *O. canum* Sims (2, 3), *O. gratissimum* L., and *O. menthaefolium* Hochst. (3). The occurrence of constituents in the Egyptian basil oils, which have not been reported previously, and their differences from basil oil samples of European origin analyzed at the same time, lead to the possibility of arranging these two Egyptian basils in separate chemical or physiological races [Dillemann (15), Rowson (16), Huerlimann (17)], the category to include those plants indigenous to and/or cultivated in Egypt. Further study of the oils of these plants in different periods of plant growth is recommended to ascertain the occurrence and percentage of their chemical constituents which may change according to climatic, edaphic, and genetic factors. These qualitative and quantitative studies are being carried out.

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Synthesis and Antitubercular Activity of Isonicotinoyl and Cyanoacetyl Hydrazones

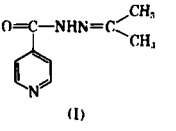
By DIPTISH CHAKRAVARTY, ARUN BOSE, and SAMIR BOSE

Seven isonicotinoyl hydrazones and four cyanoacetyl hydrazones were synthesized. Their antitubercular activities *in vitro* and *in vivo* were evaluated.

THE ANTIMICROBIAL activity of isoniazid against tubercle bacilli depends on the amount of free and unaltered isoniazid (1). The observations of Fox and Gibas (2) show that one or both of the hydrogen atoms attached to nitrogen in the hydrazine moiety of isoniazid may be replaced by a variety of groups with little loss of activity.

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Fox and Gibas (3) have shown that isonicotiny hydrazine when reacted with acetone gives 1-isonicotinyl-2-iso-propylidene hydrazine (I)



(I)

which proved to be very active against tubercle bacilli. These authors (3) have investigated systematically alkylidene derivatives of isonicotinyl hydrazone with the twofold view of dis-

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Synthesis and Antitubercular Activity of Isonicotinoyl and Cyanoacetyl Hydrazones

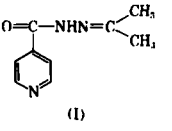
By DIPTISH CHAKRAVARTY, ARUN BOSE, and SAMIR BOSE

Seven isonicotinoyl hydrazones and four cyanoacetyl hydrazones were synthesized. Their antitubercular activities *in vitro* and *in vivo* were evaluated.

THE ANTIMICROBIAL activity of isoniazid against tubercle bacilli depends on the amount of free and unaltered isoniazid (1). The observations of Fox and Gibas (2) show that one or both of the hydrogen atoms attached to nitrogen in the hydrazine moiety of isoniazid may be replaced by a variety of groups with little loss of activity.

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Fox and Gibas (3) have shown that isonicotiny hydrazine when reacted with acetone gives 1-isonicotinyl-2-iso-propylidene hydrazine (I)



(I)

which proved to be very active against tubercle bacilli. These authors (3) have investigated systematically alkylidene derivatives of isonicotinyl hydrazone with the twofold view of dis-

TABLE I.—ISONICOTINOYL HYDRAZONES

Compd.	R	Crystallizing Solvent	Appearance	M.p., ° C.	Yield, %	Mol. Formula	N, % Calcd.	N, % Found
AT–1	(*o*)-OHC_6H_4CH	Ethanol	Colorless needles	243–244	83	$C_{13}H_{11}N_3O_2$	17.43	17.41
AT–2	(*m*)-OHC_6H_4CH	Methanol	Cream-colored rods	255–257	86	$C_{13}H_{11}N_3O_2$	17.43	17.40
AT–3	(*p*)-OHC_6H_4CH	Ethanol	Light cream-colored prisms	275–277	85	$C_{13}H_{11}N_3O_2$	17.43	17.41
AT–4	(*p*)-$(CH_3)_2NC_6H_4CH$	Dil. ethanol	Golden yellow plates	201–202	93	$C_{15}H_{16}N_4O$	20.88	20.85
AT–5	$C_6H_5—C(CH_3)$	Dil. ethanol	Colorless rods	172–173	60	$C_{11}H_{14}N_2O$	16.67	16.65
AT–6	(*p* and *m*) $(OCH_3)_2C_6H_3CH$	Dil. ethanol	Colorless prisms	188–189	70	$C_{18}H_{18}N_2O_3$	14.74	14.70
AT–7	$C_6H_5CH{:}CH{\cdot}CH$	Ethanol	Cream-colored needles	200–202	80	$C_{15}H_{12}N_2O$	16.73	16.74

TABLE II.—CYANOACETYL HYDRAZONES: $CNCH_2CONHN = R$

Compd.	R	Crystallizing Solvent	Appearance	M.p., ° C.	Yield, %	Mol. Formula	N, % Calcd.	N, % Found
AT–8	C_6H_5CH	Ethanol	Colorless rods	178–180	64	$C_{10}H_9N_3O$	22.46	22.45
AT–9	(*o*)-OHC_6H_4CH	Ethanol	Light yellow needles	166–168	69	$C_{10}H_9N_3O_2$	19.54	19.50
AT–10	(*p*)-OHC_6H_4CH	Dil. ethanol	Cream-colored plates	210–212	74	$C_{10}H_9N_3O_2$	19.54	19.55
AT–11	(*p*)-OH, (*m*)-$OCH_3C_6H_3CH$	Isopropanol	Light yellow plates	202–204	69	$C_{11}H_{11}N_3O_3$	18.03	18.04

covering superior tuberculostat. Since the isopropylidene group is a branched chain substitution, the study was extended to the straight chain alkylidenes; the 1-isonicotinyl-2-alkylidene hydrazine type, whether straight or branched chains, were actively tuberculostatic *in vivo*.

On the fragile basis of the similarity of interatomic distances between the nitrogen atoms and hydrazine moiety of cyanoacetic acid hydrazide and isoniazid, Valdecasas (4) and his co-workers conceived the idea of the probable tuberculostatic activity of cyanoacetic acid hydrazide and reported that it was indeed a remarkable tuberculostat in experimental and clinical tuberculosis comparable to isoniazid.

Shchukina, *et al.* (5), reported the synthesis of a number of isonicotinyl hydrazones from aldehydes and ketones as antitubercular agents. Their biological tests indicated that some of these isonicotinyl hydrazones are more active *in vivo* and far less toxic in animals (mice). These authors lay great emphasis on the high toxicity of isonicotinic acid hydrazide believing that the new drug does not possess a large margin of safety.

The recent results of biological tests indicate that if too much emphasis is placed on the value of isonicotinic acid hydrazide alone as a chemotherapeutic agent for the treatment of tuberculosis overlooking the possibilities of its derivatives, there is a great danger that some most interesting and valuable compounds might be missed.

This paper reports the synthesis and chemotherapeutic evaluation of antitubercular activity of (*a*) different isonicotinyl hydrazones and (*b*) cyanoacetyl hydrazones.

EXPERIMENTAL

General Procedure for the Preparation of Isonicotinoyl Hydrazones.—Isonicotinic acid hydrazide (0.05 mole) was dissolved in a minimum quantity of distilled water. To this solution, the aldehyde or ketone (0.05 mole), either as such (compound AT–1) or dissolved in a minimum quantity of ethanol (compounds AT–2, AT–3, AT–4, AT–5, AT–6, and AT–7) was added with stirring. In some cases the crystals separated within 5 minutes or after about 24 hours. However, in all cases, the crystals were separated after 24 hours by filtration. The solids were crystallized from suitable solvents and dried *in vacuo* over calcium chloride. The characteristics of the compounds synthesized following the above procedure are recorded in Table I.

General Procedure for the Preparation of Cyanoacetyl Hydrazones.—Cyanoacetic acid hydrazide (0.05 mole) was dissolved in water (25 ml.). To this solution under stirring was added the aldehyde (0.05 mole) either as such (compounds AT–8 and AT–9) or dissolved in a minimum quantity of

TABLE III.—RESULTS OF *In Vitro* TEST OF ALL TEST COMPOUNDS ON H_{37} Rv STRAIN[a]

Compd.	Response[b]
AT-1	++
AT-2	±
AT-3	++
AT-4	++
AT-5	+
AT-6	+
AT-7	±
AT-8	±
AT-9	±
AT-10	●
AT-11	±
INH	++
Control[c]	−

[a] Dose (mcg./ml.) was 0.1 and 0.2. [b] ++ = Complete inhibition of growth; + = partial inhibition of growth; ± = no inhibition of growth; − = full growth. [c] No drug.

ethanol (compounds AT-10 and AT-11). The crystals, which were filtered, separated within 24 hours at room temperature. They were crystallized from suitable solvents and dried *in vacuo* over calcium chloride. The characteristics of the compounds synthesized following the above method are shown in Table II.

Cyanoacetic Acid Hydrazide.—Hydrazine hydrate (25 ml., 60% w/v) was added dropwise to the stirred and cooled (below 5°) ethyl cyanoacetate (22 Gm.) and stirred for 3 hours. The precipitated colorless microcrystalline solid was filtered and dried *in vacuo* over calcium chloride. Yield 15 Gm.; m.p. 110–112°. The product develops color if the reaction is carried out at a higher temperature than 5°.

Anal.—Calcd. for $C_3H_5N_3O$: N, 42.42. Found: N, 42.40.

PHARMACOLOGICAL EVALUATION

Both *in vitro* and *in vivo* tests were done according to the method worked out by Sah and Peoples (6). *Mycobacterium tuberculosis* strain $H_{37}Rv$ maintained on Lowensten Jensen media and albino rats of our own breed were used.

In Vitro Test.—Compounds were incorporated with Lowensten Jensen medium in series and were kept in an inspissator at 80° for 1 hour for 3 consecutive days. Thereafter, they were inoculated with 0.1 ml. of 10-day-old culture of *M. tuberculosis* grown in Dubos medium, incubated at 37° for 3 weeks. Control tubes containing only strain culture were maintained for comparison.

Table III indicates the dose response of different

TABLE IV.—ACUTE TOXICITY OF TEST COMPOUNDS ON ALBINO RATS[a]

Compd.	Mortality
AT-1	6/6
AT-2	2/6
AT-3	0/6
AT-4	0/6
AT-5	0/6
AT-6	6/6
AT-7	2/6
AT-8	2/6
AT-9	1/6
AT-10	0/6
AT-11	2/6

[a] Six rats with an average body weight of 100 Gm. each; maximum oral dose, 1 Gm./Kg.

TABLE V.—*In Vivo* TEST ON ALBINO RATS WITH VARIED DOSE[a] AND CHANGES IN INTERNAL ORGANS

Compd.	Animals, No.[c]	Days on Test (with each dose)	Mortality, %: (a)[a]	(b)[a]	Liver[b]: (a) 0.2%	(b) 0.5%	Spleen[b]: (a) 0.2%	(b) 0.5%	Lungs[b]: (a) 0.2%	(b) 0.5%
AT-1	4 and 3	40	25	33	+	+	++++	+	+	+
AT-2	2 and 2	28	0	0	+	+	+	+	+	+
AT-3	4 and 3	40	25	33	+	+	+	+	+	+
AT-4	5 and 4	40	0	0	+	+	+	+	+	+
AT-5	4 and 3	40	0	0	+	+	+	+	+	+
AT-6	2 and 3	28	50	0	+	+	+++	+++	+++	+++
AT-7	2 and 2	28	0	0	+++	+++	+	+	+	+
AT-8	2 and 2	45	50	0	+	+	+	+	+	+
AT-9	2 and 2	45	50	50	+	+	+	+	+	+
AT-10	2 and 2	28	0	0	+	+	+	+	+	+
AT-11	2 and 2	45	0	0	+	+	+	+	+	+
INH	18 (9 groups of 2)	28	0	0	+	+	+	+	+	+
Control, no drug	35	45	95		++	++	++	++	++	++

[a] Dosage of drug diet: (a) = 0.2% daily intake of the drug by the animal weighing 100 Gm. each was estimated to be 150 mg./Kg.; (b) = 0.5% daily intake of the drug by the animal weighing 100 Gm. each was estimated to be 350 mg./Kg. [b] + = Presence of infection with enlargement of gland; − = absence of infection and no enlargement of gland. [c] Per cent drug dosage in diet was 0.2 and 0.5, respectively.

experimental samples on inhibition of *Mycobacterium* after 3 weeks.

In Vivo Test.—This test includes chronic toxicity study on albino rats of our own breed which were infected with *M. tuberculi* and subsequently took a regularly known amount of drug incorporated with their daily food intake. Both low and high doses of individual compound were administered, and the maximum period of observation was 45 days. Within this period, the therapeutic efficacy of the test compounds was studied outwardly by observing the symptoms, particularly the degree of sickness and also the degree of protection of the test animals from infection.

For investigating the effect of prolonged administration of compounds and also for examining the condition of internal organs (7–15)—particularly liver, spleen, and lungs—the duration of oral medication of certain compounds was limited to 28 days, while for others it continued to a maximum period of 45 days. During this particular period, some experimental animals died or were ultimately sacrificed for examination of internal organs. Percentage of mortality was observed to a maximum period of 45 days.

A thorough examination of internal organs of all the test animals receiving different compounds at different dose levels and time periods was conducted.

The percentage of mortality was different with individual test compounds. The therapeutic efficacy was judged by examination of the above-mentioned glands. With some compounds, the degrees of protection from infection were marked—revealed by absence of infection and nonenlargement of glands compared to other test compounds which were relatively less efficacious.

Control animals were infected with *M. tuberculi* only and no drug. They showed the maximum mortality; the glands were highly enlarged compared with all other groups receiving different test compounds. This suggests that the test compounds had definite action in protecting the infected animals from death within a certain period and also in preventing the enlargement of glands to a certain extent.

Table IV indicates the acute toxicity of test compounds, and Table V records the *in vivo* test with varied doses and changes in internal organs.

DISCUSSION

Out of seven compounds of the isonicotinoyl hydrazone series and four compounds of cyanoacetyl hydrazone series, two compounds of each series, showed some antitubercular activity. The compound AT–4 having *p*-dimethylamino group and compound AT–3 having *p*-hydroxy group as substitutions in the benzylidene ring attached to the N^2 atoms of INH molecule showed antitubercular activity comparable to that of INH. The therapeutic efficacy comparable almost to INH was observed in the compound AT–10 with *p*-hydroxy group and in the compound AT–11 having *p*-hydroxy-*m*-methoxy group in the benzylidene ring attached to the N^2 atoms of cyanoacetic acid hydrazide molecule. It suggests, therefore, that the *p*-hydroxy group in the benzylidene ring might have some antitubercular property.

SUMMARY

A series of 11 compounds was synthesized and studied for their antitubercular activity *in vivo* and *in vitro*. Therapeutic activity on sick animals and their subjective improvements and the damages of major internal organs were studied. Acute and chronic toxicities were also noted.

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Controlled Crystallization of Hydrocortisone by Ultrasonic Irradiation

By ROBERT M. COHN and DONALD M. SKAUEN*

A procedure has been developed to prepare low micron particles of hydrocortisone by direct crystallization of suitably prepared supersaturated solutions in an ultrasonic field. The solvent system employed for the preparation of the saturated solutions was observed to be a factor in controlling particle size distribution, while the effects of tenure of ultrasonic treatment and the extent of cooling the saturated solution were not as significant. A concurrent study was undertaken to determine the variables concerned with affecting crystal yields. The degree of crystallization was increased with ultrasonic treatment and with lower coolant bath temperatures. A method was developed to evaluate the effect of decreased cavitation on particle size distribution.

As a result of a series of investigations by several authors (1–16) it has been established that ultrasonic treatment increases the rate and extent of nucleation occurring from supersaturated solutions. Furthermore, it has been shown that increased nucleation results in the formation of smaller crystals.

The primary objective of this investigation was to determine the factors involved in the preparation of low micron particles of hydrocortisone[1] by direct crystallization of suitably prepared supersaturated solutions in an ultrasonic field.

EXPERIMENTAL

Since part of this study was an evaluation of the effects of solvents on crystallization induced by ultrasonics, one of the first problems was to select several solvents with satisfactory, yet slightly different solubility characteristics. The solubility of hydrocortisone in a series of solvents over a wide temperature range was studied. Isopropyl alcohol, ethyl alcohol, and a 50% blend of isopropyl alcohol and ethyl alcohol possessed all of the desired characteristics for this study and were selected as solvents to be used throughout. Representative solubility curves are shown in Fig. 1.

For the preparation of the saturated solutions of hydrocortisone in the blended solvents a standard taper three-holed flask was used. This was fitted with a water-cooled reflux condenser, a stirrer, and removable glass stopper to allow the withdrawal of samples. This procedure insured maintenance of the specific concentration of the mixed solvents in the blend.

Ultrasonic treatment of various saturated solutions of hydrocortisone in the three solvents described above was accomplished in the following manner with a schematic representation shown in Fig. 2. The Sonifier[2] was activated 10 minutes prior to beginning a run to allow sufficient warm up of its electrical and mechanical systems. The treatment vessel, a 20-ml. beaker, was placed into the coolant bath approximately 30 seconds prior to the addition of the saturated solution. The tip of the Sonifier was positioned relative to the base of the treatment vessel with the aid of a 2.3-mm. deep template. Ten milliliters of saturated solution were poured into the treatment vessel while, simultaneously, the stopwatch and Sonifier were activated. Samples were treated for 1, 2, 4, or 16-minute periods of time. Following treatment, portions of the samples were withdrawn for particle size and weight distribution analyses.

In all the test runs that were performed to evaluate the selected variables, three coolant temperatures were employed. These three temperatures, 25, 0, and −65° were chosen to allow analysis of the effect of reducing the temperature of the saturated solutions over an extremely wide range.

The effect of solute concentration on particle size distribution was studied to determine the relative

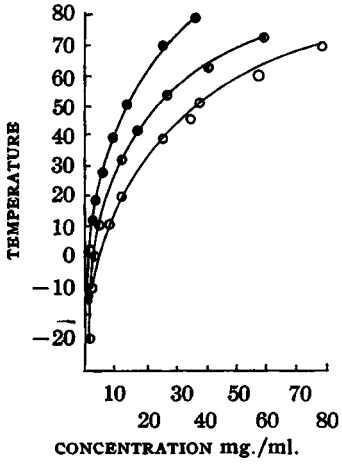


Fig. 1.—Solubility curves of hydrocortisone in the selected solvents. Key: ●, isopropyl alcohol; ○, ethyl alcohol; ◐, blend.

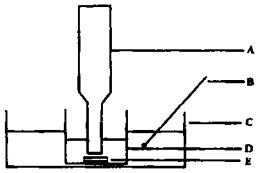


Fig. 2.—Diagram of the insonation system. Key: A, Sonifer; B, thermometer; C, coolant bath; D, treatment vessel; E, template.

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[1] We are indebted to Charles Pfizer & Co., Brooklyn, N. Y., and Merck & Co., Rahway, N. J., for a generous supply of hydrocortisone.
[2] Branson Instruments Inc., Stamford, Conn.

importance of this factor in sonic-induced crystallization. Two saturated solutions at different temperatures were prepared (hence different amounts of solute per milliliter of solution), crystallized, and evaluated.

One of the most important phases of this work dealt with determining the effect that ultrasonics has upon the crystallization procedure. Supersaturated solutions are extremely metastable and, thus, are very sensitive to mechanical shock or vibrations.

Vigorous sonic agitation is one of the most prominent manifestations of ultrasonic treatment of liquids. This is caused by the rarefaction and compression of ultrasonic waves passing into the solution. Consequently, it can be assumed that improvements in the crystallization procedures were not caused by cavitation, which is the primary effector in ultrasonic systems, but simply by the vibrational effects (stirring) produced by ultrasonics.

It was thought necessary to establish a suitable modification in the original system to reduce or, if possible, eliminate cavitation. Then, a comparison of the size frequency and weight distributions of the crystals prepared in this manner with those prepared by the normal procedures would serve to define the effect that cavitation produces in these systems. That is, if particle size and weight distribution data obtained in these modified systems were substantially inferior to the data obtained in the basic systems, this would indicate that it is sonics (cavitation) and not simple stirring that is the primary effector.

Investigators (17–19) have reported that cavitation can be substantially decreased by treating samples under pressures which have been reduced to less than 100 mm. Hg. They have observed that the ability of an ultrasonic field to induce emulsion formation, increase the rate of sonochemical reaction, destroy microorganisms, and denature proteins is suppressed by subjecting the systems to either high or low pressures.

This phenomenon can be explained in the following manner. Liquids when exposed to ultrasonics are slowly degassed; however, if this treatment is initiated while the system is being exposed to reduced pressure, degassing occurs quickly and almost completely.

Cavitation is initiated by the expansion and contraction of gas-filled nuclei within a liquid (20); therefore, since the concentration of these nuclei is significantly reduced in degassed liquids, the commencement of the catastrophic phase of cavitation is substantially hindered.

In addition, it has been observed that the catastrophic phase of cavitation occurs when the total pressures within the cavities exceed the cohesive forces of the liquid and the ambient pressure above the liquid. In reduced-pressure systems, the cavities collapse, but, because the ambient pressures have been reduced, much less energy is imparted to the system, and thus characteristic effects of sonics do not occur.

Samples of saturated solutions in isopropanol at 70° were evaluated in the usual manner regarding treatment times and coolant bath temperatures. The samples were evaluated at 114–120 mm. Hg and at 15–20 mm. Hg. Thus, data were obtained at these pressure levels for the evaluation of the effect of reduced pressure on sonic efficacy.

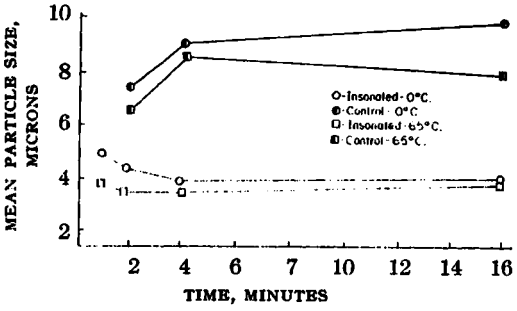


Fig. 3.—Comparison of particle size data of insonated and uninsonated samples of hydrocortisone in a 70° saturated solution in isopropyl alcohol at 0 and −65°.

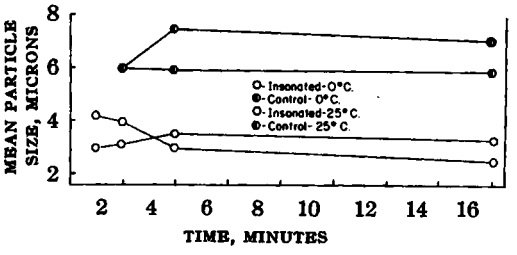


Fig. 4.—Comparison of particle size data of insonated and uninsonated samples of hydrocortisone in a 73° saturated solution in ethyl alcohol at 0 and 25°.

The Sonifier model S-75, was the ultrasonic instrument utilized in this study. This instrument is of the low-frequency (20 kc. per second) piezoelectric type and is capable of yielding high-intensity power outputs. Throughout the entire investigation, maximum power was employed because it was noted that at peak power levels the operating efficiency of the instrument was at a maximum.

For the collection of crystalline cake used in particle size and weight distribution analysis a Millipore[3] filter apparatus containing Whatman No. 42 filter paper was used. Volumetric portions of the treated samples were poured into the funnel, vacuum was applied, and the dried cake was obtained. However, filtration rates were dependent upon the particle size of the crystals formed. To prevent any change in the particle size distribution, a maximum filtration time was established. This maximum time, during which no significant particle size changes occurred, was approximately 6–8 minutes. To obtain a significant safety factor, a 2-minute filtration limit was established. Thus, depending on the estimated particle size of crystals, various sized volumetric portions (1–5 ml.) of the treated samples were placed into the Millipore funnel. The cakes of hydrocortisone after separation of solvent were weighed on an analytical balance.

The term "mean particle size" which is used throughout the paper, refers to an arithmetic mean obtained by measuring the longest diameter of the observed crystals in a manner similar to that described by Singiser (21). The following is a short synopsis of the procedures employed by this earlier worker. Photographs were taken through the barrel of a microscope fitted with a camera adapter.

[3] Millipore Filter Corp., Bedford, Mass.

A 35-mm. black and white film was utilized for recording the observations. The film was developed and projected onto a cardboard grid so prepared that the dimensions of each square of the grid were exactly the same. The projector was then placed at a specific distance from the cardboard grid. This distance was selected so that the calibrated index, when projected onto the board, aligned itself with the grid in such a manner as to be exactly superimposed upon it. This calibrated index was prepared in such a fashion that the distance between each line would represent a length of 5 μ. Thus, since this index was superimposed on the grid, each grid represented a square of 5 μ.

A single exposure was taken of each slide prepared in the following manner. A small sample of the crystalline cake was taken and placed onto the slide. The crystals were distributed uniformly with the aid of a few drops of a 2% methylcellulose 400 solution. The slide was then examined, and a picture was taken of what appeared to be a representative sample of the crystal population on that slide. Following this, a second slide of the same material was prepared, examined, and photographed in the exact manner. Thus, for each sample two different exposures were obtained. This method was employed to reduce the hazards in measuring only a small per cent of the total population.

Approximately 150 particles were counted from each exposure to give a total of 300 measured particles per sample. With this many particles and with the usual standard deviations (2–4 μ) and the usual means (2–7 μ), a 95% confidence level could be obtained between means of 0.5 μ. For example, 95% of the time observed, means of 4.5 and 5.0 μ will be significantly different.

DISCUSSION

The evaluation of the factors involved in the crystallization of hydrocortisone in an ultrasonic field established a number of extremely important

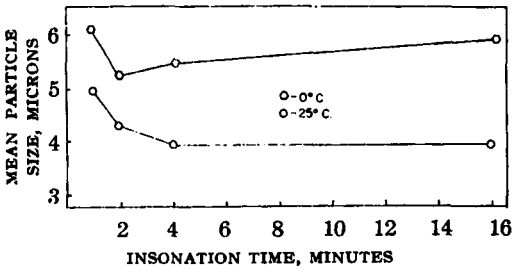


Fig. 5.—Relationship of particle size data to insonation times of samples of hydrocortisone in a 70° saturated solution in isopropyl alcohol at 0 and 25°.

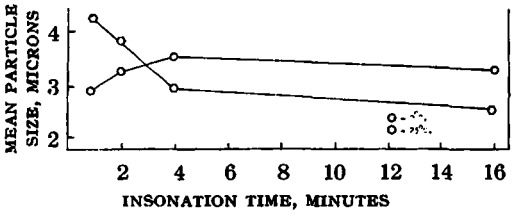


Fig. 6.—Relationship of particle size data to insonation times at 0 and 25° of samples of hydrocortisone in a 73° saturated solution in ethyl alcohol.

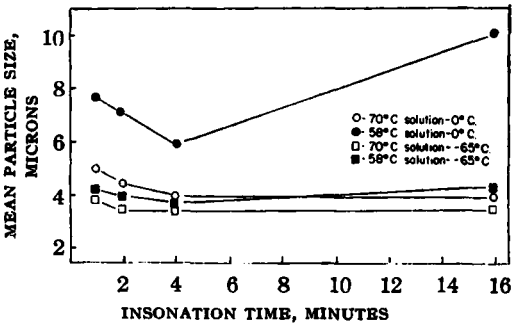


Fig. 7.—Relationship of particle size data to coolant bath temperatures of 0 and −65° for insonated samples of hydrocortisone in 70 and 58° saturated solutions in isopropyl alcohol.

facts. The results indicated that ultrasonic treatment played an extremely important part in the crystallization of hydrocortisone. It was observed that ultrasonics affected not only the particle size distributions, but also the amount of hydrocortisone which was induced to crystallize.

The discussion of the effects of ultrasonic treatment on the crystallization of hydrocortisone has been divided into three broad areas. Particle size effects are discussed first, followed by a presentation of the weight distribution analyses and, finally, a discussion of the effects of reduced pressure on sonic efficacy.

Before entering into a discussion of the specific effects of ultrasonics on the crystallization process, it was necessary to substantiate the effects of ultrasonic treatment by comparing data of treated and untreated samples.

Throughout this experiment, controls were run under the exact conditions as the treated samples. Data were thus obtained which could be used to evaluate the effectiveness of ultrasonic treatment during crystallization. The data indicate two significant facts; first, that under almost all treatment conditions, the rate of the crystallization of the controls was substantially lower than the treated samples. Occasionally, in the controls in which crystals did form, the amount of crystallate approximated that of the treated samples. In the 72 controls that were processed, 23 exhibited some crystallization and, of these 23 samples, only 10 yielded approximately the same amount of crystallization as treated samples. Thus, it is reasonably safe to say that sonics does play a significant part in enhancing the rate of crystallization.

The particle size distributions of the controls were usually larger by a factor of 2 or 3. Figures 3 and 4 illustrate these particle size effects. Hence, with regard to the two most significant factors that were investigated in this problem, reduction in particle size distribution and increased yield of crystals, ultrasonics definitely played a significant role.

Particle size effects elicited by ultrasonic treatment of saturated solutions of hydrocortisone can themselves be subdivided. Since this problem has been investigated from many different facets, the results obtained are more illustrative by analyzing each section separately.

The effects which the length of ultrasonic treatment had on the particle size distributions of the crystallates were almost negligible. Particle size

data, obtained throughout the study, indicated that there were very few cases of actual modifications in the particle size distribution of samples which had been treated for varying periods of time. A perusal of Figs. 5 and 6 clearly indicates this occurrence.

This effect can probably be explained by consideration of the treated system. In the treatment vessel there is, in addition to the crystals, a supersaturated solution containing excess molecules of solute. Normally, if the system is not disturbed, these crystals grow until they reach a finite size. This can be observed in the control experiments. However, because sonics itself has the ability to cause attrition of crystals, the growth effect is hampered. In addition, since sonics has been shown to increase the extent of nucleation, the degree of supersaturation of solution is markedly reduced and, thus, there is less of a tendency for the growth of crystals to occur.

The effect of the different coolant temperatures on particle size distribution can be observed in Figs. 7 and 8. There is very little difference in the particle size distributions obtained at the different temperatures. Except for a few samples prepared with isopropanol, particle size distributions were relatively unaffected by the differential in temperature of the saturated solutions.

These data serve to enhance further the importance of ultrasonic treatment of saturated solutions in invoking fine particle crystallizations. Usually, as a supersaturated solution is cooled, the crystals become smaller. Hence, when the −65° coolant bath was employed, smaller crystals should have been formed. Since this was not observed, this indicated that sonics was the primary influence in the preparation of these low micron crystals and not the extent of cooling.

Three solvent systems were employed in this study to determine what effect, if any, the solvent might have on the formation of low micron crystals.

A comparison of Figs. 5, 6, and 9 graphically illustrates this effect. It can be seen readily from an analysis of these data that a relationship exists between the three solvent systems. The lowest particle size distributions are obtained when ethyl alcohol is used as a solvent. Crystals obtained from isopropyl alcohol are generally the largest, while the blend yields crystals intermediate in size between the two pure solvents.

This relationship can be explained on the basis of solubility data. Hydrocortisone is most soluble in ethyl alcohol, least soluble in isopropyl alcohol, and intermediately soluble in the blend. This is the exact relationship of the particle size data of the solvents. Hence, the particle size distribution of

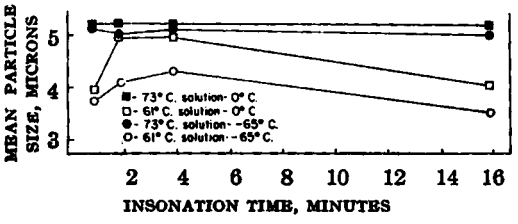


Fig. 8.—Relationship of particle size data to coolant bath temperatures of 0 and −65° for insonated samples of hydrocortisone in 73 and 61° saturated solutions in the blend.

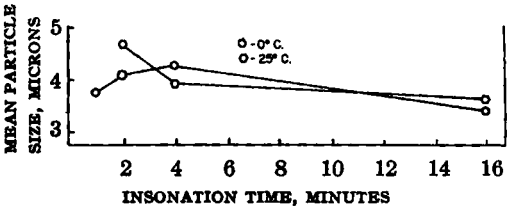


Fig. 9.—Effect of the solvent system on the particle size distributions of insonated samples of hydrocortisone in a 73° saturated solution in the blend.

hydrocortisone can be described as being a function of the extent of its solubility in each of the solvents.

This effect is further substantiated by an analysis of Figs. 7 and 8, which graphically illustrate particle size data as a function of the temperature at which the saturated solutions were prepared. It can be observed that smaller crystals occurred when the higher temperature saturated solution was treated.

This relationship can be explained best on the basis of known solubility and supersolubility theories. Since hydrocortisone is most soluble in ethyl alcohol, it is obvious that when these saturated solutions are cooled to approximately the same degree as the other solvents, they will exist in a higher unstable state. Thus, saturated solutions prepared from ethyl alcohol would be most prone to nucleate and to form smaller crystals.

There appears to be a disagreement in the data derived from this series of experiments when compared to the data obtained from the effects of different coolant temperatures on particle size distributions. As previously reported, the different coolant temperatures did not seem to affect the particle size data significantly; but a careful analysis of the problem indicates that this is probably not normally the case.

This occurrence can possibly be explained in the following manner. If one considers that a saturated solution is prepared with X molecules of solute, then there are X number of molecules which, under suitable conditions, can be caused to nucleate and form crystals. However, no matter how low the temperature is reduced, there are still only X number of molecules in the solution to invoke nucleation. If a saturated solution is prepared composed of $X + Y$ molecules, then, under the same conditions, $X + Y$ molecules are available to nucleate.

It has been observed by the authors that nuclei formation is strongly enhanced by ultrasonic treatment. In addition, it has been reported (22) that maximum nuclei formation has been demonstrated to be advantageous for the crystallization of low micron particles. Thus, the solution that nucleates to a greater extent most probably would yield smaller crystals. If we assume, as is reasonable in the light of the established data, that sonics is the primary factor in inducing nucleation, then, even though the saturated solutions are exposed to different coolant temperatures, a stimulation effect on crystallization would occur. Since the systems are induced to nucleate by sonics to the same degree, the saturated solutions, prepared with more solute (a higher-temperature saturated solution)—for example, the $X + Y$ saturated solutions—would be more prone to nucleate and, therefore, form smaller crystals.

Ultrasonics has been shown to increase the rate of

crystallization of hydrocortisone from saturated solutions.

Since ultrasonic treatment imparts energy to the system in the form of heat, this effect is even more significant. It is well known that yields from the usual crystallization procedure are primarily dependent upon the extent of reduction in temperature. The larger the temperature differential, the more pronounced are the yield differentials. However, in almost all of the treated samples, the yields were greater than in the untreated samples, even though the differential in the temperature of the treated samples was less.

The effect of the length of ultrasonic treatment on the amount of the crystallates formed is illustrated in Figs. 10 and 11. These data indicate that for ethyl alcohol and the blended solvent, increased treatment times somewhat increased the amounts of the crystallate. For isopropanol, increased yield occurred only for the −65° run. It is interesting to note the agreement of these data with the known solubility data of the three solvents. Hydrocortisone is most soluble in ethyl alcohol, least soluble in isopropyl alcohol, and intermediately soluble in the blend. A review of the data indicates that this is the same relationship as the yields of the crystallate.

The various coolant temperatures employed in this study markedly affected the yields of the crystallate. In almost every treated sample, the yields of the crystallate were directly related to the coolant temperature. That is, when −65° was utilized as the coolant temperature, maximum yields occurred, and when 25° was employed, minimum yields were obtained. This effect was expected because once sufficient nuclei are formed, the yields of crystals obtained in a crystallization procedure are primarily dependent upon the temperature effects.

The amount of crystallate formed was a function of the solvent system employed for the crystallization. Hydrocortisone is soluble to its maximum extent in ethyl alcohol. Hence, when the temperature of this saturated solution is reduced to the same extent as for example isopropanol (lower solubility) more crystals would be expected to form. This occurrence was observed throughout this series of experiments. The same effect was noted when a comparison of data obtained from the high and low saturated solutions of the same solvents was evaluated. This series of experiments, dealing with the yields of crystals obtained from saturated solutions which had been treated with sonics, agrees quite well with established crystallization principles.

The final area investigated dealt with the determination of the effects produced by reducing the external pressure of the system. As previously noted (17–19), a reduction in external pressure brings about a reduction in sonic efficacy. Employment of this procedure should offer information relative to the actual effect of ultrasonics on the crystallization procedures. That is, if the particle size data and the yields of crystallates obtained in this series of experiments are inferior to the data obtained in the normal treated samples, this would indicate that sonics effects (cavitation) are primarily responsible for the superior results obtained in the treated samples.

A series of experiments clearly defined the effect of reduced pressure on particle size distributions. The data indicate that, as the external pressure is

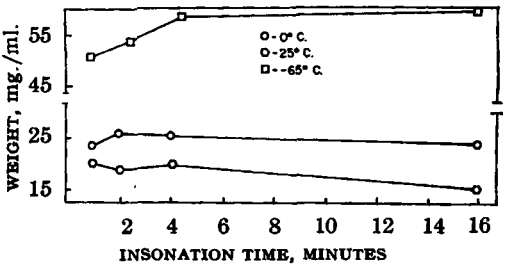


Fig. 10.—Relationships of crystal yields to insonation times at 0, −65, and 25° of samples of hydrocortisone in a 70° saturated solution in isopropyl alcohol.

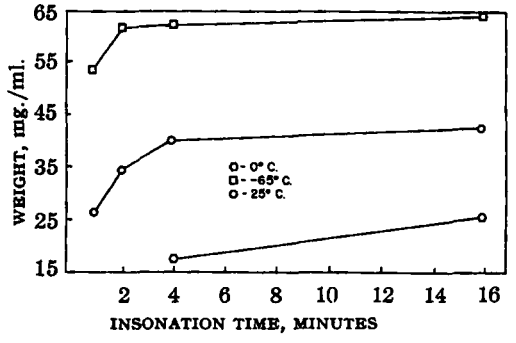


Fig. 11.—Relationship of crystal yields to insonation times at 0, −65, and 25° of samples of hydrocortisone in a 73° saturated solution in the blend.

reduced, the particle size distributions increase. Figures 12 and 13 denote this increase in particle size distribution as a function of the reduced external pressure.

The application of a vacuum to the system to effect a reduction in pressure caused an increase in the yield of the crystallate. This effect occurred even though cavitation was substantially reduced.

An explanation of this occurrence is based upon the fact that the solvent is removed from the system because of the reduced pressure, and because of the temperature of the treatment vessel. Vacuum crystallizations have been successfully employed for the preparation of numerous crystalline materials. However, as the data indicate, to achieve increased yields of crystallizations, the particle size frequency of the crystallate must be sacrificed to some extent.

SUMMARY AND CONCLUSIONS

The purpose of this investigation was to evaluate the factors involved in the preparation of low micron particulates of hydrocortisone by the application of ultra sound. A concurrent study was carried forth to determine the variables concerned with increasing crystal yields.

(*a*) Ultrasonic treatment of saturated solutions of hydrocortisone in isopropyl alcohol, ethyl alcohol, and a 50% v/v blend of these two solvents indicated that, under suitable conditions, low micron crystals can be obtained.

(*b*) The length of ultrasonic treatment of the samples has been observed not to be a factor in affecting the particle size distributions of the crystals.

(*c*) The particle size distributions of samples, sub-

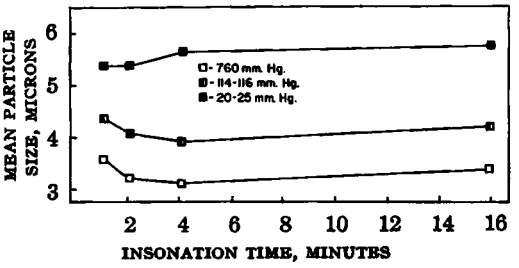


Fig. 12.—The effect of reduced pressure on the particle size distribution of insonated samples of hydrocortisone in a 70° saturated solution in isopropyl alcohol at −65°.

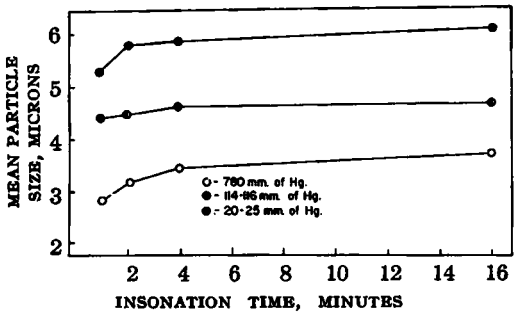


Fig. 13.—The effect of reduced pressure on the particle size distributions of insonated samples of hydrocortisone in a 73° saturated solution in ethyl alcohol at 0°.

jected to ultrasonic treatment at the various coolant temperatures, were quite similar. Except for a few samples prepared with isopropanol, particle size distributions were relatively unaffected by the differential in temperature of the saturated solutions.

(*d*) The solvent system employed for the preparation of the saturated solutions was observed to be a factor in controlling particle size distributions. Crystals obtained from treated saturated solutions utilizing ethyl alcohol as the solvent were consistently smaller in size than those obtained from saturated solutions in either the blend or isopropanol.

(*e*) The degree of saturation of the solvents was observed to affect particle size distribution; the higher the level of saturation, generally, the smaller the crystals.

(*f*) The yields of crystals obtained from samples treated with sonics were in almost all cases larger than untreated samples.

(*g*) The length of ultrasonic treatment was observed to affect slightly the yields of crystals from saturated solutions in isopropanol. However, the amount of crystallate obtained from saturated solutions in both ethyl alcohol and the blend was enhanced with increased treatment time.

(*h*) The amount of hydrocortisone, which crystallized from the solvents when subjected to ultrasonic treatment was shown to be directly related to the temperature of the coolant bath employed. The lower the temperature of the coolant bath, the greater the extent of crystallization.

(*i*) The degree of saturation of the solvents was observed to affect the amount of hydrocortisone obtained from treated samples. Greater yields of crystals were obtained from more saturated solutions in the same solvent than from lower ones.

(*j*) Decreased cavitation, caused by a reduction in the external pressure of the system, was observed to yield crystals of larger particle size distribution than those obtained from the usual sonic procedures.

(*k*) Enhanced yields of crystals were obtained from samples treated under reduced pressure. The increased amounts of the crystallate were due to the removal of the solvents by the applied vacuum.

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Effects of Centrally Acting Drugs on Confinement Motor Activity

By DAVID H. TEDESCHI, PHILIP J. FOWLER, WILLIAM H. CROMLEY, JOHN F. PAULS, ROY Z. EBY, and EDWIN J. FELLOWS

A test procedure is described for use in quantitating the effects of drugs which stimulate or depress central nervous system function. Motor activity of rats is measured by photoelectric cells while the rats are confined in a small plastic chamber. The chamber is designed to permit the rats to exhibit "up and down" exploratory movements but not movement from place to place, *i.e.*, locomotor activity. Drugs tested include caffeine, *d*-amphetamine, phenmetrazine, methylphenidate, pipradrol, tranylcypromine, chlorpromazine, prochlorperazine, trifluoperazine, and chlordiazepoxide. Advantages of the test include a high degree of reproducibility, selectivity, and sensitivity. Two of the drugs mentioned, namely caffeine and tranylcypromine, were especially potent by this test, in spite of the fact that they are essentially inactive by the conventional photoelectric cell locomotor activity test.

ONE OF THE more useful test procedures available for quantitating the effects of drugs on animal behavior involves measurement of alterations in motor activity. In general, the value of a particular motor activity measuring device resides in its sensitivity, selectivity, and reproducibility. Devices which utilize the photoelectric cell counter, first introduced for this purpose by Winter and Flataker (1), have proven to be both selective and reproducible for measuring changes in *locomotor* activity, *i.e.*, movement of animals from place to place. Such devices have, however, proven to be of little value in quantitating the effects of known central nervous system stimulants such as caffeine or tranylcypromine, which cause only minor overt changes in locomotor activity. To quantitate the stimulant effects of these compounds it was necessary to exaggerate their influence on motor activity. This was accomplished by restricting the locomotor activity of rats through confinement in a photoelectric cell activity chamber small enough to permit "up and down" movements of the animals, but not movements from place to place. This report is concerned with the effects of a variety of drugs on such "confinement motor activity" and describes the advantages of this approach for the evaluation of drugs influencing animal behavior.

METHODS

All experiments were performed on adult male albino rats (150–250 Gm.) of the Sprague-Dawley strain. All of the drugs tested were administered by the oral route and were tested at their time of peak activity as determined by the confinement motor activity (CMA) test. Drugs tested included chlorpromazine (Thorazine[1]), prochlorperazine (Compazine[1]), trifluoperazine (Stelazine[1]), chlordiazepoxide (Librium[2]), caffeine, *d*-amphetamine (Dexedrine[1]), phenmetrazine (Preludin[3]), methylphenidate (Ritalin[4]), pipradrol (Meratran[5]), and tranylcypromine (Parnate[1]). Motor activity was measured as follows.

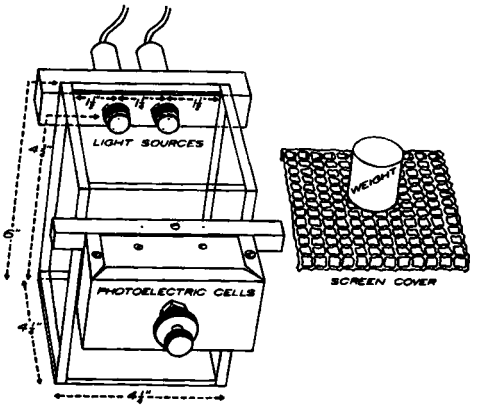


Fig. 1.—Schematic diagram of an individual photoelectric cell counting chamber.

APPARATUS

The device employed for the measurement of changes in motor activity is illustrated in Figs. 1–3. It consists of banks of five plastic chambers. The dimensions of each chamber are 4.25 × 4.25 × 6 in.[6] They are constructed of 0.25-in. thick clear plastic. Each plastic chamber is enclosed in a separate rack. Attached to this rack are two photoelectric cells and two light sources for each chamber. The center of each light source is 4.75 in. from the base of the chamber and 1.5 in. in from each side of the chamber. The centers of the light sources are thus approximately 1.25 in. apart in the same plane. Each pair of photoelectric cells

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[1] Trademark of Smith Kline and French Laboratories.
[2] Trademark of Roche Laboratories Division, Hoffmann-LaRoche, Inc.
[3] Trademark of Geigy Company, Inc.
[4] Trademark of Ciba Pharmaceutical Products, Inc.
[5] Trademark of Wm. S. Merrell Company.
[6] In a preliminary series of experiments the height of the light beams and size of the plastic chambers were varied. On the basis of the results from these experiments the parameters described above were selected as being optimal in terms of maximum sensitivity and reproducibility.

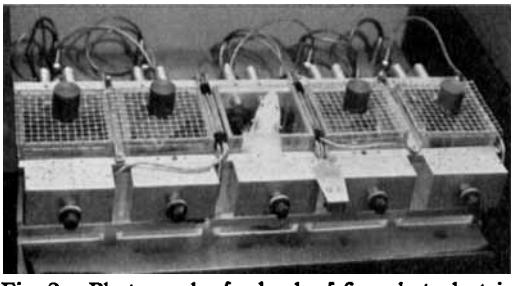


Fig. 2.—Photograph of a bank of five photoelectric cell counting chambers.

is connected to a single counter so that interruption of one or both light beams results in a single count. The top of each chamber is closed with a wire screen. A weight is attached to the screen to prevent the rat from raising it. A tray covered with paper is fitted under each bank of five chambers. The paper is changed after each occupancy to maintain an environment as constant as possible. Two hinged lids, one covering the front and one the back of each bank of five chambers, are closed to provide a minimum of light input from the room and to cut down distraction for the animals placed in the individual chambers.

Procedure for Testing Compounds Effecting a Reduction in Motor Activity

A group of 70 rats is randomly divided into seven groups. Five groups containing 10 rats each are treated with various doses of the drug under investigation. The sixth and seventh groups containing 10 rats each are pooled to make a group of 20. These are treated with equivalent volumes of the vehicle employed, water or tragacanth depending on solubility of the drug, and serve as controls. At the time of peak drug effect, determined by this same procedure, the rats are placed in the activity counting chambers for a period of 15 minutes. This test interval was selected on the basis of a series of experiments on chlorpromazine which is described under the *Results* section. The depressant dose-50 (DD_{50}) is defined as the dose of drug effective in reducing the average 15-minute counts of the treated rats to 50% of the average 15-minute counts of the control rats tested concomitantly.

Procedure for Compounds Producing an Increase in Motor Activity

The procedure is similar to that described for motor activity depressants, except that the rats are placed in the chambers 15 minutes before the time of peak drug effect and are kept in the chambers for a period of 25 minutes rather than 15 minutes. This test interval is based on a series of experiments performed on *d*-amphetamine which is described under *Results*. Only counts taken during the last 10 minutes of this 25-minute period are evaluated. The stimulant dose-200 (SD_{200}) is defined as the dose of drug which causes a 200% increase in the average 10-minute counts of treated rats, over and above the average 10-minute count of control rats tested concomitantly.

Procedure for Statistical Analysis of Data

Graphs were made for each of several drugs by plotting the mean count of activity at each dose against log dose. The resulting log dose-response curves were consistently concave upward. Furthermore, it was noticed that there was a definite tendency for the variability of the activity counts to increase as the mean activity count increased. These two observations suggested that a transformation of the activity counts would be necessary for proper analysis of the data. A logarithmic transformation provided dose-response curves of satisfactory linearity and uniformity of variability among different groups.

For each drug a straight line was fitted to the mean log activity counts *versus* log dose by the usual least-squares procedure. The parameters of this straight line and the mean log activity count of the control group were used to estimate the dose of the drug which would produce the desired change in activity from control counts by means of the following formulas:

$$x_0 = \log SD_{200} = \frac{\bar{y}_c + 0.47712 - a}{b}$$

or

$$x_0 = \log DD_{50} = \frac{\bar{y}_c - 0.30103 - a}{b}$$

In these formulas $\bar{y}_c$ is the mean log activity count of the control group, a is the intercept, and b is the slope of the fitted straight line; 0.47712 is log 3 and 0.30103 is log 2. The form of this estimate made possible the use of Fieller's theorem (2) to calculate fiducial limits for the log SD_{200} or log DD_{50} and thus for the SD_{200} or DD_{50} itself. Application of this theorem resulted in the following formula for the fiducial limits:

$$x_0 + \frac{(x_0 - \bar{x})g}{1 - g} \pm \frac{ts}{b(1 - g)} \left[(1 - g)\left(\frac{1}{n_c} + \frac{1}{\sum_{i=1}^{k} n_i} \right) + \frac{(x_0 - \bar{x})^2}{SS_x} \right]^{1/2}$$

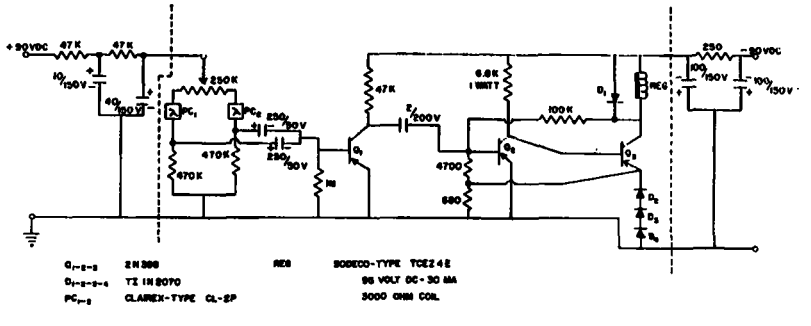


Fig. 3.—Wiring diagram for photoelectric cell and counter circuitry.

In this formula x refers to log dose; it is assumed that k doses were tested with n_i animals receiving the ith dose and n_c animals in the control group; $\bar{x}$ is thus the mean log dose tested. The symbol s stands for the pooled within-group standard deviation of the log activity counts. The symbol t stands for the value of Student's t with degrees of freedom appropriate to s and at the chosen significance level (for our purposes this was the 5% level). SS_x is customarily known as the corrected sum of squares for log dose and is computed as

$$SS_x = \sum_{i=1}^{k} n_i x_i^2 - \left[\left(\sum_{i=1}^{k} n_i x_i\right)^2 \Big/ \sum_{i=1}^{k} n_i\right]$$

where x_i is the logarithm of the ith dose. The term g is computed as

$$g = t^2 s^2 / b^2 SS_x$$

It provides an indication of the significance of the slope of the dose-response curve, and all of its terms have been defined above. In accordance with common practice, g was ignored if its value was 0.05 or less, and this resulted in the following simpler formula for the fiducial limits:

$$x_0 \pm \frac{ts}{b}\left[\frac{1}{n_c} + \frac{1}{\sum_{i=1}^{k} n_i} + \frac{(x_0 - \bar{x})^2}{SS_x}\right]^{1/2}$$

Although either of the fiducial limit formulas can be simplified somewhat in particular situations, the form in which they are presented here applies to any spacing of the doses and any inequality in number of animals per dose.

RESULTS

Determination of Optimum Periods of Both Acclimation in the Chamber and Actual Testing in the Chamber

Two series of trial experiments, each consisting of seven different tests, were performed on *d*-amphet-

TABLE I.—INFLUENCE OF VARYING ACCLIMATION PERIOD AND TEST PERIOD ON SD_{200} OF *d*-AMPHETAMINE

Acclimation Period, Min.	Test Period, Min.	SD_{200} (95% Fiducial Limits) mg./Kg. Oral	Ratio: Upper Confidence Limit / Lower Confidence Limit
None	0–15	12.6 (2.5 to 2600)	1,032.0
		2.7 (0.9 to 17.5)	19.2
		4.1 (1.1 to 87.3)	80.8
		7.1 (1.5 to 517.8)	340.6
		5.2 (0.9 to 4899)	5,512.0
		1.7 (0.6 to 10.3)	17.4
		7.7 (0.2 to 302.5)	1,681.0
None	0–25	2.2 (0.8 to 11.0)	13.7
		1.0 (0.4 to 2.9)	6.2
		1.6 (0.6 to 5.6)	8.7
		2.7 (0.9 to 21.7)	25.3
		2.0 (0.5 to 32.5)	62.5
		0.8 (0.3 to 2.8)	8.0
		2.4 (0.9 to 11.9)	13.4
5	6–15	0.7 (0.2 to 5.2)	31.7
		0.7 (0.2 to 2.5)	11.2
		1.1 (0.4 to 4.5)	11.3
		1.5 (0.6 to 6.5)	11.1
		0.9 (0.3 to 5.2)	19.3
		0.8 (0.3 to 3.3)	12.2
		1.4 (0.5 to 9.3)	21.7
15	16–25	0.17 (0.04 to 0.46)	10.7
		0.24 (0.11 to 0.47)	4.1
		0.46 (0.23 to 0.93)	4.0
		0.44 (0.22 to 0.93)	4.3
		0.39 (0.05 to 0.88)	16.3
		0.19 (0.06 to 0.47)	4.4
		0.36 (0.15 to 0.80)	8.4
None	Ratio of 16–25 min. count / 0–15 min. count	0.063 (0.00003 to 0.3932)	12,528.8
		0.128 (0.0268 to 0.3519)	13.1
		0.640 (0.274 to 1.662)	6.1
		0.360 (0.152 to 0.810)	5.3
		0.249 (0.0005 to 4.3798)	9,418.1
		0.1687 (0.0010 to 1.2768)	1,244.3
		0.2476 (0.0528 to 0.8083)	15.3
None	Ratio of 16–25 min. count / 0–5 min. count	0.0571 (0.0003 to 0.2950)	1,011.0
		0.1238 (0.0308 to 0.3142)	10.2
		0.6044 (0.2988 to 1.2956)	4.3
		0.3211 (0.1377 to 0.6949)	5.0
		0.2061 (0.0030 to 1.6679)	552.6
		0.1387 (0.0034 to 0.7203)	209.0
		0.2291 (0.0635 to 0.6228)	9.8

TABLE II.—INFLUENCE OF VARYING ACCLIMATION PERIOD AND TEST PERIOD ON DD_{50} FOR CHLORPROMAZINE

Acclimation Period, Min.	Test Period, Min.	DD_{50} (95% Fiducial Limits) mg./Kg. Oral	Ratio: Upper Confidence Limit / Lower Confidence Limit
None	0–15	3.8 (1.9 to 7.2)	3.8
		5.0 (2.2 to 11.4)	5.1
		4.8 (2.5 to 9.0)	3.6
		6.4 (3.0 to 14.4)	4.8
		3.8 (1.7 to 7.8)	4.5
		6.2 (3.0 to 13.1)	4.3
		3.0 (1.4 to 6.0)	4.2
5	6–15	4.5 (1.5 to 13.5)	9.0
		5.0 (1.1 to 21.8)	19.0
		3.8 (1.2 to 6.9)	3.5
		6.1 (2.7 to 14.4)	5.3
		2.6 (0.8 to 7.0)	9.3
		6.4 (2.4 to 18.3)	7.7
		2.0 (0.8 to 4.6)	5.9

amine and chlorpromazine, respectively. Rats treated orally with various doses of *d*-amphetamine were placed in the chambers 15 minutes after drug administration; counts were then taken at 5-minute intervals for a total of 25 minutes. Rats treated orally with various doses of chlorpromazine were placed in the chambers 270 minutes after drug administration. Counts were also recorded for these animals at 5-minute intervals for a total of 25 minutes. In both instances groups of 20 control rats were tested concomitantly.

Mean counts, averaged for the seven tests, were determined for each dose of drug tested for each interval analyzed. In addition, the ratios of mean counts for two different intervals, again averaged for the seven tests, were also determined. The mean counts for a specific interval or the ratios of mean counts for two different intervals were then plotted against log dose and evaluated graphically. On the basis of this preliminary analysis several intervals or ratios of intervals which demonstrated a reasonable dose-response relationship were selected for a detailed statistical analysis. The results of this analysis are presented in Tables I and II. It is apparent from the data presented that maximum potency with minimum variability occurred in the tests on *d*-amphetamine when the rats were acclimated for a period of 15 minutes and tested 10 minutes thereafter. In the case of chlorpromazine, the optimum test period was 15 minutes with no period of acclimation.

A number of stimulants and depressants were evaluated by these procedures; the results of these experiments are presented in Tables III and IV, respectively.

DISCUSSION

The principle involved in measuring the motor activity of rats in a restricted area of movement has several inherent advantages. Rats placed in such an environment demonstrate exaggerated attitudes of behavior involving increased exploration during the beginning of their confinement and markedly reduced exploration or complete inactivity toward the end of their confinement. Thus, drugs which reduce motor activity may be tested against a background of increased activity and, conversely, drugs which increase motor activity may be tested against a background of markedly reduced activity. These alterations in baseline activity are more or less "physiological" in nature in contrast to increases or decreases in baseline activity which are brought about by drug treatment, nociceptive stimulation, etc. Another, perhaps more practical, advantage of this test method is the compactness and relatively small cost of the equipment involved. Ten CMA testing chambers cost about the same as two conventional photoelectric cell counting chambers; the latter are 13 in. in diameter and contain six light sources and six photoelectric cells. Similarly, 10 CMA chambers occupy the same amount of space as two conventional counting chambers.

The stimulant effects reported for caffeine and tranylcypromine testify to the enhanced sensitivity of the CMA test procedure. Caffeine was tested previously in doses of 5 to 350 mg./Kg. by the conventional photoelectric cell method (Tedeschi, unpublished observation) and was ineffective in producing an increase in locomotor activity. Indeed, a graded decrease in activity was observed in doses of 100 to 400 mg./Kg. This is in sharp contrast to the 200% increase caused by caffeine in the CMA test at a dose of 5 mg./Kg. Similarly Green and co-workers (3) reported that daily administration of tranylcypromine to rats in a dose of 5 mg./Kg. twice a day failed to produce a consistent or significant effect on motor activity. These workers employed the conventional photoelectric cell counting chamber described above. As shown in Table III, an oral dose of 5 mg./Kg. of tranylcypromine in the CMA test was effective in producing a 200% increase in activity.

d-Amphetamine was especially potent in the CMA test. Its SD_{200} of 0.24 mg./Kg. makes it at least 10 times as potent as any of the other stimulants tested. It is of interest that in the conventional motor activity test, an oral dose of 5 mg./Kg. of *d*-amphetamine induces only a 40% increase in activity (Tedeschi, unpublished observations).

TABLE III.—STIMULANT DOSE-200 FOR VARIOUS CENTRAL NERVOUS SYSTEM STIMULANTS

Compd.	Pretreatment Time,[a] Min.	SD_{200} (95% Fiducial Limits) mg./Kg. Oral
Caffeine	75	5.1 (1.5 to 15.8)
d-Amphetamine	15	0.24 (0.11 to 0.47)
Phenmetrazine	15	4.2 (1.7 to 9.7)
Methylphenidate	15	5.1 (2.5 to 9.7)
Pipradrol	15	3.0 (1.0 to 8.8)
Tranylcypromine	525	5.0 (3.1 to 8.2)

[a] Refers to the time after drug administration that the rats were placed in the counting chambers. Counts were recorded during a 10-minute interval following a 15-minute acclimation period in the chamber.

TABLE IV.—QUANTITATION OF THE EFFECTS ON CONFINEMENT MOTOR ACTIVITY OF VARIOUS CENTRAL NERVOUS SYSTEM DEPRESSANTS IN RATS

Compd.	Pretreatment Time, Min.	DD_{50} (95% Fiducial Limits) mg./Kg. Oral
Chlorpromazine	270	6.8 (4.5 to 10.4)
Prochlorperazine	270	4.3 (2.3 to 8.7)
Trifluoperazine	300	1.2 (0.6 to 3.5)
Chlordiazepoxide	60	13.2 (6.6 to 25.4)

The CMA test is also effective for quantitating the effects of drugs which reduce motor activity. The sensitivity of this test for the three phenothiazines evaluated is not, however, significantly greater than that of the conventional photoelectric cell method. For example, it was previously reported (4) that trifluoperazine, prochlorperazine, and chlorpromazine had oral DD_{50}'s of 1.5 (0.9–5.7), 5.3 (3.5–8.8), and 8.5 (5.2–16.5) mg./Kg. These doses were expressed in terms of the bases of these compounds and should, of course, be compared with their DD_{50}'s in terms of the base by the CMA test. Conversion of DD_{50}'s in Table IV to bases resulted in the following for the above-mentioned compounds, respectively, 1.0 (0.5–3.0), 3.6 (1.9–7.3), and 6.1 (4.0–9.3) mg./Kg. Although the DD_{50}'s by the CMA test are consistently less than the DD_{50}'s by the conventional procedure, there is no significant difference in potency for any one drug between the two tests.

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(1) Winter, C. A., and Flataker, L., *J. Pharmacol. Exptl. Therap.*, 103, 93(1951).
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Extrapolation of Appearance of Tablets and Powders from Accelerated Storage Tests

By J. THURO CARSTENSEN, J. B. JOHNSON, W. VALENTINE*, and J. J. VANCE

A descriptive system for tablet and powder appearance utilizing tristimulus reflectances allows formal treatment of appearances. Cases are cited where responses to accelerating conditions can be treated by Arrhenius treatment.

WE HAVE, for a period of years, attempted to find a standard means of describing the appearance of a tablet in a numerical fashion, and to find "average" storage times at 55, 45, and 37° which would correspond to 2 years at 25°. Fully realizing that this will vary from compound to compound, it is nevertheless important to have a standard storage period if a large number of compounds have to be screened for compatibility with common tablet ingredients.

Several investigators (1–4) have found dyed liquid or tablet decompositions to be first order in part or *in toto*, in response to light and heat stress.

The question of whether a white tablet or powder mixture would adhere to such a scheme has been treated below by (*a*) visual observation and use of a suitable scoring system and by (*b*) reflectance measurements.

EXPERIMENTAL

As part of a routine compatibility program, the compatibility of new investigational compounds with various excipients or lubricants is tested in the following manner. The drug is mixed and ground with the excipient in question, and half of the powder mixture is transferred to glass vials which are plugged and sealed. To the other half is added 5% water, careful mixing is performed in a mortar, and the moist mix is transferred to vials as above. The plugged aluminum sealed vials are wax sealed to insure an adequate moisture barrier. As shown in Table II, 11 such two-component systems are prepared. In addition, the drug is set up *per se*. The ratio of drug to excipient is 1:5 by weight, or, in the case of lubricants, 20:1. The samples are stored at 55, 37, 25, and 5° and observed at various time intervals. The accelerated samples are stored at 5° after 10 days at 55° and 2 months at 37° to enable retrieval of the sample at later times for further comparison.

The following scoring system is used:

Degree of Color.—*1*, Unchanged; *2*, hardly noticeable darker; *3*, very slightly darker; *4*, slightly darker; *5*, darker; *6*, much darker (color change). When change toward lighter color occurs, a similar scoring system is used.

Degree of Fineness.—*1*, Unaltered; *2*, very slight particle size increase, does not adhere to glass, flow characteristics slightly altered from control; *3*, slight increase in particle size, movement not quite free flowing, and/or tendency for particles to adhere to glass; *4*, definitely increased in particle size, but still free flowing (like a granulation); *5*, caked—does not move or moves as an entity and discrete particles detectable; *6*, fluidized and solidified—no discrete particles detectable.

In the case of the photometric reflectance measurements a Color Coder[1] was used. Flat-faced bevelled tablets of two different drugs (*a*) one containing 60 mg. of active drug per 170-mg. tablet weight ($^9/_{16}$ in.) and (*b*) one containing 1 mg. of active drug per 100 mg. tablet weight ($^1/_4$ in.) were ex-

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posed to (*i*) one month at 55°, (*ii*) 3 months at 45°, (*iii*) 6 months at 37°, (*iv*) room temperature, and (*v*) 5°. The samples stored at the elevated temperatures were frozen (for reference) after the prescribed periods were elapsed. Samples were retrieved after (*a*) 16 months and (*b*) 21 months and reflectance measurements taken (ten duplications) of all samples.

TABLE I.—RESULTS OF DRUGS *per se* AFTER PROLONGED ROOM TEMPERATURE STORAGE AND 10 DAYS AT 55° C.

Compd. No.	Time at Room Temp., Mo.	Room Temp. Dry	Room Temp. Wet	10 days at 55° C. Dry	10 days at 55° C. Wet
1	27	1	4	1	2
2	27	3	3	1	1
3	27	5	1	2	1
4	26	1	3	1	1
5	26	1	5	2	2
6	25	1	1	1	1
7	25	1	1	1	1
8	25	1	1	1	1
9	25	1	3	1	2
10	22	4	6	4	6
11	21	1	2	1	1
12	21	5	4	5	3
13	20	1	1	1	1
14	20	1	1	1	1
15	20	1	1	1	2
16	19	1	3	1	4
17	18	3	1	2	2
18	18	1	1	1	1
19	18	4	6	2	4
20	17	1	1	1	1
Totals		38	49	31	38

RESULTS AND DISCUSSION

Table I shows the results of the drug *per se* after prolonged room temperature storage and after 10 days at 55° for 20 chemically unrelated drugs. It is quite apparent that, on the average, 10 days at 55° is not so strenuous a test as prolonged room temperature storage.

In the case of a dry powder, *e.g.*, 100 mg. in a 10-ml. vial, the moisture loss required to saturate the space above the powder at 55° would be about 1 mg. and would, even with hermetic seals, give rise to drying of the powder. This might explain the dry phenomena in Table I, but not the wet.

In Table II each category (wet, dry, 25°, 55°) has been tabulated for the two-component systems described above. It is also apparent here that the 10 days at 55° do not adequately represent (on the average) prolonged room temperature storage. The total scores are listed in the last column. Each category (wet, dry, 25°, 55°) has a highest possible score of $12 \times 20 \times 6 = 1440$ and a minimum possible score of 240. The increases for

TABLE II.—CATEGORIES FOR TWO-COMPONENT SYSTEMS

		Identical	17–27 Mo. at 25° C. Worse	10 Days at 55° C. Worse	Total Score 25°	Total Score 55°
Drug *per se*	Dry	15	4	1	38	31
	5% H_2O	9	8	3	49	38
Drug + mag. stearate	Dry	16	3	1	34	30
	5% H_2O	15	4	1	43	35
Drug + ca. stearate	Dry	13	4	3	37	32
	5% H_2O	12	5	3	38	35
Drug + stearic acid	Dry	15	5	0	42	31
	5% H_2O	7	11	2	60	38
Drug + talc	Dry	14	5	1	38	30
	5% H_2O	10	8	2	45	34
Drug + acid washed talc	Dry	12	8	0	44	31
	5% H_2O	10	9	1	49	35
Drug + lactose	Dry	12	5	3	38	32
	5% H_2O	9	7	4	65	56
Drug + $CaHPO_4$ anhydrous	Dry	12	6	2	46	36
	5% H_2O	9	8	3	66	53
Drug + cornstarch	Dry	12	5	3	39	34
	5% H_2O	10	5	5	40	37
Drug + mannitol	Dry	10	7	3	39	31
	5% H_2O	8	7	5	47	45
Drug + terra alba	Dry	14	6	0	41	28
	5% H_2O	11	6	3	50	45
Drug + sugar 4x	Dry	12	6	2	41	34
	5% H_2O	9	7	4	63	61

TABLE III.—REGRESSION CONSTANTS

Storage, Days	Pretreatment	Total Score	Increase *d*	% Retention (*R*)	Log *R*	Regression ($days^{-1}$)
665 at 25°	Dry	447	207	83	1.919	1.22×10^{-4}
10 at 55°	Dry	380	140	88	1.944	56×10^{-4}
665 at 25°	+5% H_2O	615	375	69	1.839	2.42×10^{-4}
10 at 55°	+5% H_2O	512	272	77	1.887	113×10^{-4}

TABLE IV.—RESULTS OF TABLET A STORED FOR 6 MONTHS AT 37° C.

Test No.	X	Y	Z	$X+Y+Z$	X %[a]
1	80.8	77.0	72.8	230.6	35.04
2	83.0	79.0	74.8	236.8	35.05
3	87.0	82.8	78.0	247.8	35.11
4	83.2	79.2	75.0	237.4	35.05
5	84.2	80.0	75.2	239.4	35.17
6	85.8	81.8	76.8	244.4	35.11
7	86.0	82.0	77.5	245.5	35.03
8	84.2	80.2	75.5	239.9	35.10
9	86.8	82.5	77.8	247.1	35.13
10	86.0	82.5	77.0	245.5	35.03

[a] $\bar{x}$ = 35.08; S.D. $x \simeq 0.05$; S.D. $\bar{x} \simeq 0.02$.

The above system is subjective and the photometric measurements reported in the following were initiated in an attempt (*a*) to eliminate the subjectivity and (*b*) to allow extrapolation.

Before discussing the photometric results of Tables V and VI it might be appropriate to investigate the reproducibility of the instrument. It was found that positioning of the tablet greatly influenced the X, Y, and Z component (but not the percentages referred to below by small letters, x, y, and z) (5–7). A special holder was therefore constructed allowing precise positioning, horizontally and vertically. Since, however, rotation and tablet-to-tablet variation would also be significant, the ten tests run on each storage condition were run on

TABLE V.—DRUG B

Temp., t °C.	$10^3/T$ °K^{-1}	Mo. Stored	x-Value, %	Log x	Log x/x_0[a]	$100k$ (Mo.$^{-1}$)
5	...	21	34.44	1.53706	0.00000	...
55	3.05	1	34.59	1.53859	0.00151	1.51 ± 0.7
45	3.145	3	34.59	1.53859	0.00151	0.50 ± 0.23
37	3.225	6	34.65	1.53958	0.00252	0.42 ± 0.12
25	3.355	21	34.59	1.53895	0.00189	0.09 ± 0.03

[a] 5° value used for reference.

TABLE VI.—DRUG A

Temp., t° C.	$10^3/T$ °K^{-1}	Mo. Stored	x-Value, %	Log x	Log x/x_0[a]	$100k$ (Mo.$^{-1}$)
5	...	21	34.78	1.54133	...	...
55	3.05	1	35.06	1.54481	0.00348	3.48 ± 0.7
45	3.145	3	35.06	1.54481	0.00348	1.15 ± 0.23
37	3.225	6	35.07	0.54494	0.00361	0.62 ± 0.12
25	3.355	16	34.88	1.54258	0.00125	0.08 ± 0.04

[a] 5° value used for reference.

each category are shown as d in Table III. The per cent retention of initial appearance is expressed as $R = (1200 - d)/12.0$, and the logarithm of R is also shown. The regressions shown in the last column of Table III are $k = (1/t) \cdot (2 - \log R)$, where t is time in days.

Hence, 10 days at 55° would correspond to 560/1.22 = 459 days at room temperature (on the average) for the dry samples and 1130/2.42 = 467 days at 25° for the samples containing water. The storage period at 55° corresponding to 2 years at room temperature would be 0.089/0.0056 = 15.9 days, and for the sample containing water would be 0.177/0.0113 = 15.6 days.

Whether such a semilogarithmic treatment is justified cannot be asserted from the treatment above, but the fairly close correlation between the two correlation times, 15.9 days for the dry samples and 15.6 days for the moist samples, is an indication of its utility. It would appear that for rapid screening by the visual procedure 16 days at 55° would be the most meaningful storage time on the average. In relation to the rule of thumb that "reaction rates double to triple with each 10° increase in temperature," (8) this average rule does not give the same result as above since storage times in this case should be in an 8:1 to 27:1 ratio between 55 and 25°.

It is realized that grand averaging, as carried out above, has limitations, and that individual drugs may deviate from the calculated optimal storage periods.

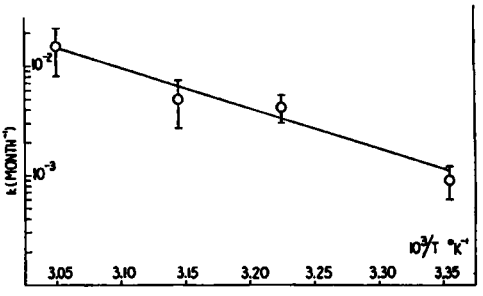


Fig. 1.—Logarithm of apparent rate constants k of drug B, taken from Table V plotted as a function of reciprocal absolute temperature.

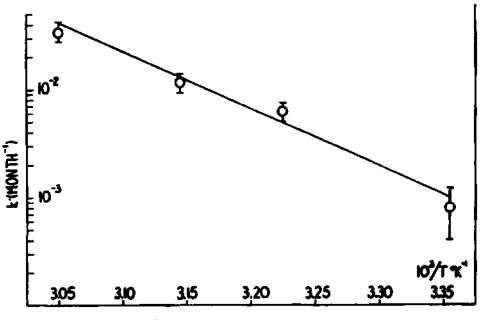


Fig. 2.—Logarithm of apparent rate constants k of drug A, taken from Table VI plotted as a function of reciprocal absolute temperature.

TABLE VII.—TABLETS OF ALGINIC ACID WITH 4% MAGNESIUM STEARATE

Days at 70°	x-Value	Initial x_0	Log x/x_0
2	35.768	35.064	0.00864
3	35.879	35.009	0.01066
4	35.876	34.890	0.01210
5	36.295	34.947	0.01644
6	36.866	34.893	0.02389
7	36.904	34.906	0.02417
8	37.457	34.942	0.03018

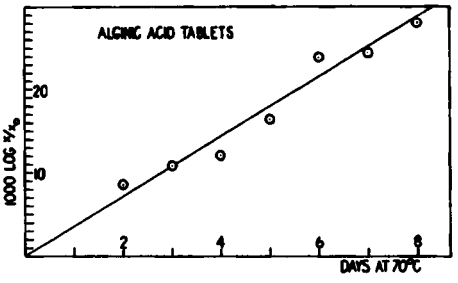


Fig. 3.—Alginic acid tablets. x-Values of reflectance are plotted as log x/x_0 *versus* days at 70°, x_0 being the original reflectance x-value.

is obeyed, (*b*) that degradation is by zero-order mathematics, and (*c*) that the sum of the X, Y, and Z components (denoted Σ below) is relatively time independent. In this case

$$\log x/x_0 = \log X - \log X_0 - \log \Sigma + \log \Sigma_0 \simeq \log X - \log X_0 = k \cdot C_d$$

where C_d is some measure of the "decomposed substance." If a first-order pattern is adhered to, then the above would also hold true in the initial stages since $\log (1 - \gamma) \simeq -\gamma$, where γ is any small number. For larger values, first-order relations would have to be tested as

$$(a) \qquad \log x/x_0 \propto C_d = \beta(1 - e^{-\alpha t}) \text{ or}$$

$$(b) \quad \frac{\partial}{\partial t}\{\log x/x_0\} = \frac{\partial}{\partial t}\{\log x\} = \beta \cdot \alpha \cdot e^{-\alpha t} = \beta \cdot \alpha \cdot 10^{-0.43\alpha t}$$

$$(c) \quad \log\left\{\frac{\partial}{\partial t}[\log x]\right\} = (\log \alpha + \log \beta) - 0.43\,\alpha t$$

Hence, if a linear relationship of type (*c*) is observed, the degradation would follow a first-order pattern. The above presupposes that the drug goes from white to some x value in degrading. The subsequent data justify this approximation.

TABLE VIII.—AVERAGE x-VALUES OF COMPOUND C TABLETS

Days at 70°	Sum of 5x Values	Av. $\bar{x}$	log $\bar{x}/\bar{x}_0$	log $\bar{x}$	Δ log $\bar{x}/\Delta t$	t, Days
4	185.06	37.012	0.02741	1.56834		
					0.00296	3.5
3	183.80	36.760	0.02445	1.56538		
					0.00402	2.5
2	182.11	36.422	0.02043	1.56136		
					0.00779	1.5
1	178.87	35.774	0.01264	1.55357		
					0.01264	0.5
0	173.74	34.748	0	1.54093		

ten different tablets. The results of tablet A stored for 6 months at 37° are shown in Table IV.

The standard deviations of this set hence are 0.02% for the average $\bar{x}$ and 0.05% for the individually measured x-value. Standard deviations for the other storage conditions are comparable and for simplicity the above values (which are in line with the reading accuracy of the instrument) have been used for the analysis below.

The results of the various storage conditions are shown in Tables V and VI. The data have been treated assuming the change in the x-value to be zero order. k is calculated from two points only, so the plots are average plots.

Since $1/t \times \log x/x_0$ is the parameter evaluated, the relative error of this term is of significance, where x is the x-value at time t. x_0 is the reference x-value (in this case the sample stored at 5°). The values are all close to 34.5 and hence log 34.54 − log 34.50 = 0.0005 would be a measure of the error on both log x and log x_0, using $2 \times 0.02\% = 0.04\%$ as the standard error of x.

The error of log x − log x_0 hence would be $10^{-4}\sqrt{25 + 25} = 0.0007$ (9). The tolerances so calculated are listed in the last column of Tables IV and V, and are also shown in the Arrhenius graphs, Figs. 1 and 2.

A provision for the above is that (*a*) Beer's law

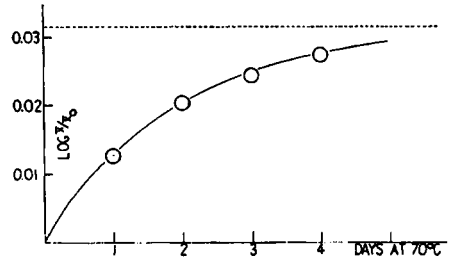


Fig. 4.—Compound C tablets. Average x-values of five lots plotted as logarithmic increase plotted *versus* days stored at 70°.

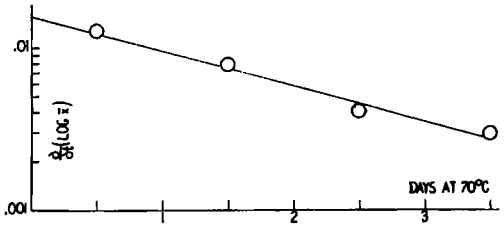


Fig. 5.—The logarithm of the slopes from Fig. 4 plotted *versus* days stored at 70°. The slope of the line is −0.4343 α, the intercept is (log α + log β). (See text for significance of α and β.)

TABLE IX.—COMPOUND C

Days at 70°	$\log \bar{x}_\infty - \log \bar{x}$	$\mu = \frac{\log \bar{x}_\infty - \log \bar{x}}{0.03166}$
4	0.00425	0.1343
3	0.00721	0.2278
2	0.01123	0.3548
1	0.01902	0.6008

Tablets were made of an alginic acid[2] containing 4% magnesium stearate. Since these tablets were difficult to make of even thickness (and hence would be difficult to position), initial values were taken of each tablet tested. Tablets were placed at 70° and withdrawn after various time periods with the results shown in Table VII (all figures being averages of 10).

These values are shown graphically in Fig. 3 and demonstrate the zero or first-order nature of the change.

As an example of a "degradation" of the first-order type, a third compound, denoted C, was tableted.

These tablets were stored at 70° for 1, 2, 3, and 4 days; the average x-values from five batches are listed in Table VIII. As Fig. 4 shows, the log x/x_0 *versus* t relationship is not linear in this case. The derivative function $(\partial/\partial t)[\log \bar{x}]$ is shown by midpoint differences in Table VIII and the log $\{(\partial/\partial t)[\log \bar{x}]\}$ is seen to be linearly related to time as shown in Fig. 5. To plot this conventionally, log x at full conversion (denoted log $\bar{x}_\infty$) would have to be known. From Fig. 5 α is 0.490 and β is calculated from the intercept to be 0.03166.

At the asymptote of graph 4 the factor $(1 - e^{-\alpha t})$ will be 1 ($t = \infty$). Thus $\log \bar{x}_\infty - \log x_0$ equals $\beta = 0.03166$, and the relations can now be expressed in a conventional manner (10), in that what appears as "fraction retained" on a first-order ordinate in this case would be

$$\mu = \frac{\log \bar{x}_\infty - \log \bar{x}}{\log \bar{x}_\infty - \log \bar{x}_0} = \frac{\log \bar{x}_\infty - \log \bar{x}}{0.03166}$$

Values of μ are tabulated for compound C in Table IX and the logarithmic relationship log $\mu = -k^*t$ is quite evident from Fig. 6.

[2] Marketed as Landalgine P 2235 by Edward Mendell Co., Inc., Yonkers, N. Y.

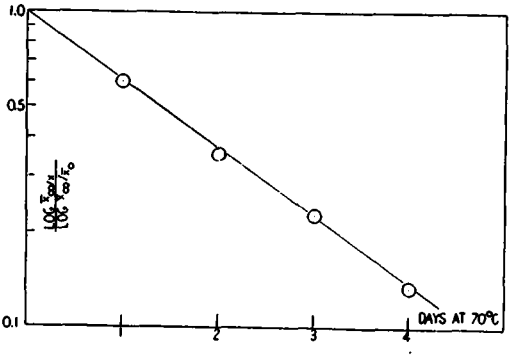


Fig. 6.—Compound C tablets. Degradation at 70° expressed as logarithm of per cent "retained" *versus* days stored at 70°.

SUMMARY

An average storage time for evaluation of tablet appearances of 16 days at 55° corresponds to 2 years at room temperature as judged by 20 different drugs.

Photometric reflectance measurements may be used to describe tablet appearances.

Photometric x, y, or z-value changes appear to be first order or zero order and may be extrapolated by formal Arrhenius treatment.

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Central Nervous Depressants VIII

Structure-Activity Relationship of Some New Azlactones

By P. K. SHARMA, M. K. MENON, and P. C. DANDIYA

Five azlactones having trimethoxybenzene structure were synthesized and their influence on blood pressure of anesthetized dogs, spontaneous motor activity of rats, pentobarbital hypnosis, and conditioned avoidance response in trained rats were studied. All the compounds possessed mild central nervous system depressant action. Two compounds—namely, azlactone of 3,4,5-trimethoxybenzylamino-2,3,5-trimethoxycinnamic acid and azlactone of 3,4,5-trimethoxybenzylamino-*p*-dimethylaminocinnamic acid—exerted a transient hypotensive action. A structure-activity relationship has been indicated.

SINCE THE structure of reserpine was established, the problem of synthesis of reserpine analogs has been investigated by various workers, to search for compounds of simpler chemical structure having similar pharmacological actions but devoid of undesirable side effects. The reserpine molecule possesses an indole nucleus and a trimethoxybenzene group in its chemical structure; it was assumed that compounds built upon these structures may reproduce the pharmacological activities of reserpine. Lassalo and Jordan (6), Ramasastry and Lassalo (9), Borsy (2), and Dandiya *et al.* (4) have synthesized some compounds having trimethoxybenzene as the basic structure. Dandiya *et al.* (4) have also studied the structure-activity relationship of the compounds synthesized by them. Another interesting development in this field was the isolation of two active principles of *Acorus calamus*, a plant indigenous to India. Both of these principles, asarone and β-asarone, are simple trimethoxybenzene derivatives (1) and exerted marked central nervous depressant effects (3).

Lately, in the course of investigations of new compounds with actions on the central nervous system, trimethoxybenzoyl-glycine-diethylamide (Riker 548, trimeglamide) has also been shown to possess tranquilizing properties (11).

Since Riker 548 could be considered as a derivative of a saturated azlactone, Robison and Schueler (10) have worked out the pharmacological activity of a number of azlactone derivatives and reported that they were capable of prolonging the duration of hexobarbital-induced hypnosis in mice, although by themselves, these compounds exhibited convulsant properties.

Since most of the compounds of the azlactone series have a prominent action on the central nervous system, it was considered desirable to synthesize a few azlactones possessing a trimethoxy group in the benzene ring. These azlactones (Table I) were prepared by reacting the substituted glycine with the appropriate aldehyde in the presence of acetic anhydride and sodium acetate. 2,4,5-Trimethoxy- and 2,3,4-trimethoxybenzaldehyde were prepared by the method described by Deohra (5).

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EXPERIMENTAL

Chemical.—The following four intermediary compounds were prepared.

2,4,5-Trimethoxybenzaldehyde.—Freshly distilled $POCl_3$ (15 ml.) was added to dimethylformamide (10 ml.) previously cooled to 5°. The mixture was then added to 1,2,4-trimethoxybenzene (16.7 Gm.), dissolved in dimethylformamide (10 ml.), and heated on a steam bath for 4 hours. The mixture was diluted with water, neutralized with sodium bicarbonate, and allowed to stand for 1 hour. It was then filtered and washed with water and recrystallized from hot water (m.p. 114°, 2,4-dinitrophenylhydrazone, m.p. 252°).

2,3,4-Trimethoxybenzaldehyde.—A mixture of $POCl_3$ (15 ml.), dimethylformamide (10 ml.), and 1,2,3-trimethoxybenzene (16.7 Gm.) in dimethylformamide (10 ml.) was heated on a steam bath for 2 hours, then diluted with water and neutralized with sodium bicarbonate. It was filtered quickly after refrigerating overnight, washed with cold water, and recrystallized with hot water (m.p. 37°, 2,4-dinitrophenylhydrazone, m.p. 177–178°).

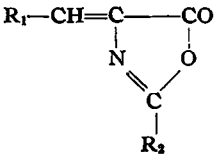
Preparation of Substituted Azlactone.—The following five compounds were synthesized.

Compound I.—(Azlactone of α-Acetylamino-2,4,5-trimethoxy cinnamic Acid).—A mixture of 2,4,5-trimethoxybenzaldehyde (10 Gm.), acetylglycine (7 Gm.), acetic anhydride (5 ml.), and anhydrous sodium acetate (4.1 Gm.) was warmed on a water bath with occasional shaking for 15–20 minutes, and the whole mixture was boiled for 1 hour and cooled by refrigeration for 4 hours. The mass was then stirred with 60 ml. of cold water, filtered, and recrystallized from carbon tetrachloride (m.p. 106–108°, yield 7.5 Gm.).

Anal.—Calcd. for C, 60.64; H, 5.41; N, 5.05. Found: C, 61.25; H, 5.93; N, 5.68.

Compound II.—(Azlactone of 3,4,5-Trimethoxybenzoyl-α-amino-2,4,5-trimethoxycinnamic Acid).—A mixture of 2,4,5-trimethoxybenzaldehyde (10

TABLE I.—SUBSTITUTED AZLACTONE DERIVATIVES AND RESULTS OF THEIR PHARMACOLOGICAL TESTING



Compd.	Substitution at R_1	Substitution at R_2	M.p., (uncorrected), °C.	Emperical Formula	Mean Sleeping Time (in Min. ± S.E.) in Mice Due to 20 mg./Kg. of Compd. +40 mg./Kg. of Pentobarbital Sodium (Mean Sleeping Time Due to Pentobarbital Alone 62.4 ± 13.5), Min.	Conditioned Avoidance Response 50 mg./Kg. Loss of CAR, %	Conditioned Avoidance Response 50 mg./Kg. Loss of Escape Phenomenon, %	Spontaneous Activity[a] in Rats, 50 mg./Kg.	Blood Pressure of Anesthetized Dogs, 10 mg./Kg. Blood Pressure Decrease, mm. Hg	Blood Pressure of Anesthetized Dogs, 10 mg./Kg. Duration of Action, Min.
I	2,4,5-Trimethoxyphenyl	Methyl	106–108	$C_{14}H_{15}NO_5$	94.8 ± 12.0	20	20	−	No effect	
II	2,4,5-Trimethoxyphenyl	2,4,5-Trimethoxyphenyl	195–197	$C_{22}H_{23}NO_8$	80.6 ± 9.6	20	0	0	No effect	
III	2,3,4-Trimethoxyphenyl	Methyl	198–199	$C_{14}H_{15}NO_5$	112.1 ± 10.0 ($P < 0.05$)	30	0	0	20–30	2–3
IV	Phenyl	3,4,5-Trimethoxyphenyl	158–159	$C_{19}H_{17}NO_5$	58.0 ± 10.2	20	0	0	...	...
V	*p*-Dimethylaminophenyl	3,4,5-Trimethoxyphenyl	194–195	$C_{21}H_{22}NO_5$	70.2 ± 8.6	20	0	+	20–30	2–3

[a] Key: 0, no effect; −, reduction; +, increase.

Gm.), 3,4,5-trimethoxybenzoylglycine (12.4 Gm.), acetic anhydride (7 ml.), and anhydrous sodium acetate (4.5 Gm.) was warmed with shaking for 10 minutes, then boiled for 1 hour, cooled in the refrigerator overnight, stirred with cold water, and filtered. Recrystallized from carbon tetrachloride (m.p. 195–197°, yield 6 Gm.).

Anal.—Calcd. for C, 61.53; H, 5.36; N, 3.26. Found: C, 60.97; H, 5.75; N, 3.15.

Compound III.—(Azlactone of α-Acetylamino-2,3,4-trimethoxycinnamic Acid).—A mixture of 2,3,4-trimethoxybenzaldehyde (10 Gm.), acetylglycine (7 Gm.), acetic anhydride (5 ml.), and anhydrous sodium acetate (4.1 Gm.) was taken and subjected to the procedure mentioned above (m.p. 198–199°, yield 8 Gm.).

Anal.—Calcd. for C, 60.64; H, 5.41; N, 5.05. Found: C, 60.96; H, 5.53; N, 5.25.

Compound IV.—(Azlactone of 3,4,5-Trimethoxybenzoylaminocinnamic Acid).—A mixture of redistilled benzaldehyde (4 Gm.), 3,4,5-trimethoxybenzoylglycine (8.5 Gm.), acetic anhydride (7 ml.), and anhydrous sodium acetate (2.5 Gm.) was warmed for 15–20 minutes with occasional shaking and boiled for 45 minutes. It was then cooled in a refrigerator for 4 hours, stirred with 100 ml. of water, filtered, and recrystallized from ethyl acetate and liq. petroleum (m.p. 158–159°, yield 2.5 Gm.).

Anal.—Calcd. for C, 67.25; H, 5.01; N, 4.12. Found: C, 66.98; H, 5.03; N, 4.25.

Compound V.—(Azlactone of 3,4,5-Trimethoxybenzoyl-α-amino-*p*-dimethylaminocinnamic Acid).—A mixture of *p*-dimethylaminobenzaldehyde (7.5 Gm.), 3,4,5-trimethoxybenzoylglycine (12.5 Gm.), acetic anhydride (7 ml.), and anhydrous sodium acetate (4 Gm.) was warmed for 10 minutes, boiled for 1 hour, cooled in the refrigerator overnight, stirred with a minimum volume of water, filtered with suction and recrystallized from carbon tetrachloride (m.p. 194–195°, yield 3.8 Gm.).

Anal.—Calcd. for C, 68.47; H, 5.97; N, 3.80. Found: C, 68.40; H, 5.88; N, 3.56.

Pharmacological Study.—The compounds were administered as a fine suspension in 3% polysorbate 80. Control experiments were always performed with the solvent-treated animals.

Blood Pressure of Anesthetized Dogs.—Ten mongrel dogs were anesthetized with morphine (5 mg./Kg.) and urethane (1.4 Gm./Kg.) administered intramuscularly. The left common carotid artery was cannulated, and blood pressure was recorded on a slowly moving kymograph. The compounds (10 mg./Kg.) were administered through the cannulated femoral vein; changes in blood pressure were observed.

Spontaneous Motor Activity.—Fifteen albino rats (Haffkine) were employed for each experiment. They were divided into three groups of five animals each and kept in individual cages. The first group of animals was treated with the compound (50 mg./Kg.), the second group received 3 mg./Kg. of chlorpromazine, and the third group was treated with the solvent which served as control. The animals were observed for 4 hours; changes in spontaneous activity and effect of tactile stimuli on their activity were compared with the control animals and to chlorpromazine-treated animals. All injections were made intraperitoneally.

Pentobarbital Hypnosis.—A set of 30 albino mice (Haffkine) was employed for each experiment. They were divided into three groups of 10 animals each. Group I was treated with pentobarbital sodium (40 mg./Kg.). Groups II and III were pretreated with the solvent and the compound (20 mg./Kg.), respectively. Pentobarbital was injected 15 minutes after the pretreatment. The injections were made intraperitoneally. The sleeping time of each individual mouse was the time taken by the animal to regain the righting reflex from the time it was lost after the administration of the anesthetic agent. The average sleeping time of the control group of animals was compared with the sleeping time of the group which received the compound in addition to pentobarbital sodium.

Conditioned Avoidance Response.—This experiment was similar to that employed by Dandiya and Sharma (3). Albino rats were trained to jump on the wall of a special cage when a bell rang to avoid an electric shock which followed. Two types of responses were observed—the conditioned avoidance response (CAR), in which the animal jumped after hearing the bell, and the escape response, in which the animal jumped only after the electric shock. Ten trained rats in which CAR was developed were employed for each experiment. The compound was administered intraperitoneally in a dose of 50 mg./Kg., and the effect on the CAR was observed at 1-hour intervals for 4 hours.

RESULTS

The results of pharmacological screening are given in Table I.

Maximum potentiation of pentobarbital hypnosis was shown by compound III, in which the sleeping time was increased by 80%, which was statistically significant. Although compounds I and II raised sleeping time, due to pentobarbital, the effect was not significant. The other two compounds (IV and V) were comparatively ineffective in influencing the sleeping time of mice.

All of the compounds exerted a moderate influence on the conditioned avoidance response of trained rats. In this experiment maximum effect was shown by compound III. Thirty per cent of the animals lost CAR, the escape response being unaffected. Other compounds blocked CAR in 20% of the animals. In addition to the loss of CAR in 20% of the animals, compound I also blocked the escape response of these animals.

There was no marked change in the spontaneous activity of rats treated with these compounds. Moderate depression, indicated by decreased alertness to tactile and auditory stimulus, was more marked in animals treated with compound I. Compound V had a slight stimulant action in rats.

None of these compounds showed marked effect on blood pressure of anesthetized dogs. A transient hypotensive effect was observed in dogs in which either compound III or V was administered.

DISCUSSION

Compared to many other trimethoxybenzene derivatives, the azlactones exerted only minimal effect on the cardiovascular system. Compounds like 2,4,5-trimethoxy-1-propenylbenzene (12), *N*-acetyl 3,4,5-trimethoxybenzamide (7), and 3,4,5-

trimethoxy amphetamine (8) have been reported to exert moderate hypotensive action in anesthetized animals. Trimeglamide, which can be considered as an analog of azlactone, also exerts only a moderate and transient fall in blood pressure (11). The relative absence of cardiovascular action may be advantageous because these compounds may be more specific in their central effect.

The effect of these compounds on spontaneous motor activity, pentobarbital hypnosis, and conditioned avoidance response shows that all of the five compounds exerted a mild central nervous system depressant action. Compound III was more potent and, in addition to its effect on the three above-mentioned experiments, it also exerted a mild and transient hypotension. The effect on conditioned response is noteworthy because of the specific blockage of CAR without affecting escape response. Only compound I acted in a nonspecific way in blocking both CAR and escape response.

A study of the structure activity relationship has shown that substitution of R_1 and R_2 by trimethoxyphenyl group does not bring about marked change in pharmacological activity. Substitution of the methyl group at position R_2 and the 2,4,5-trimethoxyphenyl group at position R_1 prolongs the pentobarbital sleeping time. A study of compounds I and III has shown that trimethoxy groups at 2,3,4-position in R_1 substitution exert a hypotensive effect as opposed when trimethoxy groups are attached to 2,4,5-position. Substitution of 3,4,5-trimethoxyphenyl group at R_2 position does not bring about change in the biological activity in the molecule. Substitution of *p*-dimethylaminophenyl group at position R_1 with 3,4,5-trimethoxyphenyl group at position R_2 in the compound exerts a hypotensive effect with slight increase in spontaneous activity. These properties are not present when the substituent is the phenyl group at the R position.

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Incorporation of Proline-C^{14} into the Principal Alkaloids of *Datura stramonium* var. *tatula* and *Datura innoxia*

By GERALD SULLIVAN and MELVIN R. GIBSON

Proline-C^{14} made available to root cultures of *Datura stramonium* L. variety *tatula* Torrey and *Datura innoxia* Miller was incorporated into hyoscyamine and scopolamine. Root growth was affected in both species.

EXTENSIVE RESEARCH has been accomplished on the genus *Datura* in an attempt to elucidate the biosynthetic pathway or pathways to alkaloid production. Various amino acids have been used to gain clearer and more precise information concerning the production of alkaloids. James (1) reported significant increases in alkaloid content when *Atropa belladonna* L. leaves were supplied *l*-arginine and *l*-ornithine. Later, van Haga (2), working with sterile root cultures of *A. belladonna*, confirmed that additional amounts of arginine and ornithine yielded increased alkaloid production. However, these and other investigators (2–4) found proline to be ineffective in increasing the plant production of hyoscyamine. Work in this laboratory (5) on *D. stramonium* variety *tatula* in which glutamic acid was added to isolated root cultures caused an increase in growth of the roots and a small but statistically significant decrease in alkaloid content of the roots. In this instance it is possible that the glutamic acid entered the citric acid cycle, contributing to growth, but not the ornithine cycle, leaving unaffected tropane alkaloid production. Other work in this laboratory (6) on the same species showed that tested quantities of *l*-proline inhibited total alkaloid production in isolated root culture, but in one concentration appeared to change relative concentrations of hyoscyamine and scopolamine in favor of scopolamine. This latter work suggested that proline plays a dynamic role in the synthesis of

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The authors are indebted to Professor R. Hegnauer, University of Leiden, for verifying the identification of *Datura innoxia* Miller, a species which is often confused with other members of the genus.

trimethoxy amphetamine (8) have been reported to exert moderate hypotensive action in anesthetized animals. Trimeglamide, which can be considered as an analog of azlactone, also exerts only a moderate and transient fall in blood pressure (11). The relative absence of cardiovascular action may be advantageous because these compounds may be more specific in their central effect.

The effect of these compounds on spontaneous motor activity, pentobarbital hypnosis, and conditioned avoidance response shows that all of the five compounds exerted a mild central nervous system depressant action. Compound III was more potent and, in addition to its effect on the three above-mentioned experiments, it also exerted a mild and transient hypotension. The effect on conditioned response is noteworthy because of the specific blockage of CAR without affecting escape response. Only compound I acted in a nonspecific way in blocking both CAR and escape response.

A study of the structure activity relationship has shown that substitution of R_1 and R_2 by trimethoxyphenyl group does not bring about marked change in pharmacological activity. Substitution of the methyl group at position R_2 and the 2,4,5-trimethoxyphenyl group at position R_1 prolongs the pentobarbital sleeping time. A study of compounds I and III has shown that trimethoxy groups at 2,3,4-position in R_1 substitution exert a hypotensive effect as opposed when trimethoxy groups are attached to 2,4,5-position. Substitution of 3,4,5-trimethoxyphenyl group at R_2 position does not bring about change in the biological activity in the molecule. Substitution of *p*-dimethylaminophenyl group at position R_1 with 3,4,5-trimethoxyphenyl group at position R_2 in the compound exerts a hypotensive effect with slight increase in spontaneous activity. These properties are not present when the substituent is the phenyl group at the R position.

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Incorporation of Proline-C^{14} into the Principal Alkaloids of *Datura stramonium* var. *tatula* and *Datura innoxia*

By GERALD SULLIVAN and MELVIN R. GIBSON

Proline-C^{14} made available to root cultures of *Datura stramonium* L. variety *tatula* Torrey and *Datura innoxia* Miller was incorporated into hyoscyamine and scopolamine. Root growth was affected in both species.

EXTENSIVE RESEARCH has been accomplished on the genus *Datura* in an attempt to elucidate the biosynthetic pathway or pathways to alkaloid production. Various amino acids have been used to gain clearer and more precise information concerning the production of alkaloids. James (1) reported significant increases in alkaloid content when *Atropa belladonna* L. leaves were supplied *l*-arginine and *l*-ornithine. Later, van Haga (2), working with sterile root cultures of *A. belladonna*, confirmed that additional amounts of arginine and ornithine yielded increased alkaloid production. However, these and other investigators (2–4) found proline to be ineffective in increasing the plant production of hyoscyamine. Work in this laboratory (5) on *D. stramonium* variety *tatula* in which glutamic acid was added to isolated root cultures caused an increase in growth of the roots and a small but statistically significant decrease in alkaloid content of the roots. In this instance it is possible that the glutamic acid entered the citric acid cycle, contributing to growth, but not the ornithine cycle, leaving unaffected tropane alkaloid production. Other work in this laboratory (6) on the same species showed that tested quantities of *l*-proline inhibited total alkaloid production in isolated root culture, but in one concentration appeared to change relative concentrations of hyoscyamine and scopolamine in favor of scopolamine. This latter work suggested that proline plays a dynamic role in the synthesis of

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The authors are indebted to Professor R. Hegnauer, University of Leiden, for verifying the identification of *Datura innoxia* Miller, a species which is often confused with other members of the genus.

tropane alkaloids. Cromwell (7) found that proline stimulated formation of hyoscyamine in *A. belladonna* roots when injected into the stem. In the same plant Schermeister *et al.* (8) found that proline was present in large amounts and accumulated in roots when the alkaloid concentration declined. These workers also found that neither arginine nor ornithine was present in detectable quantities and concluded that proline was a more direct precursor of the tropane alkaloids. Katz *et al.* (9) reported that *l*-proline-C^{14} was a precursor of hydroxyproline and ketoproline in the actinomycin peptides formed by *Streptomyces antibioticus*. Steward and Pollard (10) found that proline can be directly utilized in protein synthesis. When proline-C^{14} was supplied to growing tissue cultures of carrot root phloem, almost all of the radioactivity was found in the proline and hydroxyproline residues of the tissue protein, very little C^{14} being lost as CO_2. More recently, Miller and Kempner (11) found that proline-C^{14} was incorporated into the protein by *Candida utilis*. The ready incorporation of carbon from proline into carrot explants has been shown by Pollard and Steward (12), who demonstrated that radioactive carbon supplied as proline can be detected in the protein 15 minutes after its addition to the external solution.

From the work cited above it appeared obvious that further study was necessary to elucidate the role of proline in the biosynthesis of the tropane alkaloids. This is the purpose of this investigation. The study was limited to the two principal alkaloids of *Datura*, hyoscyamine and scopolamine. Of the two *Datura* species chosen for this investigation, each produces these alkaloids but differ in the relative quantity of each produced (13–16). *D. stramonium* L. variety *tatula* Torrey produces primarily hyoscyamine, whereas *D. innoxia* Miller produces primarily scopolamine.

EXPERIMENTAL

Growth and Processing of Root Cultures.—The methods and procedures followed in this portion of the investigation were those developed and utilized by French and Gibson (5). The seeds were obtained from plants grown in the Washington State University pharmacy greenhouse. The seeds of *D. stramonium* variety *tatula* and *D. innoxia* were sterilized with 3% hydrogen peroxide and shaken on a Kahn automatic shaker for 25 and 15 minutes, respectively. The sterilized seeds were placed in Petri dishes in the incubator for germination at 25 to 27° using sterile techniques. Upon germination, the young root radicle (1.0 to 1.5 cm. in length) was cut from the seed and placed in the appropriate nutrient solution. The root was allowed to grow for 7 days, then transferred to fresh nutrient solution and allowed to grow for 7 more days. The root was then removed and blotted dry on a paper towel, placed in a tared glass vial, and dried at 55 to 60° until constant weight was obtained.

The control root cultures were grown in White's nutrient solution (17); the experimental root cultures were grown in the same nutrient supplemented with: (*a*) 0.001 mg. of unlabeled *l*-proline per 50 ml. of nutrient solution, (*b*) 0.001 mg. of uniformly labeled *l*-proline-C^{14} [1] per 50 ml. of nutrient solution, and (*c*) 0.002 mg. of uniformly labeled *l*-proline-C^{14} per 50 ml. of nutrient solution.

Extraction and Separation of Alkaloids.—The extraction apparatus employed for this procedure was devised by French and Gibson (18). The powdered root was placed in the extractor and moistened with a few drops of a 1:1 solution of 10% ammonia and 95% alcohol. The moistened powder was allowed to stand for 1 hour. One milliliter of chloroform for every milligram of powdered root was poured into the solvent reservoir up to a maximum of 25 ml. The rate of flow of the solvent through the extractor was regulated to coincide with the evaporation of the chloroform on the warm counting planchet. The counting planchet was heated to a temperature of 58 to 60° by a hot plate. Upon completion of the extraction, the counting planchet was allowed to cool to room temperature and radioactivity determined.

The separation of the alkaloids was accomplished using thin-layer chromatography. The total alkaloid extract was subjected to five 200-μl. washings of chloroform. A 25-μl. portion of each chloroform washing was placed on a thin-layer plate. An 8 by 8 by $^1/_8$ in. glass plate with 0.5-mm. thickness of silica gel G [2] was employed. The thin-layer plate was then developed with the developing solvent of 15 ml. of diethylamine and 85 ml. of chloroform. The developing times varied between 55 and 60 minutes. At the conclusion of the developing period, the chromatographic plate was removed and placed in an air circulating oven for 30 minutes at 55 to 60°. The plate was removed from the oven and allowed to cool. It was then sprayed with iodoplatinic acid reagent (19). The alkaloids appeared as dark purple spots. The hyoscyamine and scopolamine spots were removed from the plate by removing all the silica gel from around the spots, using a spatula and a camel's hair brush, the alkaloid spots were scraped and brushed into separate counting planchets. The planchet containing the alkaloid on the silica gel was wetted with 1 drop of distilled water and stirred with a micro spatula until a uniform slurry was formed. The planchet was then placed under an infrared heating lamp and dried for 5 minutes. After cooling, the planchet was ready for counting.

Determination of Radioactivity of the Alkaloids.—The detection and recording of the activity of the radioactive alkaloids was accomplished with a windowless Nuclear-Chicago D-47 gas flow counter and a Nuclear-Chicago model 181B scaler. Nuclear-Chicago "Q-gas" (98.7% helium, 1.3% butane) was used. The planchet containing the radioactive alkaloid was inserted into the lead-shielded counting chamber and counted for a period of 30 minutes at 8 lb. pressure and 1300 v. The counts recorded by the scaler were converted and recorded as counts per

[1] Obtained from Nuclear-Chicago Corp., Des Plaines, Ill.
[2] E. Merck AG., Darmstadt, Germany (obtained from Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.).

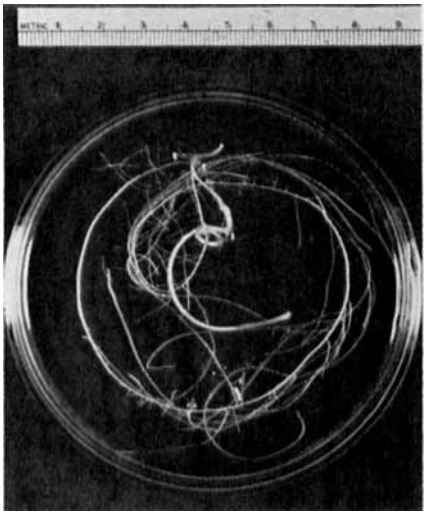


Fig. 1.—*D. stramonium* variety *tatula* root culture grown in control nutrient solution.

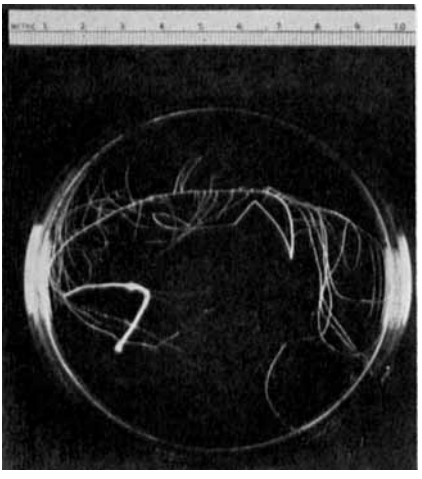


Fig. 2.—*D. innoxia* root culture grown in control nutrient solution.



Fig. 3.—*D. stramonium* variety *tatula* root culture grown in 0.001 mg. of unlabeled *l*-proline per 50 ml. of solution.

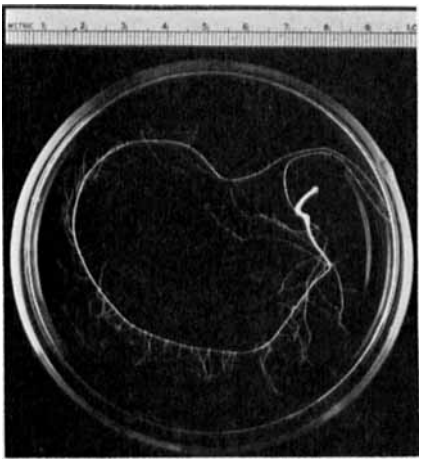


Fig. 4.—*D. innoxia* root culture grown in 0.001 mg. of unlabeled *l*-proline per 50 ml. of solution.

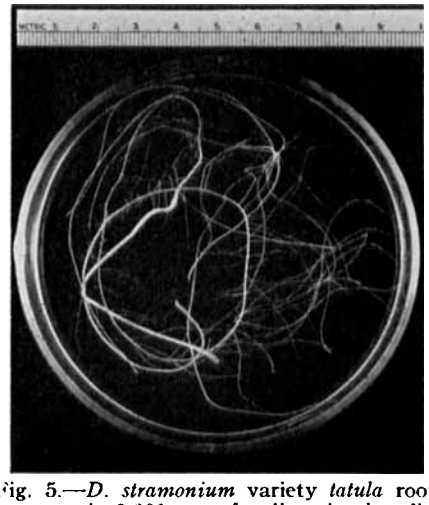


Fig. 5.—*D. stramonium* variety *tatula* root culture grown in 0.001 mg. of radioactive *l*-proline per 50 ml. of solution.

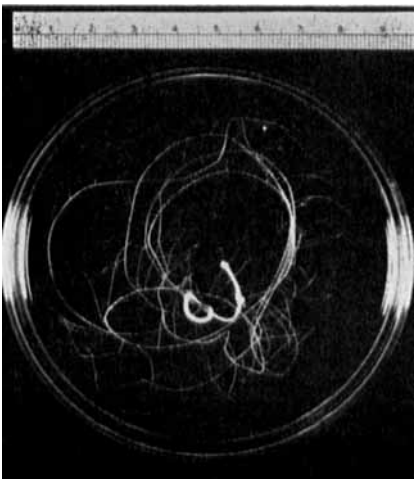


Fig. 6.—*D. innoxia* root culture grown in 0.001 mg. of radioactive *l*-proline per 50 ml. of solution.

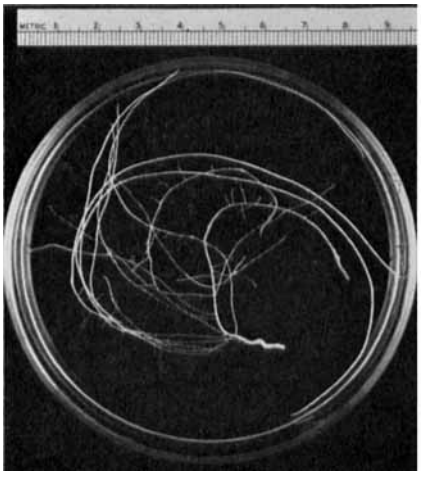


Fig. 7.—*D. stramonium* variety *tatula* root culture grown in 0.002 mg. of radioactive *l*-proline per 50 ml. of solution.



Fig. 8.—*D. innoxia* root culture grown in 0.002 mg. of radioactive *l*-proline per 50 ml. of solution.

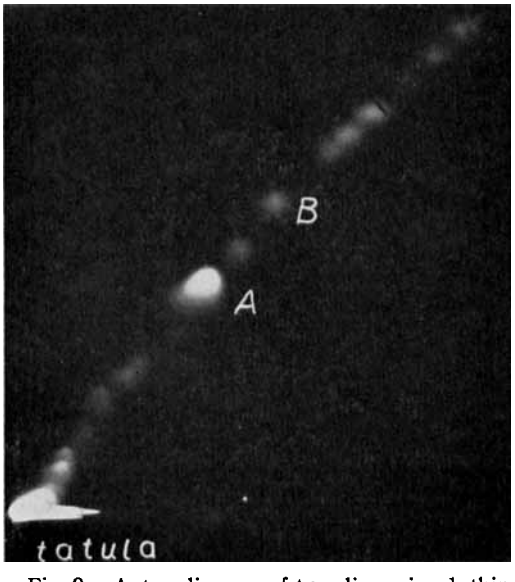


Fig. 9.—Autoradiogram of two-dimensional, thin-layer chromatographic separation of the components of an extract of a *D. stramonium* variety *tatula* root culture grown in a solution containing radioactive proline. Key: A, hyoscyamine; B, scopolamine.

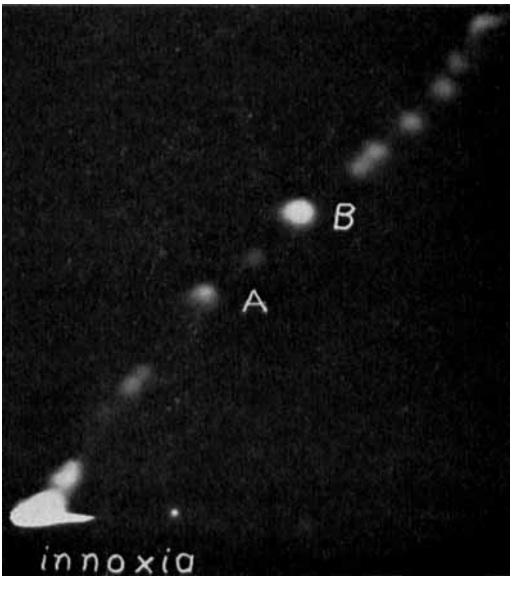


Fig. 10.—Autoradiogram of two-dimensional, thin-layer chromatographic separation of the components of an extract of a *D. innoxia* root culture grown in a solution containing radioactive proline. Key: A, hyoscyamine; B, scopolamine.

TABLE I.—DATA FOR DRY WEIGHT OF ROOTS OF *D. stramonium* VARIETY *tatula* AND *D. innoxia* GROWN IN CONTROL AND PROLINE-FORTIFIED NUTRIENT SOLUTIONS

	No. of Roots		Mean, mg.		Variance		95% Confidence Interval, mg.	
Nutrient Soln.	*D. stramonium tatula*	*D. innoxia*	*D. stramonium tatula*	*D. innoxia*	*D. stramonium tatula*	*D. innoxia*	*D. stramonium tatula*	*D. innoxia*
Control	102	107	14.6	9.2	52.84	14.19	16.0–13.2	9.9–9.5
Proline, 0.001 mg. 50 ml.	30	18	15.5	7.0	24.59	11.02	17.3–13.7	7.9–6.1
Proline,[a] 0.001 mg. 50 ml.	46	22	17.9	7.0	29.30	5.1	19.5–16.3	8.0–6.0
Proline,[a] 0.002 mg.	11	12	10.9	5.6	8.84	6.23	12.9–8.9	7.0–4.2

[a] Radioactive.

minute. The background count throughout this investigation ranged from 23 to 29 counts per minute.

Autoradiography was also employed in this investigation for the determination of the radioactivity of the individual alkaloids to amplify the direct-count data. X-ray exposure holders were constructed to conform to the size of the 8-in. square thin-layer chromatogram plates. All procedures were performed in a photography dark room. The developed thin-layer chromatogram was inserted into the X-ray exposure holder and 2 in. cut from the 8 by 10-in. Kodak medical X-ray, tinted, safety base, duplitized, no-screen film. The 8-in. square film was then placed directly on the thin-layer chromatogram and the X-ray exposure holder securely closed to prevent any light leakage. The sealed holder was weighted down and stored in a dark dry cabinet for an exposure period of 30 days. At the conclusion of the exposure period, the exposed film was removed from the holder and developed for 5 minutes in Kodak D19 developer solution. The resulting autoradiograms showed that all the alkaloids separated from the extract were radioactive.

A two-way autoradiogram was run for *D. stramonium* variety *tatula* and *D. innoxia* extracts of roots grown in 0.002 mg. of *l*-proline-C^{14} in 50 ml. of media for 2 weeks. The first and second developing phases were, respectively, 10:90 and 15:85 of diethylamine:chloroform. Exposure time was 5 months.

RESULTS

D. stramonium variety *tatula* and *D. innoxia* control roots grown in White's media are shown in Figs. 1 and 2. Roots grown in unlabeled *l*-proline are shown in Figs. 3 and 4, in *l*-proline-C^{14} in Figs. 5 and 6, and in double strength *l*-proline-C^{14} in Figs. 7 and 8. The autoradiograms of the root extracts are shown in Figs. 9 and 10.

The dry weight determinations indicate that *D. stramonium* variety *tatula* control roots had a mean dry weight of 14.6 mg.; roots grown in 0.001 mg. unlabeled proline, 15.5 mg.; roots grown in 0.001 mg. labeled proline, 17.9 mg.; and roots grown in 0.002 mg. labeled proline, 10.9 mg. *D. innoxia* control roots showed a mean dry weight of 9.2 mg.; roots grown in 0.001 mg. unlabeled proline, 7.0 mg.; roots grown in 0.001 mg. labeled proline, 7.0 mg.; and roots grown in 0.002 mg. labeled proline, 5.6 mg. These results are summarized in Table I.

Radioactivity determinations of isolated hyoscyamine and scopolamine of both species are shown in Tables II and III. It is obvious that the roots of both species were able to incorporate and metabolize the radioactive carbon of proline into hyoscyamine and scopolamine. It is also interesting to note that *D. stramonium* variety *tatula* which produces predominantly hyoscyamine incorporated more proline into this compound than into scopolamine. The ratio of radioactivity of hyoscyamine compared to scopolamine approaches 2:1. However, in *D. innoxia*, where the plant produces predominantly scopolamine, more proline is incorporated into this compound. The ratio of radioactivity is hyoscyamine compared to scopolamine approaches 1:2. This difference in proline incorporation in the two species is further borne out by the autoradiograms, Figs. 9 and 10.

DISCUSSION

From this study and a previous one (6), the conclusion that exogenous proline affects metabolism in the *Datura* species tested can be made. This effect is seen both in the habit of growth of the roots and total root growth. The response is associated in some unknown way with the concentration of the supplied exogenous proline, in this investigation made more complex by the degree of radioactivity of the supplied proline. The inhibitory action of

TABLE II.—DRY WEIGHT AND RADIOACTIVITY OF ALKALOIDS OF *D. stramonium* VARIETY *tatula* ROOTS GROWN IN 0.001 mg. LABELED PROLINE PER 50 ml. OF SOLUTION

Root No.	Root Wt., mg.	Counts Per Min.[a] Hyoscyamine	Hyoscine	Root No.	Root Wt., mg.	Counts Per Min.[a] Hyoscyamine	Hyoscine
1	10.0	12	17	24	15.8	31	15
2	11.9	16	19	25	17.0	21	8
3	19.6	14	16	26	21.5	27	7
4	11.4	25	8	27	27.1	11	6
5	31.1	22	30	28	20.3	21	13
6	30.2	12	5	29	20.6	39	10
7	21.6	33	25	30	23.0	33	20
8	9.2	30	14	31	24.1	32	20
9	23.7	28	42	32	15.9	40	13
10	8.5	10	18	33	14.8	32	15
11	18.9	18	15	34	19.9	36	17
12	15.8	34	23	35	14.5	34	12
13	12.3	27	9	36	18.8	18	24
14	19.6	13	3	37	23.9	41	18
15	15.4	31	3	38	13.0	26	15
16	11.0	29	29	39	16.8	22	21
17	24.8	41	21	40	19.8	50	16
18	14.2	8	0	41	19.3	41	19
19	18.3	21	17	42	16.0	18	3
20	21.1	28	9	43	26.2	25	24
21	13.2	13	9	44	12.8	39	13
22	20.2	25	13	45	18.9	26	12
23	10.2	12	0	46	14.8	20	7
Mean					17.9	26	15

[a] Half-hour counts.

TABLE III.—DRY WEIGHT AND RADIOACTIVITY OF *D. innoxia* ROOTS GROWN IN 0.001 mg. LABELED PROLINE PER 50 ml. OF SOLUTION

Root No.	Root Wt., mg.	Counts per Min.[a] Hyoscyamine	Hyoscine
1	3.7	2	6
2	6.6	7	7
3	9.2	5	8
4	3.2	0	0
5	8.8	9	16
6	7.7	3	15
7	10.0	9	23
8	8.6	8	13
9	5.9	2	4
10	4.2	2	7
11	6.5	0	2
12	4.9	2	5
13	5.5	1	9
14	11.3	7	13
15	7.5	6	16
16	5.8	0	0
17	9.3	7	23
18	3.5	3	2
19	8.9	6	16
20	8.9	12	18
21	6.4	0	0
22	7.5	1	9
Mean	7.0	4	10

[a] Half-hour counts.

amino acids on plants is not new (20, 21). Klein and Linser (22) found that in cut tobacco plants in proline-containing nutrient media the nicotine content increased in leaves and decreased in stems in some instances; whereas in others the media increased the nicotine content of both stems and leaves. The mechanism of these functions is unknown.

Some insight into the fate in plants of exogenous proline has been supplied by studies utilizing proline-C^{14}. The ready incorporation of carbon from proline in carrot explants has been shown by Pollard and Steward (12). They concluded from this study that the proline which enters the cell is either incorporated immediately into protein or, escaping this fate, is respired away. That the bulk of the proline appears as protein rather than as carbon dioxide suggests that protein synthesis, or at least the synthesis of proline-containing protein, occurs in the nonparticulate cytoplasm fraction before the proline ever gets to the principal site of the respiratory process—namely, the mitochondrial particles.

Thus, it can be anticipated that proline from an exogenous source is incorporated into the metabolic pool of *D. stramonium* variety *tatula* and *D. innoxia*. This is borne out by several observations. The dried roots of each are highly radioactive. The extract prepared as described in this paper is highly radioactive, but less so than the entire root. The hyoscyamine and scopolamine as well as other unidentified compounds are radioactive, separating out on the chromatogram as evidenced on the autoradiograms. The hypothesis that proline enters into the normal metabolism of the plant also is suggested by the fact that in the "hyoscyamine plant" (*D. stramonium* variety *tatula*) the greater radioactivity was in the hyoscyamine, whereas in the "scopolamine plant" (*D. innoxia*) the greater radioactivity was in the scopolamine.

SUMMARY

When *l*-proline-C^{14} was supplied to isolated roots of *D. stramonium* variety *tatula* and *D. innoxia*, growth was stimulated in the former using 0.001 mg. per 50 ml. of nutrient solution; however, growth was inhibited in the former at the 0.002-mg. concentration. Inhibition of growth was seen in *D. innoxia* at both levels of concentration.

The radioactive proline was picked up from the solution by the roots of both species and incorporated into hyoscyamine and scopolamine. The *D. stramonium* variety *tatula* roots which produce primarily hyoscyamine, compared to scopolamine, incorporated more radioactive proline into the principal alkaloid. *D. innoxia* roots which produce primarily scopolamine, compared to hyoscyamine, incorporated more radioactive proline into the principal alkaloid. There is evidence that the proline enters into the metabolism of these plants, possibly by way of protein. The work suggests that proline is an important compound utilized by these plants in the biosynthesis of the tropane alkaloids.

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Hydrolysis of Idoxuridine

By LOUIS J. RAVIN, CHARLES A. SIMPSON, ALFRED F. ZAPPALA, and JOHN J. GULESICH

Data are presented for the hydrolytic degradation of idoxuridine (5-iodo-2′-deoxyuridine) in aqueous solution over a wide range of hydrogen ion concentration (pH 1.3 to 12.0). The over-all reaction rate was experimentally equal to $k_1 \frac{[H^+]}{[H^+] + K_a} + k_2 \frac{K_a}{K_a + [H^+]} + k_3 \frac{K_a}{K_a + [H^+]} [OH^-]$. The heat of activation for the hydrolysis of idoxuridine at pH 6.0 was found to be approximately 25 Kcal. per mole. A probable pathway for the hydrolysis of idoxuridine in acid and alkaline solution is discussed.

IDOXURIDINE (5-iodo-2′-deoxyuridine) has been reported to be an effective chemotherapeutic agent for the treatment of herpetic keratitis (1). Its chemical, biological, and pharmaceutical properties have been described previously (2). Results of clinical studies have also been reported (3, 4). There are several reports that discuss the enzymatic pathways for the metabolism of idoxuridine and related pyrimidines (5–7). However, the literature is lacking in information concerning its chemical degradation. The scope of the present work was concerned with the hydrolytic degradation of idoxuridine in acidic and alkaline solution.

EXPERIMENTAL

Materials.—The idoxuridine, 5-iodouracil, 2′-deoxyuridine, and uracil used in this study were obtained from California Corporation for Biochemical Research, Los Angeles, Calif. All reagents used were of analytical grade.

Apparatus.—A Cary model 11 recording spectrophotometer, Beckman zeromatic pH meter, constant temperature oven, and Pyrex glass chromatographic columns 19 mm. in diameter and 25 cm. long fitted with a stopcock were used.

Chromatographic Columns.—The chromatographic columns were packed according to the previously described procedure (8).

Assay.—A satisfactory partition chromatography system was developed and subsequently used to separate the ingredients of the hydrolyzed samples (8). A typical chromatogram of a known equimolar mixture of idoxuridine, 5-iodouracil, 2′-deoxyuridine, and uracil is shown in Fig. 1. The chromatogram of an artificially decomposed sample is also shown in Fig. 1. The results indicate that idoxuridine can be separated from its breakdown products by this procedure.

Hydrolysis Experiments.—In most cases the following procedure was used: 100 mg. (0.0028 mole) of idoxuridine was dissolved in 100 ml. of the appropriate buffer solution. The solution was placed in 10-ml. ampuls. The ampuls were sealed and heated at 60° in a constant temperature oven Ampuls were removed at the appropriate time intervals, cooled, and analyzed.

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RESULTS AND DISCUSSION

Order of Reaction.—The rate of disappearance of idoxuridine from the solution was an over-all first-order reaction with respect to idoxuridine at pH values from 1.3 to 12.0 at constant temperature. There was a linear relationship between time and logarithm of residual idoxuridine concentration as shown in Fig. 2. The observed rate constants at the respective pH values are listed in Table I. There was no significant change in the apparent first-order rate constant when the initial concentration of idoxuridine was varied.

Primary Salt Effect.—A primary salt effect should be expected where a reaction between two charged species is involved in a hydrolytic process. Since idoxuridine exists primarily in the unionized form in highly acidic and relatively neutral solutions, one would expect that the primary salt effect would be negligible. Experiments carried out at pH 1.3 and 6.3 showed that there was no primary salt effect. However, at pH 12.0, where idoxuridine exists in the ionized form, a primary salt effect was expected. This was borne out during the study so that all

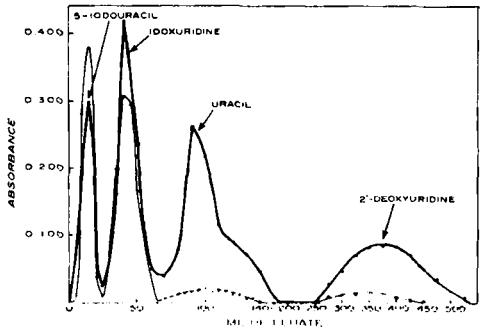


Fig. 1.—Comparison of partition chromatograms for a known equimolar mixture of idoxuridine and its possible degradation products and an artificially degraded sample stored for 18 months at 37°. 0.1 *N* HCl was used as the internal phase; a mixture consisting of five parts chloroform and one part *n*-butanol was used as the eluant. Data for the known mixture were previously reported (8). Key: ● known mixture; O, artificially degraded sample.

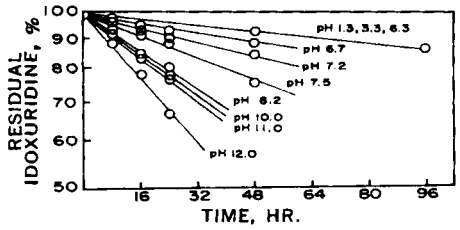


Fig. 2.—Plot showing the over-all first-order character of the hydrolysis of idoxuridine at different pH values at 60°. For the actual runs, the pH was adjusted as follows: 0.1 *N* hydrochloric acid (pH 1.3), 0.02 *M* citrate (pH 3.3), 0.036 *M* phosphate (pH 6.3, 6.7, 7.2, 7.5), 0.06 *M* borate (pH 8.2), and 0.1 *N* NaOH (pH 10.0, 11.0, and 12.0).

experiments at pH values above 10.0 were carried out in solutions adjusted to constant ionic strength with sodium chloride.

pH Dependence.—The pH-rate profile indicated in Fig. 3 shows the relationship between pH and the observed reaction rate over a pH range from 1.3 to 12.0. The nature of the curve suggests that the over-all degradative rate for the hydrolysis of idoxuridine represents a summation of the following reactions:

$$\text{Idoxuridine} \xrightarrow{k_1} \text{products}$$

$$\text{Idoxuridine}^- \xrightarrow{k_2} \text{products}$$

$$\text{Idoxuridine}^- + \text{OH}^- \xrightarrow{k_3} \text{products}$$

The over-all reaction velocity is equal to the sum of the rates of these reactions and can be represented by

$$k_{obs.} = k_1 \frac{[H^+]}{[H^+] + K_a} + k_2 \frac{K_a}{K_a + [H^+]} + k_3 \frac{K_a}{K_a + [H^+]} [OH^-] \quad \text{(Eq. 1)}$$

The theoretical rate of disappearance of idoxuridine was calculated for the pH range of 1.3 to 12.0 using the following coefficients in the above rate equation: $k_1 = 0.0015$ hour^{-1}; $k_2 = 0.0105$ hour^{-1}; $k_3 = 0.5600$ hour^{-1}; K_a (acid dissociation constant) $= 10^{-8.0}$.[1] The calculated rate appears as the solid line in Fig. 3. It is in good agreement with the experimentally determined rate.

The data indicate that the rate of breakdown is independent of pH over the range 1.3 to 6.3. In this region $[H^+]$ is high compared to K_a; therefore, the values of $k_2 \frac{K_a}{K_a + [H^+]}$ and $k_3 \frac{K_a}{K_a + [H^+]} [OH^-]$ are negligible compared to $k_1 \frac{[H^+]}{[H^+] + K}$ and may be omitted, thus

$$k_{obs.} = k_1 \frac{[H^+]}{[H^+] + K_a} \quad \text{(Eq. 2)}$$

Between pH 6.3 and approximately 9.0 there is a sharp increase in the observed reaction rate. This coincides with an increase in the ionized form in solution. $k_3 \frac{K_a}{K_a + [H^+]} [OH^-]$ is still negligible in this region. Thus in pH range 6.3 to 9.0, Eq. 1 may be expressed as

$$k_{obs.} = k_1 \frac{[H^+]}{[H^+] + K_a} + k_2 \frac{K_a}{K_a + [H^+]} \quad \text{(Eq. 3)}$$

Between pH 9.0 and 11.0 the observed reaction rate tends to level off. In this region idoxuridine exists mainly in the ionized form. k_1 is small compared to k_2 and k_3, and the $[OH^-]$ is low, which makes the values of $k_1 \frac{[H^+]}{[H^+] + K_a}$ and $k_3 \frac{K_a}{K_a + [H^+]} [OH^-]$ negligible compared to $k_2 \frac{K_a}{K_a + [H^+]}$, and they may be disregarded giving

$$k_{obs.} = k_2 \frac{K_a}{K_a + [H^+]} \quad \text{(Eq. 4)}$$

Above pH 11.0 the reaction rate increases rapidly. In this region $k_1 \frac{[H^+]}{[H^+] + K_a}$ is negligible and may be eliminated from Eq. 1 which may be written

$$k_{obs.} = k_2 \frac{K_a}{K_a + [H^+]} + k_3 \frac{K_a}{K_a + [H^+]} [OH^-] \quad \text{(Eq. 5)}$$

In this pH range the reaction is base catalyzed and changes from first order to pseudo first order.

TABLE I.—EXPERIMENTAL RATE CONSTANTS FOR THE HYDROLYSIS OF IDOXURIDINE AT 60° C.

pH	Observed First-Order Rate Constant hr.$^{-1}$ × 10^3	
1.3	1.51	
3.3	1.51	
6.3	1.51	1.54[a]
6.7	2.44	
7.2	3.31	
7.5	5.73	
8.2	8.80	
10.0	10.35	
11.0	10.90	
12.0	16.70	

[a] Initial concentration, 0.075% (0.0021 *M*).

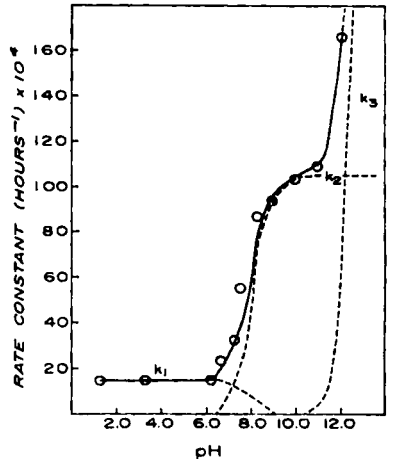


Fig. 3.—Plot showing the pH-rate profile for the hydrolysis of idoxuridine at 60°. Key: O, experimental results; ———, theoretical pH-rate profile; -------, contribution of k_1, k_2, and k_3 at any pH value.

[1] Determined spectrophotometrically at 60°.

TABLE II.—COMPARISON OF APPROXIMATE R_f VALUES OBTAINED FOR IDOXURIDINE, SOME POSSIBLE DECOMPOSITION PRODUCTS, AND AN ARTIFICIALLY DECOMPOSED ALKALINE SOLUTION OF IDOXURIDINE

Material	Known Soln.	Artificially Decomposed Alkaline Soln., (pH 10.0)
Unknown	...	0.00 (significant)
2′-Deoxyuridine	0.28	0.28 (small)
Uracil	0.33	...
Idoxuridine	0.34	0.36 (large)
2-Deoxyribose	0.42	...
5-Iodouracil	0.55	...

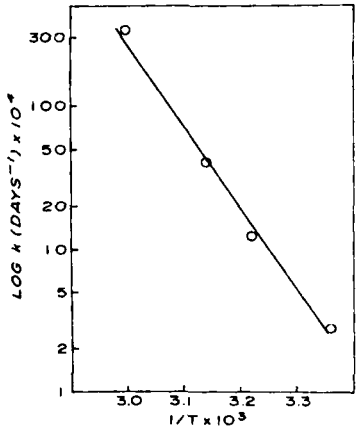


Fig. 4.—Typical Arrhenius-type plot showing the temperature dependency for the hydrolysis of idoxuridine at pH 6.0.

Probable Pathway for the Hydrolysis of Idoxuridine.—Data were presented in an earlier report (9) indicating that 5-iodouracil and 2-deoxyribose were the principal products formed during the hydrolysis of idoxuridine in acid solution. Preliminary work has indicated that alkaline hydrolysis at pH 10.0 results in a complex mixture. A paper chromatogram of this mixture (system: pH 8 buffer-2-methyl-3-butyn-2-ol) showed that the principal breakdown product is one which has lost its ultraviolet absorption characteristics (U.V. absorbance) but retained its deoxyribose moiety (gives a red coloration with cysteine in sulfuric acid). This suggests that the pyrimidine ring has been destroyed. 2′-Deoxyuridine was also found to be a product of alkaline hydrolysis. This is indicated in Table II, which contains a comparison of the R_f values of idoxuridine, some possible decomposition products, and an artificially decomposed alkaline solution (pH 10.0) of idoxuridine. The R_f values were obtained by paper chromatographic analysis using a *n*-butanol-3 *N* ammonia system.

On the basis of the previous data (9) we can assume that idoxuridine is hydrolyzed in acid solution at the riboside linkage to form 5-iodouracil. Garrett and Suzuki (10) presented data suggesting that uracil was the principal product of hydrolysis in strong acid solution. Uracil was not detected in an appreciable amount under the conditions of our study. In alkaline solution the ultraviolet absorption spectrum disappears, suggesting that the pyrimidine ring is destroyed. Since some 2′-deoxyuridine is found, the pathway for degradation may proceed through 2′-deoxyuridine.

Temperature Dependency.—Rate constants for the over-all disappearance of idoxuridine were obtained over the temperature range from 25 to 60° at pH 6.0. A typical Arrhenius-type plot of the data is presented in Fig. 4. From these data the apparent heat of activation for the process was determined as approximately 25 Kcal. per mole.

SUMMARY

(*a*) The hydrolysis of idoxuridine was first order with respect to idoxuridine over a wide range of hydrogen ion concentration (pH 1.3 to 12.0).

(*b*) The over-all rate of disappearance of idoxuridine in aqueous solution as a function of pH can be described using

$$k_{obs.} = k_1 \frac{[H^+]}{[H^+] + K_a} + k_2 \frac{K_a}{K_a + [H^+]} + k_3 \frac{K_a}{K_a + [H^+]} [OH^-]$$

A theoretical pH-rate profile calculated using the above equation is in good agreement with the experimentally determined values.

(*c*) The probable pathway for the hydrolysis is presented. The data suggest that 5-iodouracil is the principal product of acid hydrolysis. Alkaline hydrolysis results in a complex mixture containing 2′-deoxyuridine and a compound with no ultraviolet absorption spectrum. The data suggest that the pyrimidine ring is destroyed during alkaline hydrolysis.

(*d*) The heat of activation (ΔH_a) for the reaction was approximately 25 Kcal. per mole at pH 6.0.

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Laxatives II

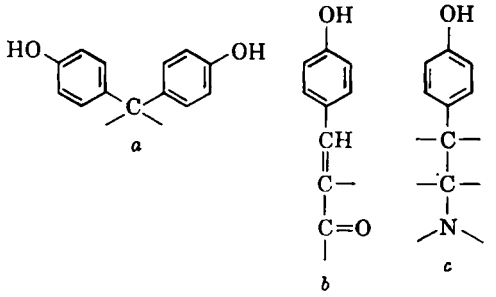
Relationship Between Structure and Potency

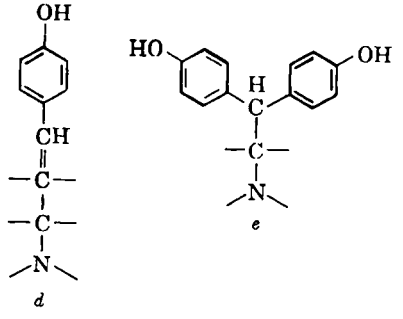
By MAX H. HUBACHER and SIDNEY DOERNBERG

The Relative Laxative Potency of a series of compounds was determined. These were mainly phenols containing certain groupings. We find two phenolic HO-groups, preferably in the configuration (*p*) HO-C_6H_4-C-C_6H_4-OH (*p'*) in the synthetic organic laxatives most commonly employed commercially. The two exhibiting the highest potency also contained a nitrogen ring. The Macaque monkey (*Macaca mulatta* and *M. speciosa*) gave the most consistent and meaningful potency values, data which could be applied successfully to humans in laxative action.

IN A PREVIOUSLY published paper on this subject (1), the observation was recorded that of 36 compounds tested, only those which had at least two phenolic hydroxyl groups had laxative properties, preferably those with the grouping *a* in their molecule. In the present work, this concept was explored further with the preparation and testing of additional compounds.

Included in this investigation were three phthaleins—an isomer of *o*-(4,4'-dihydroxybenzhydryl) benzyl alcohol, the latter previously found to be an excellent laxative (1); a mono-, di-, and tetrahydric alcohol; commercially available sennoside (2), which is a mixture of the naturally occurring glucosides extracted from senna; and other compounds having either of the groupings *a*, *b*, *c*, *d*, or *e* in their molecule.





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Methods of preparation are given for new compounds or for known compounds when the method either gives a better product or a better yield than the published one.

LAXATIVE POTENCY

The laxative potency of these compounds was determined on monkeys, as previously described (1). Until recently, the rhesus monkey (*Macaca mulatta*) was used exclusively because of the close similarity to human reaction to laxatives. Recently, Kling and Orbach (3), also Hensley and Richmond (4), described their experiences with a subspecies, *Macaca speciosa* or stump-tailed monkey. On the basis of their findings regarding the anatomical and behavioral characteristics of this species, it was decided to determine their suitability as a subject in laxative potency tests.

Results show that this animal makes an excellent subject and compares favorably with rhesus monkeys. Their response to laxatives is consistent, they take their doses willingly, are more docile and more resistant to infection than the rhesus monkey.

The laxative potency was determined by the equieffective dose method, compared to the same dose of a phenolphthalein N.F. XI, whose purity and laxative response had been determined on animals and humans. The doses were administered in food, mixed with cereal, or placed in wedges of apple, banana, melon, etc. The bottom of the cages are provided with a raised grid with 0.5-in. mesh opening, so that the soft stools are deposited on the supporting tray and can be easily counted. Three consecutive days of hard stools must intervene between administration of doses. If the initial dose produces no soft stools, it is increased by 1 mg./Kg. increments at 3-day intervals until a response of from two to five soft stools over a 3-day period is obtained. This dose is a direct relationship to the Unit Standard Dose for that animal and the Relative Laxative Potency (R.L.P.) is expressed in units obtained by the formula

$$\text{R.L.P.} = \frac{\text{Minimum Laxative Dose of Standard in mg./Kg.}}{\text{Minimum Laxative Dose of Unknown in mg./Kg.}}$$

This method is somewhat modified from previously published procedures (5–7) and has given consistent and comparable results.

For our purpose, a compound whose R.L.P. was

less than 0.1, *i.e.*, if a dose 10 times the minimum effective dose of phenolphthalein N.F. XI did not produce laxative effect, the compound was inactive for practical purposes.

EXPERIMENTAL[1]

Preparation of Compounds

Phenolthymolphthalein or 3-(4-Hydroxyphenyl)-3-(2-methyl-4-hydroxy-5-isopropyyphenyl)phthalide (I).—A mixture of 60.5 Gm. (0.4 mole) of thymol,[2] 48.4 Gm. (0.2 mole) of 2-(4-hydroxybenzoyl)benzoic acid (10), 20 Gm. of anhydrous zinc chloride, and 0.16 ml. of concentrated sulfuric acid was stirred for 2 hours at 120°. Dilute hydrochloric acid then was added and the unreacted thymol was steamed out (28 Gm.). The crude phthalein (70.5 Gm., m.p. 270–275°) was dissolved in 1600 ml. of boiling ethanol. On cooling, four crops of crystals totaling 67.8 Gm. (90%), m.p. 284–286°, were obtained.

The pure phthalein (I) formed colorless needles, melting at 285–286° [Ghatak (11) gives a m.p. of 231–232° and Kin (12) gives a m.p. of 276° for this phthalein]; it dissolved in normal sodium hydroxide with a deep purple color.

Anal.—Calcd. for $C_{24}H_{22}O_4$: C, 76.98; H, 5.92. Found: C, 77.14; H, 6.27.

Subjecting 7.5 Gm. of I to the Friedländer oxime splitting (10), yielded 1.4 Gm. of 2-(4-hydroxybenzoyl)benzoic acid and 1.1 Gm. of an amine, m.p. 177–179°, which was identical with 2-isopropyl-4-amino-5-methylphenol (13). This finding proves that both HO-groups in I are in the *para* position to the center carbon atom of the triphenylmethane molecule.

Diacetylderivative of I.—This compound, recrystallized from ethanol, formed colorless crystals melting at 156.3 to 157.1°.

Anal.—Calcd. for $C_{28}H_{26}O_6$: C, 73.36; H, 5.67; mol. wt. 458. Found: C, 73.28; H, 5.37; mol. wt. 480.

The Dimethylether of I.—This compound, prepared from I by either the methyl iodide or the dimethyl sulfate method, after crystallization from 41% ethanol (1 Gm. in 240 ml.) formed colorless, fine needles, melting at 141.3 to 142.1° [Kin (12) reported a m.p. of 122°].

Anal.—Calcd. for $C_{26}H_{26}O_4$: C, 77.58; H, 6.51. Found: C, 77.20; H, 6.22.

Phenolcatecholphthalein or 3-(p-Hydroxyphenyl)-3-(m,p-dihydroxyphenyl)phthalide (II).—This phthalein was not easily purified since it did not crystallize readily. Therefore, patience was needed to obtain seed crystals.

The following procedure gave a good product in fair yields. A mixture of 22.0 Gm. (0.2 mole) of catechol, 48.4 Gm. (0.2 mole) of 2-(4-hydroxybenzoyl)benzoic acid (10), 20 Gm. anhydrous zinc chloride, and 0.1 ml. of concentrated sulfuric acid were stirred for 2 hours at 120°. To remove the dark, sticky mass from the flask, it was refluxed with 200 ml. of ethanol until dissolved, requiring 1 to 2 hours. The solution was poured into 1 L. of water. The precipitated, pliable mass became hard after standing for several days (56–61 Gm., m.p. 192–198°). It was crystallized from 50% acetic acid (1 Gm. in 2 ml.) or from 20% ethanol (1 Gm. in 20 ml.), stirring the solution while cooling at a rate of 1° an hour; 45–50 Gm. (67–75%), m.p. 203–206°, was obtained.

Sometimes, even after seeding, no crystals could be obtained. In such cases, the amorphous mass was acetylated and the purified triacetyl derivative hydrolyzed as described below.

The pure phenolcatecholphthalein (II) melted at 203.5–208.0° [Ghatak (11) recorded a m.p. of 148–149°, while Kin (12) could not obtain it in crystalline form].

Anal.—Calcd. for $C_{20}H_{14}O_5$: C, 71.85; H, 4.22; mol. wt. 334. Found: C, 71.48; H, 4.46; mol. wt. 339.

The dark reddish-brown solution of II in 1 *N* sodium hydroxide turned brown on exposure to air at 25° for several weeks, and 2-(4-hydroxybenzoyl)-benzoic acid was isolated from the solution.

The trimethylether of II was obtained as a non-crystallizable oil.

Triacetylderivative of II.—This compound was prepared from II, acetic anhydride, and a trace of sulfuric acid. After crystallizations from ethanol (1 Gm. in 16 ml.), it melted at 154.5 to 155.6° [Kin (12) recorded a m.p. of 148°].

Anal.—Calcd. for $C_{26}H_{20}O_8$: C, 67.82; H, 4.38; mol. wt. 460. Found: C, 67.45; H, 4.73; mol. wt. 451.

This triacetylderivative was best hydrolyzed by refluxing for 1.5 hours a solution of 6 Gm. in 30 ml. of ethanol and 0.05 ml. of sulfuric acid. The solvent was evaporated *in vacuo* and 100 ml. of water was added to the residue. The amorphous precipitate of II slowly became crystalline.

Alkaline hydrolysis is not recommended because of the instability of II in alkaline solutions.

Phenoltoluenephthalein or 3-(p-Hydroxyphenyl)-3-(p-tolyl)phthalide III.—A mixture of 48.0 Gm. (0.2 mole) of *o*-(*p*-toluoyl)benzoic acid (14), 28.2 Gm. (0.3 mole or 50% excess) of phenol, 20 Gm. anhydrous zinc chloride, and 0.5 ml. concentrated sulfuric acid were stirred for 8.5 hours at 120–125°. The hot, viscous reaction mass was poured into 1 L. of water, removing the part adhering to the flask with hot ethanol. The crude phthalein was washed five times with hot water, cooling and decanting the water each time. The phthalein (60–65 Gm., m.p. 121–140°), which now had become hard, was crystallized from acetic acid (1 Gm. in 2.5 ml.). If no seed crystals are available, then crystallization may not set in for many weeks. Two crops of crystals, totaling 37.3 to 41.8 Gm. (59–66%), m.p. 149–153°, were obtained.

This phthalein may also be crystallized from benzene (1 Gm. in 8 ml., recovery 93%), from which solvent it crystallizes with 1 mole of solvate benzene, which it loses slowly at 25°, rapidly at 120°. Calcd. for $C_{21}H_{16}O_3 \cdot C_6H_6$: benzene 19.8%; loss at 120°: 20.4 ± 1.1%.

The pure III melts at 154.1 to 155.9°.

Anal.—Calcd. for $C_{21}H_{16}O_3$: C, 79.73; H, 5.10; mol. wt. 316. Found: C, 79.70; H, 5.90; mol. wt. 319.

A solution of III in 5 *N* sodium hydroxide is colorless, whereas a solution in sulfuric acid is orange. III is very soluble in acetone, ethanol, *n*-butanol, cyclohexanone, and in chloroform.

[1] All melting points are corrected. Molecular weights determined by the Signer method as described by Clark (8). Color designations are by the Munsell color system (9).

[2] This is an excess of 100% over theory, the excess acting as a solvent.

TABLE I.—COMPOUNDS AND THEIR LAXATIVE POTENCY

No.	Name	Contains Group	Prepn.	M.p., °C. cor.	Potency (N.F. Phenolphthalein = 1)
I	Phenolthymolphthalein	...	Described in text	285–286	Inactive
II	Phenolcatecholphthalein	...		203.5 to 208.0	1.4
III	Phenoltoluenephthalein	...		154.1 to 155.9	2.0
IV	*o*-(4-Hydroxy-4′-methylbenzhydryl) benzoic acid	...		221.7 to 222.5	Inactive
V	Pyromellityl alcohol	...		191.2 to 191.8	Inactive
VI	3,6-Dihydroxy-9-xanthone	...		>320	Inactive
VII	1,1-Bis(*p*-hydroxyphenyl)-cyclohexane	*a*		187.8 to 188.9	Inactive
VIII	2-Phenyl-4-(*p*-hydroxybenzylidene)-5-oxazolone	*c*		221.5 to 221.9	Inactive
IX	*p*-Hydroxybenzylidene-hydrazide of the bis(*p*-hydroxyphenyl)acetic acid	*a*, *e*		262.5 to 263 0	Inactive
X	2-(*p*-Hydroxystyryl)pyridine	*d*		216.4 to 217.4	Inactive
XI	2,6-Bis(*p*-hydroxystyryl)-pyridine	*d*		258.5 to 259.0	Inactive
XII	2-(*p*-Hydroxyphenyl)-1,3-indandione	...		177.1 to 178.1	Inactive
XIII	*o*-(2,4-Dihydroxybenzhydryl)-benzoic acid	...	Hubacher, M. H., *J. Org. Chem.*, **23**, 1402 (1958).	186.9 to 187.4	Inactive
XIV	*o*-(2,4-Dihydroxybenzhydryl) benzyl alcohol	...	*Ibid.*	169.8 to 170.4	2.1
XV	Ricinoleyl alcohol	...	Marketed as Aldol by Archer-Daniels-Midland Co., Cleveland, Ohio	Colorless oi	Inactive
XVI	Phthalyl alcohol	...	Nystrom, R. F., and Brown, W. G., *J. Am. Chem. Soc.*, **69**, 1198(1947).	63.6 to 64.1	Inactive
XVII	Sennosides Sandoz	...	A mixture of equal parts Sennoside A and B (2)	220–250	6.1
XVIII	10,10-Bis(*p*-hydroxyphenyl)-9-anthrone	*a*	Blicke, F. F., and Patelski, R. A., *J. Am. Chem. Soc.*, **60**, 2636(1938).	311–314	Inactive
XIX	4,4′-Dihydroxybenzil	...	Hubacher, M. H., *J. Org. Chem.*, **24**, 1950 (1959).	250.2 to 251.4	Inactive
XX	Ethyl bis(*p*-hydroxyphenyl)-acetate	*a*	*Ibid.*	163.8 to 165.7	Inactive
XXI	Hydrazide of the bis(*p*-hydroxyphenyl)acetic acid	*a*, *e*	Nathansohn, G., *ibid.*, **26**, 1421(1961).	252.0 to 253.6	Inactive
XXII	4,4′-Dihydroxytriphenyl-acetic acid	*a*	*J. Med. Chem.*, **7**, 571(1964).	257–259 dec.	Inactive
XXIII	4,4′-Dihydroxytetraphenyl-methane	*a*	Gomberg, M., and Jickling, R. L., *J. Am. Chem. Soc.*, **37**, 2580(1915).	294–296	Inactive
XXIV	1,5-Bis(*p*-hydroxyphenyl)-1,4-pentadiene-3-one	*b*	Zincke, T., and Mühlhausen, G., *Ber.*, **36**, 131(1903).	245.6 to 246.1	Inactive
XXV	4,4′-Dihydroxychalcone	*b*	Vorländer, D., *ibid.*, **58**, 128(1925).	201.2 to 202.1	Inactive
XXVI	2-(*p*-Hydroxybenzylidene)-1-indanone	*b*	Feuerstein, W., *ibid.*, **34**, 413(1901).	222.3 to 224.6	Inactive
XXVII	2-(*p*-Hydroxybenzylidene)-1,3-indandione	*b*	v. Kostanecki, S., and Laczkowski, *ibid*, **30**, 2141(1897).	240.8 to 242.2	Inactive
XXVIII	2-(*p*-Hydroxybenzoyl)-1,3-indandione	...	Horton, R. L., and Murdock, K. C., *J. Org. Chem.*, **25**, 940(1960).	280–283 dec.	Inactive
XXIX	α-(*p*-Hydroxybenzylidene)-hippuric acid	*c*	Erlenmeyer, E., Jr., and Halsey, J. T., *Ann.*, **307**, 140(1899).	238–240 dec.	Inactive
XXX	3-Phenyl-4-(*p*-hydroxybenzylidene)-5(4)-isoxazolone	*b*, *d*	Wahl, A., and Meyer, A., *Compt. Rend.*, **146**, 639(1908).	213.0 to 213.5	Inactive
XXXI	2,4-Diamino-6(*p*-hydroxystyryl)-*s*-triazine sulfate	*d*	v. Humnicki, *Zentralbl., II*, 706(1907).	290	Inactive
XXXII	di(*p*-Hydroxyphenyl)-(2-pyridyl) methane	*a*, *c*	Karl Thomae, G.m.b.H., Brit. pat. 730,-243; through *Chem. Abstr.*, **50**, 6515*f* (1956).	239–241	24

When 5 Gm. of III and 25 Gm. of KOH were heated in a small distilling flask for 5 minutes at 225°, toluene distilled off. From the cooled melt dissolved in water, 0.91 Gm. benzoic acid and 0.44 Gm. *p*-hydroxybenzoic acid were isolated.

Acetylderivative of III.—This compound, prepared from III and acetic anhydride, was obtained as an oil which became crystalline on rubbing with ethanol. It was purified further by dissolving it in absolute ether (1 Gm. in 10 ml.); on adding petroleum ether (10 ml.) to the solution, fine needles were obtained, which melted at 93–97°.

Anal.—Calcd. for $C_{23}H_{18}O_4$: C, 77.08; H, 5.06. Found: C, 77.17; H, 5.16.

Methylether of III.—This compound was made from III by either the dimethyl sulfate or methyl iodide method. It was crystallized from ethanol (1 Gm. in 2 ml.) or from absolute ether (1 Gm. in 8

ml.), formed rectangular plates, and melted at 94–99°.

Anal.—Calcd. for $C_{22}H_{18}O_3$: C, 79.94; H, 5.49; mol. wt. 330. Found: C, 80.32; H, 5.61; mol. wt. 357.

Phenoltoluenephthalin or o-(4-Hydroxy-4-methylbenzhydryl Benzoic Acid (IV).—To a solution of 15.8 Gm. of III in 60 ml. of 2.5 *N* sodium hydroxide, 5.0 Gm. of powdered Raney alloy (Ni-Al) was added over a period of 1 hour. The temperature of the reaction solution went up to 45–55°. The mixture was stirred for another 30 minutes, the nickel filtered off, and the filtrate acidified. The washed precipitate was redissolved at 25° in 110–140 ml. 2 *N* sodium carbonate, the aluminum hydroxide filtered off, and the filtrate acidified. The crude acid (15.0 to 15.8 Gm., m.p. 198–211°) was crystallized from 41% ethanol (1 Gm. in 20 ml.) or from 50% acetic acid (1 Gm. in 30 ml.). By sublimation at 190° and 10-μ pressure, and crystallizing the sublimate from 41% ethanol (1 Gm. in 31 ml.), a very pure acid (IV) was obtained, colorless needles melting at 221.7 to 222.5°. Its solution in dilute alkalies is colorless. It was very soluble in acetone and ethanol, soluble in acetic acid, and insoluble in water, benzene, or chloroform.

Anal.—Calcd. for $C_{21}H_{18}O_3$: C, 79.21; H, 5.70; mol. wt. 318. Found: C, 79.26; H, 5.78; neut. equiv. 315.

The Acetylderivative of IV.—3.18 Gm. of IV, 0.5 Gm. of anhydrous sodium acetate, and 5 ml. of acetic anhydride were heated for 1 hour to 120°. The compound was recrystallized from ethanol; crystals were also obtained by adding petroleum ether (6 ml.) to its solution in benzene (1 Gm. in 6 ml.). This acetylderivative formed colorless crystals melting at 179.7 to 181.0°.

Anal.—Calcd. for $C_{23}H_{20}O_4$: C, 76.64; H, 5.59. Found: C, 76.51; H, 5.08.

2-Methyl-9-acetoxy-19(p-acetoxyphenyl)anthracene (IVa).—When 0.05 ml. concentrated sulfuric acid was added to a suspension of 3.18 Gm. of IV in 5 ml. of acetic anhydride, the mixture became hot, and a dark green, clear solution was obtained. It was heated for a few minutes at 100°, cooled, and the crystal mush poured on ice. The greenish crude compound, after crystallization from ethanol (1 Gm. in 46 ml.) or from acetone, formed faint yellow crystals, m.p. 180.8 to 182.0°. Its solutions exhibited bluish fluorescence.

Anal.—Calcd. for $C_{25}H_{20}O_4$: C, 78.11; H, 5.24. Found: C, 78.23; H, 5.44.

A suspension of 5.0 Gm. of IVa in 5 ml. of ethanol and 26 ml. of 2 *N* sodium hydroxide was refluxed for 2 hours. On acidification of the dark solution, an amorphous, dark precipitate was obtained (3.2 Gm.). Since it could not be made to crystallize, it was mixed with 20 Gm. zinc dust and heated in an atmosphere of hydrogen to 300°. The small quantity of sublimate, after several crystallizations from ethanol, formed needles of faint yellow, m.p. 175.3 to 176.1°.

Anal.—Calcd. for $C_{15}H_{10}O_2$: C, 81.08; H, 4.53. Found: C, 81.03; H, 4.66.

This compound was proved, by the mixed melting point method, to be 2-methylanthraquinone.

Pyromellityl Alcohol or 1,2,4,5-Tetrahydroxymethylbenzene (V).—The isolation of this alcohol is difficult because of its insolubility in organic solvents and its great solubility in water. Therefore, the specified quantities of water and acid must be used. Our method of isolation of this alcohol is simpler than that described by Benning and Grosskinsky (15).

The thimble of an extraction apparatus was charged with 12.4 Gm. (0.4 mole) of pyromellitic acid tetramethyl ester (m.p. 141–142°) and the flask contained a solution of 9.2 Gm. $LiAlH_4$ in 400 ml. of absolute ether. After the thimble content was dissolved, refluxing was continued for an additional 20 hours. Then 160 ml. of water was added dropwise, at the same time distilling out the ether, and finally 90 ml. of 10 *N* sulfuric acid to dissolve the aluminum hydroxide. The warm solution was cooled overnight to room temperature, and the crystals which had formed were filtered off and washed with a little ice water.

The dried compound (6.7 to 8.3 Gm.) was purified by extracting it in a thimble extractor with 60 ml. of methanol. The crystals formed in the solvent weighed 6.5 to 6.9 Gm. (80–87%), m.p. 190–191°.

This alcohol also could be crystallized from water (1 Gm. in 5.4 ml.) or sublimed at 195° and 6-μ pressure. It melted at 191.2 to 191.8° [literature 191–192° (15)].

3,6-Dihydroxy-9-xanthone (VI).—A slurry of 3.0 Gm. of 2,2′,4,4′-tetrahydroxybenzophenone (m.p. 197–200°) in 30 ml. of water was heated at 220–230° in a sealed tube for 14 hours. The cooled mixture contained long needles (2.62 Gm.) which when crystallized from a considerable quantity of 20% ethanol, formed fine silky needles which did not melt even at 320°. Its alkaline solutions exhibited strong bluish fluorescence. Its diacetylderivative melted at 206.5 to 207.0° (from ethanol) [literature 204° (16)].

1,1-Bis(p-hydroxyphenyl)cyclohexane (VII).—This compound was prepared in 20% yield by condensing phenol with cyclohexanone in the presence of concentrated sulfuric acid. After crystallizations from 1,2-dichloroethane (1 Gm. in 17 ml.) and drying at 120°, VII melted at 183°, then solidified and melted again at 187.8 to 188.9° [literature 186° (17)].

2-Phenyl-4-(p-hydroxybenzylidene)oxazol-5-one (VIII).—This compound has been made in low yields either by demethylation of its methyl-ether or by heating (*p*-hydroxybenzylidene)hippuric acid at 200° (18).

The following method produced pure VIII in high yields: (*p*-hydroxybenzylidene)hippuric acid (14.15 Gm., m.p. 238° dec.) (19) and 25 ml. of thionyl chloride were heated slowly to 85° until evolution of HCl ceased. The excess $SOCl_2$ was distilled off *in vacuo*. The yellow cake was treated with 50 ml. of benzene and filtered: 10.7 to 11.1 Gm. (80–85%) VIII, m.p. 215–218°, was obtained.

After crystallization from 1,4-dichloroethane (1 Gm. in 50 ml.) or sublimation at 200° and 6μ pressure, VIII was obtained as crystals of strong yellow color, melting at 221.3 to 221.9°. Its solution in 0.1 *N* sodium hydroxide was unstable at room temperature. The initially strong orange of the solution soon faded and on acidification, (*p*-hydroxybenzylidene)hippuric acid precipitated.

Propionylderivative of VIII.—This compound made from VIII, propionic acid, and a drop of con-

centrated sulfuric anhydride, after crystallization from considerable ethanol, formed fine, light yellow needles melting at 161.2 to 161.8°.

Anal.—Calcd. for $C_{19}H_{15}O_4N$: Theory: C, 71.02; H, 4.70; N, 4.35. Found: C, 70.92; H, 4.51; N, 4.83.

p-Hydroxybenzylidenehydrazide of the Bis(p-hydroxyphenyl) Acetic Acid (IX).—A solution of 2.53 Gm. of the hydrazide of the bis(*p*-hydroxyphenyl)acetic acid (20) (m.p. 252.1 to 253.1°) and 1.22 Gm. *p*-hydroxybenzaldehyde in 25 ml. of 41% ethanol were refluxed for 2 hours. The fine needles (3.57 Gm.) were crystallized from 20% ethanol (1 Gm. in 35 ml.). Pure IX sintered at 215–220°, loosing thereby 5.0% water (theory for $C_{21}H_{18}N_2O_4 \cdot H_2O$ = 4.7% H_2O), then melted at 262.5 to 263.0°.

Anal. (of compound dried at 220° *in vacuo*)—Calcd. for $C_{21}H_{18}N_2O_4$: C, 69.61; H, 4.97; N, 7.73. Found: C, 69.70; H, 5.13; N, 7.40.

2-(4-Hydroxystyryl)pyridine or 4'-Hydroxystilbazole (X).—A solution of 18.6 Gm. (0.2 mole) of α-picoline, 24.4 Gm. (0.2 mole) of *p*-hydroxybenzaldehyde, and 41 Gm. (0.4 mole) of acetic anhydride were refluxed for 12 hours (153–160°). The unreacted picoline and acetic acid were distilled off *in vacuo*. After adding 150 ml. of 3 *N* HCl and refluxing for 1 hour, water was added and the solution adjusted to pH 7. The greenish-gray precipitate weighed 21.7 Gm. (55%) and melted at 197–207°.

Crystallized from ethanol (1 Gm. in 18 ml.) and then sublimed at 200° and 6-μ pressure, X melted at 216.4 to 217.4° [literature 219–220° (21)]. The compound had a faint yellow tinge, dissolved in 0.1 *N* NaOH with a light yellow and in 0.1 *N* HCl with a light greenish color.

Acetylderivative of X.—This was prepared from X, acetic anhydride, and a trace of sulfuric acid. It was crystallized by adding petroleum ether to its saturated benzene solution. It melted at 109.2 to 110.3°.

Anal.—Calcd. for $C_{15}H_{12}O_2N$: C, 75.31; H, 5.43; N, 5.85. Found: C, 75.67; H, 5.35; N, 6.02.

2,6-Bis(p-hydroxystyryl)pyridine (XI).—A mixture of 2,6-bis(*p*-acetoxystyryl)pyridine (30.0 Gm.; m.p. 183–184°) (21) in 90 ml. of acetic acid, 30 ml. of water, and 3 ml. of sulfuric acid was kept for 12 hours at 110–120°. The yellow compound, after crystallization from isopropanol, melted at 258.5 to 259.0°, formed yellowish-orange crystals and dissolved in aqueous alkali with a strong green color. For analysis, it was dried at 150° in high vacuum.

Anal.—Calcd. for $C_{21}H_{17}O_2N$: C, 80.00; H, 5.39; N, 4.44. Found: C, 79.14; H, 5.61; N, 4.43.

2-(p-Hydroxyphenyl)-1,3-indandione (XII).—A 10.0-Gm. quantity of 2-(*p*-anisyl)-1,3-indandione [m.p. 154–155° (22)] and 20.0 Gm. of pyridine hydrochloride were heated for 2 hours at 180°. The cooled melt was dissolved in water, the solid filtered off, washed, dried (9.2 to 9.4 Gm.), and crystallized from 1,2-dichloroethane (1 Gm. in 20 ml.). The compound formed faint yellowish needles, which melted at 177.1 to 178.1° to a red melt. The color of its solution in 0.1 *N* sodium hydroxide was dusky red. The crystals contained solvate solvent which they lost at 100°.

Anal.—Calcd. for $C_{15}H_{10}O_3 \cdot \frac{1}{2}\ ClCH_2CH_2Cl$: Dichloroethane 17.2. Found: loss of weight at 100° = 17.1. A sample dried at 100° *in vacuo* was analyzed: $C_{15}H_{10}O_3$: C, 75.62; H, 4.20; mol. wt. 238. Found: C, 75.13; H, 4.19; mol. wt. 248.

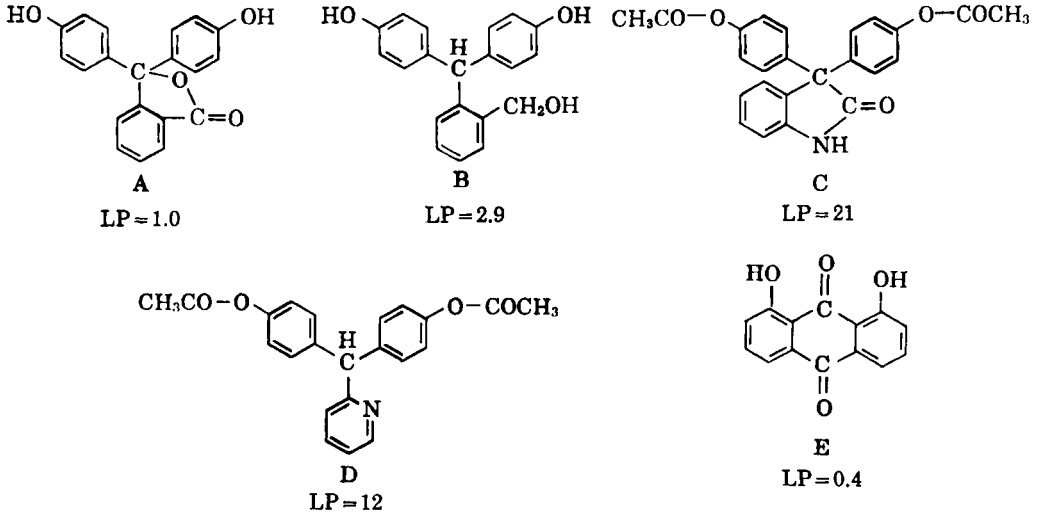
DISCUSSION

Many compounds prepared and found to be inactive, namely VII, VIII, XVIII, XXIII, and XXIV, were reported by Schmidt and Seeger (23) to be active when tested on rats in rather huge doses. Unfortunately, many compounds in the literature described as laxatives, actually are inactive in humans. Therefore, in evaluating these substances, it must be emphasized again that the test animals and dosages must be considered.

The distinct laxative action of sennosides, the principal active ingredient isolated in pure form from senna (2), was confirmed as being six times more potent than phenolphthalein.

None of the ten compounds having either grouping *b*, *c*, or *d* in their molecule was active.

The two phthaleins, II and III, possess laxative properties. It was surprising that III, which has only one HO-group, was active.



Scheme I

Of the four mono- and polyhydric alcohols tested, namely V, XIV, XV, and XVI, only the one which also has two phenol hydroxyls (XIV) showed distinct laxative properties. It is noteworthy that its position isomer, compound B in Scheme I, had previously been found to be a good laxative (1). These findings indicate that the phenolic HO-group is of greater importance than the alcohol hydroxyl, and that new laxatives might be found among the diphenolic benzyl alcohols.

Scheme I shows the structural formulas of the five synthetic organic compounds which are being used presently as active ingredients in commercial laxatives:

Phenolphthalein (*A*).—This is the most widely used of the five, is odorless, tasteless, and practically devoid of untoward effects. Studies on various animals have attested repeatedly to its low order of toxicity, all investigators having failed to establish LD_{50} (24–26).

Phenolphthalol (*B*) (1, 27).—This is used mainly in West Germany under the name of Egmol, and likewise is nontoxic (26).

3,3-Bis(p-acetoxyphenyl)oxindole (*C*) (28).—This compound is known as Isacen and is the most potent of all five. Baum *et al.* (29) claimed to have detected "diphenylisatin" or deacetylated compound C in canned prune juice. Neither we nor others were able to detect any 3,3-bis(*p*-hydroxyphenyl)oxindole in such juice. A private communication[3] states: "An exhaustive examination of prune juice failed to reveal the presence of isatin derivatives. Bioassays for the laxative effect of prune fractions have shown that the laxative action is in the fraction containing magnesium."

Bis(p-acetoxyphenyl)-(2-pyridyl)methane (*D*) (23, 27).—This is known as Dulcolax, the newest laxative, and is administered rectally as well as orally.

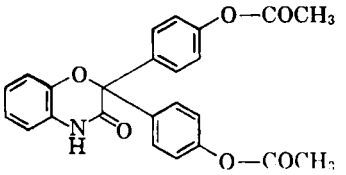
1,8-Dihydroxyanthraquinone (*E*) (30).—This compound is known under such names as chrysazin, Istin, Istizin, dantheon, or Dorbane, is related to the naturally occurring laxatives such as emodine, chrysophanic acid, and rhein, all of which have HO-groups in position 1 and 8 of anthraquinone.

It is interesting to note that compounds A, B, and C were first synthesized by A. Baeyer, but their therapeutic value was discovered much later by others.

Compounds A, B, C, and D contain group *a*, which has been called the laxaphoric group (31), and in addition, a third ring, benzene or pyridine, attached to the center C atom. Acetylation of the two HO-groups decreases the potency, exemplified by compounds C and D, which possess only half the potency of the parent compounds.

The two nitrogen containing compounds, C and D, are the most potent laxatives and contain group *e*. Recently, Schmidt and Seeger (32) synthesized several derivatives of 1,4-benzoxazoline with group *e* in their molecule. They found compound F to have approximately three times the potency of phenolphthalein when tested on rats.

[3] Letter dated July 18, 1963, from the Fruit and Pharmacology Laboratories, Western Regional Research Laboratory, Albany, Calif.



F

Our compounds with group *e*, IX and XXI, were inactive. Schraufstätter (33) synthesized four compounds with group *e* and found them all to be devoid of laxative properties.

Several other compounds with the laxaphoric group *a* are good laxatives, *i.e.*, 4,4′-dihydroxytriphenylmethane, the 1,8- and the 2,1-phenolnaphthaleins (1), and several compounds described by Schmidt and Seeger (23). However, the majority of compounds with group *a*, made and tested by us and others, were inactive.

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Complex Formation Influence on Reaction Rate II

Hydrolytic Behavior of Riboflavin in Borate Buffer

By DEODATT A. WADKE* and DAVID E. GUTTMAN*

A study was made of the hydrolytic behavior of riboflavin in aqueous solutions buffered with a borate buffer system. It was observed that the vitamin decomposed slower in the presence of the buffered rather than unbuffered solutions of equivalent hydroxide-ion concentration. Rate studies suggested that a reversible 1 : 1 association occurred between riboflavin and a component of the buffer to yield a product which underwent hydrolytic cleavage of the isoalloxazine ring at a slower rate than the parent compound. Similar studies with lumiflavin failed to demonstrate this effect and showed that the ribityl side-chain of riboflavin was involved in the association.

Evidence was presented in a previous communication (1) which demonstrated that the velocity of the base-catalyzed decomposition of riboflavin in aqueous solution was influenced by the incorporation of caffeine in the reaction mixture. The rate was shown to be slowed and dependent on the concentration of caffeine. The effect was rationalized and effectively quantitated by hypothesizing that the riboflavin-caffeine complex, which is known to form in such systems, possessed little or no reactivity toward hydroxide ion and that only the noncomplexed form of the vitamin underwent observable decomposition. It is the objective of this communication to describe a related but somewhat more complex behavior which was observed in solutions of riboflavin buffered with a boric acid buffer. Here an association between the vitamin and a buffer component apparently took place. However, in contrast to the caffeine system, here both the free and combined forms of the vitamin appeared to undergo hydrolytic breakdown but at different rates.

Other workers have shown previously that riboflavin and boric acid do undergo some type of rapid reversible union. Surrey and Nachod (2), for example, demonstrated that aged alkaline solutions of riboflavin exhibited a depressant action on cardiac and visceral muscles and that this was due to the 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid which resulted from hydrolysis of the vitamin. They reported, however, that similar solutions which contained boric acid failed to show any hypotensive activity even after prolonged standing. The reasons for this apparent stabilization were not pursued. Other reports, including those of Takata *et al.* (3) and Frost (4), indicated a reaction by noting that riboflavin was more soluble in aqueous borate solutions than in water alone. Frost also found that the degree and direction of optical rotation exhibited by riboflavin in aqueous solution was altered by the addition of boric acid. He suggested on the basis of this and other evidence that the ribityl side-chain combined with the boron compound in some way. Data obtained in the present investigation support this conclusion and demonstrate that the rate of hydrolysis of the isoalloxazine ring of the vitamin is significantly reduced as a result of association.

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EXPERIMENTAL

Reagents.—Riboflavin was obtained from Eastman Organic Chemicals. Lumiflavin was prepared by decomposing riboflavin in sodium hydroxide solution (pH 12.0–12.5) in the presence of light. The reaction mixture was acidified (pH 4.5–5.0) and extracted with chloroform. The solvent was removed to obtain lumiflavin which was washed several times with 0.5 *N* hydrochloric acid and finally with distilled water. The melting point was 332–334° with decomposition (uncorrected).

Procedure.—The reaction mixture was prepared by placing 50 ml. of appropriate buffer or sodium hydroxide solution of desired normality in a 100-ml. volumetric flask together with about 25 ml. of distilled water. The flasks were suspended in a Sargent Thermonitor constant temperature bath. Twenty milliliters of riboflavin stock solution was added to each flask and the volume made up to the mark with distilled water. Five-milliliter samples were withdrawn periodically and immediately mixed with 5 ml. of 2 *N* acetic acid to quench the hydrolysis. Samples were analyzed spectrophotometrically at 450 mμ using a Beckman model DU spectrophotometer. All samples were protected from light at all times. In studying the effect of buffer concentration on the hydrolysis of riboflavin and lumiflavin, ionic strength was maintained constant at 0.3 by adding potassium chloride.

RESULTS

A preliminary indication that the hydrolytic behavior of riboflavin was somewhat unusual in borate

buffer was obtained by comparing the pH profile for the reaction determined in unbuffered systems to that obtained using buffers. All solutions exhibited a first-order disappearance of riboflavin, but the rates of disappearance were slower in the buffered solutions than in corresponding unbuffered solutions of identical pH. Figure 1 illustrates this observation. Here, the logarithm of the first-order rate constant determined for a particular solution was plotted as a function of the logarithm of the hydroxide ion concentration. It can be seen that the rate constants for the borate systems are smaller than would be anticipated on the basis of their hydroxide-ion concentration. Subsequent experiments showed that the effect was not due to differences in ionic strength. The velocity of the reaction was, however, specifically influenced by the concentration of boric acid in the buffer. Figure 2 illustrates this effect. Here, for three different pH conditions the experimentally determined rate constants were plotted as a function of borate buffer molarity. It can be seen that initial, relatively small additions of buffer resulted in a rather marked reduction in the magnitude of the rate constant. Subsequent additions had a lesser effect, and each curve approached an asymptotic value. Rate constants at zero buffer concentration were estimated by extrapolation of the curves to the y-axis and determination of the intercept value. Rate constants at infinitely high concentrations of buffer components were estimated from the plots illustrated in Fig. 3. Here rate constant was plotted as a function of the reciprocal of the buffer molarity. The resulting curves were extrapolated to the y-axis and the intercept value determined. The constants representing the concentration extremes and those for intermediate concentrations are tabulated in Table I. Similar experimentation was conducted to study the influence of buffer molarity on the rate of disappearance of lumiflavin from aqueous solution. The results are shown in Table II and in Fig. 4, where a reference curve for riboflavin at the same pH is drawn for comparative purposes. The rate of lumiflavin disappearance was within experimental error, independent of the molarity of buffer. Spectrophotometric determinations confirmed the observation made by Frost (4) that the visible and ultraviolet spectra of riboflavin were no different in solutions buffered with borate than in unbuffered solutions.

DISCUSSION

The nature of the observed dependency of reaction rate of the buffer molarity intuitively suggested that a reversible reaction or interaction between

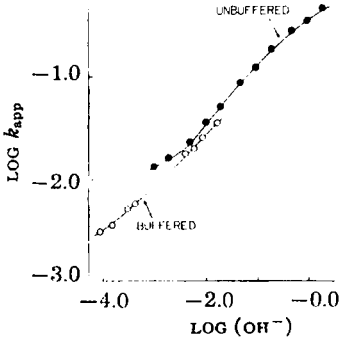


Fig. 1.—The influence of hydroxide-ion concentration on the rate of hydrolysis of riboflavin at 35° C. in the presence and absence of borate buffer.

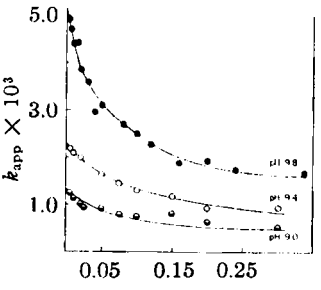


Fig. 2.—The influence of borate buffer concentration on the rate of hydrolysis of riboflavin at different pH values at an ionic strength of 0.3.

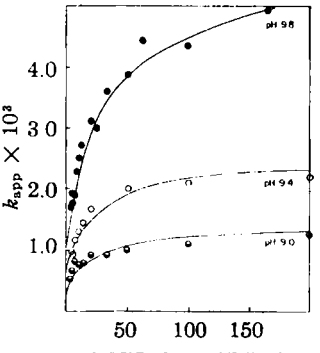
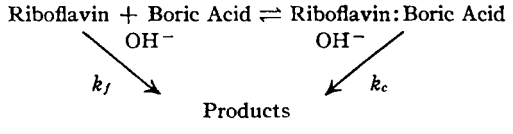


Fig. 3.—The influence of borate buffer concentration on the rate of hydrolysis of riboflavin at different pH values at 35° C. and at an ionic strength of 0.3.

riboflavin and a buffer component occurred to form a new species of riboflavin which could not be differentiated from the original by the assay method employed. Examination of the data also suggested that both the free and combined forms of the vitamin which were in equilibrium underwent hydrolytic cleavage of the isoalloxazine ring but at different rates. The suspected mechanism is summarized by:



As it will be seen, a mathematical model based on this over-all mechanism was consistent with the experimental observations.

A rate expression for the postulated situation can be derived from the following considerations. All solutions exhibited a first-order disappearance of the riboflavin chromophore, *i.e.*,

$$-d(R_t)/dt = k_{app}(R_t) \qquad \text{(Eq. 1)}$$

where (R_t) = stoichiometric concentration of riboflavin, k_{app} = pseudo first-order rate constant, and t = time. If riboflavin and boric acid, or another form of boric acid, underwent a rapid reversible 1:1 association to form a product which was also susceptible to hydrolysis of the ring, then the rate equation becomes

$$-d(R_t)/dt = k_f(R_f) + k_c(R_c) \qquad \text{(Eq. 2)}$$

where k_f = rate constant for the degradation of free riboflavin, (R_f) = concentration of free riboflavin, k_c = rate constant for the degradation of the riboflavin-boric acid product, and (R_c) = concentration of riboflavin-boric acid product. Both (R_f) and (R_c) can be expressed in terms of the stoichiometric concentration of riboflavin and the equilibrium constant for the reversible combination, *i.e.*,

$$(R_f) = (R_t)/[1 + K(B)] \quad \text{(Eq. 3)}$$

and

$$(R_c) = (R_t)K(B)/[1 + K(B)] \quad \text{(Eq. 4)}$$

where (B) = concentration of free boric acid which may be approximated by the stoichiometric concentration if it is large compared to the concentration of riboflavin, K = equilibrium constant = $(R_c)/(R_f)(B)$.

The rate equation can then be written

$$-d(R_t)/dt = (R_t)\{k_f/[1 + K(B)] + k_cK(B)/[1 + K(B)]\} \quad \text{(Eq. 5)}$$

The dependency of rate constant on buffer concentration is thus predicted by

$$k_{app} = k_f/[1 + K(B)] + k_cK(B)/[1 + K(B)] \quad \text{(Eq. 6)}$$

Equation 6 can be rearranged to yield

$$(k_f - k_{app})/(k_{app} - k_c) = K(B) \quad \text{(Eq. 7)}$$

If the basic assumptions are valid, then a plot of

TABLE I.—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE DISAPPEARANCE OF RIBOFLAVIN IN BORATE BUFFER AT CONSTANT IONIC STRENGTH OF 0.3 AND A TEMPERATURE OF 35° C. ± 0.01° C.

Molarity of Borate	k_{app}, Hr.$^{-1}$ × 10^3
pH 9.0	
0.0	1.3[a]
0.005	1.24
0.01	1.13
0.02	1.01
0.03	0.935
0.05	0.935
0.075	0.795
0.1	0.75
0.15	0.805
0.2	0.64
0.3	0.53
infinite	0.23[a]
pH 9.4	
0.0	2.31[a]
0.005	2.19
0.01	2.09
0.02	1.98
0.05	1.64
0.075	1.43
0.1	1.30
0.15	1.17
0.2	0.92
0.3	0.96
infinite	0.65[a]
pH 9.8	
0.0	5.14[a]
0.003	4.9
0.006	4.7
0.01	4.34
0.016	4.42
0.02	3.85
0.03	3.58
0.04	2.97
0.05	3.1
0.08	2.69
0.1	2.51
0.12	2.27
0.16	1.88
0.2	1.93
0.24	1.74
0.336	1.67
infinite	1.05[a]

[a] Value was obtained by extrapolation.

TABLE II.—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE DISAPPEARANCE OF LUMIFLAVIN IN BORATE BUFFER AT pH 9.8 ± 0.05, AT CONSTANT IONIC STRENGTH OF 0.3 AND A TEMPERATURE OF 35° C. ± 0.01°C.

Molarity	k_{app}, Hr.$^{-1}$ × 10^3
0.005	1.97
0.01	1.88
0.02	2.03
0.03	2.23
0.05	2.25
0.1	2.16
0.2	1.77
0.3	1.88

$(k_f - k_{app})/(k_{app} - k_c)$ *versus* the concentration of boric acid should yield a straight line with a slope of K. In treating the data for such a plot, the value of k_f used was that obtained by extrapolating rate constant to zero buffer concentration where free riboflavin would be the primary species. The value of k_c used was that obtained by extrapolating the rate constant to infinitely high buffer concentrations where the combined form of riboflavin would be expected to predominate. The concentration of boric acid was not used in testing this relationship. Rather, the total molarity of buffer was used. This was necessary because the exact nature of the boron-containing reactant is not known. The molarity of buffer does, however, provide a first-order reflection of the concentration of boric acid, borate ion, and other forms of boric acid, therefore, they can be used to gain an indication of whether the relationships under consideration are valid. The plot which tests the relationship expressed by Eq. 7 is illustrated in Fig. 5 and represents the data obtained for the pH

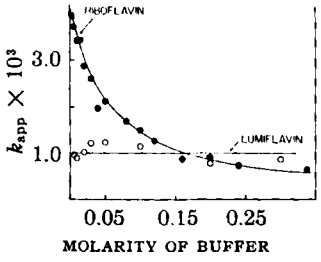


Fig. 4.—The influence of borate buffer concentration on the rates of riboflavin and lumiflavin hydrolysis at a pH value of 9.8, ionic strength of 0.3, and a temperature of 35° C.

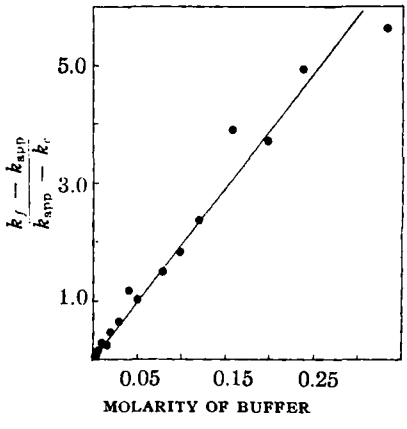


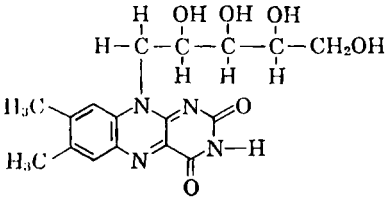
Fig. 5.—A plot of the relationship expressed by Eq. 7 (see text) for riboflavin at a pH value of 9.8, constant ionic strength of 0.3, and at 35° C.

TABLE III.—EQUILIBRIUM CONSTANTS FOR THE RIBOFLAVIN-BORIC ACID ASSOCIATION AT DIFFERENT pH VALUES AND AT A TEMPERATURE OF 35° C. ± 0.01° C.

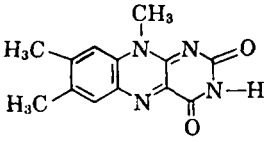
pH	K, L. mole^{-1}
9.0	10.0
9.4	14.5
9.8	19.5

condition of 9.8. It can be seen that excellent linearity over a wide range of buffer molarity was followed. The equilibrium constant which can be graphically estimated from this plot is not a thermodynamic value, but can be considered as an apparent value. This relationship was also tested with the data collected at the two other pH conditions. In both cases the expected linearity was achieved. The apparent equilibrium constants calculated from such plots are presented in Table III as a function of the pH condition. The graphical tests provide support for the model which was assumed, and indicate that such a model is reasonable and consistent with the observed behavior.

Numerous reports (5, 6) have shown that riboflavin undergoes charge-transfer interactions with a number of nucleophilic agents. The absence of changes in the spectral characteristics of the vitamin in the presence of borate buffer precludes this occurrence in the present study. That the ribityl side-chain was involved in a combination with a species of boric acid is apparent from the observation that, in contrast to riboflavin (I), the rate of reaction of lumiflavin (II) was not influenced by buffer concentration. The observed reduced rate of reaction exhibited by riboflavin in borate buffer must then be due to its partial and easily reversible conversion to a derivative formed by the combination of boric acid with the side-chain.

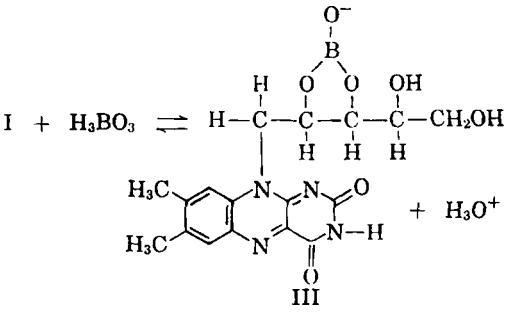


I. Riboflavin



II. Lumiflavin

On the basis of various reports on the reactivity of boric acid with polyhydroxy compounds (7, 8) we suggest that a borate ester might have formed in a manner similar to that represented by III. Such an



III

ester would be expected to be less susceptible toward hydroxide-ion attack on the ring because of a repulsive effect arising from the formal negative charge carried by the side-chain. The small decrease in the value of the apparent equilibrium constant with a decrease in pH can be considered as indirect support for such an effect. Protonation of the anionic group would occur as the pH was decreased and would result in a decreased repulsion and subsequent decrease in the apparent equilibrium constant determined in this way.

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Gastric Ulcers Induced by Phenylephrine in Certain Pharmaceutical Compositions

By THOMAS A. LYNCH, WILBUR L. HIGHLEY, and ALBERT G. WORTON

The incidence of gastric ulcers in mice was 100 per cent within 24 hours after a single oral sublethal dosage of an antihistamine-decongestant liquid pharmaceutical. Such ulcers persisted for more than 4 days. Investigation of the components of this and similar preparations indicated that phenylephrine was the primary ulcerogenic factor. The flavoring and sweetening agents, but not the antihistamines, significantly potentiated the ulcerating effect of phenylephrine. The results suggest that the composition of the base as well as the active agents in sympathomimetic amine-containing liquids may be important in eliminating the potential side effects of such preparations. The study also presents a reproducible and rapid method of ulcer induction for the investigation of antiulcer agents.

Acetylsalicylic acid, cortisone, histamine, cinchophen, and reserpine induced varying degrees of gastric ulceration in animals. Iodoacetamide fed to rats for up to 90 days produced chronic gastric ulceration without perforation or serious nutritional disturbances, suggesting a useful method in the studies of pathogenesis (1). Food reduction and drug-induced starvation caused gastrointestinal ulceration (2). Cutaneous necrosis resulted from intravenous infusion of norepinephrine in 61 human cases as reported by Close and Frackelton (3). Peirce and Polley (4) reported in two human cases that phenylephrine hydrochloride causes sloughing following intravenous infusion. No known reports of gastrointestinal ulceration are associated with the oral use of phenylephrine. Since mild gastrointestinal symptoms are occasionally observed with large doses, therapy with phenylephrine is recommended after meals (5).

Gastric ulceration, observed as a side effect of an antihistamine-sympathomimetic liquid preparation in an acute toxicity study, prompted further investigation, as presented in this report.

METHODS AND MATERIALS

Three liquid decongestant formulas containing a sympathomimetic and antihistamines were studied, as well as certain components, singularly and in combination. The drugs were administered either orally or subcutaneously in nonfasting Swiss mice (19–41 Gm.) and Wistar rats (198–369 Gm.). The oral dosage was usually 25 ml./Kg. Animals were sacrificed at 1 to 7 days after administering the drug. The per cent ulcer incidence was determined grossly. The degree of severity was expressed as the percentage of the pyloric stomach area found ulcerated.

The three basic formulas investigated contained the following active principles:

Formula A.—Each 5-ml. quantity contained 5 mg. of phenylephrine hydrochloride, 4.16 mg. of methapyrilene fumarate, 4.16 mg. of pyrilamine maleate, 4.16 mg. of pheniramine maleate, and 5% ethanol in a sweetened flavored base.

Formula B.—Each 5-ml. quantity contained 5 mg. of phenylephrine hydrochloride, 2.0 mg. of chlorprophenpyridamine maleate, 13.5 mg. of chloroform, 1.0 mg. of *l*-menthol, and 5% ethanol in a sweetened flavored base.

Formula C.—Each 5-ml. quantity contained 12.5 mg. of phenylpropanolamine hydrochloride, 6.25 mg. of pheniramine maleate, and 6.25 mg. of pyrilamine maleate in a sweetened flavored base.

RESULTS

Evaluation of the Active Constituents.—Gastric ulceration was induced by orally administering to mice 25 ml./Kg. of Formula A containing phenylephrine hydrochloride and three antihistamines as indicated in Table I. The 48-hour ulcer incidence ranging from 44.4 to 100% appeared to have a dose-response relationship. The ulcers, found only in the pyloric part of the stomach, consisted of multiple areas of epithelial erosion and hemorrhage. As much as 90% of the pyloric stomach area was eroded but without perforation. Hepatic hemorrhage and congestion and stimulation of the central nervous system were generally the associated side effects. The three antihistamines of Formula A were not ulcerogenic. Phenylephrine alone, given at four times the dosage as contained in Formula A, induced only one-half the incidence of gastric ulcers.

Formula B containing phenylephrine and one antihistamine produced 20% the incidence of gastric ulcers as equivalent dosages of Formula A. The addition of the three antihistamines of Formula A to Formula B did not increase the ulcer incidence. Formula C containing phenylpropanolamine and two antihistamines did not induce gastric ulceration.

Phenylephrine given orally to female rats starting at 100 mg./Kg. and terminating at 300 mg./Kg. per day for 3 days produced intestinal hyperemia, hepatic hemorrhage, and weight loss, but no ulcers as shown in Table II. One rat died following 100 mg./Kg. on the first day, but none died following 200 and 300 mg./Kg. on subsequent days, suggesting the development of drug tolerance. Gastric ulceration was produced in two male rats given 100 mg./Kg. of phenylephrine orally per day for 2 days. Epitaxis, rhinorrhea, and central nervous system depression were observed following the dose on the first day only. One rat died after the second dose.

As illustrated in Table III, combinations of phenyl-

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TABLE I.—INDUCTION OF GASTRIC ULCERS IN MICE 48 HOURS AFTER ORAL ADMINISTRATION OF VARIOUS PREPARATIONS

Prepn.	Mice, No.	Sex	Dosage, ml.(mg.)[a]/Kg.	Mortality, %	Incidence, %
Normal saline	10	M	25(25)	0	0
Formula A[b]	30	M	25(25)	0	100
Formula A	10	F	25(25)	30	100
Formula A	10	M	6.3(6.3)	10	44.4
Formula A	10	F	6.3(6.3)	0	50.0
Formula A-1[c]	5	M	25(25)	0	0
Formula B[d]	10	M	25(25)	0	20
Formula B-1[e]	5	M	25(25)	0	0
Formula C[f]	10	M	25(25)	0	0
Phenylephrine-2%	15	M	5(100)	26.7	45.5
Phenylephrine-2%	10	M	10(200)[g]	100	...
Phenylephrine-2%	5	F	5(100)	0	60

[a] Figure in parenthesis is the dosage of the sympathomimetic in mg./Kg. [b] Each 5-ml. quantity contains 5 mg. of phenylephrine hydrochloride, 4.16 mg. of methapyrilene fumarate, 4.16 mg. of pyrilamine maleate, 4.16 mg. of pheniramine maleate, and 5% ethanol in the Formula A base. [c] The antihistamines in aqueous solution as contained in Formula A. [d] Each 5-ml. quantity contains 5 mg. of phenylephrine hydrochloride, 2 mg. of chlorprophenpyridamine maleate, 13.5 mg. of chloroform, 1 mg. of *l*-menthol, and 5% ethanol in a sweetened flavored base. [e] The antihistamines as contained in Formula A added to Formula B. [f] Each 5-ml. quantity contains 12.5 mg. of phenylpropanolamine hydrochloride, 6.25 mg. of pheniramine maleate, and 6.25 mg. of pyrilamine maleate in a sweetened flavored base. [g] Given subcutaneously.

TABLE II.—TOXIC AND ULCEROGENIC EFFECTS OF 2% PHENYLEPHRINE HYDROCHLORIDE BY ORAL ADMINISTRATION IN WISTAR RATS (198–369 Gm.)

Time, Hr.	Rats, No.	Sex	Dosage, ml./Kg.	Remarks	Autopsy[a]
0	3	F	5(100 mg.)	One death after 2 hr. Lost 8 Gm. body weight.	Hyperemia of small intestine.
24	2	F	5(100 mg.)	Lost 8 Gm. body weight.	Autopsied one rat. No obvious pathological changes.
48	1	F	10(200 mg.)	No symptoms.	
72	1	F	15(300 mg.)	No symptoms.	No gross pathological changes except for hepatic hemorrhage.
0	3	M	5(100 mg.)	Epitaxis, rhinorrhea, C.N.S. depression persisting for 4–6 hr.	
24	3	M	5(100 mg.)	Symptoms as above. One death[b] occurred.	One rat with one pyloric stomach ulcer; one rat with three pyloric stomach ulcers.

[a] Sacrificing carried out 24 hours after the previous dosage. [b] Decomposition was too advanced for autopsy.

TABLE III.—INDUCTION OF GASTRIC ULCERS FOLLOWING ORAL ADMINISTRATION[a] IN MICE OF VARIOUS FORMULA A ACTIVE CONSTITUENTS

Prepn.	Mice, No.	Sex	Time of Autopsy, Hr.	Mortality, %	Ulcer Incidence, %
Normal Saline	5	F	48	0	0
Ph-Phen[b]	5	F	48	0	20
Ph-Meth[b]	5	F	48	20	0
Ph-Pyr[b]	5	F	48	20	0
Ph-Phen-Meth[b]	5	F	48	40	0
Ph-Phen-Pyr[b]	5	F	48	20	0
Ph-Meth-Pyr[b]	5	F	48	0	0
Normal Saline	5	M	24	0	0
Ph-Phen[c]	5	M	24	80	0
Ph-Meth[c]	5	M	24	100	...
Ph-Pyr[c]	5	M	24	80	100
Ph-Phen-Meth[c]	5	M	24	60	50
Ph-Phen-Pyr[c]	5	M	24	60	0
Ph-Meth-Pyr[c]	5	M	24	60	0
Formula A[d]	7	F	48	0	71.5
Formula A-2	7	F	48	0	100
Formula A-3	8	F	48	0	100

[a] All preparations were administered in 25 ml./Kg. dosages. [b] Ph, 0.10% phenylephrine hydrochloride; Phen, 0.25% pheniramine maleate; Meth, 0.25% methapyrilene fumarate; Pyr, 0.25% pyrilamine maleate. Two antihistamines in combination; each is 0.125%. [c] Same as *b*, except concentrations for phenylephrine and antihistamines are 2.67 times greater. [d] A, Each 5-ml. quantity contains 5 mg. of phenylephrine hydrochloride, 4.16 mg. of methapyrilene fumarate, 4.16 mg. of pyrilamine maleate, 4.16 mg. of pheniramine maleate, and 5% ethanol in Formula A base. A-2, 0.1% sodium pyrosulfite added as preservative. A-3, order of mixing altered.

ephrine with one or two antihistamines of Formula A in aqueous solution at 25 ml./Kg. orally induced ulcers in one group only. The over-all incidence of ulcers by such treatment was 3.3%. Increasing the concentration and dosage by 2.67 times increased the incidence of ulcers and mortality.

Adding a preservative or changing the order of mixing did not alter the ulcerogenic effect of Formula A.

The results suggested that some factor in the ulcerogenesis besides phenylephrine was indicated in Formula A.

Evaluation of the Vehicular Constituents.—As demonstrated in Table IV, the ulcerogenic action of phenylephrine in Formula A was potentiated by lime, ethanol, sucrose with glucose, sucrose with glucose and glycerin, and vanillin with glucose. When the ethanol and glycerin, sucrose, lime, and vanillin were deleted from Formula A, the ulcerogenic effects were eliminated. Substituting 2.5 mg. of phenylpropanolamine for 1.0 mg. of phenylephrine nearly eliminated the ulcerogenic effect of Formula A. The ulcer incidence was less than 7%, and with only one pinpoint size ulcer in each of two mice.

Figure 1 shows that the duration of gastric ulceration induced by 25 ml./Kg. of Formula A persists for more than 4 days. The peak in severity occurred at about 2 days.

TABLE IV.—INDUCTION OF GASTRIC ULCERS IN MICE BY VARIATIONS IN FORMULA A INERT CONSTITUENTS[a]

Formula[b]	Mice, No.	Sex	Mortality, %	Ulcer Incidence, %
A	15	M	0	100
A	15	F	20	100
A-I-Su	5	M	20	0
A-I-Gl	5	M	0	0
A-I-Gly	5	M	20	0
A-I-Van	5	M	20	0
A-I-Li	5	M	0	40
A-I-Et	5	M	0	20
A-I-Blue	5	M	0	0
A-I-Red	5	M	0	0
A-I-Su-Gl	5	M	0	80
A-I-Su-Gl-Gly	5	M	0	100
A-I-Gl-Red-Blue	5	M	60	0
A-I-Gl-Red-Blue	5	F	40	0
A-I-Gl-Red-Blue-Li/2-Van	5	M	0	20
A-I-Gl-Red-Blue-Li/2-Van	5	F	60	50
A-I-Gl-Red-Blue-Li/2-Cyc	5	M	20	50
A-I-2 Gl	5	F	0	80
A-C[c]	15	M	0	0
A-C	15	F	6.7	14.3[d]

[a] All preparations were orally administered in 25 ml./Kg. doses. Autopsy was performed 48 hours after oral dosage. [b] Formula A, each 5-ml. quantity contains 5 mg. of phenylephrine hydrochloride, 4.16 mg. of methapyrilene fumarate, 4.16 mg. of pyrilamine maleate, 4.16 mg. of pheniramine maleate, and 5% ethanol (Et) in a base of 50% sucrose (Su), 35% liquid glucose (Gl), 6.3% glycerin (Gly), 0.025% vanillin U.S.P. (Van), 1% lime flavor (Li), Blue, FD&C Dye No. 1 (Blue), and Red, FD&C Dye No. 2 (Red) in water. Cyc, cyclamate sodium. A-I, phenylephrine and the three antihistamines of Formula A in aqueous solution plus various vehicular agents. Li/2, one-half the amount of lime as contained in Formula A. 2 Gl, two times the amount of glucose as contained in Formula A. [c] A-C, substituting 2.5 mg. of phenylpropanolamine hydrochloride in Formula A for 1 mg. of phenylephrine hydrochloride. [d] One pinpoint size ulcer found in each of two mice only.

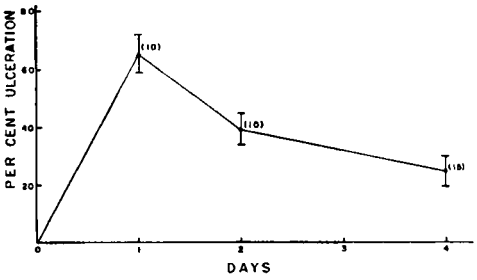


Fig. 1.—Pattern of gastric ulceration induced by oral administration of 25 ml./Kg. of Formula A in female mice. Per cent ulceration is based on the area of the pyloric stomach affected. I, mean and standard error. Number of animals in parentheses.

DISCUSSION

The mechanism of near lethal doses of phenylephrine, in producing gastric ulcers may be related to its effects on the autonomic nervous system, which include vasoconstriction of the gastric mucosa and constriction of the pyloric sphincter (6). Vasoconstriction results in ischemia and cellular anoxia and may lead to necrosis of the localized tissues as suggested by Close *et al.* (3). Ischemia and anoxia of the gastric mucosa, in the presence of retained gastric secretions, may be factors in the induction of gastric ulcers by phenylephrine in this study.

The possible role of the Formula A base in potentiating the ulcerogenic effects of phenylephrine is less obvious. Carbohydrates are reported to inhibit gastric motility, presumably by duodenal mucosa contact, which reflexly stimulates the release of the gastric inhibitory substance, enterogastrone (7). More specifically, Fenton (8) demonstrated that the volume of chyme emptied from the stomach was inversely proportional to the square root of the concentration of glucose orally fed to rats. This evidence suggests that the Formula A base inhibits gastric emptying and augments phenylephrine in producing a type of chemical pyloric ligation resulting in gastric ulcers, similar to the Shay rat (9).

The development of gastric ulcers, particularly in mice, is an acute toxic manifestation of phenylephrine and has questionable relationship to higher mammals. It is suggestive as a factor in the occasional mild gastrointestinal symptoms produced by large doses of such preparations.

The results indicate that the choice of apparent inert and active principles may be important in improving the gastrointestinal tolerance of sympathomimetic-antihistaminic liquid formulations.

The induction of gastric ulcers by phenylephrine in combination with high concentrations of certain sweetners, flavors, and solubilizing agents as contained in Formula A offers a rapid and reproducible method in the study of antiulcer drugs in mice, obviating thermocautery (10), chronic drug administration (1), and surgery, as with the Shay rat (9).

SUMMARY

Gastric ulcers were induced in mice by a single oral administration of liquid decongestant pharmaceutical.

Various liquid formulas containing a sympathomimetic drug and antihistamines, as well as different

combinations of active and inert principles, were evaluated for ulcerogenic effects in mice.

Phenylephrine hydrochloride was the initiating factor in the development of gastric ulcers.

Sucrose, glycerin, glucose, ethanol, lime, and vanillin potentiated phenylephrine-induced gastric ulcers in mice.

Phenylpropanolamine hydrochloride, similarly studied, produced little or no gastric toxicity.

These observations suggest that the composition of vehicle as well as the active principles may be important in reducing or eliminating the potential gastrointestinal side effects of sympathomimetic amine-containing liquid preparations.

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Effect of Chronic Poisoning by Emetine on Oxidative Process in Rat Heart I

Effects on Lipid Metabolism and Oxidative Phosphorylation

By GLENN D. APPELT* and HAROLD C. HEIM

Emetine at a concentration of $3 \times 10^{-3}M$ completely inhibited endogenous respiration of rat heart homogenates as well as the respiration in the presence of added butyrate or β-hydroxybutyrate. At a concentration of 10^{-4} *M*, the alkaloid elicited no effect. The inhibition observed was not specific for emetine, since quinine, at the same concentrations, was equally as effective in inhibiting respiration. Hearts from animals chronically poisoned by emetine, but not by quinine, showed impaired ability to oxidize these substrates. Chronic poisoning by emetine did not impair oxidative phosphorylation, nor did 10^{-3} *M* emetine added to mitochondria prepared from hearts of unpoisoned animals.

ABNORMALITIES in cardiac function following the use of emetine as an amebacide have been reported by numerous investigators. Ventricular fibrillation (1), diminished systolic force (2), and tachycardia (3) have been observed; other studies have shown that emetine may elicit a progressive cloudy swelling and fatty degeneration of the heart (4). Electrocardiographic changes have been induced by the drug in dogs and cats, evidenced by a widening of the initial P-R complex and by a frequently observed inversion of the T wave. These changes suggested a marked disturbance in energy utilization by the myocardium (5). Several studies have been conducted relative to the effects of emetine on cellular metabolism. It has been postulated that emetine may interfere with the enzyme systems which convert glycogen to contractile energy in the heart (6). Electrocardiographic alterations have been induced in the guinea pig by sublethal doses of the alkaloid, and these effects could not be prevented or abolished by the administration of diphosphopyridine nucleotide (7). It has also been shown that emetine evoked no effect on the synthesis of cocarboxylase by rat liver (8). The repeated administration of the alkaloid to young rats resulted in a reduction of food intake and marked inhibition of growth, findings which led to the suggestion that the drug caused nutritional and metabolic changes in certain tissues (10). A rapid depletion of liver glycogen has been reported to occur in young rats after a single administration of 0.1 to 0.2 mg. emetine/Kg. (11). Synthesis of glycogen by livers of emetine-poisoned rats was markedly depressed, and there also was observed a significant decrease in phosphorylase and aldolase activity in livers from the poisoned animals. Vitamin metabolism in emetine-poisoned rats has been studied; it was observed that the emetine-treated animals stored smaller amounts of thiamin and folic acid in the liver than did pair-fed controls. Poisoning by emetine did not alter metabolism of vitamin A, riboflavin, nicotinic acid, or biotin (12). The

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respiration of rat heart homogenates was inhibited by emetine in the presence of added glucose, pyruvate, malate, or fumarate (13). Oxygen uptake in the presence of added succinate, however, was augmented by emetine. Evidence was presented which suggested that the increase in oxygen consumption was due to a suppression of oxaloacetate accumulation, possibly through an inhibitory effect of emetine on malic dehydrogenase (14).

Although the toxic effects of emetine on the myocardium are prominent, it is not yet possible to explain the mechanism responsible for these phenomena. Some evidence exists, however, in support of the concept that the alkaloid may interfere with metabolism by the heart in such a manner as to reduce the efficiency of biochemical processes. To extend and explore this concept in more detail, a series of experiments was conducted in which the effects of emetine on fatty acid metabolism by heart homogenates and upon oxidative phosphorylation by mitochondria were investigated.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were used as the experimental animals and were young adults weighing approximately 200 Gm. unless otherwise noted. The animals were stunned and exsanguinated, the hearts were immediately removed; the ventricles were dissected away, washed quickly in cold water, blotted dry, and weighed. The ventricles were then transferred to a chilled Ten Broek tissue grinder containing sufficient cold 0.1 *M* potassium phosphate buffer, pH 7.4, so that the homogenates contained 80 mg. fresh ventricle per milliliter. The homogenates were pipeted into chilled Warburg vessels containing substrates and cofactors listed in the tables and figures under *Results*. After a 10-minute equilibration period, oxygen consumption was measured for 90 minutes according to conventional manometric techniques (15).

To determine the effect of chronic poisoning by emetine on the oxidation of the substrates used in this study, young animals weighing approximately 100 Gm. were separated into three groups of eight animals each and given intraperitoneal injections daily for 17 days as follows: Group I, 0.2 mg. emetine hydrochloride in 0.7 ml. water; Group II, 25 mg. quinine hydrochloride in 0.7 ml. water; and Group III, 0.7 ml. water. The animals were provided with food and water *ad libitum;* on the seventeenth day, they were sacrificed, the hearts removed, the ventricles homogenized as outlined above, and the oxygen consumption of the homogenates determined. Blood samples were collected at the time the animals were sacrificed and glucose levels were determined according to the method of Somogyi (16).

All results represent the average of data obtained with at least eight animals, and each determination of oxygen consumption was performed in duplicate. The pH of the contents of a representative number of flasks was determined at the end of the experimental period to verify that the observed effects were not caused by a change in pH during the experiment. Unless otherwise noted, the results are significant at the 99% probability level ($p < 0.01$) determined by the Fisher t test.

The effect of emetine on oxidative phosphorylation by heart mitochondria was determined by the method of Hunter (17). The mitochondria were isolated according to methods used by Plaut (18), and centrifugal separation was accomplished with a Servall RC-2 refrigerated centrifuge equipped with an SS-34 rotor and at 0°. Mitochondria representing 500 mg. fresh myocardium were added to each Warburg vessel, and the inorganic phosphate was determined by the method of Fiske and SubbaRow (19) with the Beckman DU spectrophotometer at 660 mμ.

RESULTS AND DISCUSSION

Figures 1–3 present data which show that the endogenous respiration of homogenates and the

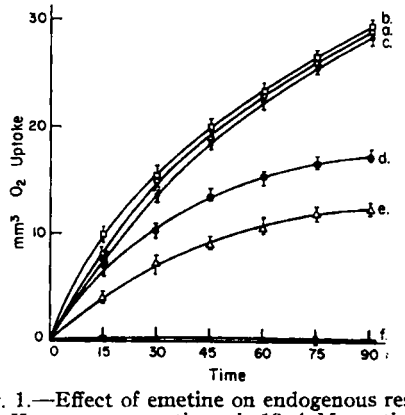


Fig. 1.—Effect of emetine on endogenous respiration. Key: *a*, no emetine; *b*, 10^{-4} *M* emetine; *c*, 2×10^{-4} *M* emetine; *d*, 5×10^{-4} *M* emetine; *e*, 10^{-3} *M* emetine; *f*, 3×10^{-3} *M* emetine. Flasks contained 0.5 ml. homogenate; 0.1×10^{-4} *M* cytochrome C; 0.1 ml. 2×10^{-2} *M* malate; 0.1 ml. 2×10^{-2} *M* ATP; 0.1 ml. 1.5×10^{-2} *M* nicotinamide; 0.1 ml. 10^{-3} *M* $MgCl_2$; 0.3 ml. H_2O; 1.0 ml. 0.1 *M* phosphate buffer, pH 7.4; 0.3 ml. emetine to yield concentrations listed; H_2O to make 3.0 ml. Center wells contained 0.2 ml. 10% KOH. Gas phase, air. Temperature, 30°C.

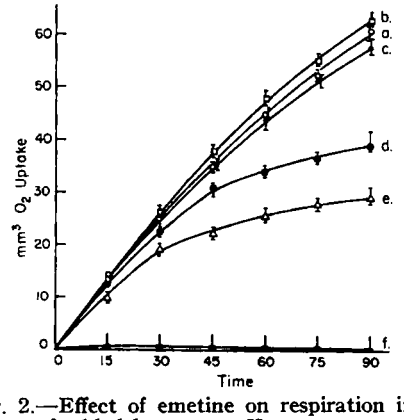


Fig. 2.—Effect of emetine on respiration in the presence of added butyrate. Key: *a*, no emetine; *b*, 10^{-4} *M* emetine; *c*, 2×10^{-4} *M* emetine; *d*, 5×10^{-4} *M* emetine; *e*, 10^{-3} *M* emetine; *f*, 3×10^{-3} *M* emetine. Experimental conditions and flask contents are identical to those in Fig. 1, except that 0.3 ml. 0.1 *M* butyrate was added instead of 0.3 ml. H_2O.

respiration in the presence of added butyrate or β-hydroxybutyrate were inhibited by emetine. The inhibition was complete at concentrations above 3×10^{-3} *M*, while at 1×10^{-4} *M* or 2×10^{-4} *M*, no significant inhibition was observed. At concentrations of 5×10^{-4} *M* and 1×10^{-3} *M* the inhibition was quite marked, being of the order of 40 to 60%. Concentrations of these magnitudes are high so as to be unrealistic when compared to the emetine found in the heart after administration of the drug to experimental animals (20), and the question arises as to whether the observed effects are specific for emetine. Figure 4 shows that quinine, at concentrations identical to those of emetine, inhibited the respiration of

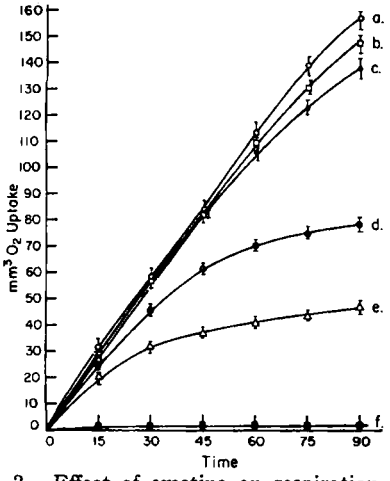


Fig. 3.—Effect of emetine on respiration in the presence of added β-hydroxybutyrate. Key: *a*, no emetine; *b*, 10^{-4} *M* emetine; *c*, 2×10^{-4} *M* emetine; *d*, 5×10^{-4} *M* emetine; *e*, 10^{-3} *M* emetine; *f*, 3×10^{-3} *M* emetine. Experimental conditions and flask contents are identical to those in Fig. 1, except that 0.3 ml. 0.1 *M* β-hydroxybutyrate was added instead of 0.3 ml. H_2O.

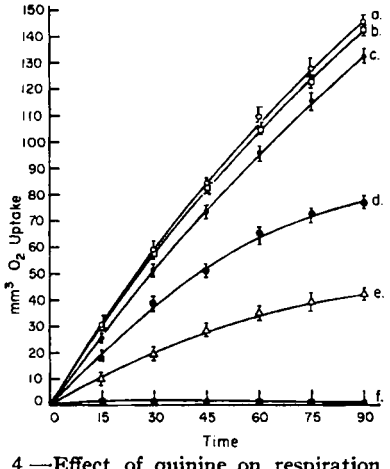


Fig. 4.—Effect of quinine on respiration in the presence of added β-hydroxybutyrate. Key: *a*, no quinine; *b*, 10^{-4} *M* quinine; *c*, 2×10^{-4} *M* quinine; *d*, 5×10^{-4} *M* quinine; *e*, 10^{-3} *M* quinine; *f*, 3×10^{-3} *M* quinine. Experimental conditions and flask contents are identical to those in Fig. 3, except that 0.3 ml. quinine hydrochloride solution, pH 7.4, was added instead of emetine to yield concentrations listed.

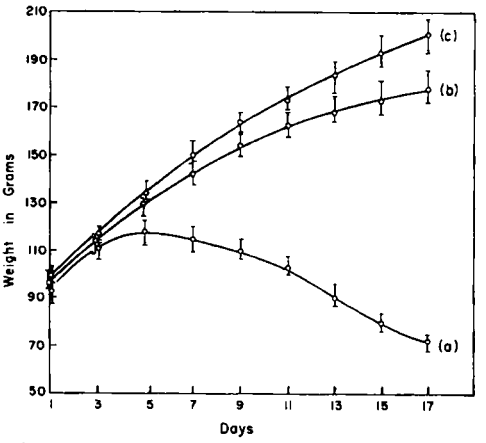


Fig. 5.—Effect of daily administration of emetine and quinine on weight gain of young rats. Key: *a*, emetine; *b*, quinine; *c*, sham-injected controls.

TABLE I.—EFFECT OF PROLONGED ADMINISTRATION OF EMETINE OR QUININE ON OXYGEN UPTAKE BY HEART HOMOGENATES[a]

Substrate	μl. O_2 Uptake/90 Min.	Variation from Controls, %
Sham-Injected Controls		
Butyrate	54 ± 4	...
β-Hydroxybutyrate	147 ± 9	...
None	29 ± 3	...
Emetine Treated		
Butyrate	35 ± 3	−35
β-Hydroxybutyrate	93 ± 6	−37
None	19 ± 3	−35
Quinine Treated		
Butyrate	61 ± 6	+11[b]
β-Hydroxybutyrate	138 ± 12	−10[b]
None	28 ± 3	+4[b]

[a] Water added to flasks in place of emetine or quinine, but other experimental conditions identical to those listed with Fig. 1. [b] Not significantly different from values obtained with sham-injected control animals.

homogenates in the presence of added β-hydroxybutyrate. Endogenous respiration, as well as that in the presence of added butyrate, was inhibited by quinine in the same concentrations; the magnitude of the inhibition was practically identical to that observed with emetine. Such observations suggest that the inhibition of respiration by high concentrations of emetine may be nonspecific and, therefore, of little value in explaining the cardiotoxic action of the drug. It would appear to be of greater importance to study the respiration of heart preparations made from animals chronically poisoned by emetine and quinine. Figure 5 shows that doses of 0.2 mg. of emetine daily caused a marked deterioration of the experimental animals evidenced by a pronounced weight loss. Animals given daily injections of 25 mg. of quinine gained weight almost as rapidly as did the sham-injected controls and, at the end of the experimental period, did not show the marked deterioration observed with the emetine group. Blood glucose levels of the emetine-treated animals were not significantly different from those of the control group or the quinine-treated group. Similar observations have been made by other in-

TABLE II.—EFFECT OF ADDED EMETINE AND OF CHRONIC EMETINE POISONING ON OXIDATIVE PHOSPHORYLATION BY HEART MITOCHONDRIA IN THE PRESENCE OF α-KETOGLUTARATE[a]

	Microatoms O Consumed	μm. P Consumed	P:O Ratio
Sham-injected animals			
No emetine added to flasks	5.8 ± 0.4	21.8 ± 3.1	3.8 ± 0.4
Sham-injected animals			
Flasks contained 10^{-3} *M* emetine	4.1 ± 0.5	15.7 ± 1.9	3.8 ± 0.3[b]
Emetine-poisoned animals	5.3 ± 0.4	19.0 ± 2.2	3.6 ± 0.3[b]

[a] Flasks contained in the main compartments 0.4 ml. 0.1 *M* potassium phosphate buffer, pH 7.4; 0.1 ml. 0.3 *M* $MgSO_4$; 0.1 ml. 4×10^{-4} *M* cytochrome c; 0.1 ml. 5×10^{-2} *M* adenosine triphosphate; 0.2 ml. 0.1 *M* α-ketoglutarate; 0.1 ml. 0.3 *M* potassium malonate; 0.8 ml. 0.25 *M* sucrose; 0.3 ml. H_2O (or emetine to yield molarity listed); and 0.4 ml. mitochondrial suspension, representing 500 mg. fresh ventricle. Sidearms contained 0.1 ml. 0.8 *M* glucose and 0.1 ml. purified hexokinase preparation (Sigma Chemical Co., St. Louis, Mo.) representing 600 units. Center wells contained 0.2 ml. 10% KOH together with a pleated strip of filter paper. Temperature, 30°; gas phase, air. Control flasks removed from bath at end of 5-minute equilibration period and reaction stopped by adding 0.5 ml. 30% trichloroacetic acid. Oxygen uptake measured in remaining flasks for 50 minutes, after which time the reaction was stopped with trichloroacetic acid and the inorganic phosphate determined. [b] Not significantly different from values obtained with sham-injected animals. Theoretical P:O ratio = 4.0 (8).

vestigators, who found that chronic poisoning by emetine did not evoke a change in blood glucose concentration (13). However, the intestinal tracts of the quinine-treated animals showed marked edema and the livers were discolored. Doses of quinine above 25 mg. per day caused death in an appreciable number of the animals and, for this reason, 25 mg. per day was chosen as the amount to be administered.

Table I shows that the oxygen consumption of heart homogenates prepared from emetine-poisoned animals is significantly lower than that observed either with the sham-injected controls or the quinine-treated animals. The heart has been reported to oxidize several substrates as energy sources among which are included pyruvate, glucose, lactate, and fatty acids (21). It has been demonstrated further that, under fasting conditions, the heart derives energy almost exclusively from the oxidation of fatty acids (22, 23). The rats poisoned by daily administration of emetine lost weight rapidly and resembled, in this respect, animals subjected to severe starvation. Furthermore, these animals consumed much less food than did the sham-injected or the quinine-treated animals. The hearts from the emetine-poisoned animals did not oxidize butyrate or β-hydroxybutyrate as efficiently as did the hearts from the unpoisoned animals or those given large daily doses of quinine. Such observations indicate that the effect of emetine on the heart is different from that which would be expected in animals subjected to severe starvation.

The efficiency of cellular oxidative processes is related not only to the actual utilization of oxygen but also to the concomitant production of high-energy phosphate, *i.e.*, oxidation is coupled to phosphorylation. Previous studies have shown that emetine enhances the oxidation of succinate by heart homogenates, an observation similar to that made by other investigators (24), who studied the effect of thyroxin on succinate oxidation. Thyroxin, under certain conditions, has been shown to uncouple oxidation from phosphorylation (25), but the results obtained in this study and set forth in Table II reveal that emetine evokes no significant effect on the P:O ratio when added to heart mitochondria respiring in the presence of α-ketoglutarate. Furthermore, oxidative phosphorylation by mitochondria prepared from the hearts of animals chronically poisoned by emetine was not significantly different from that observed with mitochondria from the sham-injected animals.

Although the chronic poisoning of experimental animals with emetine appears to impair the ability of hearts from these animals to oxidize fatty acids *in vitro*, such an effect does not necessarily account for the cardiotoxic action of this alkaloid. The oxidation of fatty acids ultimately involves the processes of the citric acid cycle, and it appears conceivable that emetine may elicit effects at various points on this "main line" of oxidation. Furthermore, the fact that the endogenous respiration of homogenates prepared from the chronically poisoned animals is lower than that observed with homogenates prepared from the control group indicates that the depression of oxidative processes is not limited to those involving butyrate or β-hydroxybutyrate. Indeed, preliminary experimentation indicates that the inhibitory effects of emetine *in vitro* can be reversed by the addition of nicotinamide adenine nucleotide coenzymes. Additional work in this area is now in progress and will be the subject of a subsequent publication.

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Evaluation of Antispasmodic Activity in the Intact Dog II

By JOHN P. RYAN*, HOWARD J. JENKINS, and SUMNER M. ROBINSON

Results obtained with a method refined to determine more accurately the comparative effectiveness of the active components of an antispasmodic formula and their combinations are presented. Accompanying these results are data pertaining to the comparative antisialagogue activity of these components and their combination in the same kind of animal preparation. In both original and refined methods, a selected submaximal dose of methacholine chloride in mcg./Kg. (Me_{50}) is, in effect, used as a common reference point, so that a comparison may be made of the relative activities of the agents involved in terms of dose equivalents. These results indicate that the combination of components containing phenobarbital constitutes a potentiating union with respect to antispasmodic effectiveness but not with respect to inhibition of salivary secretion.

ATROPINE and many of its chemically related parasympatholytic agents have been known for well over 100 years. *In vitro* methods have been used for the most part in testing the cholinolytic activity of these and like substances. Methods of *in vitro* testing such as those reported by Luduena and Lands (1) provide the means which are in general acceptance today for the pharmacologic screening of parasympatholytic agents.

For testing the total quantitative response of the gastrointestinal tract to parasympatholytic agents, *in vivo* methods such as those described by Ingelfinger (2) in 1943 and Code *et al.* (3) in 1952 are superior to the *in vitro* methods. The tachyphylactic depression of gastrointestinal sensitivity to these blockers, as described by Seevers *et al.* (4) in 1954 and by Quigley *et al.* (5) in 1937, represents an obvious source of error in *in vivo* testing which must be recognized in the development of such bioassay methods. Even so, the studies of Turkanis and Jenkins (6) in 1960 demonstrated the feasibility of properly scheduled *in vivo* methods for quantitating parasympatholytic activity in the gastrointestinal tract (of the unoperated dog).

As originally conceived, the method emerging from these studies entailed initially the determination of the antispasmodic effectiveness of atropine, hyoscyamine and hyoscine individually and collectively, in the latter instance as two different combinations (CBA and CBB),[1] in terms of the dose equivalent with respect to the response of the canine gastrointestinal tract to an established standard dose of methacholine chloride (see Table V). The original method entailed further the statistical comparison of the sum of the individual dose equivalents (calculated), actually a weighted mean, with each collective equivalent (obtained) for the purpose of determining whether potentiation is inherent in the combinations. This method was implemented in the work of Turkanis and Jenkins (6), who determined the response durations which largely dictated the procedural approach to the problem.

It was discovered in the attempt to provide additional data for the original investigation that the ability to obtain a consistent dose-response relationship with this method was limited by some factor or factors then unknown. When a dose-response relationship involving the stimulus, methacholine chloride, and gastrointestinal activity was obtained at the outset of a determination, data consistent with those of the original investigation were obtained; but all too often this relationship was absent and a consequent low yield of valid data resulted from the determination.

In an effort to determine the underlying difficulty, the effect of the anesthetic agent on the response to the various antispasmodics under study was probed. It then became apparent, as other investigators have confirmed (7, 8), that the pentobarbital employed as the anesthetic agent is capable of ganglionic blocking activity, which increases proportionally to the depth of anesthesia and hence contributes varyingly, depending on anesthesia level, to the antispasmodic effectiveness of the compounds undergoing testing.

Although no attempt was made in the former investigation to determine the effect of the combination of the component antispasmodic compounds on a side effect, this was accomplished in the study herein reported. The parameter selected was salivary secretory activity and

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[1] Kindly supplied as Donna and Donnatal, respectively, by A. H. Robins Co., Inc., Richmond, Va.

though the attendant data were collected after the motility data, the procedures employed were identical in the two instances.

This phase of the study indicated that there is no potentiation of antisialagogue activity in the combination of alkaloids.

EXPERIMENTAL

The sensing device used in this investigation was a rubber sheath which could be inflated, after positioning, through the lateral openings of the blind end of a partially inserted Cantor tube (D 111, 12 French). The opposite end of the Cantor tube, leading from the rubber sheath, was attached at its opening to a glass T-tube, the latter being connected in turn with a rubber tube to a sensitive Sanborn transducer. The side-arm of the glass T-tube was provided with a rubber extension and clamp, and thus served as a valve for the inflation and deflation of the sheath when it was positioned in the gastrointestinal tract.

When the proper amount of initial pressure was contained in the unit-tubing-transducer system (determined by using the same strain gage dial setting each time), the recording was begun. To assess the influence of respiratory excursions on gastrointestinal pressure changes, respiratory activity was recorded along with gastrointestinal responses with a pneumograph, a second Sanborn transducer and strain gage amplifier.

The test animals employed were mongrel dogs of either sex ranging in weight from 12 to 22 Kg. During the course of the study, they were fed once daily and supplied with water *ad libitum*. Each dog was placed on a strict dietary schedule to promote regularity in bowel movements, so that a time might be arranged when there was little or no likelihood of the presence in the lower tract of amounts of fecal matter which would result in interference with the response to the drug. (There was almost always a great increase in the response to the spasmogen when considerable fecal matter was present.)

Tests were conducted to evaluate the possibility of the existence of tachyphylaxis in the response to the spasmogen and/or variable effects of the antispasmodics within the framework of the original procedure. No significant discrepancies were discovered at this point, however. The findings of Nash *et al.* (7) strongly implicate pentobarbital sodium as a blocker of parasympathetic ganglia. Such an action on the part of this substance, which was used as an anesthetic agent in the original procedure, would undoubtedly affect the results of the method if the anesthesia level were subject to variation during the course of the testing. The work of Koppanyi *et al.* (8) substantiates the (*para*-sympathetic) ganglionic blocking activity of pentobarbital sodium. These investigators demonstrated a dose-response relationship with respect to the antispasmodic activity of this barbiturate.

In view of this information, it seemed advisable to change the anesthetic agent from pentobarbital sodium to one which would have no appreciable effect upon autonomic ganglia and would be reasonably satisfactory in other respects. Several anesthetic agents, such as chloral hydrate and urethane, were tested but found unsatisfactory for one reason or another. Finally, chloralose (α-D-glucochloralose) was tried and determined to be the most suitable of the lot for this procedure.

All drugs were administered intravenously by injecting their solutions in the rubber tubing connecting an 18-gauge in-dwelling hypodermic needle with a 50-ml. saline-filled buret. A constant volume (3 ml.) of saline was released from the buret to flush each dose into the circulatory system of the animal. In this manner, the administration rate and interval of the compounds under study did not vary appreciably.

In accord with the data of the previous study, the average mean duration of the response to methacholine chloride was 8 minutes, with a range of 4 to 10 minutes. An interval of 15 minutes between doses of the spasmogen was adhered to throughout the entire series of tests. It had been determined in the early work with the original procedure that this interval is adequate for the avoidance of the cumulative effects of methacholine chloride.

In all cases, the onset of antispasmodic activity was exhibited by a fall in the base line of the record. The activity of atropine and hyoscyamine became apparent in the record within 5 minutes of the intravenous administration of these cholinergic blocking agents. And their entire effects were demonstrated in every instance within a 10-minute postinjection period. The establishment of the (partial) cholinergic block of hyoscine required at least 30 minutes following intravenous administration because of its more variable onset of activity. Based upon these findings, the first postantispasmodic dose of methacholine chloride was administered 10 minutes after atropine and hyoscyamine and 30 minutes after hyoscine, CBA, and CBB.

The antispasmodic activity of atropine and related belladonna alkaloids in doses somewhat less than that dose required to produce complete block of parasympathetic myoneural receptors persisted in this test method for periods up to 24 hours. To avoid cumulative effects, it was therefore necessary to administer a submaximal dose of these alkaloids no more frequently than once every 24 hours. Thus, only one antispasmodic dose was injected for each determination involving several methacholine chloride doses.

The latter agent, serving as the stimulus or spasmogen, was administered in varying doses following a given antispasmodic dose with the intent of obtaining a methacholine chloride$_{50}$ (Me_{50}), a dose of methacholine chloride the response to which is reduced by the antispasmodic to 50% of that to a preantispasmodic dose of this drug. The extent of the inhibition of each methacholine chloride dose by the antispasmodic was determined and expressed as a per cent of the preantispasmodic response (Table I).

A minimum of three postantispasmodic doses of methacholine chloride was employed to obtain the Me_{50} for a single antispasmodic dose: a high dose with an inhibition of less than 50%, a low dose with an inhibition of more than 50%, and a middle dose with an inhibition of approximately 50%. For every antispasmodic dose, each of the three or more doses of methacholine chloride, in mcg./Kg.,

was then plotted against the corresponding per cent inhibition of the preantispasmodic response (to methacholine chloride) on logarithmic probability paper. The line to fit these data points was drawn by inspection and the χ^2 test was applied to determine the goodness of fit. From this line of the plotted data, the Me_{50}, in mcg./Kg., for a given antispasmodic dose was read directly.

In a particular determination, two or three preantispasmodic doses of methacholine chloride were employed at 15-minute intervals, their responses averaged, and the per cent of this average represented by the response to each postantispasmodic dose of methacholine chloride evaluated.

Each antispasmodic was assigned a relative potency value which was based on the reciprocal of its Me_{50} 5.60 value (mg./Kg.) multiplied by the Me_{50} 5.60 value of atropine sulfate, 0.0300 mg./Kg. In this manner atropine was given a value of unity; the other agents, including CBA and CBB, were listed according to their relative potencies as multiples of this number.

The Me_{50} 5.60 value of each antispasmodic, atropine sulfate, hyoscyamine sulfate, and hyoscine hydrobromide was weighted by its relative concentration in the combination, CBB. The products so obtained were totaled. This total was then divided by the sum of the relative concentrations of the antispasmodic under study, and the resultant quotient represented the calculated Me_{50} 5.60 values of CBA and CBB.

Three Me_{50} dose determinations were made for each dose level of each of the two combinations of antispasmodics, CBA and CBB, to insure representative results at each level (Table I). Three such determinations per dosage level were not obtained in the case of each component alkaloid, since the ratios derived from the single values agreed with the concensus ratios for these alkaloids appearing in the literature.

The mean Me_{50} dose values for the various antispasmodic dosage levels were situated in separate distributions, as demonstrated by the fact that no mean value fell within the confidence limits of any other in either the CBA or CBB determinations.

To obtain a mean value with 95% confidence limits for the Me_{50} 5.60 values of each combination, CBA and CBB, three separate dose-response curves were drawn. Each curve represented one or the other of the following: the low responses, the medium responses, or the high responses to each of the three doses of the combinations. As opposed to this, the initial curve of each combination was drawn from the mean of the three Me_{50} doses (of methacholine chloride) per combination dose level. On the multicurve basis described previously, the antispasmodic values for the Me_{50} (5.60 mcg./Kg.) of the outside curves (low re-

TABLE I.—ANTISPASMODIC ACTIVITY OF BELLADONNA ALKALOIDS

Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]	Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]
dl-Hyoscyamine (atropine) sulfate	0.30	5.60	2.16	14.9	CBA	0.016	6.22	5.68	40.0
			5.54	60.4				11.36	80.0
			15.82	72.1				22.72	91.5
l-Hyoscyamine sulfate	0.009	2.87	1.16	20.5	CBA	0.016	6.40	3.03	33.4
			3.10	61.3				5.68	48.9
			5.54	69.8				9.47	56.0
l-Hyoscyamine sulfate	0.012	5.00	3.10	17.9				18.94	87.8
			5.54	64.6	CBA	0.016	6.50	5.55	45.9
			10.85	83.3				11.03	62.7
l-Hyoscyamine sulfate	0.015	7.75	5.29	46.5				22.06	76.0
			9.67	50.0	CBB	0.009	4.95	3.30	25.9
			20.90	79.1				5.57	55.6
l-Hyoscine (scopolamine) hydrobromide	0.005	5.15	2.86	26.7				13.30	96.3
			5.53	70.0	CBB	0.009	5.57	2.75	29.8
			9.93	86.7				5.49	37.8
l-Hyoscine hydrobromide	0.007	16.40	5.59	15.3				10.99	83.8
			11.20	38.9	CBB	0.009	3.99	2.98	28.2
			22.40	58.9				5.49	74.4
l-Hyoscine hydrobromide	0.009	33.70	5.54	8.7				11.19	97.4
			10.87	12.5	CBB	0.012	9.70	5.54	32.9
			21.70	42.5				10.93	51.2
CBA	0.009	2.31	1.10	13.1				19.50	75.7
			2.94	61.8	CBB	0.012	10.00	5.54	30.7
			5.55	94.6				10.93	50.0
CBA	0.009	2.20	1.07	25.0				19.50	75.0
			2.86	58.3	CBB	0.012	8.40	5.56	45.0
			5.54	87.5				11.20	53.4
CBA	0.009	2.30	1.10	20.6				18.65	66.7
			2.53	54.5	CBB	0.016	16.50	5.55	31.9
			5.54	83.4				22.06	36.2
CBA	0.012	3.86	2.38	26.7				29.40	89.4
			5.60	66.7	CBB	0.016	15.20	5.37	24.2
			9.52	93.4				10.75	44.0
CBA	0.012	3.45	2.36	28.1				26.88	60.0
			5.50	74.1				32.20	71.2
			11.02	88.9					
CBA	0.012	3.54	2.42	33.3	CBB	0.016	16.10	5.56	20.8
			5.52	69.5				16.00	46.0
			12.10	90.8				32.00	73.2

[a] A = Methacholine chloride mcg./Kg. [b] B = Per cent response.

TABLE II.—ANTISIALAGOGUE ACTIVITY OF BELLADONNA ALKALOIDS

Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]	Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]
dl-Hyoscyamine (atropine) sulfate	0.030	18.2	11.29	14.8	CBA	0.009	11.6	5.55	10.9
			16.62	47.0				9.97	35.9
			24.92	73.1				16.62	75.2
l-Hyoscyamine sulfate	0.006	4.1	1.33	11.1	CBA	0.012	15.0	5.55	5.0
			2.99	32.0				13.29	40.0
			5.55	65.4				24.92	70.0
l-Hyoscyamine sulfate	0.009	9.9	5.55	16.4	CBA	0.012	18.0	5.55	7.0
			9.30	30.9				13.29	27.6
			3.29	81.0				24.92	69.1
			18.24	90.7	CBA	0.012	19.0	5.55	8.3
l-Hyoscyamine sulfate	0.012	15.0	5.15	5.6				13.29	30.9
			8.31	19.9				24.92	65.6
			16.62	54.8	CBB	0.006	6.6	5.55	38.1
			24.92	76.9				7.64	62.3
l-Hyoscine (scopolamine) hydrobromide	0.005	7.5	2.83	7.8				9.97	71.6
			5.55	31.8	CBB	0.006	8.6	5.55	27.2
			9.97	65.3				7.64	39.5
l-Hyoscine hydrobromide	0.007	8.6	5.55	29.8				9.97	60.5
			9.97	42.8	CBB	0.006	8.5	5.55	26.8
			16.62	87.0				7.64	38.3
l-Hyoscine hydrobromide	0.009	16.2	5.55	2.3				9.97	63.1
			9.97	18.1	CBB	0.009	13.2	5.55	2.3
			16.62	53.7				9.97	34.5
			26.58	81.4				22.60	85.5
CBA	0.006	8.15	5.55	22.6	CBB	0.009	11.8	5.55	11.7
			7.64	47.16				9.97	26.0
			9.97	64.15				22.60	89.6
CBA	0.006	9.0	5.55	25.57	CBB	0.009	11.5	5.55	10.2
			7.64	41.0				9.97	37.0
			9.97	55.17				22.60	89.8
CBA	0.006	10.5	5.55	22.1	CBB	0.012	14.2	5.55	6.0
			7.64	31.6				13.29	38.0
			9.97	49.0				24.92	87.0
CBA	0.009	10.8	5.55	8.5	CBB	0.012	17.8	5.55	1.5
			9.97	52.1				13.29	41.0
			16.62	74.4				24.92	68.0
CBA	0.009	12.2	5.55	10.7	CBB	0.012	13.9	5.55	7.0
			9.97	32.1				13.29	36.5
			16.62	73.2				24.92	88.0

[a] A = Methacholine chloride, mcg./Kg. [b] B = Per cent response.

sponse and high response) of each combination represented the broadest spread of such values possible. Consequently, the 95% confidence limits of the mean of the three values (low, medium, and high response curves) per combination represented the greatest possible range. Thus, greater significance attaches to the failure of the calculated antispasmodic value to fall within these confidence limits of the obtained value in the case of the CBB combination.

As a further check on the validity of the results, the *r* value (9) was calculated for each of the above-mentioned regression lines to determine whether the lines deviate significantly from parallel. Results from this work indicated no significant deviation from parallelism by these lines.

In the antisialagogue aspect of the study, Wharton's duct was cannulated *via* the oral cavity of the chloralose-anesthetized dog with polyethylene tubing (No. 10) which was connected to a saline-filled extension of glass tubing mounted on a millimeter stick. The data of this aspect of the study were treated in the same manner as those of the antispasmodic aspect. (Tables II–VI).

TABLE III.—COMPUTATION OF CALCULATED VALUE OF Me_{50} 5.60 mcg./Kg. FOR CBA (ANTISPASMODIC)

Alkaloid	Concn. in CBA			Actual Me_{50} 5.60, mcg./Kg.
l-Hyoscyamine sulfate	1037 × 0.01272	=		13.19064
dl-Hyoscyamine sulfate	194 × 0.00300	=		5.82000
l-Hyoscine hydrobromide	65 × 0.00543	=		0.35295
	1296			19.36359[a]

[a] 19.36359/1296 = 0.01493 mg./Kg. of CBA is the calculated value of the Me_{50} 5.60 mcg./Kg. Calculated value of CBB same as calculated value of CBA since phenobarbital in amount used has no activity.

TABLE IV.—COMPUTATION OF CALCULATED VALUE OF Me_{50} 18.2 mcg./Kg. FOR CBA (ANTISIALAGOGUE)

Alkaloid	Concn. in CBA			Actual Me_{50} 18.2, mcg./Kg.
l-Hyoscyamine sulfate	1037 × 0.01303	=		13.5121
dl-Hyoscyamine sulfate	194 × 0.0300	=		5.8200
l-Hyoscine hydrobromide	65 × 0.00882	=		0.5733
	1296			19.9054[a]

[a] 19.9054/1296 = 0.01536 mg./Kg. Calculated value of CBB same as calculated value of CBA since phenobarbital in amount used has no activity.

TABLE V.—ANTISPASMODIC ACTIVITY IN COMPARISON WITH ATROPINE SULFATE

Alkaloid	ED_{50} 5.60, mcg./Kg.	Potency Compared to Atropine Sulfate
dl-Hyoscyamine sulfate	0.0300	1.00
l-Hyoscyamine sulfate	0.01272	2.36
l-Hyoscine hydrobromide	0.00543	5.52
CBA[a]	0.01507	2.00
CBB[b]	0.00952	3.15

ED_{50} 5.60, mcg./Kg. calcd. for [a] CBA = 0.01493, for [b] CBB = 0.01493.

TABLE VI.—ANTISIALAGOGUE ACTIVITY IN COMPARISON WITH ATROPINE SULFATE

Alkaloid	ED_{50} 18.2, mcg./Kg.	Potency Compared to Atropine Sulfate
dl-Hyoscyamine sulfate	0.0300	1
l-Hyoscyamine sulfate	0.01303	2.30
l-Hyoscine hydrobromide	0.00882	3.40
CBA[a]	0.01350	2.22
CBB[b]	0.01443	2.08

ED_{50} 18.2, mcg./Kg. calcd. for [a] CBA = 0.01536, for [b] CBB = 0.01536.

DISCUSSION

The original procedure for the assay method under consideration was not entirely satisfactory because it yielded a low percentage of valid data. This procedure was operating properly when the results obtained were consistent with a previously established dose-response relationship. But on several occasions the collected data lacked coherence, at which time it was judged that the procedure was not functioning in the manner of which it was capable.

That the original procedure and the modification of this work constitute bioassay methods is attested by the fact that the potency ratios of atropine, hyoscine, and hyoscyamine obtained with them agree with the consensus values for these agents.

The task of the present investigation was involved then with increasing the yield of valid data from the original procedure through proper modification of it. Of those changes in the original procedure, the only productive one, as it turned out, was the substitution of α-chloralose for the ganglion-influencing pentobarbital as the anesthetic agent. This has served to bring about the desired end of increase in the percentage of valid tests by making considerably less critical the level of anesthesia required during testing.

The results of the work of Turkanis evidence a potentiation in the combination, CBA, which the results of this investigation did not show. This difference is not irreconcilable, however. Pentobarbital, as noted, was the anesthetic agent in the Turkanis procedure, while α-chloralose was used in that capacity in the present investigation. As has been mentioned previously, evidence is available to indicate that pentobarbital in proper dosage possesses ganglion-blocking activity, which activity is manifest in the gut by a decrease in motility. Thus, the effectiveness of any antispasmodic should be increased in the presence of the ganglion-influencing doses of pentobarbital employed in the original procedure; this is apparent when the methacholine chloride-antispasmodic ratio of the Turkanis work is compared with this ratio of the present study. The higher ratios of the calculated and the obtained values for the combination CBA in the Turkanis work, opposed to those of this study, reflect the assist provided the antispasmodic in every instance by the pentobarbital administered. That the obtained value ratio should be higher than the calculated value ratio in the Turkanis work reflects also the ability of the pentobarbital to potentiate when the combination of alkaloids is involved. In the study of this report, there was no evidence of increase in the antispasmodic effectiveness of CBA over the effectiveness of the sum of the components. In neither the CBA nor the individual alkaloid determination of this study was a barbiturate present.

The conclusion to be drawn from the results obtained with the modification of this investigation is that while the combination consisting of the alkaloids alone does not represent, with respect to the antimotility activity, a potentiating mixture in the absence of the phenobarbital of CBB, it does so in the presence of this phenobarbital. The further conclusion that neither combination amounts to enhancement of the total antisialagogue activity of the components is warranted by the results obtained.

SUMMARY

The relative antispasmodic effectiveness of atropine sulfate, hyoscyamine sulfate, and hyoscine hydrobromide both alone and in combination have been determined in a biological assay employing the surgically intact dog. The effect of the addition of phenobarbital to the combination has been evaluated.

In the procedure employed, the combination of antispasmodic alkaloids was observed to be no more effective than the sum of their individual activities when the dose of phenobarbital appearing in the combination, CBB, was absent. When this dose of phenobarbital was present, the combination of alkaloids demonstrated potentiation. Thus, while the combination of alkaloids alone does not appear to constitute a potentiating mixture, in so far as antimotility activity is concerned, the combination in the presence of phenobarbital does. At the same time, however, there does not appear to occur in either mixture an enhancement of the total antisialagogue activity of the component alkaloids.

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Suspending Potential of Gum Systems and Their General Flow Properties

By GREGORY CATACALOS and JOHN H. WOOD

Plotting of the logarithm of shear stress against logarithm of shear rate should be used to recognize whether the rheogram of a pseudoplastic gum system will become Newtonian or display a yield value at zero shear rate. Either Fitch or Casson plots give a reasonable measure of the yield value, although in general neither are found to hold rigidly. Rheograms may also be used to determine an index of pourability by considering the torque at some suitable higher shear rate. In this work a pourability index is defined as the logarithm of the torque at 50 seconds^{-1}. Since Chong has shown yield value to be a measure of suspending capability, suspendability is similarly defined as the logarithm of yield value. Increasing gum concentration generally leads from Newtonian to semipseudoplastic to pseudoplastic behavior. Yield value only occurs as a limiting case following this transition. The addition of a second agent such as Cab-O-Sil can develop the yield value at levels where neither it nor the other gum displays such behavior. A plot of yield value against concentration shows clearly that there is a critical concentration required to develop this parameter. A plot of yield value against pourability index permits a relative assessment of the suspendability obtainable for a given pourability. These plots, or tables derived from them, facilitate the choice of the most suitable gum system to give a desired degree of suspending capability.

THICKENED SYSTEMS suitable for the stable suspension of insoluble particles of pharmaceutical value have two rheological shear rate regions of practical importance. The moderate shear rate range, 20 to 100 seconds^{-1}, may be considered (1) the region of pouring. The other, the very low shear rate range, defines the existence of absence of a yield value term. Earlier, the importance of yield value for the permanent suspending of such diverse systems as golf balls, marbles, and sand was shown clearly (2) and the possible relation between yield value and sedimentation considered (3). More recently, Chong (4) demonstrated clearly the relationship between the suspending medium and the suspended particle in terms of minimum yield value required to prevent motion of particles of various radii and relative density gradients. He demonstrated the applicability of yield value as a criterion of suspendability (4, 5).

In the study of rheological curves as normally obtained, there is frequently a problem in extrapolation from low shear readings to a yield value. Several methods of yield value extrapolation have been presented in the literature. Normally these are stress (S) *versus* shear rate (D) curves (6), from S *versus* $D^{1/2}$ plots (7), and from $S^{1/2}$ *versus* $D^{1/2}$ plots (8). The latter two have been justified on theoretical grounds by their originators. Hereafter the latter two will be designated as Fitch and Casson plots, respectively. An alternate to graphical procedures has been the use of yield values calculated from equations of state (9). In general, this procedure is too laborous to be of practical value.

Suspending systems which display yield value character tend to be non-Newtonian, specifically pseudoplastic, with increasing shear. Accordingly, for each system a different shear dependency exists, so that materials of similar apparent viscosity at low shear rates can have notably different apparent viscosities at higher shear rates.

It, therefore, seemed desirable to set up from a direct plot of rheological curves parameters which permit the recognition and determination of yield values and pourability.

EXPERIMENTAL

All rheological studies were made at 25° on either the modified Brookfield LVT or the RVT as described elsewhere (10). Although ascending and descending rheograms were run, only the ascending rheograms are considered here since yield value suspension is a static force before flow, and pouring involves no shear in excess of that conveyed by that action. Of course, if pouring into another container for storage is involved and yield value recovery is not rapid, obviously this must be kept in mind in the rheological pattern of measurement. All data were plotted on logarithmic paper. Four basic types of rheograms are obtained and are shown in Fig. 1. First are those curves of type A; these are of slope unity and are therefore by definition, Newtonian, the intercept being the viscosity. The second, type B, is also linear through the range of operations and typifies the Power law behavior $T = k\,D^n$, where k is the intercept and n is the slope. As long as the Power law is obeyed, there is no yield value. In the type C is the well-known behavior of many pseudoplastic systems, the shifting to Newtonian at low shear rates. In general, any downward curvature from a type B toward a type C plot will yield a limiting slope of unity. The last, type D, displays

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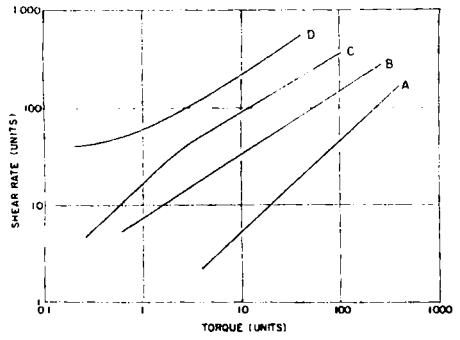


Fig. 1.—Basic classifications of rheograms. Key: A, Newtonian; B, typical Power law; C, Power law going Newtonian; and D, Power law with yield value.

curvature upwards from B and is a mathematical necessity for a definite yield value to exist, regardless of the extrapolation procedure to be used to determine that value.

A shear rate of 50 seconds^{-1} for pouring was taken as an arbitrarily suitable one from Henderson's estimates (1). Now both Scott-Blair (11) and Fryklof (12) have shown that the logarithm of the viscosity is directly related to the subjective evaluation of the consistency of a lotion or paste. Since apparent viscosity is directly proportional to shear stress for any shear rate, the logarithm to the base 10 of the shear stress at 50 seconds^{-1} was defined as the pourability index. Thus, an index of 2 has a viscosity of 2 poises, of 3, 20 poises, and of 3.5, 60 poises. This is the range of medium oils or of chilled 90 to 100% glycerol.

The composition of the gum systems examined rheologically and the essential elements of their preparation are given in the following paragraph. All solvent to gum ratios are on a w/w basis. Either Sorbo[1] (70% sorbitol)–water mixtures or glycerol–water mixtures were used, depending on the implication from the manufacturer's literature as to the preferred system with that gum. No attempt was made to use the same gum in both solvents.

All gum systems were hot hydrated with adequate stirring and then diluted to the desired concentrations. Where the pH was not in the neutral range when finished, the individuals were adjusted in pH as noted. Carbopol 941,[2] a cross-linked synthetic polymer, in water to Sorbo ratios of 40/60, 50/50, 60/40, 70/30, and 80/20 (pH adjusted to 7); the same in 50/50 with 0, 0.5, and 1.0% Cab-O-Sil,[3] a pyrogenic silica (pH adjusted to 7); carboxymethylcellulose (CMC 12 HP[4]) in 50/50 glycerol–water with 0, 0.25, 0.5, and 1.0% Cab-O-Sil; the following in 50/50 water–Sorbo: Guartec GF[5] and Jaguar[6] (neutralized to pH 8.9) both guar gums; the following in 50/50 water–glycerol: karaya,[7] a guar gum (neutralized to pH 8.9), Kelcosol,[8] a sodium alginate, Keltose,[8] an ammonium calcium alginate, Sea-

[1] Atlas Chemical Industries, Wilmington, Del.
[2] B. F. Goodrich Company, New York, N. Y.
[3] Godfrey L. Cabot, Inc., Boston, Mass.
[4] Hercules Powder Company, Wilmington, Del.
[5] General Mills, Inc., Minneapolis, Minn.
[6] Stein Hall & Company, Inc., New York, N. Y.
[7] S. B. Penick & Company, New York, N. Y.
[8] Kelco Company, New York, N. Y.

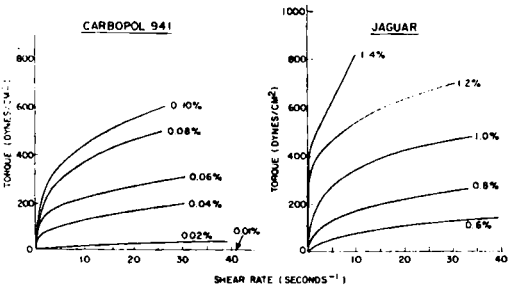


Fig. 2.—Rheograms of Carbopol 941 and Jaguar as a function of concentration.

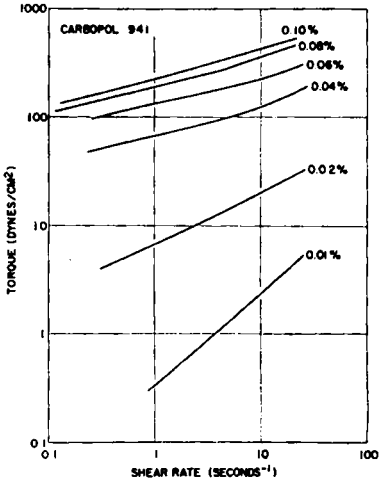


Fig. 3.—Logarithmic plot of Carbopol 941 rheograms.

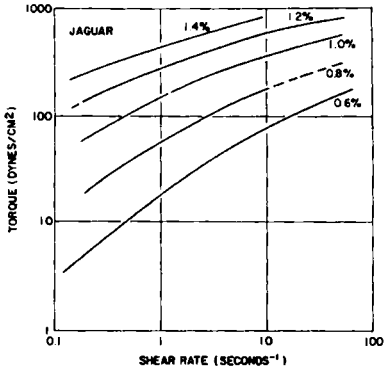


Fig. 4.—Logarithmic plot of Jaguar rheograms

Kem,[9] an Irish moss, tragacanth (Grade A),[7] an Irish moss (neutralized to pH 8.9 to 9.4), Viscarin,[9] a carrageenan, and TIC Colloid-600,[10] a sulfated Irish moss. The latter was hydrated at temperatures of 25, 40, and 60°, since cold hydration was implicitly possible.

RESULTS

Figure 2 shows typical rheograms for two gum systems, neutral Carbopol 941 and Jaguar as a func-

[9] Marine Colloids, Inc., New York, N. Y.
[10] Tragacanth Importing Company, New York, N. Y.

tion of gum concentration. Figures 3 and 4 show the corresponding logarithmic plots. These are of the type D for Carbopol 941 and type C for the Jaguar. In Figs. 5 and 6 these two gum systems are shown in both the Casson and Fitch plots. In Fig. 5 the yield value is clearly apparent, while in Fig. 6 the torques at shear rates below 4 seconds^{-1} suddenly drop off toward zero. The data from the various gum systems were treated similarly. Unless noted, the data reported are usually for 2 days after making of the mucilage. In the systems studied, in no case was a time of aging dependency noticed within the first 10 days.

Table I shows the effect on the yield value and pourability index for two of the Carbopol 941 concentrations of the variation in the ratio of water to Sorbo used.

In Table II the result of the addition of Cab-O-Sil on the rheological parameters is given. Its addition developed yield value and raised the pourability index.

The CMC systems become Newtonian at low shear rates, thus displaying type C properties. The addition of Cab-O-Sil raises the limiting zero shear rate viscosity until finally a yield value appears (see Table III). This is paralleled by a raising of the pourability index.

With karaya no yield value character was observed. With Tragacanth and Jaguar the curves extrapolated to yield values by both procedures, although the behavior was in curve C of Fig. 1. In all gum systems but CMC, increasing concentration resulted in a shift from Newtonian character toward pseudoplastic at low shears with the subsequent evolution at higher concentrations, except for karaya, of a yield value.

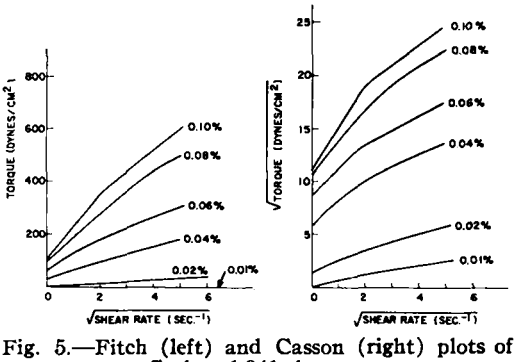


Fig. 5.—Fitch (left) and Casson (right) plots of Carbopol 941 rheograms.

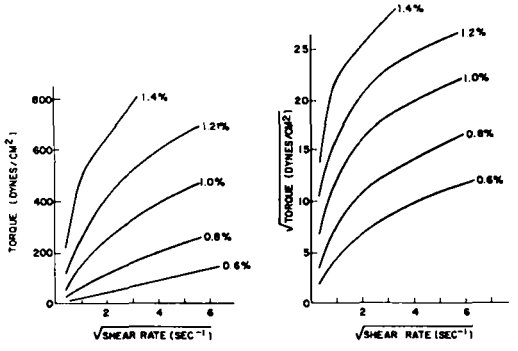


Fig. 6.—Fitch (left) and Casson (right) plots of Jaguar rheograms.

TABLE I.—EFFECT OF RATIO OF WATER TO SORBO ON YIELD VALUE AND POURABILITY INDEX OF TWO CONCENTRATIONS OF CARBOPOL 941 (NEUTRAL), MEASURED AT 4 HOURS AFTER MAKING

Ratio Water/ Sorbo	Yield Value (dynes/cm.²) 0.04%	0.08%	Pourability Index 0.04%	0.08%
40/60	11	80	2.14	2.79
50/50	32	97	2.38	2.81
60/40	18	69	2.11	2.59
70/30	21	80	2.15	2.60
80/20	28	78	2.18	2.58

TABLE II.—EFFECT OF CAB-O-SIL ON YIELD VALUE (dynes/cm.²) AND POURABILITY INDEX OF CARBOPOL 941 (NEUTRAL IN 50/50 SORBO/WATER), MEASURED AT 8 DAYS

Carbopol 941 Concn.	Cab-O-Sil Concn. 0.0% Y.V.	P.I.	0.5% Y.V.	P.I.	1.0% Y.V.	P.I.
0.02%	...	0.97	...	1.39	3	1.74
0.04	...	1.51	16	2.16	9	2.18
0.06	4	1.90	21	2.39	26	2.47
0.08	14	2.19	34	2.60	40	2.65
0.10	34	2.44	43	2.70	72	2.74

Increased temperature of preparation for TIC Colloid-600 raised both the yield value and the pourability index.

Figure 7 shows the dependence of yield value upon the gum concentration for all systems. Included are the apparent values obtained from extrapolation of tragacanth and Jaguar, even though these are of the type C classification. It should be noted that in general the onset of finite yield value occurs for a critical concentration for each gum and that further concentration increases result in extremely high yield values. Only the 60° data are given for TIC Colloid-600. An average value is given for the Carbopol 941 and for the CMC systems.

Table IV gives values characterizing the critical parameters of each gum system. The suspendability is defined by analogy to the pourability as the logarithm of the yield value. Chong (4) found the logarithm of yield values to be related to the suspending requirements.

In Fig. 8 yield value is related to pourability. It is quite apparent that for a given gum the increase in yield value is paralleled by an increase in the pourability index. Only one line each was generated by the Carbopol and CMC systems, and by the different temperatures for the TIC Colloid-600 system. Since the lines are not all parallel, it also follows

TABLE III.—EFFECT OF CAB-O-SIL ON YIELD VALUE (dynes/cm.²) AND POURABILITY INDEX OF CMC-12 HP IN 50/50 GLYCEROL/WATER, MEASURED AT 8 DAYS

CMC-12 HP Concn.	Cab-O-Sil Concn. 0.0% P.I.[a]	0.25% P.I.[a]	0.50% P.I.[a]	1.0% Y.V.	P.I.
0.75%	2.03	2.06	2.08	4	2.34
1.00	2.29	2.32	2.38	10	2.59
1.25	2.52	2.60	2.60	12	2.79
1.50	2.69	2.77	2.87	15	2.96
1.75	2.90	2.99	3.04	19	3.06
2.00	3.04	3.18	3.13	21	3.19

[a] No yield value observed.

that the best suspending agent at lower values is not also the best, automatically, at high yield stresses, considering ease of pouring as the criterion of judgment.

DISCUSSION

When yield values were calculated by both the Casson and Fitch procedures, *i.e.*, by the $T^{1/2}$ *versus* $D^{1/2}$ and T *versus* $D^{1/2}$ methods, respectively, the Casson plot inevitably was slightly higher in resultant yield value than the Fitch plot. The agreement was always good. In some systems, one yielded a better straight line than the other; and in some neither was too satisfactory.

In general, when the Power law index is small, the Casson plot permits better straight lines. For the higher indexes, the Fitch plot is concave to the abscissa and the Casson convex. Thus the consideration of both plots leads to a more valid yield value.

The need for obtaining relatively low shear rates for yield value extrapolation is clearly evidenced by the logarithmic plot. Unless upward curvature has been established, the yield value obtained by

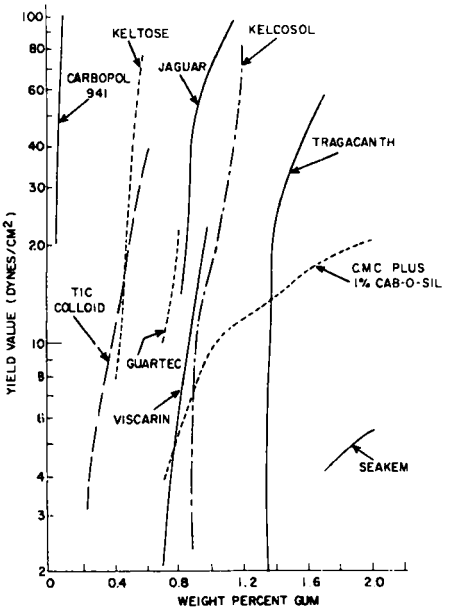


Fig. 7.—Dependency of yield value on gum concentration.

TABLE IV.—CRITICAL PARAMETERS FOR THE GUM SYSTEMS, RELATING POURABILITY INDEX (P.I.), SUSPENDABILITY (S), AND CONCENTRATION

Gum	Concn. for $S = 1$, %	P.I. $S = 1$	P.I./S ($S > 1$)	S/P.I. ($S > 1$)
Carbopol 941	0.01	2.0	1.0	1.0
Keltose	0.4	2.1	0.7	1.5
Jaguar	0.8	2.6	0.3	3.5
Kelcosol	0.9	2.7	0.7	1.5
Tragacanth	1.3	2.8	0.3	3.5
TIC Colloid 600	0.4	2.2	0.4	2.5
Guartec GF	0.7	2.2	0.3	3.5
Viscarin	0.9	2.3	0.3	3.5
Seakem	(2.7)	...	...	...
CMC (+1% Cab-O-Sil)	1.0	2.6	1.8	0.5

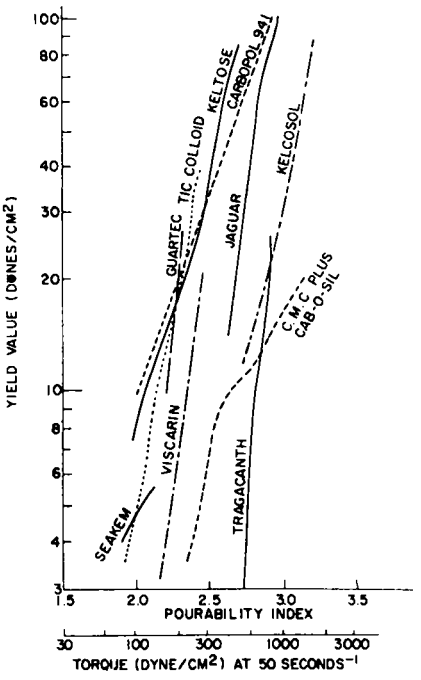


Fig. 8.—Dependency of yield value on pourability for various gum systems.

mathematical or graphical extrapolations of low shear points is of no significance.

Thus in Figs. 5 and 6, if the lowest available shear rates had been for $D^{1/2}$ of 2, obviously quite different results would have been obtained. The Carbopol 941 yield values would have been much higher and the Jaguar ones finite and appreciable. Similar results were recently reported by Charm (13), who found yield values of different orders of magnitude for foods when measured on high and low shear rheometers.

The use of viscosity alone, without benefit of a yield value, cannot lead to a pourable stable suspension. Accordingly, the ability of an agent like Cab-O-Sil, which does not alone at low concentrations give a suitable system, to build yield value into such different systems as Carbopol 941 and CMC 12HP is of distinct value in the developing of practical suspending systems. It should be particularly noted that for low concentration of the primary gum, the Cab-O-Sil displayed no inherent yield value.

Recognizing that suspending power can be built into an otherwise desirable but inadequate suspending medium permits a latitude in formulation. Obviously, other systems than Cab-O-Sil may be considered, including the bentonite and montmorillonite clays, depending on the end use of the product. Thus for certain external applications Baymal[11] might well be considered.

The rheology of mixed systems is virtually an untouched area, although occasionally used in formulative practice on a "feel" basis.

The pourability index, as defined, is a suitable measure of personal judgment of pouring. Others might prefer to define it at any shear rate other than

[11] E. I. du Pont de Nemours & Company, Inc., Wilmington, Del.

our 50 seconds^{-1}, the choice in this work being a practical working range for extrapolation on the log stress-log shear rate plot for the rheometer used.

In the judgment of relative merits of different suspending systems, two ratios can prove valuable for comparison. These are the ratios of the suspendability to the pourability index and the reciprocal of this ratio. The first is a measure of suspendability of a given degree of flow, the other the ease of pouring for a definite degree of suspending ability. The first ratio defines the relative ordinate value in Fig. 8 for a given abscissa and the reciprocal the abscissa for a given value of the ordinate.

Both are useful terms to use to characterize in tabular form the data of Fig. 8 for given values of pourability or suspendability. The ratio, its parameters, along with the critical concentration to give a suspendability of unity, permit an excellent system characterization as is seen in Table IV.

SUMMARY

The use of log shear stress-log shear rate for low shear rate data is suggested as desirable for determining whether yield value character is, or is not, implicit in a given rheogram.

The practical difficulty of extrapolation to a yield value may, in practice, be estimated by either a Casson or Fitch plot.

Many gum systems, on increase in concentration of gum, show the development of yield value characteristics. These characteristics may be markedly enhanced by a second agent whose own yield value will be developed at low concentration.

Since yield value is a direct measure of suspendability and viscosity of pourability, a suspendability-pourability relationship has been developed for a series of gum systems. Tables and plots suitable for suspending gum characterization are given.

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Action and Interaction of Gibberellic Acid and B995 on *Datura innoxia*

By ROGER P. JAMES* and LEO A. SCIUCHETTI

Thirty-three-day-old *Datura innoxia* seedlings were treated weekly for a period of 4 weeks with 50 mcg. of GA, 100 to 1000 p.p.m. solutions of B995, or combinations of both. The GA-treated plants indicated significantly increased height, increased stem dry weight, and generally decreased alkaloid concentration. Plants treated with the combination of the growth factors closely paralleled those treated with GA. Plants treated with B995 closely resembled controls, except for a decrease in alkaloid concentration in the roots at the last harvest. The results of a modified Dragendorff analysis are reported.

RESEARCH recently completed on several different compounds revealed that B995, known chemically as *N*-dimethylaminosuccinamic acid, produced growth retardation in plants (1). Numerous chemical compounds having the specific effect of retarding stem elongation have recently been described (2–9). These growth retardants cause a marked decrease in stem and petiole elongation and exert relatively little influence on root development or leaf expansion, unless administered in high doses. Lockhart (7) has indicated that several of the growth retardants interact competitively with gibberellin on stem growth, and that they act to retard stem elongation by partially blocking the system which provides active gibberellin to the growth mechanism. Others have indicated apparent interactions between gibberellin and certain retardants in *Ulothrix* growth (10), cell division of chrysanthemum (11), and in bean internode growth (12).

Most growth inhibitors are known to stimulate growth at dilute concentrations and inhibit at high concentrations (4, 10, 13). Low concentrations of Amo-1618 stimulated the growth of cucumber seedlings, whereas higher concentra-

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tions retarded growth (4). A similar effect was noted when onion roots were treated with maleic hydrazide (13) and when *U. subtillisma* was treated with AMAB (10).

The following general gibberellin effects have been reported in the solanaceous plants producing tropane alkaloids: increased internodal elongation, taller and spindlier plants, slightly chlorotic leaves, increased stem growth, and reduction in the concentration of alkaloids in the aerial parts (14–20).

The purpose of this investigation was (*a*) to determine whether B995 would retard the growth and/or influence the biogenesis of alkaloids and other components in *Datura innoxia;* (*b*) to ascertain whether this plant responded to gibberellic acid (GA) as did other solanaceous plants; and (*c*) to determine whether interaction occurred when the plant was treated with a combination of growth chemicals which generally induce opposite effects on stem growth.

EXPERIMENTAL

Procedure.—Ninety-six *D. innoxia* seedlings which were approximately 33 days old were used in this study. The seedlings were grown from a 1961 seed crop at Oregon State University and were kept under greenhouse conditions at a temperature ranging from 65 to 85° F. throughout the experiment. The seedlings were transplanted to individual 1-gal. metal cans 9 days before the experimental period began and were randomized on a greenhouse bench. The soil mixture consisted of one part sand, two parts loam, and 5 Gm. of Organic Morcrop[1] fertilizer per can.

The experimental period started on July 5, 1962 (zero time), and lasted 28 days. Three harvests were made, at 0, 2, and 4 weeks. Each harvest consisted of eight plants from each of the four groups, *viz.*, control plants, GA-treated plants, B995-treated plants, and plants receiving a combined treatment. The first harvest, at zero time, however, consisted of 32 plants, which were considered controls since none had been treated. Weekly thereafter, the following treatments were instituted: the GA-treated group received 50 mcg. of GA in a volume of 0.02 ml., which was applied with a micropipet onto the growing apex of the plant. The B995 group received increasing concentrations of the chemical, since visible growth retardation was not observed initially with the lower concentration of the growth inhibitor. At zero time, a 100 p.p.m. solution of B995[2] was used. The next week a 500 p.p.m. solution was employed. The last two treatments consisted of 1000 p.p.m. solutions. The chemical was dissolved in distilled water, and two drops of Triton X100, a nonionic emulsifier, was added to each liter of solution to provide better spreading properties. The treatment was a spray to "run-off." Specially prepared paper shields prevented the solutions from entering the soil. Plants receiving the combined treatment were administered the gibberellic acid 2 days before treatment with B995. The latter was administered weekly in the same concentration as that used on the plants receiving it alone.

At harvest time, each plant was immediately washed, cleaned, and the moisture was removed with towels. Then the plant was divided into leaf-tops, roots, and stem portions. The leaf-tops and stems were cut into small segments, and the fresh weight of each portion was taken immediately. Dry weights were taken after drying 48 hours in a forced air drier at 48.5° C. The plant parts were then ground to a No. 40 powder in a Wiley mill, pooled according to treatment and harvest, and stored in colored glass containers in a desiccator until subsequent analyses were performed.

Growth Effects.—The GA and combination-treated plants grew more rapidly and matured faster than the others. More and larger lateral buds and shoots were noted on these plants than in the controls and B995-treated plants. The stems of the latter were not so woody, nor did these plants bloom so fast as the GA and combination-treated plants.

Height measurements, taken twice weekly, showed that there was no difference between GA and combination-treated plants, which grew significantly taller and more rapidly than controls and the B995-treated plants (Fig. 1). Throughout the experiment, the growth pattern of the controls and B995-treated plants were similar. At the end of the experimental period, both GA and combination-treated plants had attained heights about 146% of controls, whereas B995-treated plants were about 10% shorter than controls (Fig. 1).

Fresh and Dry Weights.—The total dry weight of the GA-treated group was about 15% greater than control plants at the second and fourth weeks. This increase was due to about a 60% gain in stem weight (Table I). The leaf-tops weight of this

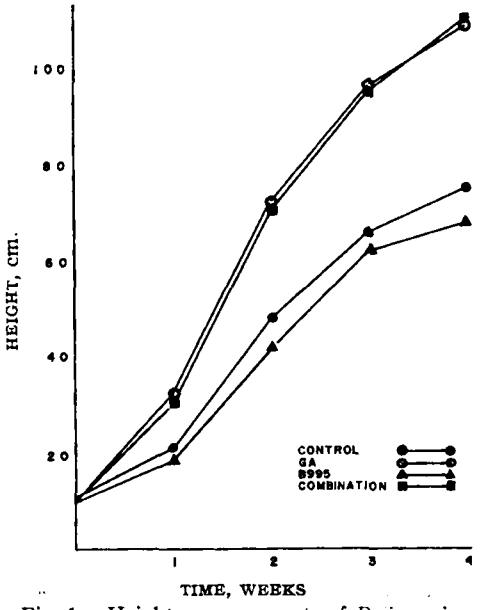


Fig. 1.—Height measurements of *D. innoxia.*

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (analysis 5% of total nitrogen, 3% available phosphate, 2% available potash).

[2] The B995 employed in this study was furnished through the courtesy of Dr. J. A. Riddell, Plant Physiologist, Naugutuck Chemical, Betheny, Conn.

TABLE I.—WEIGHTS OF *Datura* PLANT PARTS[a]

Treatment and Harvest Time, Wk.	Total Wt. Fresh, Gm.	Total Wt. Dry, Gm.	Total Wt. Control Dry Wt., %	Leaf-Tops Fresh, Gm.	Leaf-Tops Dry, Gm.	Leaf-Tops Control Dry Wt., %	Stems Fresh, Gm.	Stems Dry, Gm.	Stems Control Dry Wt., %	Roots Fresh, Gm.	Roots Dry, Gm.	Roots Control Dry Wt., %
Control, 0[b]	4.18	0.276	...	3.11	0.223	...	0.57	0.028	...	0.50	0.025	...
Control, 2	55.92	5.43	...	32.62	3.38	...	14.72	1.35	...	8.58	0.70	...
GA, 2	63.46	6.18	114	27.87	3.39	100	29.41	2.21	163	6.18	0.58	83
B995, 2	53.93	5.58	103	32.12	3.71	109	13.88	1.19	88	7.93	0.68	97
Combination, 2	60.59	5.40	99	27.31	3.20	92	26.98	1.16	123	6.30	0.54	77
Control, 4	118.90	18.99	...	47.50	7.64	...	39.70	6.99	...	31.70	4.36	...
GA, 4	118.10	21.80	115	46.40	8.08	106	56.40	11.13	160	15.30	2.59	59
B995, 4	116.60	18.75	99	50.90	7.52	98	36.40	6.22	89	29.30	5.01	115
Combination, 4	109.10	19.41	102	40.90	7.08	93	54.20	10.02	143	14.00	2.31	53

[a] Mean weights per group of eight plants. [b] Mean weights of all four groups which were harvested but not treated at 0 time.

group gained slightly, whereas the root weight was markedly reduced. The decrease of 41% in the dry weight of the roots at the fourth week was considered significant. In general, the total dry weight and the dry weights of the individual organs of the B995 group approximated that of the controls. Although the total dry weight of the group receiving the combined treatment was about the same as the controls, significant differences were found in stem and root dry weights (Table I). Stem dry weights increased 23 and 43% at the second and fourth weeks, respectively. The increased stem weight of this group was not so great as that of the GA-treated group. Root dry weights were reduced 23 and 47% in the combination group at the second and fourth weeks, respectively. These decreases were larger than that noted in the group treated with GA.

Analysis for Alkaloids.—The dried plant parts, using pooled samples, were assayed for alkaloid concentration, expressed as scopolamine, according to the Brummett-Sciuchetti method (16). Two extractions were made per group, and each group was analyzed in duplicate. When duplicate determinations did not agree, two further extractions were made for each group.

The concentration of alkaloids (mg./Gm.) in the organs of the GA-treated plants was markedly reduced, except that a gain was noted in the leaves of this group at the terminal harvest (Table II). The B995-treated plants at the final harvest indicated about a 17% increase in the leaves and a 35% decrease in the roots, with no appreciable change in the stems. All organs of this group contained less alkaloid at the second week harvest. Significant decreases were noted in all organs of the plants receiving a combination treatment at the second and fourth week harvests (Table II). The reductions in the plant organs of this group were greater than either of the other two groups receiving treatments. In general, the gibberellin effect appeared to be potentiated in plants receiving the combination treatment.

Total Plant Alkaloids.—The total alkaloids per plant and per plant organ were obtained by multiplying the dry weight of the plant part by the per cent of alkaloids obtained from the alkaloid analyses, and expressing the results in milligrams. GA-treatment induced a slight decrease in the total alkaloids per plant (Table III). However, the accumulation of alkaloids in the leaf-tops was increased, whereas total root alkaloids were significantly reduced. The B995 group approximated the controls in total alkaloids per plant. However, appreciable increases were noted in leaf and root alkaloids at the final harvest. In general, significant reductions per plant and per plant organ were noted in the total alkaloids of plants receiving the combination treatment.

Selective Solvent Extraction.—Duplicate 2-Gm. samples of powdered leaf material from each group were selectively extracted in a Soxhlet apparatus in sequence with the following solvents: petroleum ether U.S.P., anhydrous ether C.P., ethyl alcohol U.S.P., distilled water, sodium hydroxide solution (0.2%), and 1% hydrochloric acid according to a modified Dragendorff method (21). The powdered material was subjected to each solvent until completely extracted. Second duplicate determinations were carried out when agreement was not obtained between two samples from the same group. The weights of the dry extracts, obtained

TABLE II.—CONCENTRATION OF ALKALOIDS[a] IN *Datura* PLANT PARTS

Treatment and Harvest Time, Wk.	Leaf-Tops Alkaloids, mg./Gm.	Leaf-Tops Controls, %	Stems Alkaloids, mg./Gm.	Stems Controls, %	Roots Alkaloids, mg./Gm.	Roots Controls, %
Control, 0	3.07	...	8.50	...	5.01	...
Control, 2	4.44	...	5.77	...	5.50	...
GA, 2	4.04	91	3.79	66	4.86	88
B995, 2	4.40	99	4.88	85	4.80	87
Combination, 2	2.98	67	3.31	57	3.34	61
Control, 4	2.56	...	3.49	...	6.12	...
GA, 4	3.24	126	2.28	65	4.00	65
B995, 4	2.99	117	3.68	105	4.00	65
Combination, 4	1.50	59	2.18	62	4.68	76

[a] Expressed as scopolamine.

TABLE III.—TOTAL ALKALOID CONTENT[a] (mg.) OF *D. innoxia*

Treatment and Harvest Time, Wk.	Per Plant		Leaf-Tops		Stems		Roots	
	Total Alkaloids	Controls, %	Totals Alkaloids	Controls, %	Total Alkaloids	Controls, %	Total Alkaloids	Controls, %
Control, 0	1.17	...	0.80	...	0.25	...	0.12	...
Control, 2	25.65	...	15.01	...	6.79	...	3.85	...
GA, 2	24.88	91	13.70	91	8.36	123	2.82	73
B995, 2	25.39	99	16.32	109	5.81	86	3.26	85
Combination, 2	16.83	66	9.54	64	5.49	81	1.80	47
Control, 4	65.96	...	18.79	...	25.79	...	21.38	...
GA, 4	61.92	94	26.18	139	25.38	98	10.36	48
B995, 4	70.42	107	22.48	119	22.89	89	25.05	112
Combination, 4	43.27	66	10.62	56	21.84	85	10.81	51

[a] Calculated from dry weight and alkaloid analyses data; per = leaf-tops + plant stems + roots.

by evaporation of the solvent on a water bath, followed by oven-drying at 48.5° C. for 24 hours, are given in Table IV.

Considerable changes in the concentrations of the various extractives of the leaf-tops were noted in the treated groups. Considering the plants gathered at the final harvest as indicative of specific effects from the treatments, the following differences from controls were noted. Decreases of 17, 26, and 31% were found in the petroleum ether extract of the GA, B995, and combination groups, respectively. This suggests that lipid synthesis and/or accumulation was reduced in the leaf-tops. The ether extractive of the GA group only was affected (a 25% decrease). About a 14% reduction was observed in the alcohol-soluble extractive of the B995 and combination groups, whereas a slight increase was noted in the GA group. Reductions of about 22% were noted in the water extracts of the GA and combination groups; an 11% decrease was found in the B995 group. The sodium hydroxide extract was increased about 14% in the GA group, while decreases of 28% were noted in the B995 and combination extracts, respectively. No appreciable changes were found in the hydrochloric acid extracts. Where appreciable changes occurred in the various fractions, the B995 and combination groups indicated decreases from controls. The reductions were of a greater magnitude in the group receiving a combined GA and B995 treatment than in the group treated with B995 alone.

RESULTS AND DISCUSSION

Slight growth retardation was induced by B995. This group of plants was about 90% as tall as the controls. Although some fluctuations were noted in the dry weights of the various organs of the B995 group, the general growth pattern was similar to the controls. Inconsistent trends were noted regarding alkaloid concentration. All of the organs of the plants treated with B995 had a lower alkaloid concentration at the second week, while at the fourth week a 17% increase was found in the leaf-tops and a decrease in the roots. The total alkaloid content per treated plant approximated the controls. At the final harvest appreciable decreases were noted in the petroleum ether, alcohol, and sodium hydroxide extracts. This indicates that treatment with B995 did markedly affect the quantity of components soluble in these solvents. It is the authors' opinion that more striking effects might have been induced on internode elongation (height) had a much higher concentration of B995 been employed during the treatment period.

D. innoxia responded to GA-treatment, regarding growth and concentration of alkaloids in the aerial parts, in a manner similar to that previously reported for *D. stramonium* (14–16, 19) and *D. meteloides* (18). The general trends in growth and effect on alkaloid concentration as reported by Kapoor and Kaul (20) in *D. innoxia* were confirmed. However, the concentration of alkaloids in the leaf-tops and roots of our plants was about twice as high in both treated and untreated groups. This difference was most likely due to divergences in the environmental conditions and the experimental design of the two investigations. In addition to a favorable effect on growth (as indicated by increased dry weights) the GA-treated plants flowered earlier than controls. The stems also appeared to be more woody. The total alkaloid content per plant at the final harvest was about 6% less than controls. This was due to significant reductions in the concentration of alkaloids in the stems and roots accompanied by a significant increase in leaf-tops alkaloids. About a 20% decrease was noted at the final harvest in the petroleum ether, ether, and water-soluble extractives of the leaf-tops of the GA group. Slight increases were noted in the alcohol-soluble and sodium hydroxide-soluble extractives. The increase in the former correlates with the increased concentration of alkaloids found in the leaf-tops of the GA group.

TABLE IV.—RESULTS[a] OF SELECTIVE SOLVENT EXTRACTION OF LEAF-TOPS OF *D. innoxia*

Treatment and Harvest Time, Wk.	Petroleum Ether	Ether	Alcohol	Water	NaOH	HCl
Control, 2	34.8	15.3	213.4	101.7	142.2	88.5
GA, 2	36.1	13.4	245.6	109.3	151.0	89.8
B995, 2	34.6	17.0	214.0	115.6	147.7	92.5
Combination, 2	33.0	12.0	266.5	144.5	190.1	106.5
Control, 4	26.4	11.5	367.1	158.9	305.9	85.2
GA, 4	21.9	8.6	390.4	123.9	348.8	81.3
B995, 4	19.5	11.9	316.1	140.9	217.7	82.2
Combination, 4	18.3	10.4	319.8	123.8	210.9	76.9

[a] Concentrations given in mg./Gm.

A most interesting aspect of this experiment was the apparent synergistic effect treatment with B995 had when it was combined with GA. The gibberellin effects (on internode elongation and on alkaloid concentration) generally appeared to be potentiated. Plants treated with B995 only were shorter than controls. GA-treatment induced about a 46% increase in height. Plants receiving the combined treatment were also approximately 46% taller than controls. In this case, as well as with the decrease in root dry weight, the combination treatment apparently potentiated the effect noted with GA alone. The stem alkaloid concentration at the last harvest was about 65% of controls in the GA group, 105% in the B995 group, and 62% in the group receiving the combined treatment. In general, when reduced concentrations of alkaloids were found in the organs of the GA group, the decreases were of a greater magnitude in the combined group. The total alkaloid content per plant among the treated groups was the greatest in the B995 group, considerably less in the GA group, and the least in the combined group. Where appreciable changes were noted in the various selective solvent extracts of the B995 group, the variations were greater when B995 was combined with GA than when B995 was employed alone.

CONCLUSIONS

B995 had a slight effect on the retardation of stem elongation at the concentrations employed. The total alkaloid content of this group approximated the controls. However, results from the selective solvent extraction procedure indicated marked decreases in the quantity of the ether, alcohol, and sodium hydroxide extracts from the leaf-tops.

This plant responded to GA-treatment in a manner similar to other solanaceous plants. The following characteristic gibberellin effects were noted: taller and spindlier plants, significantly increased internodal elongation, slightly chlorotic leaves, woodier stems, a significant increase in stem weight, and a general reduction in the concentration of alkaloids in the aerial parts of the plant. Also, decreases of about 20% were found in the petroleum ether, ether, and water-soluble extractives of the the leaf-tops.

A most interesting interaction occurred when the plants were treated with a combination of growth chemicals. Only slight growth retardation, as indicated by height, was noted in *D. innoxia* treated with B995. Significantly increased height was induced with GA. However, the gibberellin effect predominated or was apparently potentiated when both treatments were employed. A similar trend was usually noted in the alkaloid concentrations of the various plant organs. This suggests that the concentration of B995 used in this experiment was not sufficiently high to induce definite growth retardation, but was low enough to demonstrate a slight stimulatory (gibberellin) effect. Others (4, 10, 13) have shown that growth inhibitors at dilute concentrations stimulate growth, whereas at high concentrations growth is inhibited.

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Disposition of S^{35}-Prochlorperazine in the Rat

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Prochloroperazine was shown to be distributed in the organs of the rat much like other weak organic bases. Drug absorption was retarded in repeatedly treated rats and certain tissues appeared to play a storage role. Excretion studies revealed that the fecal route plays the major role in prochlorperazine excretion, less than 25 per cent of the administered dose being excreted in the urine of intraperitoneally dosed control rats. A trend toward decreased urinary and increased fecal excretion was observed in repeatedly treated intraperitoneally dosed and orally dosed rats. Excretion *via* the bile was shown to be a major route in intraperitoneally dosed rats. Unchanged prochlorperazine and three metabolites were demonstrated in the urine.

IN RECENT YEARS numerous reports have appeared concerning the metabolic fate of a variety of *N*-substituted phenothiazines. However, with the exception of the recent reports of Symchowicz *et al.* (perphenazine) (1, 2) and Flanagan *et al.* (prochlorperazine) (3), no investigations have been reported regarding the disposition in animals of the piperazine-linked analogs of this class of compounds. The present report deals with studies of the distribution and excretion of prochlorperazine. Since the development of tolerance to this compound following prolonged administration has been reported (4), a portion of the study was designed to demonstrate any changes in drug disposition which might occur following repeated administration. We have reported previously (5) on the excretion of this agent in the experimentally stressed and jaundiced rat.

METHODS AND MATERIALS

Male albino Holtzman rats of from 250 to 350 Gm. body weight were employed in these studies. All animals were allowed food and water *ad libitum* up to the time of drug administration. Repeatedly treated rats received single intraperitoneal daily doses of 25 mg./Kg. of unlabeled prochlorperazine base (as the dimaleate) for 13 days. They were utilized on the fourteenth day. No attempt was made to determine if a pretreatment regimen of this nature would result in the development of tolerance.

S^{35}-Labeled prochlorperazine dimaleate was used in these studies. It was administered orally or intraperitoneally as a 1.25% suspension in 4% acacia in a dose of 25 mg./Kg. (of the base), as was the unlabeled drug. Each rat received 10 μc. of S^{35} per 275 Gm. of body weight. Within the individual studies, all animals were dosed from the same freshly prepared suspension of drug.

Distribution, metabolism, and excretion studies were conducted on orally dosed, acutely intraperitioneally dosed, and repeatedly intraperitoneally dosed rats. Aside from the method of administration and the tissues sampled, the same techniques were employed in each study.

Drug distribution was studied in three groups of 21 animals, each divided into seven groups containing three rats each. In each group the animals were sacrificed at intervals of 1, 2, 4, 8, 16, 24, and 48 hours after drug administration. Samples of blood, brain [brain samples from intraperitoneally dosed rats were divided into the four areas described by Emmerson *et al.* (6), only cerebral tissue was assayed from orally dosed animals], lung, liver, kidney, spleen, fat, heart, skin, and muscle were obtained from each rat. Tissues were prepared for liquid scintillation counting by adding 3 ml. of a 3:1 mixture of approximately 1 *M* hyamine hydroxide and 30% aqueous potassium hydroxide to approximately 100-mg. portions of tissues contained in Wheaton vials. The vials were then heated at 60° for 2 hours. After cooling at room temperature for 12 hours, 1 ml. of glacial acetic acid and 14 ml. of a scintillator solution (XDC) containing naphthalene, 80 Gm./L; 2,5-diphenyloxazole, 10 Gm./L, and 1,4-di [2-(5-phenyloxazolyl)] benzene, 0.5 Gm./L; in a 1:3:3 mixture of xylene, dioxane, and ethylene glycol monoethyl ether were added.

The urinary and fecal excretion of S^{35} was investigated in three groups containing six rats each. After administration of the labeled drug, the rats were placed in stainless steel metabolism cages. Urine excreted by these rats was collected under toluene at 4, 8, 12, 24, 36, 48, 60, 72, 84, and 96 hours; feces were collected at 24, 48, 72, and 96 hours after dosing. Biliary excretion of S^{35} was studied in five animals by inserting a polyethylene cannula into the bile duct under ether anesthesis. When the animal began to recover from the anesthesia, the labeled drug suspension was administered intraperitoneally, and the animals were restrained in Bollman cages (7). Bile excreted by these animals was collected for 24 hours. Urine and bile samples were prepared for liquid scintillation counting as described previously (8). Feces samples were prepared by adding 3 ml. of the hyamine hydroxide-potassium hydroxide mixture to 50-mg. portions of finely ground feces powder in Wheaton vials. The vials were then heated at 60° for 5 hours. After cooling to room temperature, 4 drops of 30% hydrogen peroxide were added to each vial. When the evolution of gas was complete, the vials were heated at 60° for 2 additional hours. After standing at

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* U.S.P.H.S. predoctoral fellow.

room temperature for 12 hours, 2 ml. of glacial acetic acid and 14 ml. of XDC scintillator solution were added to each vial.

The samples prepared as above were cooled to 0° and counted in a liquid scintillation spectrometer for a period sufficient to give less than 2% counting error. Count rates obtained from these samples were corrected to absolute efficiency by a technique developed by Bruno and Christian (9) or by internal standard techniques. A C^{14} internal standard was employed to avoid the problems associated with the relatively short half-life of S^{35}. Appropriate decay corrections were made in the count rates obtained with all S^{35} samples.

Recovery of metabolites from urine and bile was accomplished by extraction of the total volume excreted by the individual rats during the 96- or 24-hour intervals with ethylene dichloride as described by Fishman and Goldenberg (10) for chloropromazine. Hydrolysis of the urine (5–25 ml.) or bile (1–10 ml.) was accomplished by adding equal amounts of 1 *M* pH 5 acetate buffer and B-glucuronidase, 2,000 units per original 5 ml. of urine or 5,000 units per original 5 ml. of bile. The mixtures were then transferred to 50-ml. conical flasks and incubated at 37° for 18 hours in a Dubnoff shaker.

Urine and bile extracts, reconstituted in 1 ml. of ethylene dichloride, were chromatographed on Whatman No. 3 chromatographic paper using the upper phase of an iso-amyl:tertiary-amyl:formic acid:water (5:5:4:8) system. Chromatograms were developed in the absence of light in a nitrogen atmosphere. Prochlorperazine and its metabolites were visualized on these chromatograms by observation under short-wave ultraviolet light and after spraying with the iodoplatinate reagent described by Smith (11).

Corrected count rates obtained in the distribution studies were treated statistically by the method of least squares so that the concentration of radioactivity in tissues with time could be expressed as a straight line. The biological half-life of S^{35} in the various tissues was calculated using first-order rate equations (12). The significance of differences between the levels of radioactivity in the several tissues in a single group of animals and between the rates of disappearance of S^{35} from those tissues was tested by the Newman-Keuls test (13); the error terms employed in these tests were determined by analysis of variance. All other differences were tested by the *t* test.

RESULTS

Distribution.—The data in Table I describe the distribution plots in the various tissues of the three groups of animals. Data for blood are not included, since in all three studies blood levels were extremely low and nonlinear.

In general, the various tissues can be divided into two categories: (*a*) those from which S^{35} leaves relatively rapidly (brain and fat) and (*b*) those from which it disappears more slowly (reticuloendothelial tissues). Kidney, heart, and skin fall intermediate between these two categories (see Table I). However, while there are roughly two rates at which S^{35} is lost from tissues, the average concentrations reached in the tissues vary widely. In orally dosed rats there is no clear-cut delineation between the rates of disappearance of S^{35} from the various tissue; but there are only minor differences resulting in a spectrum of slopes, only the extremes of which differ significantly. S^{35} disappears most rapidly from cerebrum, fat, and muscle, and more slowly from the reticuloendothelial tissues. This was because, at least in part, the levels were linear over fewer time intervals; therefore, a more conservative error term was employed. In the repeatedly treated rats, evels of S^{35} in the cerebrum are significantly greater than levels in the other brain areas examined, whereas in control rats, S^{35} levels reached in cerebrum, midbrain, and hindbrain are not significantly different.

Tests of differences between distribution plots in corresponding tissues indicated that S^{35} disappeared from liver, kidney, heart, and muscle at a significantly more rapid rate in control than in repeatedly treated rats, as illustrated by the longer half-lives in these tissues in the latter animals. In addition, in nearly every tissue of repeatedly treated rats, peak levels of S^{35} are not reached until 2 hours after drug administration, while in control animals peak levels are attained in 1 hour in almost every tissue. The result of this change in the pattern of drug absorption in the major brain areas

TABLE I.—DISTRIBUTION AND S^{35}-BIOLOGICAL HALF-LIVES

Tissue	Av. S^{35} Concn.[a]	Half-Life, Hr.
	Control-i.p.	
Cerebrum	4.21	7.08
Midbrain	3.48	7.82
Cerebellum	3.02	7.79
Hindbrain	3.49	7.79
Lung	41.05	10.75
Liver	48.55	12.06
Kidney	18.19	9.81
Spleen	23.32	11.62
Fat[b]	103.03	6.08
Heart	4.59	9.64
Skin[c]	5.95	9.69
Muscle	2.54	8.81
	Repeated-i.p.	
Cerebrum	7.55	8.66
Midbrain	5.36	8.14
Cerebellum	4.38	8.49
Hindbrain	4.69	7.79
Lung	100.46	12.15
Liver	76.54	18.83[d]
Kidney	31.76	15.10[d]
Spleen	43.65	14.66
Fat[b]	73.10	8.22
Heart	12.83	15.29[d]
Skin[c]	5.75	12.15
Muscle	3.66	10.96[d]
	Oral	
Cerebrum[c]	2.19	8.82
Lung[c]	28.02	11.03
Liver[b]	48.72	26.08
Kidney[b]	15.44	18.63
Spleen[b]	17.25	16.63
Fat[b]	5.87	12.15
Heart[b]	4.49	14.43
Skin[c]	1.98	13.64
Muscle[c]	1.48	12.70

[a] Mean concentrations over all time intervals expressed as micrograms of prochlorperazine base per gram of wet tissue. [b] Linear from 2 to 48 hours. [c] Linear from 4 to 48 hours. [d] Significantly longer (0.05) than in the corresponding tissue in control-intraperitoneal animals.

TABLE II.—MEAN CUMULATIVE PER CENT OF ORIGINAL DOSE OF S^{35} EXCRETED IN URINE AND FECES FOLLOWING ADMINISTRATION OF S^{35}-PROCHLORPERAZINE TO RATS

Route of Excretion	I. p. Control	I. p. Repeated[a]	Oral
		Urine	
4[b]	0.94	1.68	0.92
8	6.98	4.91	3.27
12	9.09	7.09	6.56
24	17.87	12.70	13.35
36	21.49	15.01	14.51
48	22.64	16.24	15.10
60	22.85	16.71	15.54
72	22.98	17.12	15.86
84	23.04	17.33	16.03
96	23.20^{I}	17.62^{II}	16.15
	$\pm 0.61^{c}$	$\pm 0.92^{c}$	$\pm 1.21^{c}$
		Feces	
24	2.75	8.64	42.87
48	56.39	66.35	71.56
72	68.10	76.42	74.27
96	71.51^{III}	78.73^{IV}	75.12
	$\pm 3.45^{c}$	$\pm 2.67^{c}$	$\pm 4.54^{c}$
Total	94.71^{V}	96.34^{VI}	91.27
	$\pm 3.59^{c}$	$\pm 2.73^{c}$	$\pm 5.61^{c}$

[a] Mean of five rats, others the mean of six rats. [b] Hours after drug administration. [c] ± Standard error; I *vs.* II, significant at the 0.01 level; III *vs.* IV and V *vs.* VI, not significant at the 0.05 level.

is that the initial peak levels obtained in control animals are never reached in the repeatedly treated rat, although levels at later time intervals are somewhat higher in repeatedly treated animals. Biological half-lives of S^{35} in tissues of orally dosed rats are 10 to 100% longer than in intraperitoneally dosed control rats, probably the result of a more prolonged drug-absorption time.

S^{35} Excretion.—Table II summarizes the results of the urinary and fecal excretion studies. These data emphasize the importance of the fecal excretion route for prochlorperazine; urinary excretion accounts for less than 25% of the total amount excreted by control rats. A significantly smaller portion of the original dose was excreted in the urine by repeatedly treated rats than by control rats; this was compensated by increased fecal excretion. In the biliary excretion study 36.79 ± 4.72% of the administered dose of S^{35} appeared in the bile in 24 hours.

Metabolite.—Four basic compounds were detected in urine extracts by paper chromatography (R_f values: I = 0.15, II = 0.20, III = 0.38, IV = 0.84). None of metabolite I appeared in the bile. Comparison of the chromatographic behavior of IV with authentic prochlorperazine in the solvent system described indicated it to be unchanged prochlorperazine. No attempt was made to identify the other metabolites. Because of the relatively small percentage of the original dose excreted in the urine, none of these represented a substantial portion of the dose administered. About 65% of the S^{35} excreted in the urine was extractable by the method employed, only an additional 7% was extractable following the treatment with β-glucuronidase. Thus, among the three groups of rats, only 10–16% of the dose of S^{35} was excreted in the urine as organic extractable material. Very little of the S^{35} present is extractable from bile with ethylene dichloride (10%), and incubation with β-glucuronidase does not result in subsequent extraction of an appreciably greater quantity (16%). Metabolites in the various tissue samples could not be determined.

DISCUSSION

The finding that blood levels were low at all time intervals is typical of many phenothiazines (1, 14, 15). One exception is that the brain levels determined in this study were much lower with respect to other tissues than were brain levels of chlorpromazine determined by Salzman and Brodie (14). Symchowicz *et al.* (1) found that the piperazine-linked phenothiazine, perphenazine, was less concentrated in the brain than in reticuloendothelial tissues, but more concentrated than in skeletal muscle. Thus, somewhat lower brain levels may be characteristic of piperazine-linked *N*-substituted phenothiazines.

These findings demonstrate that repeated administration of prochlorperazine does not result in an increased rate of disappearance of drug from the brain, although peak levels are reduced. The fact that S^{35} disappears more slowly from liver, kidney, heart, and muscle in repeatedly treated rats compared with controls suggests that these tissues may play a storage role in the repeatedly treated animal. In addition, there appears to be a decrease in the rate of absorption of the test dose in repeatedly treated rats.

It was anticipated that fecal excretion of prochlorperazine would be an important route since this is typical of organic bases in general. However, it was not expected that there would be such a marked difference in the relative importance of the two routes since Emmerson and Miya (16) found that fecal and urinary routes of excretion of chlorpromazine in the rat were of essentially equal importance. These findings are compatible with those of Flanagan *et al.* (3), although a direct comparison is not possible because of the difference in doses employed. In view of the findings of Symchowicz *et al.* (1), this pattern of excretion should be considered typical of the piperazine-linked phenothiazines. However, while the rate of urinary excretion of prochlorperazine and perphenazine during the initial 24 hours after drug administration is virtually identical, fecal excretion of prochlorperazine occurs at a much slower rate than is the case with perphenazine. Results of the excretion study in orally dosed rats are in agreement with the findings of the distribution study. The decreased urinary excretion of S^{35} and the rapid appearance of S^{35} in large quantities in the feces implies slower absorption of prochlorperazine. This suggestion is supported by the lower tissue levels of S^{35} obtained in orally dosed rats.

The excretion studies revealed a major alteration in drug disposition resulting from repeated dosing with prochlorperazine. The results are similar to the findings of Mellett and Woods (17), who observed a trend toward decreased urinary and increased fecal excretion of morphine in morphine-tolerant rats. In contrast to these findings, however, Emmerson and Miya (16) found that rats repeatedly treated with chlorpromazine excreted more of a test dose of chlorpromazine in the urine and less in the feces than control rats.

Results of the biliary excretion study imply that the disposition of material excreted into the duodenum *via* the bile does not involve simple direct excretion in the feces. Excretion of large amounts of S^{35} into the duodenum *via* the bile does not lead to fecal excretion of an appreciable quantity of S^{35} during the initial 24 hours after intraperitoneal drug administration. However, about five times as much is excreted in the feces during the first 24 hours when the drug is introduced directly into the gastrointestinal tract (oral dosing). The finding that none of the administered dose is excreted in the bile as metabolite I implies that this metabolite represents further alteration of either metabolite II or III.

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Thin-Layer Radiochromatographic Study of Prochlorperazine Photodeterioration

By SETSUYA SENO, WAYNE V. KESSLER, and JOHN E. CHRISTIAN

Procedures to determine microquantities of photochemical deterioration products of prochlorperazine were developed by using thin-layer chromatography in combination with the radioisotope tracer technique. Up to 11 products were separated on a silica gel thin-layer plate using ethylene dichloride-methanol-ammonia as the solvent. The separated radioactive spots were scraped off the plate, suspended in a Thixcin scintillator gel, and counted. Quantitative results for each product indicated that complex reactions are involved in the photochemical deterioration of prochlorperazine.

PHENOTHIAZINE DERIVATIVES are decomposed by light into various oxidative and cleavage compounds (1–3). They are metabolized by *N*-methyl and side chain cleavage and formation of sulfoxides, phenols, free radicals, and *N*-oxides (4). Several analytical procedures have been used for separating and identifying phenothiazine derivatives and metabolites, especially those of chlorpromazine (4). The most important of these was paper chromatography in combination with spectrophotometry and the radioisotope tracer technique.

Thin-layer chromatography recently has become widely recognized because of many advantages over paper chromatography, such as speed, simplicity, and high sensitivity. The combination of thin-layer chromatography and the radioisotope tracer technique is especially well adapted to very small samples. This combination was reported by Snyder and Stephens (5) in which C^{14} and H^3 labeled lipids were analyzed. The purpose of this study was to use thin-layer chromatography combined with the radioisotope tracer technique for the microquantitative study of the photochemical deterioration of prochlorperazine.

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The authors thank Dr. D. W. Blackburn and Smith Kline and French Laboratories, Philadelphia, Pa., for supplying the labeled and unlabeled prochlorperazine and the Sankyo Co., Tokyo, Japan, for financial assistance.

EXPERIMENTAL

Materials.—Fixed thickness chromatofilm assembly, ascending (model 200-1, Research Specialties Co., Richmond, Calif.) with 8 × 8-in. glass plates was used. Also used were silica gel G (Merck, Germany); prochlorperazine ethanedisulfonate and prochlorperazine-S-35 ethanedisulfonate (Smith Kline and French Laboratories, Philadelphia, Pa.) (the purity of the labeled compound was 99.1%); a Beckman model DB spectrophotometer; a Packard model 314-X Tri-Carb liquid scintillation spectrometer; and solvents and chemicals of reagent grade or equivalent.

Thin-Layer Chromatography.—Aqueous solutions of unlabeled prochlorperazine ethanedisulfonate (0.010, 0.10, and 5.0 mg./ml.) were irradiated by sunlight for 2 hours. The solutions became colored shortly after exposure and darkened further upon continued exposure. The change in the absorption

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spectrum is shown in Fig. 1. After irradiation, prochlorperazine lost the characteristic absorption maximum at 255 mμ, became colored, and showed an absorption maximum at 500 mμ. The resultant solutions which were assumed to contain intact prochlorperazine and its deterioration products were analyzed by thin-layer chromatography.

Silica gel G thin-layer plates were prepared in the usual manner. The plates were activated at 105° for 1 hour. They were spotted with 5, 10, and 25-μl. aliquots of the irradiated solutions and were developed. Twelve solvent systems were examined. It took 30 minutes to 2 hours for the solvent front to advance 10 to 15 cm., the time depending on the nature of the solvent and the temperature of the developing chamber. After the solvent had advanced 10 to 15 cm. from the starting point, the plate was removed from the chamber, dried in a hood at room temperature, and subjected to detection of the separated spots. Ultraviolet lamps (dominant wavelengths 2537 and 3660 Å.), iodine solution (1% in methanol), 2′,7′-dichlorofluorescein solution (0.2% in methanol), iodoplatinic acid solution (0.3% aqueous), sulfuric acid (20% aqueous), and antimony trichloride solution (20% in chloroform) were used. Color production by spraying with antimony trichloride solution gave the highest sensitivity. The detection limit was approximately 0.1 mcg. of prochlorperazine ethanedisulfonate. The best separation was obtained with an ethylene dichloride-methanol-ammonia (65:35:5) mixture.

Five-milliliter aliquots of aqueous prochlorperazine ethanedisulfonate solution (1.0 mg./ml.) were placed in test tubes. The test tubes were plugged with Saran wrapped corks, placed under an ultraviolet lamp (dominant wavelength 3660 Å.) 40 cm. from the filter, and irradiated for 1 to 30 hours. Irradiation caused changes in their color and absorption spectra. Changes in absorbance values at 255 and 500 mμ are shown in Fig. 2. Five-microliter aliquots of the original and irradiated solutions were spotted on a thin-layer plate and chromatographed using the ethylene dichloride-methanol-ammonia solvent. From one to eight separate spots were detected on the thin-layer plate by the color reaction with antimony trichloride. The chromatogram is shown in Fig. 3. The spots with the highest R_f value (R_f = 0.83) correspond to intact prochlorperazine. Others are those of deterioration products. They were not identified in this study.

Thin-Layer Radiochromatography and Scintillation Counting.—Prochlorperazine-S-35 ethanedisulfonate was dissolved in water to yield a concentration of 1.188 mg./ml. (specific activity 0.839 μc./ml.). Aliquots of this solution, each measuring 3.5 ml., were placed in test tubes and irradiated with ultraviolet light for 1 to 33 hours in the same manner as the unlabeled prochlorperazine. Five-microliter aliquots of the original and irradiated solutions were then chromatographed. An autoradiogram of the chromatogram was made by placing a sheet of X-ray film (Kodak No-screen) directly in contact with the silica gel layer and exposing for 80 hours. The developed autoradiogram is shown in Fig. 4.

The radioactivity of each spot located by autoradiography was measured with a liquid scintillation spectrometer. Since silica gel is insoluble and tends to settle in ordinary scintillator solutions and since the complete extraction of adsorbed radioactive material is uncertain, it was desirable to use a scintillator gel system for the radioactivity measurements. A Thixcin gel scintillator similar to that reported by White and Helf (6) was used. The gel consisted of 2.5% Thixcin R in a toluene scintillator

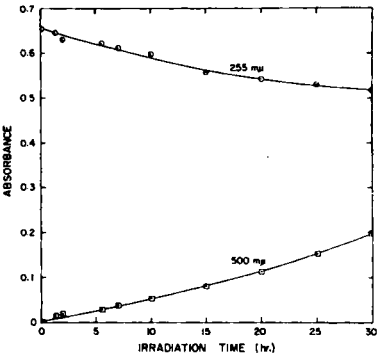


Fig. 2.—Effect of ultraviolet irradiation (dominant wavelength 3600 Å.) on the absorption spectra of prochlorperazine.

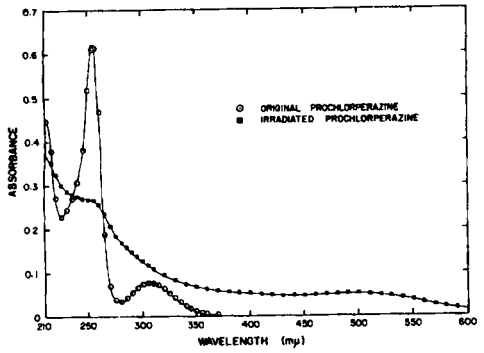


Fig. 1.—Change in absorption spectrum of prochlorperazine upon exposure to sunlight. Concentration of prochlorperazine ethanedisulfonate was 0.010 mg./ml.

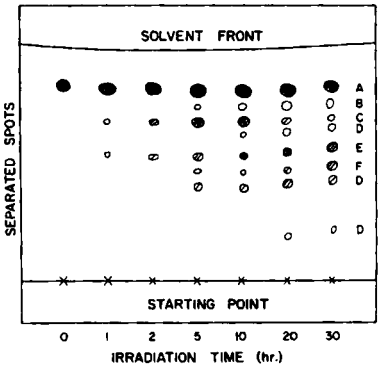


Fig. 3.—Thin-layer chromatogram of photodeterioration products of prochlorperazine. Solvent front averaged 13.3 cm. from the starting point. Color of spots (using antimony trichloride reagent) were: A, pink; B, faint pink; C, purple; D, faint violet; E, dark blue; F, violet.

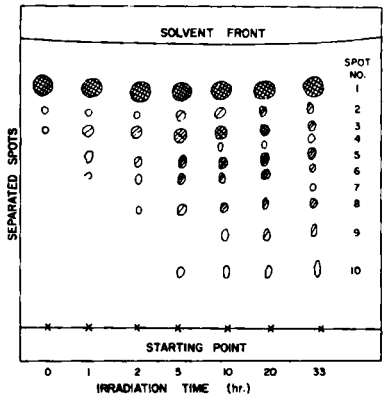


Fig. 4.—Autoradiogram of thin-layer chromatogram of photodeterioration products of prochlorperazine-S-35. Spot 1 corresponds to prochlorperazine.

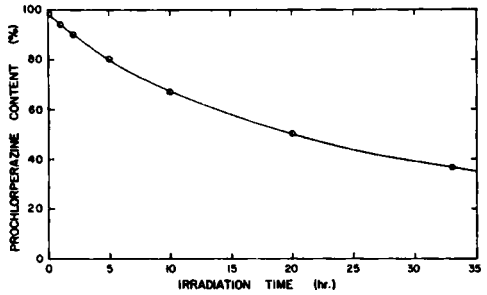


Fig. 5.—Decrease in prochlorperazine content of a sample upon ultraviolet irradiation.

containing 0.5% PPO (2,5-diphenyloxazole) and 0.03% POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene].

The adsorbed spots on the thin-layer chromatogram were scraped quantitatively into a counting vial. The silica gel powder was then suspended in 15 ml. of the scintillator gel. A slight yellow coloration of the gel caused some depression of the counting efficiency. This coloration was prevented by adding 50 μl. of dimethylformamide. All samples counted with the same efficiency. The quantity of material in a spot at each irradiation time was calculated as the per cent of the total count rate at that irradiation time.

The decrease in the prochlorperazine content of the samples upon irradiation is shown in Fig. 5. The curve is that of a first-order reaction during the initial 15 hours, but it deviates thereafter. The calculated first-order rate constant is 0.037 hour^{-1}.

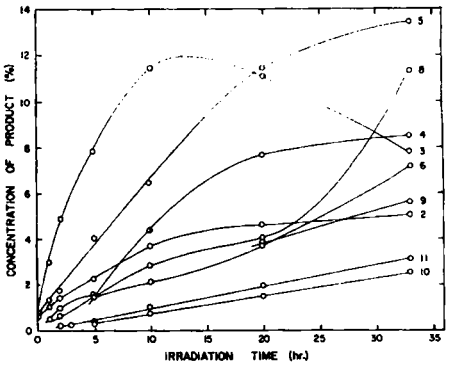


Fig. 6.—Relationships between the concentration of the deterioration products of prochlorperazine and irradiation time. Numbers on curves refer to spot numbers in Fig. 4. Spot 7 is included with spot 8 at 33 hour. Spot 11 refers to starting point.

Figure 6 shows the concentration changes of the deterioration products detected. These curves show that progressive changes of the products into new ones are occurring in the photochemical deterioration process.

CONCLUSIONS

The detection sensitivity of the separated spots was greater in the autoradiographic procedure (Fig. 4) than in the chemical procedure (Fig. 3) in which antimony trichloride was used. This was shown by the greater number of spots detected in the autoradiographic procedure. The detection sensitivity could be increased by increasing the exposure time of the autoradiogram and the specific activity of the sample.

The technique in which thin-layer chromatography is combined with autoradiography and scintillation counting is useful in the study of drug stability, especially in the early stage of deterioration. The sensitivity of the technique would be generally much greater than that of other methods of analysis.

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Physical Factors Affecting the Subcoating of Compressed Tablets I

Instrumentation

By V. N. BHATIA and A. L. BETTS

The objective of this study is to measure the effect of the many physical factors involved in subcoating; this paper deals with a description of an instrument designed for the study. The relative smoothness (or the relative absence of roughness) is being used as the index for evaluation of the subcoating. This has necessitated the development of an instrument which makes possible the quantification of roughness. The "tablet roughness recorder" is a specially designed distance transducer for recording an enlarged profile of a small part of the surfaces of a series of tablets. The area enclosed by these profiles or curves can be measured; thus, comparisons can be made between different batches of tablets. This establishes the relative significance of the physical factors. Their exact quantitative influence can then be individually studied.

IN RECENT YEARS there has been considerable interest in the development of new methods and equipment for tablet coating (1–7). However, the process of tablet coating in conventional pans has undergone very little change, particularly along the lines of better controlled and instrumented equipment and procedures. An important reason for this lack of improvement and change in the basic operations of pan coating might stem from the absence of fundamental studies aimed at elucidating the physical phenomena that affect the process. Several years ago Koren and Benton (8) showed that it was possible to standardize the method of coating. However, extensive standardization, modernization of equipment, and eventually automation could best be achieved if they were based on quantitative fundamental studies.

This study is designed to evaluate quantitatively the physical factors that affect the subcoating process. Subcoating has been selected as the first operation to be studied. Not only does it have more variables than the other steps in the coating process, but also it is the first major step in the entire coating operation—hence, it greatly affects the quality of the other steps.

EVALUATION OF THE SUBCOATING

The uniformity of deposition of the subcoating materials on the tablet appeared to be the most likely criterion for the evaluation of the efficiency of subcoating. The uniformity of the deposition would be related to the relative roughness of the subcoated tablet surface.

The evaluation of the relative roughness, however, is a more complex problem. It has necessitated the development of a special instrument and technique for recording and evaluating the roughness of the tablet surface.

The instrument (hereafter referred to as the roughness recorder) records a nine times magnified equitorval profile of a portion (4.2 ± 0.2 mm.) of the top central surface of a tablet. The area enclosed by the profile or the curve and a chosen base line is measured and serves as the index of roughness of the tablet surface. Comparison of the average indices of batches of tablets coated under varying physical conditions provides a quantitative method for the evaluation of the factors affecting the coating.

ROUGHNESS RECORDER

The roughness recorder consists of the following three main parts.

Signal Source (*S* **in Fig. 1**).—It yields a constant output of 3 v. at 1000 c.p.s.

Roughness Detector (*R* **in Fig. 1**).—The track (*T*) (shown in Figs. 1 and 2) is so arranged that a row of tablets up to $^{7}/_{16}$ of an in. can be placed to a cardboard or metal strip (14 by $1^{1}/_{4}$ in.). The strip can be moved with motor *M* at a constant speed of 3 mm. per minute.

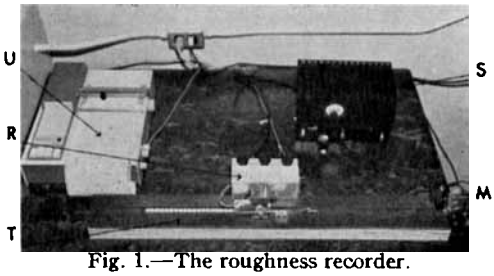


Fig. 1.—The roughness recorder.

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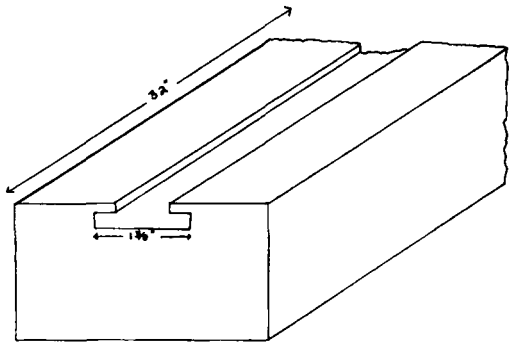


Fig. 2.—The track.

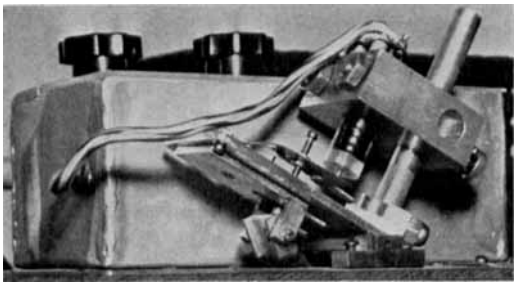


Fig. 3.—The detector.

The mechanical portion of the detector is shown in Figs. 3 and 4. It consists of the probe *A* which protrudes down over track *T* through the hinged guide *G*. The probe moves in a vertical plane as it encounters the roughness or convolutions on a tablet surface. The movements of this probe are mechanically magnified by lever *C* and transferred to a second probe *B;* this in turn moves between two coils arranged in a balancing bridge housed in assembly *D*.

Assembly *D* can be raised or lowered to the proper setting on post *E*. Lever *C* can also be raised and lowered to calibrate the span setting on recorder by turning cam *F*.

The electrical circuitry of the detector is shown in Fig. 5. The balancing bridge is driven by the input from the signal sources and feeds into a bridge detector which in turn feeds into a recorder. The movements of the second probe cause variations in the inductance of the coils, resulting in proportional change in the output voltage of the bridge. This is recorded as the tablet profile.

In operation, the bridge is balanced by first switching the input to the bridge corner at point *X* and adjusting *R*-1 and *R*-2 to a zero setting, the point at which a minimum tone is heard through the headset. The input is then switched to position *Y* and *R*-3 and *L*-1 are similarly adjusted. Finally, the input is switched back to position *X*. It may be necessary also to readjust the height of assembly *D* on post *E* from time to time to check its zero setting. This adjustment should, of course, be carried out prior to the adjustment of *R*-1, *R*-2, *R*-3, and *L*-1.

Thus, when the instrument is adjusted, there is no current flowing through the bridge. Now any movement of probe *B* will result in the increase of the inductance of one of the coils wound around the probe and a decrease in the inductance in the other, producing a voltage across the output terminals. The output voltage is fed to the rectifying bridge which has the effect of removing 1000 c.p.s. and leaving the modulation envelope to be fed to the recorder. The modulation envelope then is determined by the roughness of the surface under the probe *A*.

The Recorder (*U* **in Fig. 1**).—The recorder used in the instrument is the Beckman potentiometric strip-chart recorder. This recorder has an adjustable span which must be calibrated prior to the operation. This is done using cam *F*. Lever *C* in the detector is raised as high as possible with the cam and held in that position while the recorder pen is brought to a predetermined position in the top part of the span. The cam is then turned to allow the lever to return to its lowest position, at which point the zero setting of the instrument is fixed.

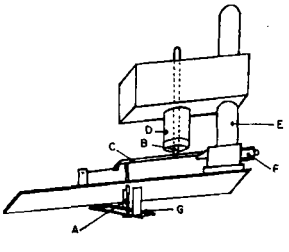


Fig. 4.—Mechanical portion of detector.

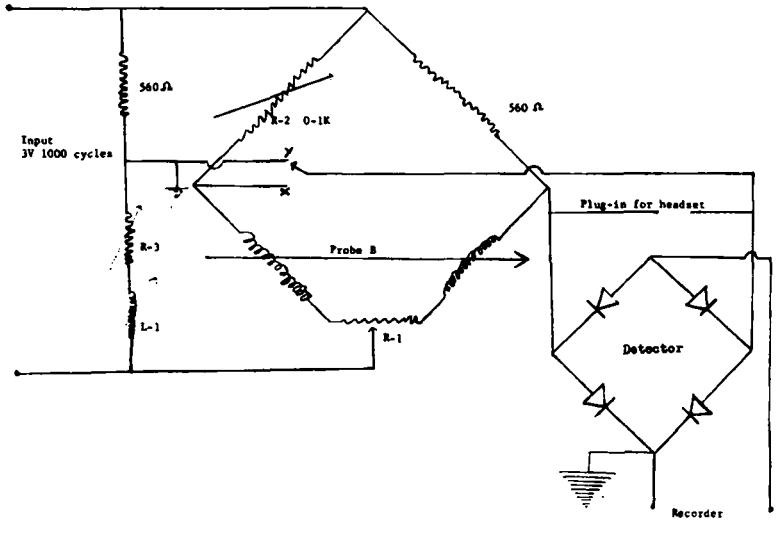


Fig. 5.—Electrical circuit of detector.

Operation

The following steps are involved in using the instrument:

A tablet strip is prepared by affixing 25 tablets from a batch on a 14 by $1^1/_4$-in. thin piece of cardboard. A drop of glue is put on each of the tablets; they are placed in a straight line along the center of the cardboard strip.

The tablet strip is placed on the track and connected to the motor which pulls it along the track.

The power supply to the signal source and the recorder are turned on and the instrument allowed to "warm up" for 10 minutes.

The zero and span settings of the recorder are set, and the detector is zero balanced.

The track motor and the recorder are started and the profile of the tablets recorded.

The operations are repeated with additional tablet strips until recordings of the desired number of tablets have been obtained. The roughness index of the batch can then be calculated.

Reproducibility of Recordings

Repeated runs made with the same tablet strip of subcoated $^3/_8$-in. deep concave tablets gave identical results. The detector portion of the instrument was so balanced that total weight on the tablet at the point where it was contacted by probe *A* was less than 4 Gm. This was insufficient to scratch or damage a tablet surface of normal hardness. Thus, not only are the records true representations of the surface roughness, but also repeat runs on the same tablet strip may be obtained if necessary.

Analysis showed that there was no significant difference between the data on roughness index obtained from several different 100-tablet samples of the same batch of tablets. However, the data on roughness index for batches of tablets coated under different conditions have been different, even when their surface roughness could not be differentiated by visual observation.

Interpretation of Data

Figure 6 shows a typical recording obtained from a subcoated tablet and the method used in reading the recorded curve. It will be noted that in the central part of the curve the recordings tend to be somewhat more linear than the normal convexity of the tablet surface because of the mechanical effect of the hinged guide (*G* in Fig. 4). A similar recording obtained from a polished metal disk having the same general shape and size as the subcoated tablets showed a smooth profile.

In order to read the curve it was first bisected by the line *AB*. Lines *CD* and *EF* were drawn parallel to *AB* and 1.5 cm. from it. Lines *CD* and *EF* were connected by line *GH*, which was so located that it touched the lowest point of the curve between *CD* and *EF* (point *X*). Line *GH* was drawn at a right angle to lines *CD* and *EF*. The area enclosed by the recorded curve and lines *CD*, *EF*, and *GH* (shaded area) was then measured with a planimeter. This procedure standardizes the area measurement by having it apply to the same portion of the recorded curve for each tablet. The average area for 100 tablets of a batch is designated as the "roughness index"

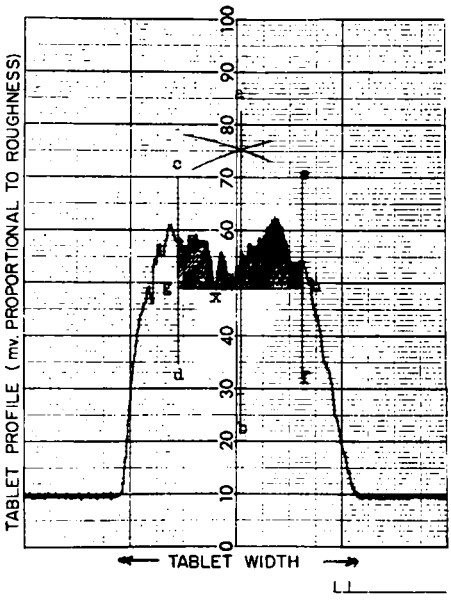


Fig. 6.—Method for reading the area.

for the batch. The index would be zero for a completely smooth surface and increase in value as the roughness increases. It can be used for comparing the degree of roughness of different batches.

The method used for reading the curve was chosen for two reasons: (*a*) the same middle portion of a curve may be read for each tablet, and (*b*) a small area of the recording may be measured because it is assumed that each tablet is uniformly coated up to the thickness represented by point *X*, and the roughness occurs above this point. This is obvious because point *X* represents the deepest depression on the subcoated tablet surface.

Another Application of the Roughness Recorder

The roughness recorder should prove useful as a control instrument for checking the roughness of coated tablets. The Beckman potentiometric recorder used in this instrument can be equipped with an external circuit controller. With this accessory, consisting of two (upper and lower limit) external circuit switches, the instrument becomes a monitor which automatically activates a warning or compensating system when the pen records surface roughness beyond predetermined limits. Each controller operates an independent microswitch that can be used to activate separate external circuits when the pen crosses a preselected point in its travel.

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Physical Factors Affecting the Subcoating of Compressed Tablets II

Preliminary Screening of Some Variables

By M. R. NAZARETH and V. N. BHATIA

A series of preliminary experiments was performed to screen several physical factors that might affect the subcoating process. On the basis of this preliminary screening, three variables were selected for further study: tablet density, pan speed, and pan load. The effect of each of these variables on the "roughness index" was studied. All three strongly influenced the roughness index value of the subcoated tablets. Two equations were developed—one showing the relationship between pan load and roughness index and the other showing the relationship between tablet density and roughness index.

THE NUMBER of physical factors that could possibly have an effect on the quality of subcoating is great. They include such variables as tablet weight, size, surface characteristics, and geometry; batch size, pan dimensions, volume, and speed; physical properties of the subcoating fluid and its constituents; physical properties of the subcoating powder; amount and frequency of addition of the fluid and the powder; temperatures involved in the process; and many others.

Obviously, it would be difficult to study such a large number of variables in a single series of experiments. Hence, the following limited number of factors were selected for the first preliminary screening: (*a*) tablet weight, (*b*) tablet diameter, (*c*) tablet thickness, (*d*) pan depth, (*e*) pan diameter, (*f*) pan volume, (*g*) rotational speed, (*h*) volume of fluid per application, (*i*) number of fluid applications, (*j*) time between applications, (*k*) weight of subcoating powder, and (*l*) pan load.

Actually (*a*), (*b*), and (*c*) vary as a single group when tablets of different sizes are used. Similarly, (*d*), (*e*), and (*f*) vary as a group with a change in pan size. Other factors (*e.g.*, temperature, viscosity and density of coating syrup, temperature of drying air, etc.) were held constant in these experiments.

The procedure of this investigation involved the following steps: (*a*) the subcoating of a number of batches of tablets under strictly controlled conditions, with the quantitative level of one or two of the physical factors sharply varied in each batch (two quantitative levels were used for each of the factors), (*b*) quantitative evaluation of the subcoating for each batch, and (*c*) further studies using the roughness recorder (1) developed for this study to establish the exact quantitative affect of the physical conditions or factors which affect the operation significantly.

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The statistical work in this paper was supervised by Dr. Thomas S. Russell, Associate Statistician, Washington State University, Pullman.

Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

EXPERIMENTAL

Materials

Subcoating Syrup.—The heavy gelatin syrup recommended by Clarkson (2) was used.

Subcoating Powder.—The subcoating powder used was the subcoating dusting powder recommended by Clarkson (2).

Tablets.—Three kinds of tablets were used in this study: (*a*) lactose tablets, (*b*) sodium bicarbonate tablets, and (*c*) zinc oxide-lactose tablets.

Lactose Tablets.—Lactose was moistened with sufficient 20% w/v acacia solution, wet granulated through a 10-mesh screen, dried, and passed through a 16-mesh screen. The granules were then lubricated with 0.75% magnesium stearate and 2% talc and compressed into 0.300-Gm. tablets on a Stokes B2 rotary machine using $^3/_8$-in. deep concave punches. The tablets had a hardness of 6 as measured with a Strong-Cobb hardness tester and a thickness of 0.435 cm.

Sodium Bicarbonate Tablets.—Sodium bicarbonate was wet granulated with 20% w/v acacia solution, dried, and passed through a 16-mesh screen. The granules were lubricated with 0.75% magnesium stearate and 2% talc and compressed into 0.600-Gm. tablets on a Stokes B2 rotary machine using $^7/_{16}$-in. deep concave punches. The tablets had a hardness of 6 as measured on a Strong-Cobb hardness tester and a thickness of 0.445 cm.

Zinc Oxide-Lactose Tablets.—Zinc oxide was wet granulated with 20% w/v acacia solution, dried, and passed through a 16-mesh screen. The granules were lubricated with 0.75% magnesium stearate.

Batches of zinc oxide-lactose tablets having different densities were made by mixing varying proportions of lubricated zinc oxide granulation and lubricated lactose granulation (similar to that used for the lactose tablets) and compressing the mixed granulation on a Stokes B2 rotary machine using $^3/_8$-in. deep concave punches. The thickness of the

tablets was maintained at 0.435 cm. The actual density of each batch of tablets was determined by dividing the mass of a tablet by the volume of liquid (liquid petrolatum) displaced by it.

Equipment and Methods

Coating Pan.—Two pear-shaped copper pans of 39.3 and 30.7 cm. maximum diameter were used. They were equipped with variable speed drives and suction and hot air ducts; the temperature of the hot air was regulated with a rheostat.

Subcoating Procedure.—Powder-free tablets were placed in the pan and preheated with hot air at 55° for 30 minutes. The pan was rotated at a predetermined rate. The hot air was turned off, and hot subcoating solution (50°) was poured over the tumbling tablets in a thin stream directed back and forth across the surface of the tablets. The tablets were immediately raked twice by hand to distribute the syrup and allowed to roll for 2 minutes (at the faster pan speeds, the time was shortened to 1 minute) before the subcoating powder was sprinkled over the tablets. The tablets were then raked again by hand and allowed to roll for 5 minutes before the hot air (55°) and suction was turned on. The tablets were allowed to roll under these conditions until a total of 20 minutes had elapsed from the time the subcoating solution was added. At the end of this period, the suction and the hot air were turned off, and the next addition of syrup was commenced. This process was repeated until the desired increase in weight was achieved. All the tablets used were subcoated until they reached a specific weight. This final weight for the subcoated tablets was 0.430 Gm. for the $^3/_8$-in. lactose tablets and 0.760 Gm. for the $^7/_{16}$-in. sodium bicarbonate tablets.

RESULTS AND DISCUSSION

Over 50 batches needed to be subcoated to complete the preliminary screening of the factors under study. The data are presented only in those instances where the factor or factors had a significant influence in the roughness index. Where the roughness index was not significantly affected by the variations of a physical factor, the negative data have not been included in the report. Wherever necessary, the results were statistically analyzed using the Student t values to test for statistical significance. In the presentation of the data, the physical factors not specifically mentioned were held to a constant value. In the interests of brevity, certain terms are used in the following statements. These terms and their meanings are:

Small Tablet—0.300 Gm. lactose tablets, 0.952 cm. diameter, 0.435 cm. thick, having a density of 1.463 Gm./cm.^{3}.

Large Tablets—0.600 Gm. sodium bicarbonate tablets, 1.111 cm. diameter, 0.445 cm. thick, having a density of 2.000 Gm./cm.^{3}.

Small Pan—pear-shaped copper coating pan, 30.7 cm. maximum diameter, 24.5 cm. deep, having a capacity of 13.7 L.

Large Pan—pear-shaped copper coating pan, 39.3 cm. maximum diameter, 30.2 cm. deep, having a capacity of 28.8 L.

Slow Pan Speed—29 r.p.m.

Fast Pan Speed—47 r.p.m.

Pan Load—unless specified, the pan load for the small pans was maintained at 3 lb. of tablets and for large pans, at 6 lb. of tablets.

Amount of Subcoating Solution per Addition.—The optimum amount of subcoating solution used per addition was determined empirically. The amount used was just sufficient to wet the entire surface of the tablets. When a smaller amount was employed, relatively rougher tablets were obtained. The increase in the roughness index as the amount of subcoating solution is decreased is predictable since the smaller amount of syrup used failed to wet the entire tablet surface and caused the subcoating powder to adhere in patches. Also, because the tablets were not completely wetted under these conditions, grooves on the edges of the tablets were observed. Therefore, this variable has an effect on

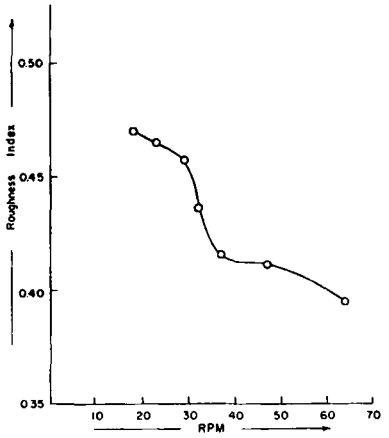


Fig. 1.—Influence of pan speed on the roughness index.

TABLE I.—INFLUENCE OF PAN SPEED ON THE ROUGHNESS INDEX OF SMALL TABLETS[a]

Pan Size	Pan Speed	Roughness Index
Small	Slow	0.4574
Small	Fast	0.4112
Large	Slow	0.4456
Large	Fast	0.3808

[a] Subcoated in small and large pans at slow and fast pan speeds.

TABLE II.—INFLUENCE OF PAN SPEED ON THE ROUGHNESS INDEX OF LARGE TABLETS[a]

Pan Size	Pan Speed	Roughness Index
Small	Slow	0.4668
Small	Fast	0.4656
Large	Slow	0.4488
Large	Fast	0.4422

[a] Subcoated in small and large pans at slow and fast pan speeds.

TABLE III.—INFLUENCE OF PAN SPEED ON THE ROUGHNESS INDEX[a]

Pan Speed, r.p.m.	Roughness Index
18	0.4706
23	0.4652
29	0.4574
32	0.4365
37	0.4158
47	0.4112
64	0.3951

[a] Small tablets and small pan.

TABLE IV.—INFLUENCE OF PAN LOAD ON THE ROUGHNESS INDEX[a]

Pan Load, lb.	Roughness Index
1.0	0.3670
1.5	0.3833
2.0	0.4083
3.0	0.4574
4.0	0.4669
4.5	0.5221
5.0	0.5343
6.0	0.5397

[a] Small tablets, small pan, slow speed.

the roughness index. However, this appears to be an easily controlled variable since it is relatively simple to arrive at the amount of subcoating solution that is just sufficient to wet the entire surface of the tablets. For this reason this factor was not selected for further study.

Amount of Subcoating Powder per Addition.—The optimum amount of subcoating powder used was determined empirically. After allowing the wetted tablets to roll until tacky, just sufficient subcoating powder was added to cause the tablets to roll smoothly again without adhering to each other or to the sides of the pan. An increase in the amount of subcoating powder per addition caused a decrease in the number of additions necessary to add on the desired weight of subcoat, but had little influence on the roughness index of the subcoated tablet. (The excess of subcoating powder often accumulated at the back of the pan and formed small granules on the next addition of syrup. The granules attached themselves to the tablets which increased the number of rejects by creating tablets which had adhering particles.)

Time Between Additions.—The time between additions of subcoating solution had little influence on the roughness index of the subcoated tablet as long as there was sufficient time to dry the previous coat. With the temperature of the hot air blast at 55°, 20 minutes between additions insured complete drying. When the time between additions was reduced to 15 minutes, the roughness index of the tablets was affected little; however, the previous coat did not dry completely and could easily be scraped off. This could create serious problems in the completely coated tablets as the trapped moisture would cause the coating to crack and peel.

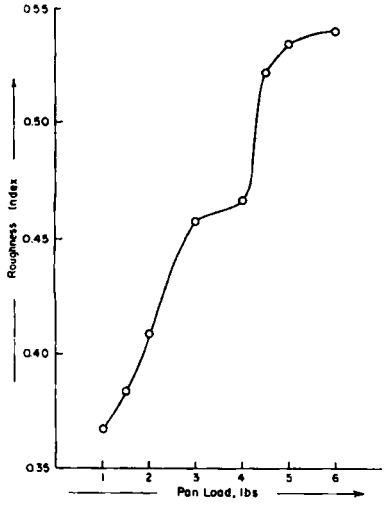


Fig. 2.—Influence of pan load on the roughness index.

TABLE V.—INFLUENCE OF TABLET SIZE ON THE ROUGHNESS INDEX OF TABLETS[a]

Tablet Size	Pan Size	Roughness Index
Small	Small	0.4574
Small	Large	0.4456
Large	Small	0.4668
Large	Large	0.4488

[a] Subcoated in small and large pans at the slow pan speed.

TABLE VI.—INFLUENCE OF TABLET SIZE ON THE ROUGHNESS INDEX OF TABLETS[a]

Tablet Size	Pan Size	Roughness Index
Small	Small	0.4112
Small	Large	0.3804
Large	Small	0.4656
Large	Large	0.4422

[a] Subcoated in small and large pans at the fast pan speed.

Pan Size.—Changing the pan size with proportional changes in pan load had no significant effect statistically on the roughness index of the coated tablet. Slightly smoother tablets were consistently obtained with the large pan compared to the smaller pan. However, this difference was not statistically significant.

Speed of Rotation.—The preliminary data on the influence of pan speed on the roughness of subcoated tablets are shown in Tables I, II, and Fig. 1. Pan speed had a considerable influence on the roughness index of the small tablets (Table I), but had little influence on the large tablets (Table II). The differences in the results for small tablets were statistically significant ($p < 0.05$); this factor was selected for further study. The effect of pan speed on small tablets in small pans is shown in Table III.

An increase in the speed of rotation caused a decrease in the roughness index of the subcoated tablets. As the speed was increased from 18 r.p.m. through 29 r.p.m., the decrease in the roughness index was relatively small. A further increase in the speed of rotation caused a sharper decrease in the roughness index until a speed of 37 r.p.m. was reached. Then as the speed of rotation was increased further, the drop in the roughness index tended to level off. This effect of pan speed on the roughness index appeared to be related to the influence of pan speed on the visually observed roll characteristics of the tablets being coated. An increase in the speed enhanced the tumbling of the tablets, causing them to roll better. The effect was more gradual until a pan speed of 29 r.p.m. was reached. Further increases in the pan speed caused a marked improvement in the roll characteristics until a speed of 37 r.p.m. was reached. An additional increase in the pan speed did not markedly change the roll characteristics.

Pan Load.—In the preliminary screening, pan load (batch size) affected the roughness index, with the smaller pan loads producing a lower roughness index. Hence, this factor was selected for further study. The results of this study are shown in Table IV. The study was made using the small tablets in the small pan at the slow speed.

TABLE VII.—INFLUENCE OF TABLET DENSITY ON THE ROUGHNESS INDEX

Density, Gm./cm.³	Roughness Index
1.463	0.4112
1.756	0.4229
2.049	0.4319
2.439	0.4597
2.927	0.4683
3.658	0.4986

A pan load of 6 lb. of tablets was used as the upper limit since this was the maximum load the pan could hold without an overflow. A pan load of 1 lb. of tablets was chosen as the lower limit because a 0.5 lb. load failed to tumble properly.

An increase in the pan load while keeping all the other factors constant resulted in an increase in rough tablets. In the case of the tablets used there was almost a linear increase in the roughness index as the pan load was increased from 1 to 3 lb. of tablets. The effect started to level off in the 3 to 4-lb. pan load range before it again increased sharply in the 4 to 5-lb. pan load range, and finally leveled off again at pan loads of 5 lb. or more.

The influence of pan load on the roughness index also appeared to correlate with the visually observed roll characteristics of the tablets. It is likely that as the pan load is increased, the number of times a tablet would tumble would be decreased. This hindrance in tumbling might be responsible for the increase in the roughness index with the increase in pan loads. There was not much difference in the visually observed roll characteristics of the tablets at pan loads of 5 and 6 lb., which accounted for the leveling off of the roughness index at these loads. To establish an approximate relationship between pan load and the roughness index, the over-all slope of the curve (Fig. 2) was determined by drawing a line between the 1 and 5-lb. values on the curve. This line corresponds to the over-all linearity of the curve, except at the 4 and 6-lb. levels, at which this linearity does not exist, and gives the following approximate relationship between roughness index and pan load.

$$\text{Roughness Index} = 0.0418 \times \text{Pan Load (lb.)} + 0.3245$$

where 0.0418 is the slope, and 0.3245 is the roughness index intercept. It would be interesting to determine if this relationship holds true for tablets of other size and geometry.

Tablet Size.—There appeared to be little influence of tablet size on the roughness index of tablets at the slow speed. (See Table V.) The roughness indices of similar batches of tablets coated at the fast speed are given in Table VI. At the higher pan speed, the roughness index of the small tablet was reduced, but there was little influence on the roughness index of the large tablet. It was considered likely that tablet density rather than tablet size was the controlling factor in determining the roughness of the subcoated tablets. From the data accumulated, there was no way of determining whether tablet density or tablet size was the main controlling factor; tablet density was arbitrarily chosen for further study.

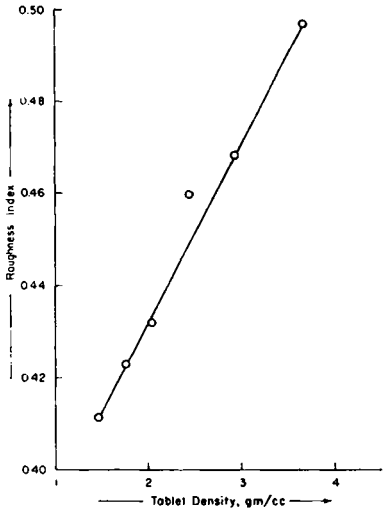


Fig. 3.—Influence of tablet density on the roughness index.

The influence of tablet density on the roughness index of subcoated tablets is shown in Table VII and Fig. 3. The study was made using 3-lb. batches of zinc oxide-lactose tablets of varying density and weight but having the same diameter, thickness, and geometry. The small pan was used at the fast speed. The fast speed was used because in the preliminary studies a greater effect was observed at this speed.

An increase in the tablet density while keeping the other factors constant resulted in an increase in rough tablets. This observation also appeared to correlate with the visually observed change in the roll characteristics of the tablets. The roll characteristics showed a progressive deterioration with increasing tablet density. A linear relationship existed between the roughness index and the tablet density under the above experimental conditions. This relationship was:

$$\text{Roughness Index} = 0.039 \times \text{Tablet Density (Gm./cm.}^3\text{)} + 0.3935$$

where 0.039 is the slope, and 0.3935 is the roughness index intercept. Additional study would be necessary to see if this relationship between the roughness index and the tablet density would hold for tablets of other size and geometry.

SUMMARY

A preliminary study of 12 factors that might affect subcoating has been carried out.

The effects of three factors—pan speed, pan load, and tablet density, which had significant effect on smoothness of the subcoating—have been studied further.

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Method For Recording Electrical Activity of the Isolated Heart

By MARVIN L. ZATZMAN and ROBERT L. RUSSELL

The simultaneous recording of electrical activity from two sites on an isolated mammalian heart by a simple and effective method is described. The use of agar-filled polyethylene tubing containing cotton wick electrodes offers distinct advantages compared to the usual platinum hook electrodes.

THE USUAL METHOD for recording electrical activity from isolated hearts has involved the use of platinum hooks (1, 2). An alternate method which has several advantageous features is described. While recording the atrial and ventricular ECG from isolated rabbit hearts in the Anderson coronary perfusion apparatus, it was found that platinum hook electrodes had limitations. Not only was there injury to the tissue but also breaks between the leads and the platinum hooks occurred repeatedly. To alleviate these occurrences, it was decided to employ cotton wick electrodes. Accordingly, the lower chamber of this unit as described by Anderson and Craver[1] (3) was altered to allow the introduction of these wicks (Fig. 1).

Two electrodes were positioned in each stopper. The wicks were made of cotton tufts saturated with the perfusing solution. Accordingly, each wick was tamped lightly into the end of the polyethylene tubing (PE 350, o.d. 0.157 in., i.d. 0.125 in.) which had been filled previously with agar (3–4% in mammalian Ringer's). Stainless steel wires or tantalum wires (0.025-in. diam.) were inserted into the agar-filled tubing for connection to a recorder with alligator clips. A piece of warmed PE 100 was crimped to the wire as a sleeve to keep the wire in place. Another adaptation is shown in the upper right corner of the figure. This was a hypodermic needle (No. 18, 1.5-in. length) inserted in the agar instead of the stainless steel wire, thereby making it possible to couple the electrode to the recorder with a banana plug.

In addition to the two openings in the lower chamber for the introduction of the stoppers containing the polyethylene tubing, a third opening or port was provided in the original apparatus. Consequently, the experimenter is enabled to insert a probe or forceps through the port in order to position the cotton wicks on specific areas of the heart as desired. After positioning the wicks, the port can be closed by inserting a stopper.

Appropriate recording apparatus was used to record atrial ECG and ventricular ECG. Coronary flow and heart rate may also be recorded simultaneously (Fig. 2). To determine any possible distorting effect of the wick electrodes on the ECG as compared to the record obtained with the usual hook electrodes, a simultaneous recording was obtained from a frog heart using both types of elec-

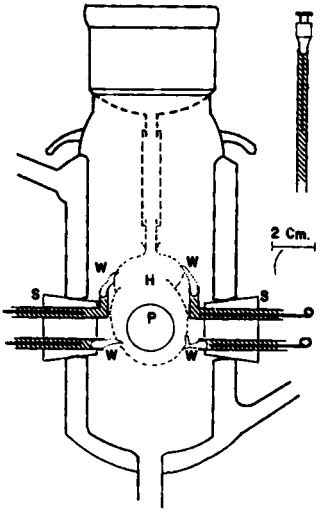


Fig. 1.—Modified lower chamber for recording of ECG from an isolated heart with wick electrodes. Key: W, cotton tuft; H, heart; S, stopper; P, port. Insert: Alternative connector to agar-electrode. (See text for details.)

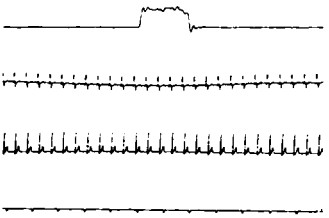


Fig. 2.—Electrical activity and coronary flow in an isolated rabbit heart. From above downward the record presents coronary flow, atrial electrocardiogram, ventricular electrocardiogram, and time in seconds. The coronary flow is indicated by the change in air pressure in a venous Statham pressure gage each time the coronary flow-meter empties.

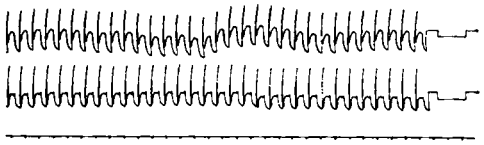


Fig. 3.—Simultaneous recording from the same sites on a frog heart. From above downward: ventricular electrical activity recorded through platinum hook electrodes, ventricular electrical activity recorded through wick electrodes, and time in seconds. Calibration indicates 2 mv.

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[1]Available from Metro Industries, Mineola, L. I., N. Y.

trodes (Fig. 3). Although the amplitude of the recorded potential was reduced somewhat using the wick electrodes, the wave forms are essentially the same. The mass of the cotton wick electrodes is extremely low and is a very flexible system, permitting the tip of the tuft to follow the movements of the heart. The wave form of the ECG of a control preparation indicates no change even after 1.5 hours of continuous recording.

Using the system described, it is possible to denote the difference between a control recording of an isolated cardiac preparation and that following experimental procedures, illustrating that the cotton wicks retain their position and that no injury is produced from their use.

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Microbiological Assay of Aspartocin in Pharmaceutical Preparations Containing Chlortetracycline

By ANTHONY ABBEY and DAVID B. HEWEL

An agar diffusion procedure, using *Staphylococcus epidermidis* ATCC No. 12,228 and 0.1 *M* calcium chloride solution as diluent for aspartocin standard and samples, was evaluated. Satisfactory standard curves for aspartocin were obtained with concentrations of compound ranging from 0.5 mcg./ml. to 50 mcg./ml.; chlortetracycline (CTC) did not interfere in the assay of aspartocin-CTC mixtures.

THE FERMENTATION, isolation, and antimicrobial properties of aspartocin have been previously reported (1–4). It is active primarily against Gram-positive bacteria; and although somewhat similar to amphomycin (5), aspartocin may be separated from this compound and identified by paper chromatography or electrophoresis (4).

For microbiological assay, an agar-plate method using *B. subtilis* and alkaline conditions was proposed (4); however, the tetracyclines are highly active against this test organism, and aspartocin-tetracycline mixtures would be difficult to assay under these conditions. With this in mind, the application of a tetracycline-resistant test organism was investigated.

Staphylococcus epidermidis ATCC #12,228 is routinely employed in this laboratory for neomycin assays, particularly with mixtures containing streptomycin and tetracycline, where resistance to these antibiotics (6) would be essential. Since the aspartocin antibacterial spectrum indicates that low concentrations inhibit staphylococci and streptococci *in vitro* (2), *Staphylococcus epidermidis* ATCC #12,228 was evaluated for use in aspartocin microbiological assays.

EXPERIMENTAL

The method outlined is a modification of FDA cylinder-plate procedures generally used in our laboratory for antibiotic assays; a more detailed description of antibiotic cylinder-plate methods appears in Grove and Randall (7).

Test Organism.—*Staphylococcus epidermidis* ATCC #12,228 (available from the American Type Culture Collection) is subcultured every 2 weeks on nutrient agar slants, incubated overnight at 30–35° and stored at 4°.

For inoculum, suspend the growth from a stock slant in sterile 0.9% saline solution and inoculate a Roux flask containing 200 ml. sterile Difco Penassay seed agar; incubate overnight at 30–35°. Harvest this growth with approximately 50 ml. sterile saline solution and store at 4°; this concentrated suspension may be used at least 5 to 6 days. Before the actual assay, determine what dilution of the concentrate will give 80% light transmission using a Lumetron model #400 colorimeter and 650 mμ filter. Use trial plates to determine the optimum concentration of diluted suspension for the assay (usually 0.5 to 1%).

Assay Medium.—Difco Penassay seed agar (antibiotic medium #1) is autoclaved 15 minutes at 121°, cooled, and adjusted to pH 8.0 with 4% NaOH solution.

Diluent.—The diluent was calcium chloride 0.1 *M* in distilled water for aspartocin standard and unknowns; anhydrous $CaCl_2$ (reagent grade) 11.1 Gm. dissolved in distilled water to make 1,000 ml. of solution (pH 5.8); it was autoclaved for 15 minutes at 121°.

Aspartocin Standard Curve.—Sodium aspartocin (Lot #31460-165A) with an assigned potency of 1,000 mcg./mg.[1] was used for the standard.

Weigh 50 to 100 mg. of the standard and dissolve in sufficient distilled water to obtain 1,000 mcg./ml. stock solution. Dilute further in calcium chloride solution to obtain 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mcg./ml.; the reference point solution is at 10 mcg./ml. The stock solution is held refrigerated and may be used at least 7 days.

Preparation of Test Plates.—The assay agar is cooled to about 45° and inoculated with the test organism. A 6-ml. volume of inoculated agar is distributed evenly into each flat-bottomed Petri dish (Pyrex #3162) equipped with an unglazed ceramic cover, and allowed to solidify on a perfectly level surface.

The agar is allowed to harden 15 to 20 minutes at room temperature before placing six stainless steel Penicylinders on each plate. These cylinder plates

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[1] Obtained from the Biochemical Research Section, Lederle Laboratories Division, American Cyanamid Co.

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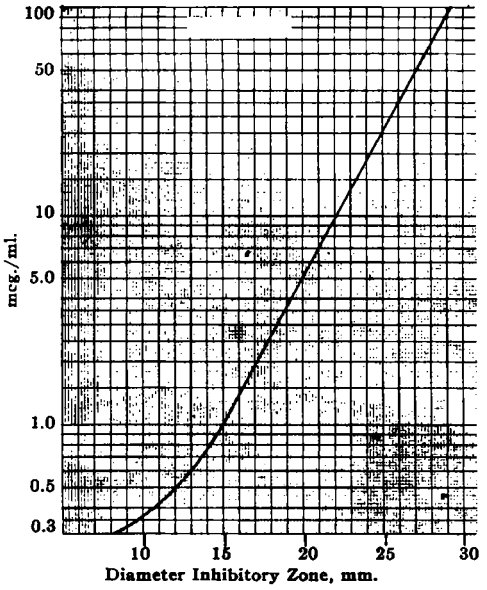


Fig. 1.—Cylinder plate standard curve for sodium aspartocin (Lot #3146C-165A) in 0.1 *M* $CaCl_2$ *vs. S. epidermidis* #12,228.

are held about 10 minutes before filling the cylinders with solutions.

CONDUCTING THE ASSAY

Aspartocin Standard Curve And Unknowns.—Three plates are set for each point on the standard curve. On each plate, fill three alternate cylinders with the 10 mcg./ml. reference solution and the other three with the standard curve solution. Incubate all plates 16–18 hours at 30–32° and measure the diameter of the inhibitory zones. (A Quebec colony counter equipped with an etched glass millimeter scale is used in this laboratory.) Average all of the reference point readings, and use this value to adjust the averages of reference point and standard solution for the three plates set for each point on the standard curve. For example, if all the readings of the reference solution average 20 mm. and, on a particular set of three plates the reference solution averages 19.8 mm., the correction is plus 0.2 mm. for the other solution's response on that same set of three plates.

Plot all points, including the reference point, on 3 cycle semilog paper, using the logarithmic scale for the potency (mcg./ml.). Draw the best straight line to fit the points (see Fig. 1).

The test sample is similarly assayed on three plates after diluting to an estimated 10 mcg./ml. in $CaCl_2$ solution. The sample zone size is adjusted to fit the curve as determined by the response of the reference point. This corrected zone size is then converted directly to mcg./ml. from the standard curve, and the value multiplied by the dilution factor for sample potency.

In the dose-response curve comparisons, the same basic procedure was employed with variations in diluent and culture media; *B. subtilis* or *B. cereus* var. *mycoides* spore suspensions were used for inoculum in specific trials, as indicated (Table I).

Aspartocin–CTC complexes or dry-powder mixtures, such as tablets or capsules, may be extracted in a Waring Blendor using 0.01 *N* HCl to obtain

TABLE I.—SODIUM ASPARTOCIN DOSE-RESPONSE CURVES

Organism	Test Conditions: Agar Medium	Dilutions[a] Prepared In	Average mm. Zone Size Per mcg./ml. Aspartocin Solution At: 50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.195	Diluent Control
S. epidermidis ATCC #12,228	Difco Penassay seed agar pH 8.0	0.1 *M* K_2HPO_4, pH 8.7	23.6	20.6	17.2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
		1% ammonium carbonate, pH 9.0	25.5	23.3	16.6	10.0	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
		0.1% ammonium carbonate, pH 8.8	24.2	22.4	18.0	13.8	Neg.	Neg.	Neg.	Neg.	...	Neg.
		0.1 *M* $CaCl_2$, pH 5.8	27.3	24.8	22.3	20.8	18.5	16.6	14.2	10.5	...	Neg.
		Distilled H_2O, pH 5.9	22.7	19.6	16.9	11.3	Neg.	Neg.	Neg.	Neg.	...	Neg.
		0.1% $Ca(OH)_2$, pH 12.0	20.6	18.9	16.5	14.1	12.5	11.2	10.4	10.2	10.4	Neg.
		0.9% NaCl, pH 5.1	22.2	19.7	17.7	13.3	9.0	Neg.	Neg.	Neg.	Neg.	Neg.
		0.1 *M* KH_2PO_4, pH 5.0	20.5	17.1	11.9	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	Difco nutrient agar pH 7.0 plus $CaCl_2$ after sterilization to obtain 0.1 *M*	0.1 *M* $CaCl_2$, pH 5.8	25.3	23.3	20.6	18.6	16.6	14.3	11.3	9.5	...	Neg.
		Distilled H_2O, pH 5.9	25.4	24.5	22.5	19.9	17.3	15.0	12.0	10.1	...	Neg.
B. cereus var. *mycoides* ATCC #11,778	Difco Penassay base pH 8.0 plus $CaCl_2$ after sterilization to obtain 0.1 *M*	0.1 *M* K_2HPO_4, pH 8.4	19.0	18.0	16.8	15.1	13.9	12.7	12.0	11.0	...	Neg.
		0.1 *M* sodium bicarbonate, pH 7.9	25.6	24.5	22.9	21.3	20.2	18.7	17.2	15.4	...	Neg.
		0.1 *M* sodium citrate, pH 7.2	24.6	23.7	21.8	20.8	19.6	18.0	17.2	15.9	...	Neg.
B. subtilis ATCC #6,633	Difco nutrient agar pH 8.0	0.1 *M* K_2HPO_4, pH 8.7	29.3	28.2	26.0	23.3	20.8	17.6	12.0	Neg.	Neg.	Neg.

[a] Diluent pH value may represent that obtained as prepared, or after adjustment with the appropriate base or acid.

TABLE II.—COMPARISON STANDARD CURVES USING 0.1% AMMONIUM CARBONATE DILUENT AND *B. Subtilis* (4, 9)

Preparation	Average mm. Zone Size Per mcg./ml. Aspartocin Solution At— 100	50	25	12.5	6.25
Sodium aspartocin Lot #50A	27.3	25.0	23.8	21.7	19.7
Calcium aspartocin Lot #85A	27.0	24.8	22.8	20.2	18.1

TABLE III.—SODIUM ASPARTOCIN ACTIVITY IN THE CTC MICROBIOASSAY TEST SYSTEM (10)

Test Conditions— Test Organism	Agar	Curve Diluent	Average mm. Zone Size Per mcg./ml. Aspartocin At— 50	25	12.5	6.25	3.12	1.56	Control
B. cereus var. *mycoides* ATCC #11,778	Difco Penassay base adjusted to pH 5.7	0.1 *M* KH_2PO_4 pH 4.7	21.8	19.3	16.8	14.3	11.5	Neg.	Neg.

TABLE IV.—MICROBIOLOGICAL ASSAY ASPARTOCIN–CTC MIXTURES[a]

Preparation Number	Theoretical Content, % Aspartocin	CTC	Activity Assay Value, % Aspartocin	CTC	Colorimetric CTC (11)
#582-43	13.5	76	12.5	68.5	...
#582-118-1	27	65	28.8	62.0	61.1
#582-118-2	27	65	24.9	61.5	59.0

[a] These products prepared by Mr. F. H. Smith of our Laboratories.

an estimated 500–1,000 mcg. aspartocin/ml.; assay dilutions are prepared in 0.1 *M* $CaCl_2$ diluent. Portions of this same extract may be tested for CTC content (Table IV).

RESULTS AND DISCUSSION

Satisfactory standard curves for aspartocin were obtained with *S. epidermidis* ATCC #12,228 and 0.1 *M* calcium chloride solution as diluent for the reference material and samples under test.

Several assay systems were compared, and the inhibitory-zone responses for graded doses of aspartocin appear in Table I.

The influence of diluent, pH, and concentration of salts in agar-diffusion streptomycin assays is well known (8). In this respect the potentiation of aspartocin activity (2) was of particular interest. When 0.1 *M* calcium chloride solution was used as a diluent, the aspartocin standard curve showed good sensitivity (Fig. 1). The addition of 0.1 *M* calcium chloride in the assay agar also appeared satisfactory; however, this made the culture medium hazy and responses at low concentrations might be difficult to measure. Calcium as calcium hydroxide and chloride as sodium chloride did not appear to enhance aspartocin activity in the agar diffusion assay. Similarly, the responses obtained with sodium and calcium aspartocin salts were not significantly different (Table II) when ammonium carbonate was used as the diluent.

The responses using the *B. subtilis* procedure, as proposed (4), appear in Table II; however, aspartocin dilutions were prepared in 0.1% ammonium carbonate in distilled water instead of 1% ammonium carbonate, and responses were less erratic (9). Although suitable standard curves may be obtained with *B. subtilis* and *B. cereus* var. *mycoides*, the tetracycline sensitivity of these organisms limits their usefulness in the assay of aspartocin–tetracycline mixtures.

In the determination of CTC in aspartocin–CTC mixtures, the response of aspartocin in the usual CTC cylinder-plate assay (10) indicated relatively low activity; aspartocin alone at 3 mcg./ml. showed a small zone of inhibition (Table III). This response may be insignificant in the presence of an equivalent weight of CTC. However, if a mixture is predominantly aspartocin with low CTC content present, a mixed standard curve (aspartocin plus CTC in an appropriate ratio) may be more useful for the assay of CTC content.

Experimental aspartocin–CTC mixtures were evaluated by the *S. epidermidis*–0.1 *M* $CaCl_2$ test system; the results of these assays (Table IV) indicated no interference in the aspartocin or CTC determinations. Chlortetracycline microbiological values were confirmed by colorimetric assay (11), and for aspartocin by nitrogen determinations.

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Colorimetric Assay of 4,6-Unsaturated-3-keto Steroids Using Sodium Picrate Reagent

By E. P. SCHULZ and M. A. DIAZ

The observation in these laboratories that 4,6-unsaturated-3-keto steroids react with alkaline picrate to form chromogenic anionic complexes has been specifically utilized to determine 6-chloro-17α-hydroxy-pregna-4,6-diene-3,20-dione-17α-acetate in pharmaceutical dosage forms. Despite the over-all lack of specificity of the reagent, this disadvantage has been offset by the sensitivity, reliability, and simplicity of the presented analytical method. As little as 40 mcg. of steroid can be quantitatively determined at room temperature color development.

THE COMPLEXATION of the reactive α,β-unsaturated lactone group of cardiotionic glycosides (1) with alkaline picric acid to form chromogenic anions, suggested the possible use of this reagent for the assay of α,β-unsaturated-3-keto steroids.

In treating various α,β-unsaturated-3-keto steroids with alkaline picric acid in methanol, it was observed that chromogens of weak intensity were produced. Greater sensitivity was noted for α,β-unsaturated-3-keto steroids with conjugation extended to ring B by the presence of an additional double bond at the 6 position. It was also observed that the introduction of chlorine to the unsaturated 6 position of the latter type steroid greatly enhanced the intensity of the steroid-picric acid chromogen. Consequently, the efforts of this investigation were directed toward the utilization of alkaline picrate as a general means of analyzing 4,6-unsaturated-3-keto steroids and as a specific means of assaying 6-chloro-17α-hydroxy-pregna-4,6-diene-3,20-dione-17α-acetate [to be referred to in the subsequent text as chlormadinone (2)].

EXPERIMENTAL

Apparatus.—Beckman model DU spectrophotometer with 1-cm. cells, and culture tubes (screw capped type), 20 × 150 mm. were used.

Reagents.—The following reagents were used: picric acid, 0.666% in methanol (w/v), prepared by dissolving 666 mg. of picric acid (Baker's analyzed grade containing 10% water) in 100 ml. of methanol; sodium hydroxide, 8.0% in distilled water, prepared by diluting 8.0 Gm. of reagent grade material to 100 ml. with distilled water; and a steroid reference standard, prepared by dissolving 20.0 mg. steroid in 50.0 ml. methanol and diluting 10/50 with methanol so that the final concentration is equivalent to 80 mcg. steroid per 1.0 ml.

Assay Procedure for Formulated Chlormadinone.—Tablets containing 2.0 mg. of chlormadinone and 80 mcg. of 17α-ethynylestradiol-3-methylether were assayed by the following procedure.

The equivalent of two ground tablets (containing as excipients cornstarch, lactose, and magnesium stearate) were stirred for 15 minutes with 50.0 ml. of chloroform in a 125-ml. glass-stoppered flask. Exactly 10.0 ml. of the filtered extract was evaporated to dryness and the cooled residue dissolved in 10.0 ml. of methanol. A 1.0-ml. portion of the methanol phase was pipeted into a culture tube and wrapped in aluminum foil to protect the reactants from incident light. To a second culture tube, also wrapped in aluminum foil, was added 1.0 ml. of chlormadinone reference standard in methanol (80 mcg. per 1.0 ml.). This was followed by the addition of 3.0 ml. of a freshly prepared 1:1 mixture of the 0.666% picric acid and 8.0% sodium hydroxide solutions. (The combined reagent was cooled to ambient temperature before addition to the steroid solutions in the culture tubes.) A third tube (reagent blank) containing 1.0 ml. of methanol and 3.0 ml. of picric acid alkali reagent was concurrently prepared.

After 120-minute color development at room temperature, the contents of the stoppered culture tubes were each transferred to three separate 1-cm. glass cells. Absorbance values at 490 ± 2 mμ were immediately made, using as a reference the prepared reagent blank.

Calculations.—$A_p/A_s \times W \times 0.025$ = mg. chlormadinone/tablet, where A_p and A_s are the respective 1-cm. absorbance values of the analytical sample and the steroid standard as read against the reagent blank at 490 mμ and W is the mcg. weight of reference standard (*ca.* 80 mcg.) per 1.0 ml. of methanol.

RESULTS AND DISCUSSION

Analyses of a typical production lot of tablets containing 2.0 mg. of chlormadinone and 80 mcg. of estrogen per tablet, resulted in assay values of 1.87 and 1.89 mg. chlormadinone per tablet. Placebo tablets gave no response to the analytical reagent, nor did 80 mcg. of the estrogen produce color. Placebo tablets, to which were added 2.0-mg. levels of chlormadinone reference standard, assayed 1.98 and 1.94 mg. chlormadinone per tablet.

Reaction Conditions.—Optimum conditions were determined only for the 6-chloro-4,6-unsaturated-3-keto steroids. Absorbance values for the other type of steroids (Table I) were determined using the conditions arrived at for the above type steroid and do not necessarily represent the maximum obtainable intensities.

Sensitivity.—The data presented in Table I indicated the following order of group sensitivity: (*a*) 6-halogenated-4,6-unsaturated-3-keto > 4,6-unsaturated-3-keto > 4-unsaturated-3-keto and (*b*) within the first group, the substitution of fluorine for chlorine at the unsaturated 6 position led to decreased sensitivity.

Specificity.—In addition to the three types of steroid found to respond to the described reagent, the data for miscellaneous steroids (80 mcg.) after 120-minute color development (*versus* reagent blank) were as follows:

(*a*) 6α,9α-Difluoro-1,4-unsaturated-3-keto steroids gave no response.

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TABLE I.—ABSORBANCE VALUES[a] FOR 80 MCG. OF VARIOUS STEROIDS

	Absorbance	Maximum, mμ
6-Chloro-17α-hydroxy-pregna-4,6-diene-3,20-dione	0.625	490
17α-Acetate of above steroid	0.564	490
6-Fluoro-17α,21-dihydroxy-16α-methyl-pregna-4,6-diene-3,11,20-trione-21-acetate	0.265	497
Pregna-4,6-diene-3,20-dione	0.356	492
17α-Hydroxy-pregna-4,6-diene-3,20-dione-17-acetate	0.311	492
6α-Methyl-pregn-4-ene-3,20-dione	0.147	497
6β-Hydroxy-pregn-4-ene-3,20-dione	0.298	493
Pregn-4-ene-3,20-dione	0.193	493

[a] Values are based on a 1-cm. cell path, as read against a reagent blank.

(*b*) 6-Chloro-1,4-unsaturated-3-keto steroids produced an absorbance of 0.030 at 492 mμ.

(*c*) 1,6-Unsaturated-3-keto steroids led to values of 0.163 at 492 mμ.

(*d*) 3-Hydroxy-4,6-unsaturated steroids gave an absorbance value of 0.052 at 487 mμ.

(*e*) 3,5-Unsaturated steroids (with no 3-keto group) exhibited values of 0.261 at 491 mμ.

(*f*) U.S.P. digitoxin gave a value of 0.140 at 476 mμ.

Solvents.—The use of either absolute or 96% ethanol in lieu of methanol to prepare the reagents and steroids standards led to a 66% decrease in intensity values.

Effect of Light.—Color development without adequate protection of the reactants from incident light led to 20% lower intensities as well as irreproducibility of color.

Color Stability.—A plot of absorbance *versus* time indicated that developed colors exhibit the least variation during the 80–140-minute interval after the addition of reagent. Each 10-minute increment during this 80–140-minute period showed an average absorbance increment of 1.8%.

Color Linearity.—Developed colors were linear within a concentration range of 40–120 mcg. of chlormadinone.

Color Development with Other Polynitro Phenols.—2,4,6-Trinitro-resorcinol; 2,4,6-trinitro-anisole; 4,6-dinitro-2-amino-phenol; and *p*-nitrophenol failed to produce chromogens under the described conditions.

Concentration of Alkali.—Variations in the sodium hydroxide content while maintaining the concentration of picric acid constant indicated that the method is particularly sensitive to the concentration of alkali. A 4 to 8% decrease in alkali content led to 7% lower absorbance values. Concentrations of sodium hydroxide greater than 8% led to precipitation of the picric acid. The use of 8.0% potassium hydroxide also precipitated the picric acid.

Concentration of Picric Acid.—Varying the picric acid concentration from 0.637 to 0.693%, while maintaining the alkali content at 8.0%, produced no significant changes in the observed absorbance values.

Stability of Reagents.—The combined reagent, after 24-hour aging of the uncombined components showed a 6% decrease in absorbance values; the combined reagent after 24-hour aging at ambient temperature showed 13% lower values.

Interferences.—Chloroform soluble carboxylic acids (stearic acid, carbapol, alginic acid, etc.) and excipients tending to precipitate picric acid (tertiary amines, proteins, etc.) interfered. Interference due to chloroform-soluble carboxylic acids was resolved by re-extracting the chloroform–steroid phase with 0.1 *N* sodium hydroxide.

The second type interference was overcome by precipitation of these materials from the chloroform phase, using a 1% solution of silicotungstic acid in 6 *N* hydrochloric acid.

The described reagent may also be applied to the analyses of residues from chloroform extracts of steroids formulated in cream bases, after the removal of interferences from the organic phase by means of 0.1 *N* aqueous sodium hydroxide. A cream base (propylene glycol, stearic acid, Spans, and Tweens) gave no response to the reagent using the above extraction technique.

Reproducibility.—Using freshly prepared reagents, the day-to-day variation of chlormadinone (80 mcg.) absorbance values ranged from 0.466 to 0.564. However, triplicate determinations of the same steroid standard solution and using the same reagents indicated that the mean average deviation of absorbance values was ±2.2%. Since steroid standards were determined concurrently with the steroid isolated from the formulated sample, the analytical error also was within the above range. The above deviation was reduced to a range of ±1.1% by using larger volumes of reactants and by minimizing the lapsed time between absorbance measurements. For example, colors developed on 3.0 ml. of steroid (240 mcg. chlormadinone) with 9.0 ml. of picric acid-alkali reagent led to the latter mean average deviation.

Purity of Steroids.—In all instances, steroids were used which indicated only one spot after thin-layer chromatography on silica gel.

Mechanism of Reaction.—The available data, though not rigorously elucidating the mode of reaction, indicated the following results:

(*a*) The bis 2,4-dinitro-phenylhydrazone of chlormadinone, as prepared by the method of Cohen and Bates (3), reacted with the alkaline picric acid reagent to form a chromogen absorbing at 490 mμ. This indicated that complexation did not occur at the unavailable 3 or 20 keto positions of the steroid molecule.

(*b*) The ultraviolet spectrum of the isolated complex showed a maximum at the same wavelength (284 mμ) as that of unreacted chlormadinone. This suggested intact structure of the 4,6-unsaturated-3-keto moiety in the complexation product.

(*c*) Conversion of the sodium form of the complex anion to the hydrogen form yielded, after column chromatography on silica gel and elution with 90:10 chloroform–methanol, a pure product which analyzed C, 54.3%, H, 4.6%, and N, 6.9%. Calculated values for a 1:1 complex of 6-chloro-17α-hydroxy-pregna-1,4-diene-3,20-dione ($C_{21}H_{27}O_3Cl$, mol. wt. 362.89) and picric acid ($C_6H_3N_3O_7$, mol. wt. 229.11) were: C, 54.7%, H 5.1%, and

N, 7.0%. This demonstrated that the reaction involves a 1:1 complexation of picric acid and chlormadinone as well as alkaline hydrolysis of the steroidal 17-acetoxy entity. This is consistent with the mechanism (4) proposed for the anionic complexation of picric acid and α,β-unsaturated lactones.

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Enzymatic Methylation of Nicotinamide by *Claviceps purpurea*

By R. C. S. AUDETTE, L. R. WORTHEN, and H. W. YOUNGKEN, JR.

Soluble supernatant fractions from two strains of *Claviceps purpurea* were tested for the presence of enzymes capable of methylating tryptamine, 5-hydroxytryptamine, tyramine, and nicotinamide. The latter compound was the only one of the substrates tested in which methylation took place.

SEVERAL PSYCHOTOMIMETIC indole substances (1), such as bufotenin and psilocybin, which are found in certain fungi are *N*-methylated tryptamine derivatives. Lysergic acid, a derivative of which is the most potent psychotogen known, also contains an *N*-methyl group, and a residue of this compound occurs in the alkaloids of ergot.

It has been reported (2) that the *N*-methyl group in the ergot alkaloids is probably derived from a transmethylation reaction from methionine.

Cantoni (3) first reported that *S*-adenosylmethionine was an active methyl donor and has studied in great detail the methylation of nicotinamide. Axelrod (4) has isolated an enzyme from rabbit lung that is capable of methylating a number of naturally occurring compounds to psychotomimetic derivatives utilizing *S*-adenosylmethionine as the methyl donor.

The literature is replete with information concerning the biosynthesis of the ergot alkaloids from *C. purpurea*, but relatively little information is available concerning the activity of the enzymes found in this organism. The present investigation has been conducted in an attempt to determine if a cell-free extract of the fungus possesses an enzyme system which can methylate tryptamine, 5-hydroxytryptamine (5-HT), tyramine, and nicotinamide, since their methylated derivatives have been associated with psychotomimetic activity.

EXPERIMENTAL

Cultures.—Two strains of *C. purpurea* (Fries) Tulasne were used in this investigation. One of the strains, designated CPM, was isolated from sclerotia obtained from the Department of Plant Pathology, University of Minnesota, Minneapolis. The other, an alkaloid producing strain, designated 47A, was obtained as a slant culture from the College of Pharmacy, University of Washington, Seattle.

The fungi were cultured in 50 ml. of Abe's medium (5) in cotton-stoppered 250-ml. conical flasks grown as still cultures in the dark at room temperature. The cultures were harvested for extraction at periods of from 21 to 27 days.

Preparation of Enzyme Fraction.—The mycelial tissue of both CPM and 47A cultures was separated from the culture medium by centrifugation, washed three times with distilled water, recentrifuged, and finally pressed between sheets of filter paper.

The enzyme fraction was prepared from 6–7 Gm. (fresh weight) of mycelial tissue, which was homogenized with 15 ml. of phosphate buffer (0.1 *M* K_2HPO_4, pH 7.9) in a VirTis 45 homogenizer for 10 minutes in a chilled vessel in an ice bath. After the homogenate was obtained, it was centrifuged at 2500 r.p.m. (755 × *g*) for 10 minutes and the resultant supernatant fraction recentrifuged at 16,000 r.p.m. (31,000 × *g*) for 20 minutes in a refrigerated Servall centrifuge at 0–3°. The soluble supernatant fraction was then separated and employed as the enzyme preparation.

In addition to the above method, three other means were tested for extraction of the mycelium: (*a*) homogenizing the mycelium with buffer as previously described, then subjecting the homogenate to ultrasonic vibrations, (*b*) grinding the mycelium in buffer solution with sand, and (*c*) grinding with alumina. After several preliminary trials, it was decided to use homogenization only as the extraction method for further investigations since this method proved most successful.

Enzymatic Activity of Soluble Fractions.—Nicotinamide,[1] tryptamine hydrochloride,[1] tyramine hydrochloride,[1] and 5-hydroxytryptamine creatinine sulfate[1] were tested with the soluble supernatant fraction using *S*-adenosylmethionine[2] as a methyl donor to determine if the soluble fraction contained an enzyme(s) capable of methylating these physiologically active naturally occurring substances.

Nicotinamide—The incubation mixture consisted of 0.005 mole of *S*-adenosylmethionine (0.2 ml.), 0.01 *M* nicotinamide (0.3 ml.), and an amount of the soluble supernatant fraction equivalent to 6

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mg. of nitrogen, all in a volume of 5 ml., using 0.1 *M* K_2HPO_4 (pH 7.9) as the diluent. This mixture was incubated in a 15-ml. glass-stoppered centrifuge tube in a shaking water bath at 25° for 1 hour. Two other tubes—one containing a similar mixture less the soluble supernatant fraction and the second tube containing the soluble supernatant fraction only—served as controls. The controls were brought to a volume of 5 ml. using the phosphate buffer described above as the diluent. An equal volume of 10% trichloroacetic acid was added to each of the tubes at the end of the incubation period to stop any enzymatic action, and the vessels were left to stand at room temperature for 30 minutes. The resultant precipitate was removed by centrifugation.

The method used for detecting the formation of *N*-methylnicotinamide in the reaction mixture was similar to that of Huff and Perlzweig (6). Glacial acetic acid (0.1 ml.) was added to 1 ml. of the reaction mixture and to the corresponding controls followed by 1.0 ml. of methyl ethyl ketone and 1.0 ml. of 6 *N* NaOH. This mixture was immediately shaken and allowed to stand for 5 minutes at room temperature, after which 6 *N* HCl was added to a pH of 3.0. The reaction vessels were placed in boiling water for 5 minutes, cooled, then 1 ml. of 20% KH_2PO_4 was added. The fluorescence of the solutions was measured in an Aminco-Bowman spectrophotofluorometer at an activation maximum of 365 mμ and a fluorescence maximum of 440 mμ.

It was determined by this method that *N*-methylnicotinamide was present in the incubation mixture, but none could be detected in either of the controls employed. A reference sample of *N*-methylnicotinamide was used in the same extraction procedure to ascertain the validity of this method. The results obtained were identical.

Tyramine, Tryptamine, and 5-Hydroxytryptamine.—The incubation mixture was composed of 0.01 *M* (0.5 ml.) of the substrate to be tested, 0.005 mole (0.2 ml.) of *S*-adenosylmethionine, and a volume of the soluble supernatant fraction containing 6 mg. of nitrogen. This was brought to a volume of 5 ml. using phosphate buffer (pH 7.9). These mixtures along with controls, consisting of the soluble supernatant fraction alone and the substrate and methyl donor without the enzyme fraction, were incubated in 15-ml. glass-stoppered centrifuge tubes on a shaking machine at 25° for 90 minutes. At the end of this period, the pH was adjusted to 10 using borate buffer. The contents were frozen and lyophilized for 36 hours, and the resulting residue then shaken with isoamyl alcohol (tryptamine and 5-HT) or *n*-butanol (tyramine). After filtration, the extract was concentrated *in vacuo* at 50° to dryness, then reconstituted to 1–2 ml. with the same solvent. All extracts, reference samples of the substrates used, and methylated derivatives of these substances were chromatographed. The materials were spotted on Whatman No. 1 paper and the papers equilibrated for 4 hours, then allowed to develop for 18 hours using a solvent system of butanol–acetic acid–water (4:1:1), using the descending method. After the papers were removed from the glass chambers and dried, they were sprayed with 2% paradimethylaminobenzaldehyde (Ehrlich's reagent) in equal parts of ethanol and 6 *N* HCl. After development, the spots produced in the extract samples were compared with those appearing in the corresponding controls and with the reference compounds. The spots found in the extract samples corresponded to those found in the controls; no spots corresponding to the methylated derivatives could be detected. The 5-hydroxytryptamine spots had an R_f value of 0.45, were blue, later changing to green; the tryptamine spots had an R_f value of 0.66 and at first were a purple-pink, later changing to green. The tyramine spots had an R_f value of 0.52 and turned yellow.

SUMMARY

Soluble supernatant fractions were prepared by homogenization and centrifugation from 21–27-day-old cultures of two strains of *C. purpurea*, one of which produced ergot alkaloids in saprophytic culture.

Tryptamine, tyramine, 5-hydroxytryptamine, and nicotinamide were incubated with these extracts using *S*-adenosylmethionine as a methyl donor in order to determine if methylated derivatives of these derivatives could be detected.

N-Methylnicotinamide was formed from nicotinamide using extracts of both strains of the fungi, but no methylated derivatives of the other substrates employed could be detected.

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Simplified 2,6-Di-*tert*-butyl-*p*-cresol Colorimetric Method for Unsaturated-3-keto Steroids

By E. P. SCHULZ, M. A. DIAZ, and L. M. GUERRERO

An improved modification of the original 2,6-DTBPC method for the determination of unsaturated 3-keto steroids has been developed. The presented technique features a simpler mode of operation, improved sensitivity, equivalent reproducibility, and extension of the original method to the analyses of 6-fluoro 3-keto steroids unsaturated at both the 1 and 4 positions. Limited application to pharmaceutical dosage forms is also discussed.

SINCE THE APPEARANCE of the original procedure (1) describing the use of 2,6-di-tertiary-butyl-*p*-cresol (DTBPC) as a reagent for the assay of unsaturated 3-keto steroids, several publications have been presented as proposed improvements of the original technique. Ansari's modification (2)—the use of freshly prepared and standardized 1.0 *N* sodium hydroxide in place of nonstandardized 5% alkali and 0.5% DTBPC in 90% ethanol rather than 0.6% in 95% ethanol—retained the desired specificity and eliminated the cumbersome heating and stirring apparatus initially described.

Bianchi's technique (3), though eliminating the use of elevated temperature for color development, resulted in a loss of specificity. Steroids containing a 4-unsaturated-3-keto group showed maxima between 380 and 385 mμ, irrespective of the substituent at the 11 position, while the original method presented distinct maxima at 471 and 625 mμ. Cross (4) also reported maxima between 373 and 375 mμ for the above type steroids by merely heating the steroid with alkali and without the incorporation of DTBPC reagent.

Bartos (5), reasoning that the agitation of reactants merely served to oxidize the DTBPC and that an oxidized form of DTBPC was the actual reagent, sought simply to include 0.03% hydrogen peroxide and successfully eliminated the necessity of agitating the reactants during color development. Maxima were reported at 460 mμ (4-unsaturated-3-keto steroids with an 11-keto group), at 500 mμ for saturated-3-keto steroids, and at 610 mμ for 4-unsaturated-3-keto steroids with a hydroxylated or unsubstituted 11 position. Bartos' modification was the first to reveal that 1,4-unsaturated-3-keto steroids would react with DTBPC, though at reduced sensitivity.

The present method permits color development within 14 to 21 minutes at 90 to 91°, avoids the necessity of having to stir the reactants during color development, does not require the use of hydrogen peroxide, results in greater sensitivity (40 to 80 mcg. of steroid) than the original method, retains the original specificity, and does not require the use of standardized alkali. The method has, however, limited applicability for the analyses of formulated steroids.

EXPERIMENTAL

Apparatus.—An improvised water bath with all component parts is described in Fig. 1. Test tubes, culture type with screw caps, 20 × 125 mm. and a microliter syringe with 50 μl. capacity were also used.

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Reagents.—The following reagents were used: *N*-butyl alcohol (Baker's analyzed reagent grade); DTBPC reagent, 2,6-di-tertiary-butyl-*p*-cresol (Eastman practical grade or Koppers' technical or food grades) 0.80% in *n*-butyl alcohol; TMAH reagent, 10% aqueous tetramethyl ammonium hydroxide (Eastman); and steroid standards, 40 mcg. of reference standard material per 1.0 ml. of *n*-butyl alcohol.

Unformulated Steroid Assay Procedure.—Using a semimicro balance, weigh to the nearest 0.02 mg. about 10 mg. of assay sample into a 50-ml. volumetric flask. Add about 30 ml. of *n*-butyl alcohol. Stopper and shake to dissolve the steroid. Dilute to the mark with solvent. Pipet 5.0 ml. of the above solution into a 25-ml. volumetric flask and dilute to the mark with *n*-butyl alcohol. Pipet 2.0 ml. of the final dilution (*ca.* 80 mcg. of steroid) into a 20 × 125-mm. culture tube.

Prepare (as above) a reference standard solution of the same steroid equivalent to about 80 mcg. of steroid per 2.0 ml. of *n*-butyl alcohol. Pipet 2.0 ml. of this solution into a second culture tube of the same dimensions as above. Add 2.0 ml. of *n*-butyl alcohol to a third culture tube which is to serve as a reagent blank.

To each of the three culture tubes add 3.0 ml. of 0.80% DTBPC in *n*-butyl alcohol, followed by the addition of 50 μl. of 10% aqueous TMAH with a 50-μl. syringe. Replace the screw caps on each of the three tubes.

Immediately insert each tube into the 20-mm. apertures in the aluminum support plate, resting on the 400-ml. beaker containing 200 ml. of magnetically stirred 90 to 91° water (Fig. 1). (The fourth opening in the plate is used to accommodate a thermometer inserted in a No. 3 rubber stopper.) The position of each Pb tube-guide should be preset (prior to the addition of reactants to the culture tubes) to allow just the bottom edges of the tubes to clear the rotating magnetic bar. The Pb strips also serve to offset the buoyant effects of the water bath on the inserted tubes.

Heat for 14 to 21 minutes at 90 to 91°, depending

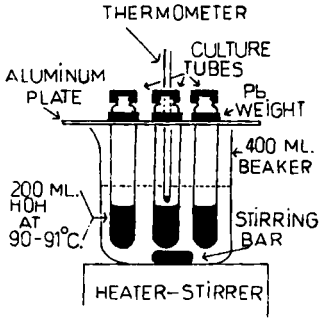


Fig. 1. — Diagram of the improvised water bath used to develop the steroid–DTBPC colors.

TABLE I.—DATA AND CONDITIONS FOR OPTIMUM DTBPC COLOR REACTION ACCORDING TO TYPE OF STEROID

Steroid	Type[a]	Max. mμ	Absorbance, 1-cm. path, *vs.* Reagent Blank	Mcg.	Heating Time, Min.
Fluocinolone acetonide	A	620	0.507, 0.503	80	21
			0.508, 0.513		
			0.258, 0.257	40	21
21-Dihydrogen phosphate monosodium salt of above compound	A	620	0.363, 0.363	80	21
Paramethasone acetate	A	620	0.292, 0.292	80	21
			0.292, 0.299		
			0.296		
Progesterone	B	620	0.474, 0.474	80	21
			0.474		
Hydrocortisone acetate	B	630	0.697, 0.736	80	21
			0.716		
Cortisone acetate	C	485	0.322, 0.322	80	14
			0.322		

[a] Type A steroids, those which feature a 6α-fluoro-11β-hydroxy-3-keto (1,4-unsaturated) structure; Type B steroids, those which show a 11-unsubstituted or 11-hydroxylated-3-keto (4-unsaturated) structure; Type C steroids, those having a 3,11-diketo (4-unsaturated) structure.

on the type steroid (Table I) being analyzed. Immediately immerse all three tubes in an ice bath at 0° for 3 minutes. Determine the 1-cm. absorbance at the particular analytical wavelength (Table I) of the steroid during the subsequent 15-minute interval, using as a reference the simultaneously prepared reagent blank. For steroids, of other types than for which data are presented, the analytical wavelengths and optimum heating times should be determined by the analyst.

Per cent purity = $A_p/A_s \times S/W \times 100$, where A_p and A_s are the respective 1-cm. absorbance values of the analytical sample and the steroid reference standard and where the respective microgram weights of the analytical sample and steroid standard in the colored solutions are W and S.

Formulated Steroid Assay Procedure.—A portion of a filtered chloroform extract of the steroid equivalent to 200 mcg. of steroid is evaporated to dryness in a 50-ml. glass-stoppered flask. The residue is stirred for 5 minutes with 5.0 ml. of *n*-butyl alcohol. Exactly 2.0 ml. of this solution, containing about 80 mcg. of steroid, is transferred to a culture tube and the color developed as above along with 80 mcg. of corresponding reference standard steroid and the reagent blank. The optimum heating time and analytical wavelength, being dependent on the type steroid, should either be predetermined or based on the data provided in Table I.

Per cent steroid = $A_p/A_s \times B/D \times C/E \times 0.00025$, where A_p and A_s remain as defined above, B is the mcg. weight of steroid standard per 2.0 ml. of *n*-butyl alcohol, C is the volume (ml.) of chloroform used to extract E ml. or E Gm. of formulation, and D is the volume (ml.) of chloroform extract evaporated to dryness and equivalent to 200 mcg. of steroid.

RESULTS AND DISCUSSION

Analyses of a tablet formulation containing 0.666% 6α - fluoro - 11β,17α,21 - trihydroxy - 16α - methylpregna - 1,4 - diene - 3,20 - dione - 21 - acetate (paramethasone acetate), led to assay values of 0.660, 0.650, and 0.644% steroid. These results correspond to an average value of 0.651% paramethasone acetate, with a mean average deviation of ±0.0054% steroid. A placebo (cornstarch, lactose, magnesium stearate, polyvinylpyrrolidone, and FD & C No. 4 and 5 dyes) gave no color at 625 mμ. Direct ultraviolet analyses (methanol at 240 mμ) of residues from chloroform extracts of these tablets led to a mean assay value of 0.669% steroid, using as a reference a correspondingly treated placebo tablet.

Scope of Reaction.—1-unsaturated-3-keto steroids (480 to 485 mμ), prednisolone (590 mμ), and prednisone (575 mμ) produced maxima of weak and irreproducible intensities. The introduction of 6α-chlorine to the prednisone molecule failed to enhance its reactivity toward the DTBPC reagent. The types of 3-keto steroid which gave the most sensitive and reproducible responses are presented in Table I.

Temperature Conditions.—No other temperatures were investigated beyond the 90–91° range used in the development of this method.

Heating Time.—The optimum reaction time at 90–91° for 3,11-diketo steroids (unsaturated at the 4 position) was established to be between 13 and 14 minutes; while 20 to 21 minutes were required for 6α-fluoro-11-hydroxy-3-keto steroids (unsaturated at both the 1 and 4 positions), for 11-hydroxy-3-keto steroids (4-unsaturated), and for 11-unsubstituted 3-keto steroids unsaturated at the 4 position.

DTBPC Concentration.—The concentration of DTBPC was varied from 1.0 to 0.60%, and colors were developed keeping the other variables constant. Increasing the concentration of reagent above 0.8% tended to increase absorbance values for the steroids examined, but led to less reproducibility of color intensity. Concentrations less than 0.8% DTBPC resulted in decreased color intensity and decreased reproducibility.

TMAH Concentration.—Increasing the volume of 10% TMAH beyond the recommended 50-μl. level led to lack of reproducibility. Levels of 100, 150, and 200 μl. of 10% TMAH were tested under the reaction conditions.

Color Linearity.—The chromogen for fluocinolone acetonide (620 mμ) was linear within the 40–80-mcg. range. Linearity of color for the other steroids was not evaluated over different concentration ranges.

Reproducibility.—The data presented in Table I indicated that the technique is suitable if a control reference standard is analyzed along with the analytical sample. The paired absorbance values

in this table represent simultaneous but not independent duplicate determinations. Each distinct paired group (or single value) represents a determination independent of the other group. Intensities of the developed chromophores in each instance exhibited a mean average deviation of ±2% or less.

Color Stability.—In all instances the color of the steroid chromogen was stable during the 15-minute interval after the cooling step. Longer time intervals were not investigated.

Interferences.—Chloroform soluble carboxylic acids (acetylsalicylic acid and stearic acid, etc.) and esters (sorbitan monostearates, methyl, and propyl parabens, etc.) tended to inhibit color formation. Consequently, cream formulations were not capable of being assayed using the described reagent.

Reagent Blank.—The 1-cm. absorbance values at 485 and 620 mμ for the reagent blank were, respectively, 0.116 and 0.008.

General Aspects.—DTBPC reagent, oxidized by refluxing and aerating for 1 hour, gave identical color intensity for fluocinolone acetonide (6α,9α-difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide) as did untreated reagent, indicating that an oxidized form of DTBPC is not necessarily the reactant.

Paper chromatograms of fluocinolone acetonide, impregnated and heated with the combined TMAH–DTBPC reagent, indicated that this reagent is not suitable for the detection of steroid spots.

Fluocinolone acetonide color obtained by the original method (1) was lower by a factor of 7.5 relative to this method. The former method was, however, 1.6 times more sensitive for 3, 11-diketo (4-unsaturated) steroids than the present method.

From the data presented the following conclusions may be made: (*a*) that type A steroids (Table I) containing a 6α, 9α-difluoro moiety (fluocinolone acetonide) produced more color than their 6α-fluoro counterparts (paramethasone acetate), and (*b*) that the molar response of 11-unsubstituted-3-keto (4-unsaturated) steroids toward the DTBPC reagent was decreased by substitution of an 11-keto function and increased by the introduction of a 11-hydroxyl group.

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Assay for Methapyrilene in Complex Mixtures

By NATHAN SCOTT

A method is presented for the estimation of methapyrilene in complex mixtures which may include other antihistamines. Briefly, it consists of treating ninhydrin with methapyrilene which has been extracted from an alkaline solution with petroleum ether.

ANTIHISTAMINES have proved their therapeutic value over the years. Methapyrilene is one of the established antihistamines which has found increased usage by itself and in combination with other ingredients.

Many methods exist for the estimation of methapyrilene. The Association of Official Agricultural Chemists (1) and "The National Formulary" (2) describe methods employing an ultraviolet absorption. Clair and Chatten (3) describe a nonaqueous method and Cox *et al.* (4) employ chromatography and electrophoresis. Another method described by Celeste and Turczan (5) uses gas chromatography after a preliminary extraction. This article is concerned with a spectrophotometric method for its determination, particularly when it is present in pharmaceutical dosage forms with other materials.

EXPERIMENTAL

Materials.—Reagents used in the experiments were of analytical grade where available and used without additional purification. The following materials were used: ninhydrin (1,2,3 triketohydrindene) (Eastman Kodak); petroleum ether; sulfuric acid; polysorbate 80 U.S.P.; methapyrilene hydrochloride N.F.; sodium hydroxide; phenylephrine hydrochloride U.S.P.; dextromethorphan hydrobromide; chlorpheniramine maleate U.S.P.; and tripelennamine hydrochloride U.S.P.

Apparatus.—Beckman model DU spectrophotometer was used.

Procedure.—The method is applicable to liquid or solid dosage forms. A portion of liquid containing about 5 mg. of methapyrilene hydrochloride is transferred to a separator. The solution is rendered alkaline with either 1 *N* sodium hydroxide solution or sufficient sodium bicarbonate. The alkaline solution is then extracted successively with 15 ml. and 3 × 10 ml. of petroleum ether. The petroleum ether extracts are passed through a cotton plug moistened with petroleum ether into a 50-ml. volumetric flask. After rinsing the separator with about 3 ml. of solvent and filtering into the flask, the liquid is brought to the mark and mixed. One milliliter of the solution, equivalent to about 100 mcg. of antihistamine, is carefully transferred to a 10-ml. flask and the petroleum ether removed with a gentle stream of nitrogen for about 5 minutes. To the residue is then added 0.25 ml. of a 1% solution of 1,2,3 triketohydrindene (ninhydrin) in concentrated sulfuric acid. This solution should be freshly prepared, but is usable for several days. The flask is rotated so that the liquid covers its surface to insure complete contact with the residue. After about 5 minutes of intermittent rotation, 5 ml. of a 5% solution of poly-

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TABLE I.—ABSORBANCE DETERMINATIONS

mcg.	Absorbance					Av. Absorbance	S.D.
20	0.086	0.089	0.094	0.083	0.094	0.089	0.004
40	0.174	0.177	0.176	0.169	0.174	0.174	0.002
60	0.265	0.259	0.264	0.254	0.268	0.262	0.004
80	0.346	0.349	0.350	0.347	0.347	0.348	0.001
100	0.435	0.438	0.439	0.447	0.441	0.440	0.003
120	0.520	0.531	0.525	0.538	0.529	0.529	0.006
140	0.611	0.609	0.611	0.604	0.619	0.611	0.005

TABLE II.—ASSAY RESULTS

	Soln.		Methapyrilene Added	Amount Recovered	% Recovered
I.	Dextromethorphan HBr	5 mg.	5 mg.	4.94 mg.	98.3
	Chlorpheniramine maleate	5 mg.		5.02 mg.	100.4
	Pyrilamine maleate	20 mg.			
II.	Phenylephrine HCl	12.5 mg.	5 mg.	4.98 mg.	99.5
	Pyrilamine maleate	5 mg.		5.03 mg.	100.6
	Chlorpheniramine maleate	1 mg.			
III.	Pyrilamine maleate	10 mg.	5 mg.	4.90 mg.	98.0
	Phenylephrine HCl	25 mg.		5.03 mg.	100.6
	Chlorpheniramine maleate	2 mg.			
IV.	Tripelennamine extract in petroleum ether	100 mcg.	100 mcg. in petroleum ether	100.5 mcg.	100.5
V.	Tripelennamine extract in petroleum ether	50 mcg.	100 mcg. in petroleum ether	101.6 mcg.	101.6

sorbate 80 in distilled water is added with gentle mixing. The solution is then read on a Beckman spectrophotometer or other suitable instrument at 525 mμ. A standard of the corresponding methapyrilene salt is prepared by a similar procedure, or an aqueous solution of methapyrilene hydrochloride is evaporated with the aid of heat and treated with the ninhydrin reagent. A reagent blank is prepared and is used to set the instrument. The red-purple solution may be read immediately, although it is stable for several hours. This color will vary somewhat. The higher the concentration, the more purplish the solution.

Powders, capsules, and tablets may be treated in the same way after first dissolving a portion of pulverized material in a specific volume. The solution is filtered where necessary, and an aliquot treated in the manner outlined above.

RESULTS

Table I gives the absorbance, average absorbance, and standard deviations of a series of experiments. Treatment of portions of a petroleum ether extract of methapyrilene hydrochloride containing 100 mcg./ml., reacted with ninhydrin according to the method outlined above, gave the results of Table I. The measurements were made at 525 mμ. Although no sharp peak is obtained with the colored solutions, a flattened peak is observable at about 525 mμ. If the results of the absorbances were graphed, a nearly straight line would result.

To determine the specificity of the method, several mixtures were prepared and tested. These mixtures simulated in composition some actual commercial products. The results are listed in Table II. As can be seen in Table II, satisfactory recovery results of added methapyrilene are obtainable by the method described.

Other solutions tested, not included in the table, but which gave good results contained vitamins, salicylamide, acetophenetidin, antibiotics, and phenylpropanolamine.

DISCUSSION

Great flexibility may be exercised in the amount of sample used, the number of extractions made, and the ultimate volume of the extracted material. Different solvents, such as chloroform, may replace the petroleum ether and solubilizing agents other than polysorbate 80 may be used.

Of the compounds tested, as shown in Table II, dextromethorphan interfered with the assay method. In instances where dextromethorphan hydrobromide is present, it may be removed by extracting the sample with several portions of chloroform after first acidifying the preparation with 5 ml. of 1 *N* hydrochloric acid. The sample is then made alkaline and extracted with petroleum ether. In general, due to the complexity of liquid preparations, it is desirable to pre-extract the acidified syrups to remove any potential interferences from these mixtures.

The accuracy and reproducibility of this procedure are satisfactory for analytical and control purposes. That the methapyrilene can be differentiated readily from the other components in complex mixtures contributes to its usefulness.

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Preliminary Studies on the Extraction of Glycosides from *Digitalis grandiflora* Seeds

By AYHAN ULUBELEN*

Enzyme-inhibiting and enzyme-favoring extractions were applied to the air-dried seeds of *Digitalis grandiflora*. By paper and thin-layer chromatographic methods, using 70 per cent methanol, native glycosides such as digitalinum verum, purpurea glycoside B, purpurea glycoside A, and strospesid were separated and colorimetrically determined. Digitoxin and gitoxin were obtained when 35 per cent methanolic solution was used.

THE PRIMARY and secondary glycosides found in the air-dried seeds of *Digitalis ferruginea* were reported earlier (1). The glycosides were identified by paper chromatography using two dependable solvent systems. Although the extract of the seeds was obtained by an enzyme-inhibiting technique, investigation showed that secondary glycosides were present in amounts approximately the same as primary glycosides. Early studies on the air-dried seeds of *D. ferruginea* were started only 1 week after their collection; therefore, the enzymatic action could not be attributed to their staying for 1 year, but to some other reason—perhaps the drying conditions.

In this paper air-dried seeds of *D. grandiflora*[1] were used. To eliminate the time factor on the degradation of the glycosides, seeds approximately 10 years old were chosen. As will be seen under *Experimental*, desglucoglycosides were detected when enzyme-favoring conditions were used and native glycosides when enzyme-inhibiting techniques were employed. No secondary glycosides were present in the seeds at the beginning.

After investigating the seeds of some other *Digitalis* species in the near future, a more precise conclusion will be reached about the presence of secondary glycosides in the seeds of *D. ferruginea*.

EXPERIMENTAL

The details of the paper chromatographic technique and the preparation of the standard curves for the colorimetric determination used in this study have been described in a previous paper (1). The extracts were prepared under the enzyme-inhibiting technique—namely, maceration with 70% methanol overnight and percolation the next day. The enzyme-favoring technique was also used and was the same procedure with 35% methanol. Raymond and Jensen reagents (2) were used to detect the glycosides on the paper; 3,5-dinitrobenzoic acid and alcoholic solution of NaOH were used for the colorimetric determination.

Fifty grams of the powdered mass of the seeds of *D. grandiflora* were extracted with petroleum ether; a high yield of oil was obtained (32.7%). The petroleum ether extracted powder was macerated overnight with 100 ml. of a 70% aqueous solution of MeOH, then percolated slowly. When this yellow extract was concentrated under a vacuum, a white precipitate was separated. This precipitate (281.0 mg.) was dissolved in MeOH and purified by recrystallization with ether or isopropyl ether. The melting point of the pure compound was 240–244°, similar to the melting point of digitalinum verum (3), and the amount was 142.7 mg. with a 50.8% yield. This precipitate turned red with the H_2SO_4 solution of *p*-dimethylaminobenzaldehyde—a characteristic reaction for digitaline (4). Paper chromatographic analysis of the crude precipitate showed the presence of two spots using Method *A* (*a*) a yellow pigment (0.02 R_f value) and (*b*) a blue spot with Raymond reagent and an R_f value of 0.083, again similar to that of digitalinum verum (0.082).

This substance was hydrolyzed with 2 *N* HCl and also with 0.1 *N* H_2SO_4, and the aglycon and the sugar parts were analyzed both by paper and thin-layer chromatography.

Gitoxigenin and dianhydrogitoxigenin were found in the aglycon; acid-hydrolyzed products of standard digitalinum verum gave the same compounds with the same R_f values. R_f values obtained from the paper chromatographic separation were: digitalinum verum, 0.08; gitoxigenin, 0.46; and dianhydrogitoxigenin, 0.75. For thin-layer chromatography the R_f values were: 0.81, 0.46, and 0.035, respectively.

Paper chromatography of the sugar of both unknown and the standard digitalinum verum proved the presence of glucose and digitalose. The upper phase of the solvent system of BuOH:H_2O:AcOH (4:5:1) (by volume) was used; the R_f values were: glucose, 0.15 and digitalose, 0.41.

The mother liquid of the precipitate was dried under a vacuum, extracted with a mixture of $CHCl_3$:MeOH (2:1) (by volume), and diluted to exactly 5 ml. When examined by paper chromatography, this extract showed several spots corresponding to purpurea glycoside A, purpurea glycoside B, strospesid, and also digitalinum verum. To obtain a good separation of these glycosides, the solvent systems were overflowed—18 hours for Method *A* and 8 hours for Method *B* (Fig. 1).

Another extract was prepared with a 35% aqueous solution of MeOH. The same precipitate was obtained when MeOH was evaporated; this precipitate proved to be the same compound. Paper chromatography of this enzyme-activated extract revealed that most of the native glycosides had been converted to the desglucoglycosides, such as digitoxin and gitoxin (Fig. 2).

It was possible to lose the secondary glycosides by overrunning the chromatograms. Development times were adjusted accordingly—5 hours for Method *A* and 1.5 hours for Method *B*. R_f values were given for Method *A* and *B*, respectively: digitalinum verum, 0.023 and 0.017; purpurea glycoside B, 0.071 and 0.068; purpurea glycoside A, 0.31 and 0.16; strospesid, 0.57 and 0.34; gitoxin, 0.75 and 0.62; and digitoxin, 0.87 and 0.77.

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[1] The author expresses her gratitude to the College of Pharmacy, University of Minnesota, Minneapolis, for supplying the seeds.

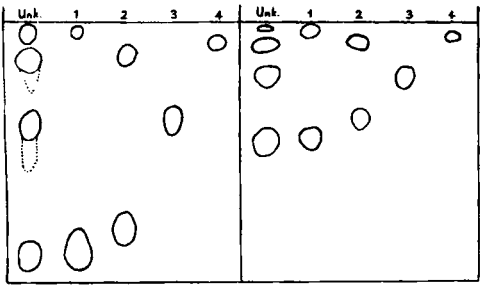


Fig. 1.—Key: *1*, Digitalinum verum, strospesid; *2*, purpurea glycoside B, digilanid A; *3*, purpurea glycoside A; *4*, gitorin; *SL*, starting line.

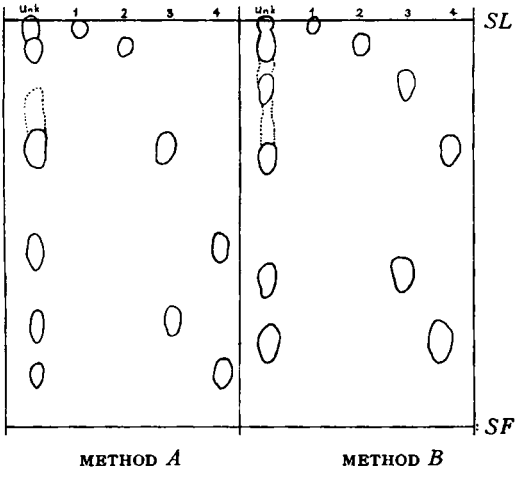


Fig. 2.—Key; *1*, digitalinum verum; *2*, purpurea glycoside B; *3*, purpurea glycoside A; *4*, strospesid, digitoxin; *SL*, starting line; *SF*, solvent front.

Thin-Layer Chromatography.—To establish the paper chromatographic findings and also as a beginning for the future studies, thin-layer chromatographic methods were developed.

Method A.—Smooth glass plates 10 × 20 cm. in size were used. These were coated with an even layer, 0.5 mm. thick, of an adsorbant mixture of silica gel G (Merck). A suspension of 15 Gm. of silica gel G and 50 ml. of water mixed for 1 minute was applied on four to six plates with a simple instrument prepared in our laboratory. After air drying, the plates were activated further by drying at 120° for 1 hour.

The solvent system used by Stahl (5) in the separation of *Digitalis*[2] glycosides was inefficient in this case. The most suitable solvent system was methylisobutyl ketone:isopropyl ether:tetrahydrofuran:methanol (10:5:10:3) (by volume). The total volume of the solvent system depends upon the size of the chromatography jars used. The solvent level should be below the starting line. In this case 20 ml. of solvent was sufficient. After a development time of 30 minutes and air drying, the plates were sprayed with Raymond or Jensen reagents, and a very good separation and small spots were obtained in a short time (Fig. 3). The amounts of standards which were used in this method were about 10 mcg. in each case. R_f values were: purpurea glycoside B, 0.085; strospesid, 0.46; purpurea glycoside A, 0.91; and digitalinum verum, 0.97.

Method B.—The glass plates were coated with a slurry of specially prepared polyamide powder (6). The preparation of the powder is as follows.

One-hundred and fifty grams of Durethan BK 31F (Bayer) is dissolved in 500 ml. of technical HCl over a water bath for 6–7 hours. After cooling, the clear solution is diluted with 250 ml. of MeOH and precipitated with tap water by continuous mixing. The precipitate is filtered and the residue suspended in about 750–800 ml. of MeOH, redissolved with 500 ml. of technical HCl, and reprecipitated as described above. It is then filtered and washed with water until neutral to litmus paper. The neutral residue is suspended in water and stored. At time of use, a part of this suspension is filtered and washed with MeOH. Fifteen grams of the moist residue is suspended in 45 ml. of ethyl acetate which contains 0.5 to 1% Subitogen 2N (Hoest). About six to eight plates can be prepared with this amount. These plates are air-dried; further drying is unnecessary.

Because this polyamide powder was used only recently in the separation of flavon glycosides (6, 7),

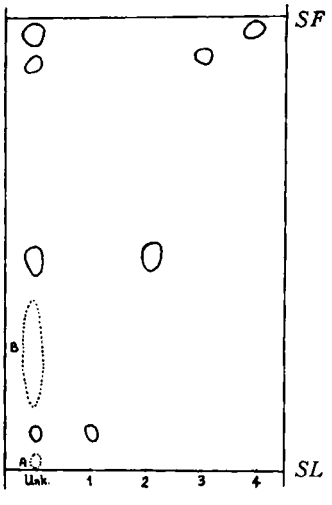


Fig. 3.—Key: *1*, purpurea glycoside B; *2*, strospesid; *3*, purpurea glycoside A; *4*, digitalinum verum; A, yellow pigment; *B*, pink pigment; *SL*, starting line; *SF*, solvent front.

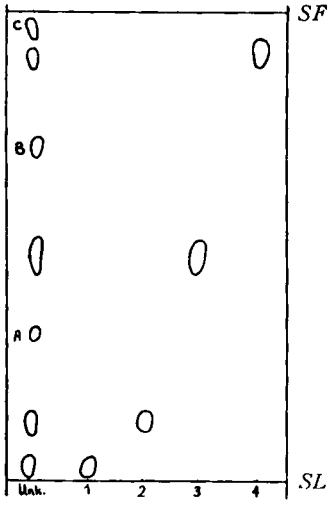


Fig. 4.—Key: *1*, purpurea glycoside B; *2*, purpurea glycoside A; *3*, strospesid; *4*, digitalinum verum; ABC, pink pigments; *SL*, starting line; *SF*, solvent front.

[2] The author thanks Dr. Yoshio Sasakawa for sending the standard digitalis samples.

TABLE I.—PERCENTAGES OF VARIOUS GLYCOSIDES IN THE SEEDS OF *D. grandiflora*

Glycosides	Absorbance[a]	mcg.[b] in 50 µl.	mg. in 5 ml.	Gm. in 100 Gm. Drug
Digitalinum verum	0.115	84	8.4	0.0168
Purpurea glycoside B	0.101	60	6.0	0.0120
Purpurea glycoside A	0.205	230	23.0	0.0460
Strospesid	0.147	134	13.4	0.0268

[a] Average of five determinations. [b] These amounts were calculated from the relationship between the concentration and absorbance which were given in previous paper (1).

it was thought that it could also be used in the separation of digitalis glycosides. After a few experiments with different solvent systems, this material was at least as effective as silica gel G. Figure 4 shows that the pigments were so well separated from the glycosides that it was possible to obtain pure glycosides by using the same powder in a column. The solvent system used was isopropyl ether:methanol (10:3) (by volume). Raymond reagent was sprayed on the plates to develop the spots. The R_f values were: purpurea glycoside B, 0.037; purpurea glycoside A, 0.12; strospesid, 0.48; and digitalinum verum, 0.9.

Quantitative Estimation.—The standard curves were prepared as described previously (1). The same reagents and technique were used.

Fifty microliters of the chloroformic solution of the crude drug was applied to the paper. After development, the areas on the paper corresponding to the native glycosides were cut out and extracted with MeOH. The colors were developed and measured as described previously. The results are shown in Table I.

CONCLUSION

This study showed that only primary glycosides are present in the seeds of *D. grandiflora*. This eliminates the time factor on the degradation of the glycosides and makes the previous findings on the seeds of *D. ferruginea* more interesting. A successful separation was obtained by thin-layer and paper chromatography, the colorimetric determination showed a rather high yield of the total glycosides.

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Effect of Aspartic Acid Salts on Exhaustion Produced by Sleep Deprivation

By ROBERT A. LEVITT and WILSE B. WEBB

Male rats were maintained on a constantly moving wheel in a study of prolonged sleep deprivation. Animals in three age groups were used, and aspartic acid salts were given to half the animals in each age group. The results showed that aspartic acid salts significantly inhibited performance of rats in this situation. Also, the same negative relationship between age and exhaustion time as that found by Webb and Agnew (2) was obtained.

A DELAY IN exhaustion of rats in a swim test as a result of potassium and magnesium aspartate administration has been reported by Rosen *et al.* (1). Webb and Agnew (2) reported a relationship between exhaustion rates and age of rats using a continuous activity wheel. The latter experimenters suggested that sleep deprivation rather than fatigue may have been the critical exhaustion variable involved.

The present investigation duplicated the Webb and Agnew procedure with the addition of aspartate experimental groups.[1] It was reasoned that the more adequately controlled activity output may furnish additional data regarding the action of aspartates. Further, because aspartates are presumably metabolized in the muscle cells, the results may clarify the role of activity *per se* in the exhaustion times reported by Webb and Agnew and hypothesized as sleep deprivation effects.

METHOD

Forty male Lashley-hooded rats from the University of Florida colony were used: Group I, 12 animals, 150 days old (weight range 300–440 Gm.); Group II, 16 animals, 175 days old (weight range 310–460 Gm.); Group III, 12 animals, 235 days old (weight range 370–490 Gm.). Each group was divided into two equal groups matched by weight and assigned to the control or experimental

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condition randomly. Group I-E received 250 mg. each of potassium and magnesium aspartate every 12 hours starting within the first hour of the experiment. The drug was administered suspended in 5 ml. distilled water directly into the stomach with a No. 18 catheter and syringe. (The potassium, but not the magnesium aspartate, would dissolve in the quantity of water used; for this reason only potassium aspartate was administered to groups II-E and III-E.) Group II-E received 200 mg. of potassium aspartate intraperitoneally in 1 ml. distilled water every 12 hours. Group III-E received 50 mg. of potassium aspartate i.p. every 12 hours in 0.5 ml. of distilled water. Each control group received an equal amount of distilled water administered by the same route as its experimental group.

The rats were placed in individual 5.5 × 9.5-in. cubicles, on wheels, two-thirds submerged in water, which rotated at a constant speed of approximately 2 r.p.m. Food trays were placed in each cubicle so that the animals could feed at any time. The animals remained on these wheels continuously except when they were removed twice a day for the drug administration. The total distance covered by an animal during the day was 0.7 mile. The rats, when exhausted, fell from the wheel into the water and were unable to remount the wheel. Animals were removed from the experiment when they fell into the water after being replaced on the wheel three times during a 15-minute period. This procedure is the same as that employed by Webb and Agnew.

RESULTS

Figure 1 shows for the experimental and control groups at each age the mean hours at which the criterion of exhaustion was reached. Data for only 34 animals are included in the analysis because six did not learn to walk on the wheel (one in Group I-C, two in Group I-E, one in Group III-C, and two in Group III-E). Three animals were removed from the wheel at 136 hours at they seemed to be interminable walkers (one in Group I-C, one in Group II-E, and one in Group II-C).

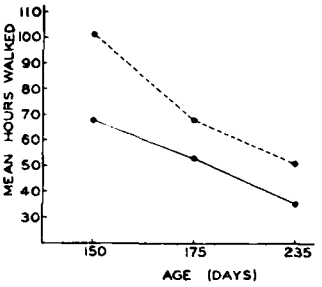


Fig. 1.—Mean hours until exhaustion for the control and experimental groups at three ages. Key: ●---●, control; ●—●, experimental.

DISCUSSION

An analysis of variance resulted in a significant age difference ($p < .01$) and a significant drug effect ($p < .05$).

First it should be recognized that the design is flawed by the changed dosage procedures in the three age groups. Despite this flaw, the consistency of the results across all three conditions supports certain general conclusions.

The previously reported relationship between age and exhaustion time was confirmed for both the experimental and the control groups. On the other hand, the results of the aspartate group (*a*) showed no differential age effect (when compared with the control groups) and (*b*) were contrary to the results reported by Rosen *et al.* (1).

The latter finding suggests a differential action of aspartic acid in a chronic exhaustion situation such as used here in contrast to the acute exhaustion procedure involved in the swim test. It is possible that aspartic acid may result in an overexpenditure of energy in a low requirement situation which reduces the possibility of resisting terminal exhaustion.

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Mineral Acid Salts of Lidocaine

By HENRY M. KOEHLER and JOHN J. HEFFERREN

Some physicochemical properties of the hydrobromide, hydrochloride, nitrate, perchlorate, phosphate, and sulfate salts of lidocaine are reported.

All local anesthetic agents with one or two exceptions are marketed as hydrochloride salts. Since these agents are generally available in aqueous or glycol solutions or ointments for parenteral or topical administration, physical properties such as hygroscopicity are not so important as those of a drug normally formulated as a tablet or capsule. For example, aqueous solutions of lidocaine hydrochloride for pharmaceutical dosage forms are prepared by adding lidocaine base U.S.P. XVI (1) to a slight molar excess of dilute hydrochloric acid, rather than by dissolving the hydrochloride salt in water (2). In his dissertation about the synthesis and characteristics of anilide-type local anesthetics, Löfgren listed the melting points of four salts of lidocaine and the solubility of the hydrochloride salt in the common organic solvents (3). Except for this work, there is little or no published information on the hydrochloride (4) or other salts of lidocaine. This, coupled with the general feeling that lidocaine hydrochloride was difficult to prepare and somewhat hard to handle (5) led to the preparation and study of the mineral acid salts of lidocaine.

The hydrobromide and hydrochloride salts were

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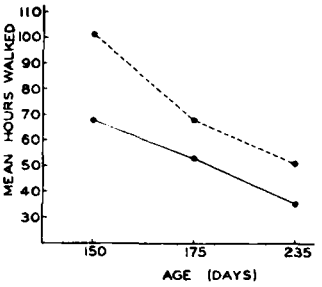


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TABLE I.—SOLUBILITY OF LIDOCAINE SALTS Gm./100 ml. AT 25° C.

	HCl	HBr	HNO_3	H_2SO_4	H_3PO_4	$HClO_4$
Water	100	100	17	25	29	1.7
Methanol	67	67	40	0.8	25	10
Ethanol	11	40	4	0.6	0.7	1.1
Acetone	1.8	2.2	1.2	<0.01	<0.01	14
Chloroform	4	2.5	20	0.02	<0.01	0.03
Carbon disulfide	<0.01	<0.01	0.01	<0.01	<0.01	<0.01
Ether	<0.01	<0.01	<0.01	<0.01	0.06	<0.01
Carbon tetrachloride	0.01	0.01	<0.01	0.01	<0.01	<0.01

prepared in a dry box with a nitrogen atmosphere by passing dry hydrogen bromide and hydrogen chloride into anhydrous ether solutions of lidocaine base. The salts precipitated immediately and were washed thoroughly with ether. After removal of the ether, monohydrates of the halides were obtained in essentially quantitative yield. The anhydrous salts were prepared by drying overnight at 40° in a pumping vacuum. The nitrate, perchlorate, phosphate, and sulfate salts were prepared by adding a slight molar excess of the concentrated mineral acid or an absolute ethanolic solution of the acid to a well-stirred anhydrous ether solution of lidocaine base. The sulfate was an equimolar salt, whereas the phosphate contained 1 mole of base per 2 moles of phosphoric acid. The salts, which were obtained in excellent yield and purity, could be recrystallized from an absolute methanol-ether mixture. The melting points of the lidocaine salts were: HBr, 127–132°; $HBr \cdot H_2O$, 94–103°; HCl, 127–132°, $HCl \cdot H_2O$, 74–78°; HNO_3, 131–133°; $HClO_4$, 204–205°; $2H_3PO_4$, 182–184°; and H_2SO_4, 210–212°. Löfgren (3) reported values of HCl, 128–129°; HNO_3, 133–134°; $HClO_4$, 205°. The $HCl \cdot H_2O$ was reported as 77–80° (4).

All the lidocaine salts prepared were white, odorless, crystalline solids. The solubilities of these salts are tabulated in Table I. All the salts, with the possible exception of the perchlorate, are highly soluble in water and fairly soluble in polar solvents. Unlike most amine halide salts, lidocaine hydrobromide and hydrochloride are relatively soluble in chloroform.

The stability of the salts in atmospheres of various relative humidities was determined by placing the powdered salts in tared aluminum moisture pans in three large desiccators, maintained at 52.9, 71.2, and 93.0% relative humidities. These relative humidities were obtained by placing saturated solutions of $Mg(NO_3)_2 \cdot 6H_2O$, $NH_4Cl \cdot KNO_3$, and $NH_4H_2PO_4$, respectively, in the bottom of the desiccators (6). The aluminum pans were weighed at 12 and 24-hour intervals until constant weight was obtained. The gain in weight of the salts expressed as moles of water per mole of salt is given in Table II. In the 93% relative humidity chamber, the halide salts liquified within 12 hours. In contrast, the monohydrates of the halide salts were relatively stable at the lower humidities.

TABLE II.—HYDRATION CHARACTERISTICS OF LIDOCAINE SALTS

	Relative Humidities at 25° C.		
	52.9%	71.2%	93.0%
Hydrochloride	1[a]	1[a]	>2[a]
Hydrobromide	1	1	>2
Nitrate	0	0	0
Perchlorate	0	0	0
Phosphate	0	0	0
Sulfate	0	0	0

[a] Increase in weight expressed as ratio of moles of water per moles of salt.

The infrared absorption of the lidocaine salts was determined in KBr pellets and in chloroform when the solubility was adequate. The carbonyl band at about 6μ offered an excellent peak for analysis. The base was extracted with chloroform from an aqueous alkaline solution and the chloroform extract dried, evaporated to a convenient volume, and then diluted to known volume. In a 1-mm. sodium chloride cell, the absorbance at 6μ of the lidocaine base was linear from 1 to 7 mg./ml. Perchloric acid titration of the salt in glacial acetic acid or a chloroform extract of the base was also a convenient analytical method.

The mineral acid salts of lidocaine base seemed to be characteristic of the salts of a number of therapeutic agents containing basic nitrogen moieties. It would appear that lidocaine hydrochloride monohydrate would present few problems in essentially dry pharmaceutical dosage forms. If extremes of humidity were anticipated, other salts such as the phosphate could be considered. Thus, with lidocaine as well as any basic drug, the potential availability of other mineral and carboxylic acid salts which may have more desirable physical properties should be considered before limiting work to the hydrochloride salt.

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Comparison of the Diuretic Activities of Benzimidazolyl-toluenesulfonamide Compounds and Acetazolamide

By THOMAS R. CASTLES and HAROLD E. WILLIAMSON

The diuretic activities of α-(2-benzimidazolyl)*p*-toluenesulfonamide (BTS) and α-(5-methyl-2-benzimidazolyl)-*p*-toluenesulfonamide (MBTS) were compared with the diuretic activity of acetazolamide using a rat diuretic assay. Acetazolamide was about 83 times more potent than BTS and about 56 times more potent than MBTS on a weight basis. Both BTS and MBTS exhibited a greater kaluretic action than acetazolamide when equivalent natruretic doses were employed, suggesting that BTS and MBTS affect renal distal tubular mechanisms to a greater degree than acetazolamide.

SOUTHWORTH (1) first reported that sulfanilamide produced an alkaline urine. Subsequently, sulfanilamide was found to inhibit carbonic anhydrase (2); this led to the development of acetazolamide as a diuretic agent (3). Miller *et al.* (4) found that *p*-toluenesulfonamide was a more potent inhibitor than sufanilamide. Hosein *et al.* (5) reported that benzimidazole was also an inhibitor of carbonic anhydrase. It was of interest to learn whether the incorporation of two structurally different inhibitory groups into the same compound would result in an addition of activities. Therefore, the benzimidazole derivatives of *p*-toluenesulfonamide, α-(2-benzimidazolyl)-*p*-toluenesulfonamide (BTS) and α-(5-methyl-2-benzimidazolyl)-*p*-toluenesulfonamide (MBTS), were examined *in vivo* using a rat diuretic assay. 2-Acetylamino-1,3,4-thiadiazole-5-sulfonamide (acetazolamide), a potent carbonic anhydrase inhibitor both *in vitro* and *in vivo* (3), was used as the reference compound.

METHODS

Drugs were evaluated by the assay procedure described by Lipschitz *et al.* (6). Male albino (Holtzman) rats, 140–200 Gm., were fasted for 16 hours prior to the experiment and given an oral load of 0.9% NaCl or water, 50 ml./Kg., at the beginning of the assay. Drugs were administered orally in the load as a solution (acetazolamide) or as a suspension (BTS, MBTS). For urine collection, three rats were placed in each metabolism cage, and urine was collected over a 5-hour period. At the end of the collection period, the bladders of the rats were emptied manually. Urine samples were analyzed for sodium and potassium with a Coleman flame photometer (model 21), and chloride was determined using a Buchler-Cotlove chloridometer.

Data in Tables I and II were analyzed using Duncan's new multiple-range test following analysis of variance (7). Figures 1 and 2 were analyzed using a four-point parallel-line bioassay (8). The 0.05 level of probability was used as the criterion of significance.

RESULTS

The results of Finney's four-point parallel-line assay comparing the natruretic actions of BTS, MBTS, and acetazolamide in rats given a saline load are shown in Figs. 1 and 2. No significant deviation from parallelism was found. Acetazolamide was calculated to be 83 times more potent than BTS (95% confidence limits, 46–310) and 56 times more potent than MBTS (95% confidence limits, 28–97) on a weight basis.

Table I summarizes the effects of BTS, MBTS, and acetazolamide on the excretion of water, chloride, and potassium in these same rats. All three compounds produced a significant increase in the excretion of water but did not alter the excretion of chloride significantly. MBTS at both doses and BTS at the higher of the two doses employed, produced a significant increase in the excretion of potassium. The small increases in the

TABLE I.—EFFECT OF BTS, MBTS, AND ACETAZOLAMIDE ON THE EXCRETION OF WATER, CHLORIDE, AND POTASSIUM IN RATS GIVEN AN ORAL LOAD OF SALINE

	Treatment, mg./Kg							Coefficient of Variability, %
Excretion of	None	BTS 75	MBTS 1.25	Acetazolamide 1.25	BTS 150	MBTS 150	Acetazolamide 2.5	
Water, % of load	47[a]	57	62	65	65	79	89	14
Chloride, % of load	67	63	61	62	60	66	62	28
	None	Acetazolamide 1.25	Acetazolamide 2.5	BTS 75	BTS 150	MBTS 75	MBTS 150	
Potassium, meq./Kg.	1.5	1.8	1.9	2.0	2.2	2.7	2.8	20

[a] Data were analyzed by Duncan's multiple-range test following analysis of variance using a randomized complete block design. Any two values in each row which are underscored by the same line are not significantly different. Any two values in each row which are not underscored by the same line are significantly different ($p < 0.05$). Five groups of rats were employed for each treatment.

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The benzimidazolyl-toluenesulfonamide derivatives were kindly supplied by Dr. E. E. Dunn, Dow Chemical Co., Midland, Mich.

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TABLE II.—EFFECT OF EQUIVALENT NATRURETIC DOSES OF BTS OR MBTS AND ACETAZOLAMIDE ON THE URINARY EXCRETION OF WATER, CHLORIDE, AND POTASSIUM IN RATS GIVEN AN ORAL LOAD OF WATER

Excretion of	Treatment: Control	BTS, 140 mg./Kg.	Acetazolamide, 1.75 mg./Kg.	Coefficient of Variability, %
Water, % of load	79[a]	91	93	8
Sodium, meq./Kg.	0.5	3.2	2.8	17
Chloride, meq./Kg.	0.5	2.4	1.5	23
Potassium, meq./Kg.	0.7	4.0	2.3	32
	Control	MBTS, 150 mg./Kg.	Acetazolamide, 2.5 mg./Kg.	
Water, % of load	93	114	119	6
Sodium, meq./Kg.	0.6	2.7	2.4	19
Chloride, meq./Kg.	0.5	0.9	1.1	23
Potassium, meq./Kg.	0.8	2.3	1.6	13

[a] Data were analyzed by Duncan's new multiple-range test following analysis of variance using a completely randomized block design. Any two values in each row which are underscored by the same line are not significantly different. Any two values in the same row which are not underscored by the same line are significantly different ($p < 0.05$). Four groups of rats were used for each treatment.

excretion of potassium seen with acetazolamide were not significantly different from the control rate of excretion of this ion.

Table II summarizes the effect of equivalent natruretic doses of BTS or MBTS and acetazolamide on the excretion of sodium, water, chloride, and potassium in rats given an oral load of water. Equivalent natruretic doses (as determined from Figs. 1 and 2) were used to compare the effects of these drugs on the excretion of potassium at equivalent rates of excretion of sodium. Both BTS and MBTS produced a greater kaluresis than acetazolamide at equivalent rates of natruresis. All three agents increased the excretion of chloride. Only the increase seen with BTS was significantly greater than that seen with acetazolamide.

DISCUSSION

Diuretic agents which act by inhibiting renal carbonic anhydrase produce a characteristic change in the urinary excretory pattern of water and electrolytes. They increase the excretion of sodium and potassium ions, bicarbonate, and water; decrease the excretion of hydrogen and ammonium ions; and do not markedly alter the excretion of chloride. The two benzimidazolyl-toluenesulfonamide compounds, when administered to saline-loaded animals, increased the excretion of sodium, potassium, and water, but did not alter the excretion of chloride. Thus, these compounds appear to induce changes consistent with inhibition of renal carbonic anhydrase.

Miller *et al.* (4) found that acetazolamide on a molar basis was about 39 times more potent an inhibitor of carbonic anhydrase than *p*-toluenesulfonamide *in vitro*. Converting our data to a molar basis shows that acetazolamide is about 64 times more potent than BTS and about 41 times more potent than MBTS. Thus, it would appear that the addition of a benzimidazole moiety does not enhance the carbonic anhydrase inhibitory activity of *p*-toluenesulfonamide.

The kaluresis seen with BTS and MBTS in the saline-loaded animals appeared to be greater than that seen with acetazolamide. This was examined further using equinatruretic doses of these agents. Both BTS and MBTS produced a greater kaluresis than acetazolamide when the natruretic responses were equal. This could be due to a difference in inhibition in various portions of the tubule. Carbonic anhydrase inhibitors have been reported to act on both the proximal and distal tubules (9–11). By blocking carbonic anhydrase in the proximal tubule, and consequently the supply of hydrogen ions for exchange with sodium, proximal reabsorption of sodium ion is depressed. By blocking carbonic anhydrase in the distal tubule, the decreased supply of hydrogen ions to the hydrogen ion-potassium ion exchange mechanism for sodium ion favors a potassium-for-sodium exchange. From this, an agent acting predominantly on the distal tubules should cause a greater kaluresis than nat-

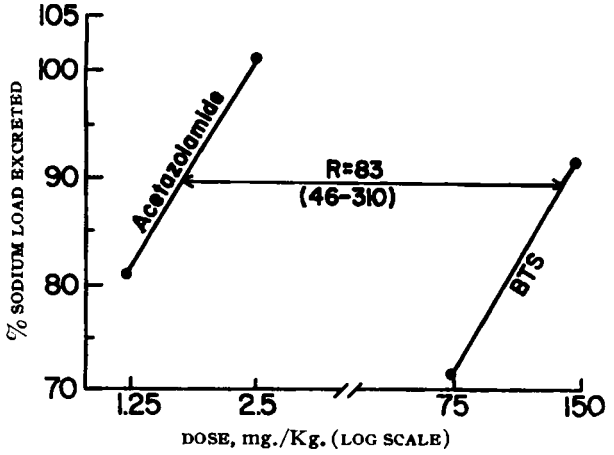


Fig. 1.—The natruretic action of BTS compared to that of acetazolamide using a four-point parallel-line bioassay. *R* equals potency ratio with 95% confidence limits in parentheses.

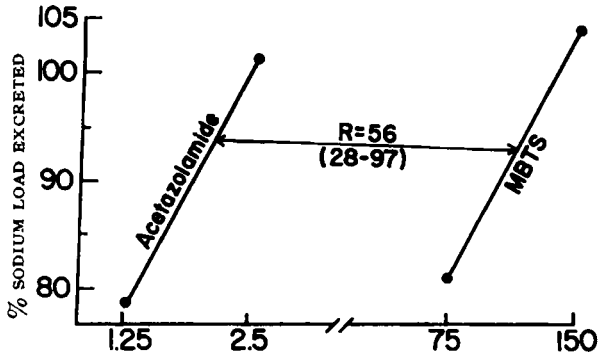


Fig. 2.—The natruretic action of MBTS compared to that of acetazolamide using a four-point parallel-line bioassay. *R* equals potency ratio with 95% confidence limits in parentheses.

ruresis. The greater kaluresis produced by BTS and MBTS when equivalent natruretic doses of these agents and acetazolamide were employed therefore suggests that these benzimidazolyl-toluenesulfonamide compounds exert a greater effect on the distally located exchange mechanism than does acetazolamide.

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Use of 3-Azabicyclo[3.2.2]nonane in the Mannich Reaction IV. Additional Derived Products

By C. DEWITT BLANTON, JR., and W. LEWIS NOBLES

Additional β-amino ketones, phenol Mannich bases, allylamines, propylamines, and γ-aminoalkyl esters involving the use of 3-azabicyclo[3.2.2]nonane in the Mannich reaction are reported. Results of biological tests are cited.

ADDITIONAL MANNICH bases derived from 3-azabicyclo[3.2.2.]nonane are reported in Tables I–V. Results of biological tests are cited.

EXPERIMENTAL

The Mannich reaction was carried out as previously described (1). Preparation of the phenolic Mannich bases was achieved by the procedure of Burckhalter *et al.* (2) or by that of Bruson and MacMullen (3). The γ-amino secondary and γ-amino tertiary alcohols used as intermediates for the preparation of the compounds reported herein were prepared as described earlier (4, 5). The procedure of Pohland and Sullivan (6), Method D, was adopted as a general one for the esterification of γ-amino secondary alcohols. The method employed for the dehydration of the tertiary alcohols was patterned after that of Adamson (7). The procedure adopted as a general one for the reduction of allylamines to propylamines was patterned after that of Adamson and Billinghurst (8). For the preparation of the allylamines 7 and 10 (Table III) the tertiary alcohols, 3 - [3 - (3 - azabicyclo[3.2.2.]-nonyl)] - 1,1 - di - (2 - thienyl)propan - 1 - ol hydrochloride and 3 - [3 - (3 - azabicyclo[3.2.2]nonyl)]-1 - (4′ - propoxyphenyl) - 1 - phenylpropan - 1 - ol hydrochloride, underwent dehydration during their isolation from the Grignard reaction medium.

BIOLOGICAL TEST RESULTS[1]

During the preliminary screening program, compounds reported in this series of papers exhibited a broad spectrum of antimicrobial activity against such organisms as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Candida albicans, Trichophyton mentagrophytes,* and *Trichomonas foetus.* The activity is particularly pronounced against *Trichophyton* and *Trichomonas;* in this regard, the compound β-3-(3-azabicyclo [3.2.2.] nonyl) - 2,5 - dimethyl-

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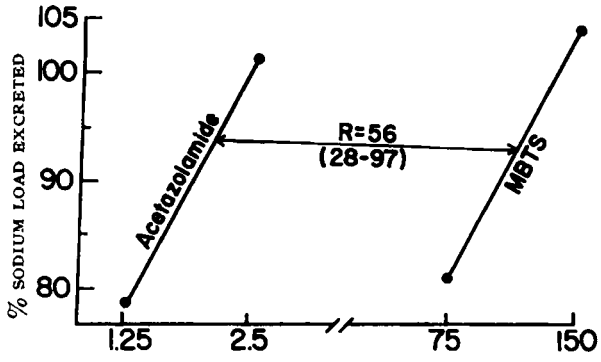


Fig. 2.—The natruretic action of MBTS compared to that of acetazolamide using a four-point parallel-line bioassay. *R* equals potency ratio with 95% confidence limits in parentheses.

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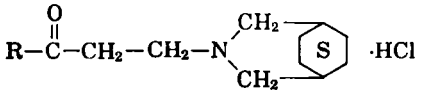
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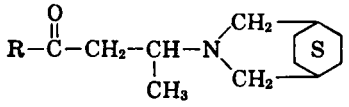
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TABLE I.—ADDITIONAL β-AMINO KETONES



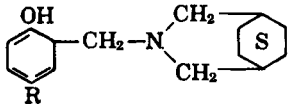
No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	*p*-Propoxyphenyl	62.1	199–201	$C_{20}H_{29}NO_2 \cdot HCl$	68.28	68.50	8.53	8.77	3.98	3.79
2	*o*-Butoxyphenyl	45.0	172–173	$C_{21}H_{29}NO_2 \cdot HCl$	68.92	69.05	8.81	8.86	3.87	4.12
3	3,4-Dimethylphenyl	52.6	216–216.5	$C_{16}H_{21}NO \cdot HCl$	70.92	71.03	8.71	8.75	4.35	4.39
4	2,5-Dimethylphenyl	36.1	210–211	$C_{16}H_{21}NO \cdot HCl$	70.92	71.25	8.71	8.81	4.35	4.36
5	2,4-Dimethylphenyl	78.3	204–206[d]	$C_{16}H_{21}NO \cdot HCl$	70.92	70.88	8.71	8.73	4.35	4.45
6	2,4,6-Trimethylphenyl	44.8	203–205[d]	$C_{26}H_{23}NO \cdot HCl$	71.51	71.37	9.00	8.89	4.17	4.22
7	*o*-Fluorophenyl	37.4	187–190[d]	$C_{17}H_{18}FNO \cdot HCl$	65.48	65.83	7.43	7.75	4.49	4.53
8	*o*-Trifluoromethylphenyl	35.6	195–200[d]	$C_{18}H_{18}F_3NO \cdot HCl$	59.75	59.94	6.41	6.69	3.87	4.07
9	*p*-Trifluoromethylphenyl	48.8	203–205[d]	$C_{18}H_{18}F_3NO \cdot HCl$	59.75	59.74	6.41	6.63	3.87	3.92



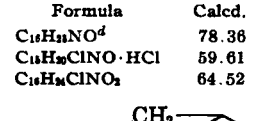
10	5-Chloro-2-thienyl	62.9	202–204	$C_{16}H_{22}ClNOS \cdot HCl$	55.17	55.28	6.66	6.49	4.03	4.01

[a] All Mannich bases in this table were recrystallized from an ethanol–acetone, ethanol, or ethanol–water solution. [b] Melting points, which are uncorrected, were taken on a Fisher-Johns melting point apparatus. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline and French Laboratories, Philadelphia, Pa. [d] Compound sublimed and melting points were taken by the sealed capillary method.

TABLE II.—PHENOL MANNICH BASES



No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	*p*-Methyl	78.0	107–108	$C_{16}H_{21}NO$[d]	78.36	78.29	9.39	9.23	5.72	5.75
2	*p*-Chloro	31.6	244–246	$C_{15}H_{19}ClNO \cdot HCl$	59.61	59.49	7.00	7.05	4.63	4.69
3	*p*-Methoxy	63.2	153–155	$C_{16}H_{21}ClNO_2$	64.52	64.20	8.12	8.18	4.70	4.46



4	2-(α-Naphthol)	51.7	195–196	$C_{19}H_{21}NO \cdot HCl$	71.79	71.37	7.61	7.62	4.41	4.29
5	1-(β-Naphthol)	88.3	155–157	$C_{19}H_{21}NO$	81.09	80.93	8.24	8.14	4.98	4.86

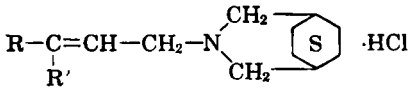
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propiophenone hydrochloride (Table I, Compound 4) was especially interesting.

For determining the antimicrobial activity, filter paper disks (6.3 mm.) are dipped in a 20 mg./ml. suspension of the test compound and tested for activity by the agar diffusion method. A Petri dish of 90-mm. diameter is employed, and the activity is determined by the zone of inhibition. For compound 4 (Table I), this zone of inhibition is greater than 90 mm. for *Trichophyton* and *Trichomonas* by the above method.

The benzenoid and heterocyclic ketonic Mannich bases displayed similar antimicrobial activity and toxicities, although activity of the heterocyclic compounds was not so pronounced. 3-Azabicyclo-[3.2.2.]nonane was observed to exhibit no activity as an antimicrobial agent. The amino alcohols, allylamines, and propylamines have not shown this marked antimicrobial activity, but are pharmacological agents having central nervous system activity. It is anticipated that future publications will be presented concerning these compounds as more details of the biological screening program become available.

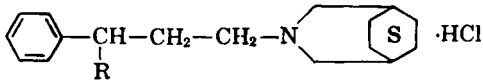
TABLE III.—ALLYLAMINES (HYDROCHLORIDE SALTS)



No.[a]	R	R′	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Phenyl	Phenyl	43.8	219–220	$C_{23}H_{28}ClN$	78.05	77.63	7.97	7.85	3.96	4.01
2	Phenyl	*p*-Fluorophenyl[d]	51.0	225–227	$C_{23}H_{27}FClN$	73.32	73.22	7.31	7.29	3.72	3.75
3	Phenyl	*p*-Chlorophenyl	73.6	225–230[d]	$C_{23}H_{27}Cl_2N$	71.13	71.48	6.96	7.24	3.61	3.76
4	Phenyl	*p*-Methylphenyl	46.4	220–225	$C_{24}H_{30}ClN$	78.34	78.22	8.22	8.24	3.81	4.03
5	Phenyl	*p*-Methoxyphenyl[e]	69.5	170–175	$C_{24}H_{30}ClNO$	73.30	73.39	7.89	7.80	3.56	3.54
6	Phenyl	*p*-Ethoxyphenyl	60.3	193–195	$C_{25}H_{32}ClNO$	75.44	75.20	8.10	8.08	3.52	3.52
7	Phenyl	*p*-Propoxyphenyl	49.0	205–207	$C_{26}H_{34}ClNO$	75.70	75.44	8.25	8.22	3.39	3.45
8	Phenyl	*p*-Butoxyphenyl	88.9	175–177	$C_{27}H_{36}ClNO$	76.12	75.93	8.52	8.31	3.29	3.20
9	Phenyl	2-Thienyl	66.9	180–183	$C_{21}H_{26}ClNS$	70.06	69.39	7.28	7.27	3.89	3.98
10	2-Thienyl	2-Thienyl	71.8	195–200	$C_{19}H_{24}ClNS_2$	62.35	61.78	6.61	6.71	3.83	3.65

[a] All allylamines in this table were recrystallized from an ethanol–acetone or ethanol–ether solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline and French Laboratories, Philadelphia, Pa. [d] Calculated for 0.25 mole of water. [e] Calculated for 0.50 mole of water.

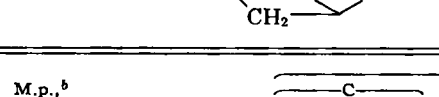
TABLE IV.—PROPYLAMINES (HYDROCHLORIDE SALTS)



No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Phenyl	75.5	280–284	$C_{23}H_{30}ClN$	77.64	77.91	8.44	8.64	3.94	4.16
2	*p*-Fluorophenyl	45.4	257–260	$C_{23}H_{29}ClFN$	73.87	73.57	7.82	7.90	3.75	3.94
3	*p*-Chlorophenyl	57.6	263–268	$C_{23}H_{29}Cl_2N$	70.76	70.74	7.49	7.61	3.59	3.61
4	*p*-Methylphenyl	46.3	270–280	$C_{24}H_{32}ClN$	77.91	77.54	8.72	8.56	3.79	3.84
5	*p*-Methoxyphenyl	52.5	240–246	$C_{24}H_{32}ClNO$	74.68	74.82	8.36	8.24	3.63	3.75
6	*p*-Ethoxyphenyl	91.3	238–245	$C_{25}H_{34}ClNO$	75.06	75.13	8.57	8.66	3.50	3.64
7	*p*-Butoxyphenyl	87.2	215–219	$C_{27}H_{38}ClNO$	75.76	75.85	8.95	9.03	3.27	3.50

[a] All propylamines in this table were recrystallized from an ethanol–acetone or ethanol–ether solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul N. Craig, Smith Kline and French Laboratories, Philadelphia, Pa.

TABLE V.—γ-AMINOALKYL ESTERS



No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Phenyl	93.0	228–230	$C_{19}H_{28}ClNO$	67.56	67.67	8.29	8.57	4.15	4.16
2	*p*-Chlorophenyl	95.2	220–223	$C_{19}H_{27}Cl_2NO_2$	61.26	61.81	7.53	7.46	3.76	3.94
3	*p*-Methoxyphenyl	98.1	205–207	$C_{20}H_{30}ClNO_3$	65.32	65.32	8.22	8.44	3.81	4.07
4	*p*-Methylphenyl	79.4	237–238	$C_{20}H_{30}ClNO_2$	68.26	68.19	8.59	8.85	3.98	4.12
5	*p*-Nitrophenyl	84.9	212–215	$C_{19}H_{27}ClN_2O_4$	59.61	59.20	7.11	7.23	7.32	7.82

[a] All γ-aminoalkyl esters were recrystallized from a methanol-ether solution. [b] Melting points are uncorrected. [c] Carbon hydrogen, and nitrogen analyses are through the courtesy of Dr, Paul N, Craig, Smith Kline and French Laboratories, Philadelphia, Pa.

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Simple Programmed Thermoregulator

By M. W. SCOTT*, C. Y. MIN, W. A. CAMPBELL, and C. M. ANDERSON

The design and construction are described for a programmed thermoregulator which was used to provide time-temperature control in nonisothermal reaction kinetics studies of product stability. The apparatus is inexpensive to construct and provides moderate programming flexibility.

POSSIBLE APPLICATIONS of nonisothermal reaction kinetics in the evaluation and prediction of product stability have been under recent investigation in these laboratories. For the experimental sections of this research, a time-temperature control was required in order to obtain a constant rate of heating in the (liquid) reaction systems selected for study. A new programmed thermoregulator was designed for this purpose. The apparatus was inexpensive to construct and performed in a reliable and predictable manner. The unit also provided moderate flexibility in the types of programs which could be established.

A photograph and an assembly drawing of the programmed thermoregulator are given in Figs. 1 and 2, respectively. The construction was based on the use of a standard slide-wire type thermoregulator.[1] This was mounted on a plastic base. A synchronous motor[2] was mounted on the same support and was used to drive (rotate) the external magnetic collar of the thermoregulator through a friction-belt connection. A simple spring and screw arrangement was provided to regulate the tension in the belt. A fixed rate of collar rotation was obtained in this way. As a result, the contact position of the slide wire within the mercury column which, in turn, governs the control temperature set on the thermoregulator, was varied at a constant rate. The thermoregulator was connected to a standard relay unit[3] and controlled the power input to heating elements in a bath surrounding the reaction vessel.

In the majority of studies, the thermoregulator was used to control the temperatures of 1–2 L. of reactants contained in a three-neck glass flask fitted with a mechanical agitator. The mercury bulb of the regulator was placed directly into the reaction vessel which was held in an agitated water bath with approximately 5-gal. capacity. A single 500-w. immersion heater operated through the thermoregulator was used in the bath.

The time-temperature programs could be varied using synchronous motors with differing rates of rotation. It was more convenient, however, to change the rate of heating by adjusting the belt ratio between the motor and the thermoregulator collar. This was accomplished by fitting different sized sleeves onto the collar.

Constant rates of cooling also could be obtained using the control apparatus. This was achieved by inverting the position of the motor (thereby

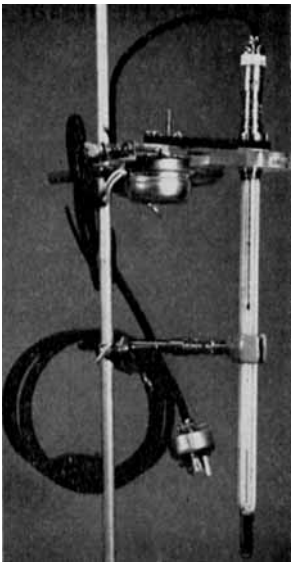


Fig. 1.—Programmed thermoregulator.

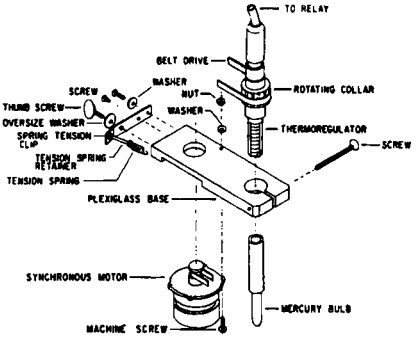


Fig. 2.—Assembly drawing of programmed thermoregulator.

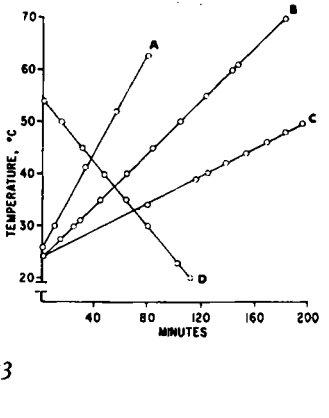


Fig. 3. — Rates of heating and cooling obtained with programmed thermoregulator. A, 0.46° per minute; B, 0.25° per minute; C, 0.13° per minute; D, 0.30° per minute (cooling).

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* Present address: Division of Research, Development and Quality Control, Maradel Products, Inc., New York, N. Y.
[1] Thermoregulator, adjustable micro-set type, Catalog No. 62537, −10 to 110°, Precision Scientific Co., Chicago, Ill.
[2] Synchron motor No. 630, 1 r.p.m., Synchron Co., Homer City, Pa.
[3] Electronic relay, self-contained control unit, Catalog No. 62690, Precision Scientific Co., Chicago, Ill.

reversing its direction of rotation) and by adding a cooling cell to the water bath to oppose the action of the heating element. Several typical plots of time *versus* the temperature of the reaction system obtained under these operating conditions are illustrated in Fig. 3. The constancy of the rates of heating and cooling is evident.

The programmed thermoregulator appears to have usefulness in a variety of applications where accurate control of rates of heating or cooling are required. The performance of the apparatus compares favorably with that specified for the more costly program devices which are available commercially.

Hydrogenation of 1-Naphthol with Rhodium Catalyst

By MORRIS FREIFELDER and GEORGE R. STONE

The low-pressure hydrogenation of 1-naphthol with rhodium is found to cause perhydrogenation. Use of this is made to produce *cis,cis* 1-decalol.

IN THE COURSE of studying the effect of rhodium catalysts in the hydrogenation of organic compounds, we investigated the reduction of 1-naphthol (I) under low-pressure conditions. Hydrogenation was interrupted after 2 *M* equivalents were absorbed and the resultant reaction mixture subjected to vapor-phase chromatography. A major peak consisting of about 70% of the total was identified as 5,6,7,8-tetrahydro-1-naphthol (II). In addition to a small amount of decalin and a few per cent of what appeared to be 1-tetralone, about 20% of another product was seen in the chromatograph. From its position it was suspected to be a decalol.

In view of this tendency toward overhydrogenation, it seemed worthwhile to study the reaction further. The mild conditions employed should lead to a predominance of *cis* products. It was hoped that *cis,cis* 1-decalol (III) would be obtained. If so, a less time consuming and more direct method than those described in the literature would be available.

Gutsche and Peter have prepared III in a three-step synthesis (1). Their material was of excellent quality judging from its melting point, but the conversion of II to III with freshly prepared platinum oxide required 4 days. Zimmerman and Mais, starting with I, also found that a long reduction period was necessary (2). The same slow uptake of hydrogen was encountered in another reduction in which decalin and mixed decalols were obtained as well as III (3). With Raney nickel at elevated temperature and pressure, mixtures were obtained (4).

Hydrogenation of I with rhodium catalyst at 60° and 3 Atm. did lead to III. Vapor-phase chromatography showed about 5% of decalin and almost 10% of a product which we assumed to be the intermediate 1-decalone, and a major peak (84.5%). On recrystallization of the residue from this reduction III was obtained. After several reductions at low pressure, it appeared more desirable to run the reaction at high pressure and at room temperature to convert the intermediate 1-decalone completely (if indeed it was present) and to cut down on hydrogenolysis. When the hydrogenation was carried out at 130 Atm., presumably the only product obtained was III. On chromatography, a small amount of impurity (*ca.* 3%) was found. Infrared examination showed the absence of OH and aromatic ring and the presence of a carbonyl peak at 5.78 μ. In the absence of any conjugation, the impurity was assumed to be 1-decalone.

Received November 18, 1963, from the Organic Chemistry Department, Research Division, Abbott Laboratories, North Chicago, Ill.
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EXPERIMENTAL

5,6,7,8-Tetrahydro-1-naphthol.—A solution of 14.4 Gm. (0.1 mole) of 1-naphthol in 50 ml. of analytical reagent methyl alcohol was hydrogenated under 3 Atm. pressure in the presence of 4.3 Gm. of 5% rhodium on alumina catalyst.[1] Uptake of 2 *M* equivalents was complete in 5 hours. The reaction was interrupted and the solution filtered from the catalyst. After concentration, the residue solidified on cooling. Vapor-phase chromatography[2] showed 0.72% decalin, 20.4% of decalol or decalols, 69% of a tetrahydronaphthol, plus about 7% of two unknowns. The mixture on infrared examination[3] showed presence of OH, aromatic ring, and a carbonyl peak at 5.9$^+$ μ (1-tetralone shows a peak at 5.98 μ).

The mixture was then dissolved in benzene and extracted with 100 ml. of 50% sodium hydroxide solution. The alkaline solution was acidified and extracted with benzene. The benzene extract was dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was treated with hexane and allowed to stand to crystallize. The product obtained melted at 68° (described 68.5 to 69°) (1) and was shown to be 5,6,7,8-tetrahydro-1-naphthol by mixed melting point and infrared absorption compared to a known standard.

cis,cis 1-Decalol.—The hydrogenation of 0.1 mole of 1-naphthol was carried out at 60° and 3 Atm. in the presence of 5.8 Gm. of 5% rhodium on carbon.[1] Uptake for 5 H_2 was complete in 12 hours. After removal of catalyst and solvent, the residue obtained solidified and melted at 77–86° with softening at 70°. Recrystallization from heptane raised the melting point to 90 to 91.5° (described 93°) (1). The crude material was examined by means of vapor-phase chromatography. Results showed 5.4% decalin, 9.6% of an unidentified fraction (possibly 1-decalone), and 84.3% of III.

A second set of runs was carried out as above. The residue was distilled. Decalin was again obtained, the remainder of the material distilled over a wide range, 210–237° at atmospheric pressure.

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[2] Carried out by P. F. Helgren of this laboratory.
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Reduction at Higher Pressure.—A solution of 72.0 Gm. (0.5 mole) of I in 250 ml. of 95% ethyl alcohol was hydrogenated at room temperature and 130 Atm. in the presence of 30 Gm. of 5% rhodium on carbon. Reaction was slightly exothermic, the temperature rising to 46°. Uptake was complete in about 1 hour. The catalyst was removed, the solution concentrated, and the residue distilled. It boiled at 237–240° [lit. 246° at 745 mm. (5)] at atmospheric pressure and solidified quickly. The product weighed 74.5 Gm. and melted at 89–91°.

To determine the purity of the product,[4] 2.5 Gm. was dissolved in a mixture of 20 ml. of 99% benzene and 1% ether, put over a silica gel column, and eluted with a 95% benzene and 5% ether mixture. Fractions were collected in 30-ml. portions. These were followed by means of thin-layer chromatography. In one fraction a minor component was seen. It was isolated and found by infrared examination to have a peak at 5.78 μ, indicative of an unconjugated carbonyl group. Since there was no evidence of aromaticity, it was assumed to be 1-decalone. One more fraction produced a mixture in which the major component predominated (85%). Thereafter only *cis,cis* 1-decalol was obtained, 1.5 Gm., m.p. 91–92°.

In a larger run (1.0 mole) at 200 Atm. pressure, the temperature rose to 54–55°. In this instance the yield of distilled III was lower (76.5%).

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Lipid Content of *Chlorella* "Aerated" with a CO_2–Nitrogen *versus* a CO_2–Air Mixture

By ROBERTSON PRATT and EVELYN JOHNSON

Contrary to some earlier reports, substitution of nitrogen for air in the CO_2–air mixture conventionally used to "aerate" cultures of *Chlorella* had little effect on dry weight or on protein and lipid content of *C. pyrenoidosa* harvested after 8 weeks, whether the cells were provided with a good nutrient solution or with one deficient in nitrogen. In a manganese-deficient medium, the yield of dry weight and the protein and lipid content of the cells produced was approximately the same whether a CO_2–air or a CO_2–nitrogen gas stream was used, but the dry weight and lipid content were markedly lower and the protein content was higher than in cultures developed in the standard complete medium.

IT HAS BEEN REPORTED (1, 2) and confirmed (3) that cells of *Chlorella pyrenoidosa* have a higher lipid content when cultured in a nitrogen-deficient mineral nutrient solution than when cultured in one with an adequate concentration of nitrogen. However, the highest level of lipid found by the later authors was markedly lower than the 65% in 4 weeks and approximately 75% in 10 weeks, reported by the earlier workers (1, 2). Seeking sources of the discrepancy is difficult because of the lack of specific details with respect to environmental conditions in the earlier papers. Since composition of the "aeration" stream might have been an important variable, this has now been investigated and is the principal subject of this report.

The "aeration" mixture customarily used in *Chlorella* experiments in this laboratory and elsewhere for many years consists of 5% carbon dioxide and 95% air. However, a personal communication from one of the earlier authors states that they "found it essential to use 5% CO_2 in *nitrogen* in order to maintain cultures in good shape for the long time needed to obtain the highest lipide content" and that "we too observed clumping of cells in old cultures in SM (nitrogen-deficient) medium grown with CO_2 in air . . . But even in the 70–80 day old, very high lipide cultures grown with CO_2 in N_2, the cells remained freely suspendible and showed negligible sticking together" (4).

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RESULTS

In three experiments (each run in duplicate) averaged in Table IA, substitution of CO_2–nitrogen for the CO_2–air stream had only minimal effect on the dry weight of cells produced and on their protein and lipid content, whether the alga was cultured in the standard medium or in the nitrogen-deficient one. Regardless of which gas mixture was provided, the lipid content (as per cent of dry weight) was about 19% higher, and the protein level was about 10% lower in the medium deficient in mineral nitrogen than in the standard. Under the experimental conditions employed, we found no evidence that composition of the culture solution affected the response of the cells to substitution of nitrogen for air in the gas stream or to support the cautiously worded suggestion that *Chlorella* may "under the special conditions producing high *R*-value (namely, mineral nitrogen starvation and supplied with 5% CO_2 in nitrogen), fix atmospheric nitrogen" (1).

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TABLE I.—YIELDS FROM *C. pyrenoidosa* IN STANDARD (STD.)[a] AND IN "HIGH-LIPID-INDUCING" (SM)[a] MEDIA AND IN THE STANDARD MEDIUM LACKING MANGANESE (−Mn)[a] PROVIDED WITH CO_2–AIR AND CO_2–NITROGEN "AERATING" MIXTURES[b]

	A				B	
	Std.		SM		Mn	
	1 Air	*2* N	*3* Air	*4* N	Air	N
Dry wt. (mg./ml. of culture)	8.393	8.725	1.115	1.198	3.463	3.583
Protein (% dry wt.)	19.8	19.8	10.1	9.5	29.3	30.6
Lipid (% dry wt.)	33.2	36.7	52.6	55.8	17.6	16.6

[a] Culture media.

	Std.	SM	Std. − Mn
KNO_3	0.025 *M*	...	0.025 *M*
$MgSO_4 \cdot 7H_2O$	0.020 *M*	0.01 *M*	0.020 *M*
KH_2PO_4	0.018 *M*	0.01 *M*	0.018 *M*
$(NH_4)H_2PO_4$	...	0.000825 *M*	...
$(NH_4)_2HPO_4$	...	0.000714 *M*	...
KCl	...	0.03 *M*	...
$FeSO_4 \cdot 7H_2O$	5×10^{-5} *M*	5×10^{-5} *M*	5×10^{-5} *M*
K citrate	5×10^{-5} *M*	5×10^{-5} *M*	5×10^{-5} *M*
Zn (as $ZnSO_4 \cdot 7H_2O$)	0.400 p.p.m.	0.400 p.p.m.	0.400 p.p.m.
Cu (as $CuSO_4 \cdot 5H_2O$)	0.004 p.p.m.	0.004 p.p.m.	0.004 p.p.m.
Mn (as $MnSO_4 \cdot 4H_2O$)	0.400 p.p.m.	0.400 p.p.m.	...
B (as H_3BO_3)	0.020 p.p.m.	0.020 p.p.m.	0.020 p.p.m.

[b] Culture conditions: Light = 1250 f.-c. (Mazda source) at the level of the flasks. Temperature: 22.5 ± 2° C. Culture period: 8 weeks. Aeration mixtures: air = 5% CO_2 + 95% air; N = 5% CO_2 + 95% nitrogen (both humidified to prevent evaporation of culture solutions).

simulated closely those used by the earlier workers, the cultures did not reduce the "residual" nitrogen level substantially below 0.001 *M*. The "residual" nitrogen level we found in the initially nitrogen-deficient cultures supplied with the CO_2–nitrogen gas stream was 0.00087 *M versus* 0.00093 *M* in similar cultures provided with CO_2–air.

In our experiments, substitution of nitrogen for air in the gas stream had no effect on the appearance or the morphology of the cells in SM medium. After 10 days to 2 weeks, cells in SM medium, irrespective of the composition of the "aeration" mixture, adhered tenaciously in clumps which could not be separated sufficiently to permit cell counts and which would not remain suspended in the medium. All SM cultures displayed the physical characteristics described previously (3).

In a preliminary study of trace elements, it was observed that omission of manganese from the standard medium resulted in cultures that, upon gross inspection, appeared very similar to those produced in the nitrogen-deficient medium. In view of that observation and of the relation of manganese to processes, *e.g.*, photosynthesis and enzyme reactions, fundamental to nitrogen metabolism, cultures in a manganese-deficient solution (−Mn) were included in the present experiments. It should be emphasized that the cultures were manganese-deficient but not manganese-free. Solutions were prepared with twice-distilled water, the last distillation being in an all Pyrex still, and cells for inoculation of the manganese-deficient cultures were rinsed twice in such water. However, undetermined amounts of intracellular manganese undoubtedly were carried into the new cultures with the inocula, and no attempt was made to remove possible trace impurities of the element from the salts used to prepare the culture medium. Estimates based on reports in the literature (5–8) suggest that in the present experiments the concentration of manganese in the −Mn medium was above 5×10^{-5} and below 5×10^{-3} p.p.m. A reasonable approximation appears to be about 5×10^{-4} p.p.m.—a reduction of roughly 800-fold below the initial concentration in the standard and in the SM media.

The experiments revealed marked differences between cells developed in the SM and in the −Mn media (Table IA, columns *3* and *4 versus* Table IB), despite the apparent similarity of the cultures upon gross visual inspection. The experiments showed also that results in the manganese-deficient medium were essentially similar whether the CO_2–air or the CO_2–nitrogen mixture was employed (Table IB).

The most striking feature of the data from cells developed in manganese-deficient cultures in comparison with data from standard cultures is the relatively high protein and low lipid content of cells from the −Mn medium (Table IB *versus* IA, columns *1* and *2*). In 8 weeks, −Mn cultures supplied with the CO_2–air mixture produced about 40% as much dry weight as cultures in the standard medium, but, relative to their dry weight, had approximately 48% more protein and about half as much lipid. In cultures receiving the CO_2–nitrogen gas stream, protein content, relative to dry weight, was 54% greater in cells from manganese-deficient cultures than in those produced in the standard medium and the lipid content was reduced to less than half. These effects are not interpreted as stemming from a direct effect of manganese on nitrogen metabolism, but rather as being indirect consequences of manganese deficiency.

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Differential Inhibition of Dihydrofolic Reductase from Different Species

Sir:

One of the key enzymes necessary for cellular reproduction is (dihydro)folic reductase; when this enzyme reduces (dihydro)folic acid to tetrahydrofolic acid, a spate of some 15 enzymic reactions follow which ultimately are involved mainly in purine and pyrimidine biosynthesis (2, 8). Although all types of cells would not be dependent on all 15 enzymic reactions, almost all cells will be dependent upon (dihydro)folic reductase and some of the subsequent reactions, unless the cell can utilize both preformed purine and thymine or their derivatives.

A variety of 2,4-diaminoheterocycles known to be inhibitors of (dihydro)folic reductase (2) are sufficiently species- or tissue-specific to be useful chemotherapeutic agents—for example, amethopterin for leukemia (5), 5-(*p*-chlorophenyl)-2,4-diamino - 6 - ethylpyrimidine (pyrimethamine) (7) and 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine (4) as antimalarials, 2,4 - diamino - 5 - (3,4,5 - trimethoxybenzyl)-pyrimidine as an antibacterial (13), and 1-(*p*-chlorophenyl) - 4,6 - diamino - 1,2 - dihydro-(4′ - methylpentamethylene) - *s* - triazine as an anthelmintic (1, 12). The selectivity of amethopterin on certain tissues can be attributed to a difference in the efficiency of the folic acid active-transport system (15). Contrariwise, the malaria parasite presumably has no active-transport system for folic acid; since pyrimethamine enters cells by passive diffusion, the host cells are protected by active-transport of folic acid or leucovorin (6).

For years many investigators in the field of comparative biochemistry have sought to establish that all species use identical biosynthetic pathways and that these enzymes are identical regardless of species. Although the biosynthetic pathways appear to be similar, small differences in specific enzymes are readily apparent. For example, (dihydro)folic reductase from most mammalian cells and *S. faecalis* (16) can utilize folate as the substrate, albeit less efficiently than dihydrofolate. The (dihydro)folic reductases from some species show no perceptible utilization of folic acid, particularly those that synthesize dihydrofolate *de novo*, such as *E. coli* (16) and presumably the malaria parasite (7). Furthermore, some (dihydro)folic reductases can use TPNH, but not DPNH, as the cofactor, whereas others can use DPNH although somewhat less efficiently (9, 10).

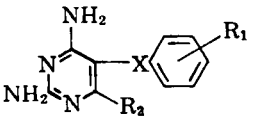
Since these differences in the (dihydro)folic reductases do exist, it would not be beyond the realm of possibility for various reversible inhibitors to show a more potent inhibition of the dihydrofolic reductase from one species compared to another. Such thirty to seventyfold differences have now been observed among the diamino pyrimidines and triazines and these are the subject of this paper.

No large differences were observed in the inhibition of dihydrofolic reductase from pigeon liver or mouse liver (Table I); it might be anticipated that less difference in the dihydrofolic reductases from the same tissue of two different species might be found than from two different tissues of the same species (3). However, large differences were observed in the inhibition of dihydrofolic reductase from pigeon liver and *E. coli*.

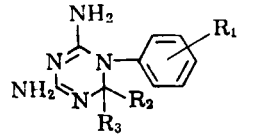
Excluding the 2-chlorophenyl triazine (IX), the structural modifications in Table I change the effectiveness on *E. coli* dihydrofolic reductase by only a factor of 10. In contrast, structural modification can change the effectiveness on pigeon liver dihydrofolic reductase by a factor of 1000. For example, the 3-chlorophenyl derivative (VII) and the 3,4-dichlorophenyl derivative (II, V) are twenty-five to fiftyfold more effective on the pigeon liver enzyme than the corresponding 4-chloro derivatives (III, VI); on the *E. coli* enzyme, the effectiveness is hardly altered. Insertion of an oxygen between the two rings (IV) causes little change on the *E. coli* enzyme, but causes a thirty-two-fold decrease in effectiveness on the pigeon liver enzyme. Thus the 5-phenoxypyrimidine (IV) is thirtyfold more effective on the *E. coli* enzyme than the pigeon liver enzyme; in contrast, the 3-chlorophenyl triazine (VII) is seventy-one-fold more effective on the pigeon liver enzyme than the *E. coli* enzyme.

If the effectiveness of pyrimethamine is primarily due to its inhibition of malaria (*P. berghei*) dihydrofolic reductase, the lack of correlation between the effectiveness of pyri-

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASES BY DIAMINO PYRIMIDINES AND TRIAZINES



Compd.	NSC No.[a]	R_1	R_2	X	—μM Concn. for 50% Inhibition[b,c]— Mouse Liver	Pigeon Liver	*E. coli* B.	Ratio *E. coli*: Pigeon Liver
I	3062	3,4-Cl_2	C_2H_5	...		0.018	0.10	5.5
II	19494	3,4-Cl_2	CH_3	...	0.010	0.032	0.50	16
III[d]		4-Cl	CH_3	...		0.85		
IV	61641	4-Cl	CH_3	O		27	0.90	0.033



		R_1	R_2	R_3				
V	3077	3,4-Cl_2	CH_3	CH_3	0.046	0.015	0.16	11
VI	3074	4-Cl	CH_3	CH_3	0.66	0.44	0.40	0.91
VII	3080	3-Cl	CH_3	CH_3	0.0076	0.0085	0.60	71
VIII	3082	3-Br	CH_3	CH_3		0.0085	0.39	46
IX	3079	2-Cl	CH_3	CH_3		160	200	1.2
X		3-Cl	C_6H_5	H		5.5	1.0	0.15
XI		4-Cl	C_6H_5	H		9.0	3.7	0.41

[a] Ascension numbers of the Cancer Chemotherapy National Service Center; we thank Dr. Harry B. Wood, Jr., for these NSC compounds. [b] The technical assistance of Rita Zielinsk and Bonnie Myers for these assays is acknowledged. [c] An 0.45 to 0.90 saturated ammonium sulfate fraction was used for assay (11) employing 6 μM dihydrofolic acid and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM Versene; 36 μM TPNH was used for *E. coli*. [d] Prepared by W. H. Myers in this laboratory.

methamine analogs as antimalarials (14) and the dihydrofolic reductases listed in Table I indicate that inhibition of the *P. berghei* dihydrofolic reductase has still a different order of effectiveness; for example, the 3,4-dichlorophenyl analog (I) and the 4-chlorophenyl analog are twenty-fold more effective than the 3-chlorophenyl analog against *P. berghei* in mice.

Whether differential inhibitions will be observed with the dihydrofolic reductases from other species or tissues that can be correlated with chemotherapeutic effectiveness remains to be determined.

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Ruticulomycins, New Anthracycline Antibiotics

Sir:

In the past decade a considerable number of anthracycline antibiotics has been described (1). Most of these compounds are aminoglycosides of various hydroxylated tetrahydronaphthacenequinones differing from one another in the oxygenation pattern of the aromatic rings and in the number and nature of the attached sugars. Because these compounds bear a formal structural resemblance to the tetracycline antibiotics and appear likely to have a similar biochemical origin (2), they have been studied intensively.

The authors present a preliminary report on the isolation of two new anthracycline antibiotics which belong to a spectral class not previously reported. The antibiotics are produced by cultivation of a strain of *Streptomyces rubrireticuli* under conventional conditions of agitation and aeration when grown in a medium composed of 1% molasses, 1% glucose, 1% N-Z Amine Type A,[1] and 0.1% calcium carbonate. Crude mixtures of the antibiotic components were isolated by solvent extraction of the filtered mash at neutral pH values. The organic layers were extracted with dilute mineral acid, the acidic extracts were neutralized, then extracted with chloroform. The chloroform extracts were dried, concentrated to small volume, and diluted with hexane. The precipitated bright-red to orange powder contains numerous minor and two major components which are partially separated by solvent partition chromatography on columns of diatomaceous earth using a system composed of cyclohexane-butanol–water-acetic acid (465:925:1100:13). Approximately equal amounts of fairly pure ruticulomycins A and B were obtained. Final purification was obtained by repartition using mixtures of benzene-petroleum ether–alcohol-water (8:12:15:5 for A, 3:1:3:1 for B). Ruticulomycin A crystallizes from acetone-hexane in dark-red plates, m.p. 183–184° dec., $\lambda_{max.}^{MeOH}$ 235, 258, 295, and 475 mμ ($E_{1cm.}^{1\%}$ 650, 300, 120, and 170, respectively), and significant infrared bands (KBr) at 3450 (hydroxyl), 1740 (ester), 1668, 1624 [hydrogen bonded quinone (3)], and 1576 cm.$^{-1}$. Reproducible analytical figures could only be obtained by drying the compound over P_2O_5 for 20 hours at 70° and 10^{-2} mm. pressure.

Anal.—Found: C, 58.52; H, 6.71; N, 1.80. (O)methyl, 7.26; (N)methyl, 4.42; mol. wt. 942 (thermistor).

Ruticulomycin B crystallizes from acetone-ether in red rosettes, m.p. 179–180° dec. The ultraviolet spectrum is virtually identical with that of A, except for some differences in $E_{1cm.}^{1\%}$ values (690, 300, 120, and 190, respectively), and the infrared spectrum differs from that of A only in minor detail. Although not so hygroscopic as A, B was dried for analysis under the same conditions.

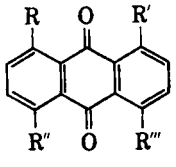
Anal.—Found: C, 57.09; H, 6.37; N, 1.84. (O)methyl, 5.87; (N)methyl, 2.13.

Both antibiotics show absorption maxima at 595 and 650 mμ when dissolved in concentrated sulfuric acid.

The number and position of the hydroxyl groups have a marked influence upon the absorption spectra of the various hydroxyanthraquinones (Table I). This fact has been used as a diagnostic tool in elucidating the hydroxylation pattern of the various anthracyclines (1). In this respect it is clear that the spectra of ruticulomycins A and B are closely related to that of 1,4-dihydroxyanthraquinone (Table I, No. *1;* broad visible maximum at 470 mμ which shifts to 545 mμ in alkaline solution). The tetracyclic nature of A and B was demonstrated by zinc dust distillation which produced a hydrocarbon whose ultraviolet absorption spectrum (maxima at 398, 420, 446, and 476 mμ) in chloroform solution was very similar to that of naphthacene (maxima at 397, 419, 444, and 478 mμ).

The common anthracyclines readily undergo acid hydrolysis to produce a mixture of sugars and an insoluble aglycone chromophore. In this respect, ruticulomycins A and B are exceptional, for they do not give rise to a nitrogen-free chromophore except under very vigorous acid conditions (heating in 48% hydrobromic

TABLE I.—VISIBLE MAXIMA OF A VARIETY OF HYDROXYANTHRAQUINONES



No.	Substance R	R′	R″	R‴	Visible max., mμ	Ref.
1	OH	H	OH	H	470 (MeOH)	(4)
2	OH	OH	H	H	430 (MeOH)	(4)
3	OH	H	H	OH	420 (MeOH)	(4)
4	OH	OH	OH	H	529,516,495,483 (cyclohexane	(5)
5	OH	OH	OH	OH	563,548,524,513,490 (cyclohexane)	(6)

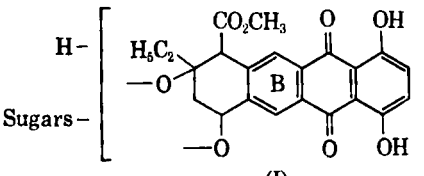
[1] N-Z Amine Type A is an enzymatic digest of casein. Marketed by the Sheffield Chemical Division, National Dairy Products Corp.

acid in glacial acetic acid after acetylation with acetic anhydride/pyridine), and then in very poor yield. Under these strenuous conditions, the anthracycline terminal ring undergoes dehydration to the fully aromatic system. Our amorphous product has so far resisted purification but possesses a visible absorption maximum at 478 mμ in methanol solution. Brockmann has shown that in many cases conversion of anthraquinones to the corresponding tetracenequinones results primarily in an increase in ultraviolet absorption intensity and has relatively little influence upon the position of the visible maxima (7). Aklavinone (II) would appear to be exceptional, for in this case a pronounced wavelength shift was obtained (8). Because this introduces an element of uncertainty in the spectral conclusions, 1,4-dihydroxynaphthacene-5,12-quinone, m.p. 285°, was prepared by the Friedel-Crafts condensation of 1,4-dimethoxybenzene and naphthalene-2,3-dicarboxylic acid anhydride.

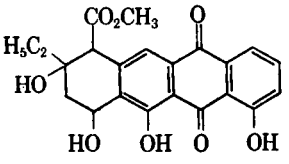
Anal.—Calcd. for $C_{18}H_{10}O_4$: C, 74.48; H, 3.47; O, 22.05. Found: C, 74.17; H, 3.68; O, 21.48.

Due to poor methanol solubility, carbon tetrachloride was used as solvent for spectral examination, and a visible maximum was observed at 466 mμ. This is in reasonable agreement with that of 1,4-dihydroxyanthraquinone (max. at 470 mμ). Thus, there seems to be little doubt that the chromophore of the ruticulomycins is as depicted in formula I. These observations serve to emphasize the uniqueness of the ruticulomycins among the anthracyclines, since all of the previously described members have at least one hydroxyl substituent in ring B (1).

Using the anthracycline parent nucleus (1) and placing the ester function by analogy to those compounds of this series whose structures are already known, it seems reasonable to propose structure I as a working hypothesis for further studies and to infer that A and B differ from one another in the sugar portion of the molecule.



(I)



(II)

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Characterization of a New Magnoliaceae Alkaloid

Sir:

In a previous communication (1) the authors reported results of preliminary studies on the alkaloids of *Magnolia acuminata* L. Fractionation of the stem extracts revealed the major alkaloids to be of the quaternary type. Five such bases were detected by paper chromatographic technique. Of these, three phenolic alkaloids—*viz.*, salicifoline, magnoflorine, and magnocurarine—were identified. Choline was revealed to be the fourth base. Identification of salicifoline and magnocurarine was confirmed by isolation of the picrates of the alkaloids and comparison with authentic samples.

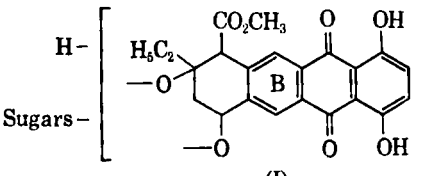
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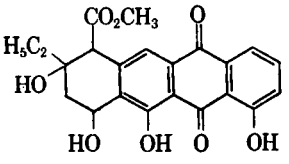
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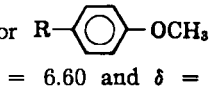
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Chromatography of the purified quaternary alkaloid fraction indicated magnoflorine to be the major phenolic base. While purifying other frac-

tions of the leaves, however, Kapadia isolated a nonphenolic tertiary alkaloid and a neutral ketone. The latter compound was isolated from petroleum ether extract of dry leaves and characterized as palmitone by elemental analysis and comparison of I.R., NMR, mass spectra, and mixed melting point of the isolated and synthetically prepared ketone.

The alkaloid was isolated from an ethanolic extract of the leaves which were previously deprived of petroleum ether and methylene chloride extractives. The residue from alcoholic extract was purified, and the alkaloidal oxalate was obtained as a colorless product from chloroform. Attempts to obtain a crystalline free base were not successful, but paper, thin-layer, and gas-liquid chromatographic examination of the product, $[\alpha]_{589}^{23} = 68.7°$ and $[\alpha]_{436}^{23} = 177.6°$ (C = 1.165, $CHCl_3$), indicated a single alkaloid to be an almost exclusive constituent. NMR spectrum (in $CDCl_3$) indicated presence of three aromatic methoxyl groups (9H at δ = 3.87, 3.80, 3.60 p.p.m.) and one *N*-methyl (3H at δ = 2.55 p.p.m.). Other peaks could most likely be assigned as four aromatic protons in an A_2B_2 system (δ = 7.07, 6.83 p.p.m.) suitable for  and one proton each at δ = 6.60 and δ = 6.10 p.p.m., each of which was also probably aromatic. Results suggested that the alkaloid was *d*-*O*-methylarmepavine. Identity of the base was confirmed with this compound by comparison with an authentic sample of *l*-*O*-methylarmepavine. Paper, thin-layer, gas-liquid chromatographic data, and U.V. and I.R. spectra of the isolated base and authentic *l*-*O*-methylarmepavine were identical. The NMR spectrum of the isolated alkaloid was also identical with that of *dl*-*O*-methylarmepavine, synthesized by a method[1] differing from that reported by Marion *et al.* (2) and Tomita and Yamaguchi (3). Preparation of crystalline hydrochloride [m.p. 198–199° (chloroform-petroleum ether)].

Anal.—Calcd. for $C_{20}H_{26}O_3NCl$: N, 3.85%. Found: N, 3.84%.

The methiodide [m.p. 135–137° (sinters at 128°); reported m.p. 135° (sinters at 127°) (4) and 137–138° (5); $[\alpha]_{589}^{24} = 91.7°$ and $[\alpha]_{436}^{24} = 249°$ (C = 0.193, MeOH)] of the isolated alkaloid and I.R. spectra identical to that of the corresponding alkaloidal hydrochloride and methiodide prepared from authentic base further confirmed the isolated compound as *d*-*O*-methylarmepavine.

Although *dl*-*O*-methylarmepavine has been synthesized (2, 3) and *d*, *l*, or *dl*- forms of the alkaloid have been obtained either as derivatives of armepavine and coclaurine (6–9) or in degradation studies of bisbenzylisoquinolines (5, 10–15), *M. acuminata* appears to be the first plant from which the alkaloid has been isolated and characterized.

Details of the methods for isolation and characterization reported here will be published at a later date.

[1] A modified synthesis of *dl*-*O*-methylarmepavine is being reported separately.

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Review Article

Oximes Antagonistic to Inhibitors of Cholinesterase

Part II

By ROBERT I. ELLIN and J. HENRY WILLS

PHARMACEUTIC ASPECTS

Experiments with 2-PAM indicated that the iodide form of the oxime would be of little practical value because of its comparatively low solubility in water. The iodide is approximately 2% soluble in water at room temperature (144), while saturated aqueous solutions at 25° contain only 4.8%. In view of the fact that 2-PAM is effective in humans in doses of 20 or more mg./Kg., a volume of at least 30 ml. would be needed for a single injection. Such volumes are obviously impractical for intramuscular injection. There is an additional inherent disadvantage in the use of the iodide form. Large doses of 2-PAM can, and have, elicited symptoms of iodism (145).

In attempting to find more soluble salts of 2-formyl *N*-methyl pyridinium oxime (2FMPO), the nitrate was made by simply adding silver nitrate to 2-PAM (146). Fundamental principles of general chemistry tell us that this reaction should go to completion readily, with the formation of insoluble silver iodide and the nitrate of the oxime. The resulting nitrate was found to be 15 times more soluble than the iodide. Other salts were subsequently synthesized. Table III shows the water solubilities and the percentages of oxime in each salt. The chloride salt (2-PAMCl) on the basis of its excellent water solubility, its high oxime content per mole of compound and, most important, its physiological compatibility, was proposed as the oxime of choice. The methanesulfonate of 2-formyl *N*-methyl pyridinium oxime (2FMPOMS), has been championed by another group of investigators (147). The latter contains 56.6% oxime per mole of compound and is about as soluble as the chloride in water. Equivalent concentrations of various oxime salts reactivate inhibited eel cholinesterase at approximately the same rate; that is, there is little or no effect due to the anion moiety.

Aqueous solutions of the chloride can be autoclaved without significant breakdown, provided that a suitable pH is maintained. Unbuffered 2.5, 10, and 20% solutions of 2-PAMCl showed less than a 4% breakdown when autoclaved at 120° (15 pounds pressure) for 15 minutes. The decomposition of the oxime occurs *via* two pH-

TABLE III.—SOLUBILITY AND PER CENT OXIME MOIETY IN METHYL PYRIDINIUM ALDOXIME SALTS (145)

2-PAM Salt	Solubility, mg./ml. of Soln. at 25°	Percentage Oxime
Chloride	640	79.5
Nitrate	675	68.9
Dihydrogen phosphate	46	58.6
Hydrogen sulfate	640	58.6
Iodide	48	51.9
Fumarate	389	70.6
Acetate	...	69.9
Tartrate	565	64.9
Lactate	1000	60.6

Received from the Physiology Division, Directorate of Medical Research, U. S. Army Chemical and Research Laboratories, Edgewood Arsenal, Md.

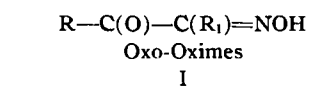
EDITOR'S NOTE: Part I of this review appeared in the September issue, THIS JOURNAL, 53, 995(1964).

dependent mechanisms. At pH values below 4, the hydrogen ion catalyzes hydrolysis of the acid form of the oxime; various states of equilibrium between 2-PAM and its hydrolytic products, pyridine-2-carboxaldehyde methiodide and hydroxylamine, are established in accord with the pH and temperature of the reaction mixture. Above pH 4, the decomposition is due either to hydroxyl ion catalysis of breakdown of the acid form of the oxime or to noncatalytic, direct attack on the oximate ion. Hydroxyl ion attack at the methine hydrogen results in the removal of a proton and the formation of a carbanion as the rate-controlling step (134). Subsequent loss of hydroxide ion from the oximino nitrogen results in the formation of a triple bond—in this case the corresponding nitrile The nitrile, depending on whether there is direct hydroxide ion attack on the cyano grouping or addition to the pyridine ring, forms carbamidopyridinium or hydroxypyridinium ions. On further reaction with hydroxide ions, the latter forms a pseudo base which loses water to form *N*-methyl-α-pyridone. Ellin (123) showed the presence of 2-cyanopyridinium ion, cyanide ion, and *N*-methyl-pyridone in alkaline hydrolysates of 2-PAM. Kosower and Patton (148) conclusively showed that 2-carbamidopyridine methiodide formed when 2-cyanopyridine methiodide was placed in alkaline media. One would then expect the formation of this amide when 2-PAM is degraded in basic solution; its presence among the products of alkaline degradation of 2-PAM has been confirmed by paper chromatographic techniques.

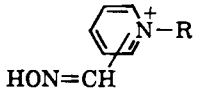
Because cyanide had been found to be a decomposition product of 2-PAM (101, 133), experiments were run to check for the presence of cyanide in aqueous solutions stored over long periods of time. Accelerated storage-stability studies established that the toxicity resulting from 10 and 20% concentrations of oxime was due only to the initial oxime concentration (145). From the established equations for the degradation of PAM, the stability of the oxime in aqueous solution at any pH and temperature may be predicted. Kinetic data show that a solution of 2-PAM, maintained at pH 4.36 at 25°, should retain half of its original concentration of oxime even after a period of 80 years.

Other oximes, such as the aforementioned TMB-4, show promise as antagonists of the toxic effects of organophosphorus compounds, but have not been investigated so extensively as 2-PAM. Experiments concerned with the stability of TMB-4 are complete and are being prepared for publication (149).

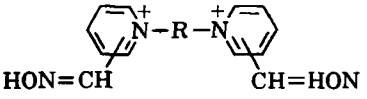
Although chemicals containing the oxime group have been found to possess activity as antibacterial, antifungal, antirickettsial, trypanocidal, tuberculostatic, anticonvulsant, centrally depressant, antispastic, insecticidal, or locally anesthetic agents, probably the most striking biological activity of this type of compound is the ability of some oximes to serve as reactivators of cholinesterase inhibited by certain organophosphorus and carbamate compounds and as antagonists of poisoning by these inhibitors of cholinesterases. The oximes that have been found to have value in these ways fall in general into one of three groups, all mentioned previously:



Oxo-Oximes
I



Monoquaternary Pyridine Aldoximes
II



Bisquaternary Pyridine Bisoximes
III

A few oximes antagonistic to organophosphorus compounds but having other ring structures, including one having the oxime group attached directly to a ring carbon, have been described. These compounds have not exceeded in activity the usual types of oximes.

TOXICITY OF OXIMES

The best known members of the oxo-oxime group (I) are MINA, DAM, and DINA; the best known of the group of monoquaternary pyridine aldoximes (II) are the chloride, iodide, and methanesulfonate salts of 2-formyl *N*-methylpyridinium oxime. Among the bisquaternary pyridine aldoximes (III), the best known is 1,3-bis-(4-formylpyridinium) propane bisoxime dibromide (B4FPBOBr$_2$ or TMB-4). Table IV gives toxicity information about a representative group of oximes. It is apparent that DINA is the most lethal of the group. O'Leary *et al.* (158) have examined the lethal effectiveness in the mouse of i.v. injection of mixtures of equal parts of pyridinium monoximes and bispyridinium bisoximes; in four cases (2FMPOI + B4FPBOBr$_2$, 2FMPOL + B4FPBOBr$_2$, 2FMPOCl + B4FPBOCl$_2$, and 2FMPOMS + B4FPBOBr$_2$), the toxicity of the mixture of

TABLE IV.—TOXICITIES OF SELECTED OXIMES

Oxime[a]	Species	Route	LD_{50}	Ref.
Oximinoacetamide	Mouse	i.p.	4200 mg./Kg.	(100)
MINA	Mouse	i.p.	150	(100)
	Rat (M)	i.p.	50	(150)
	Rat (F)	i.p.	74	(150)
DINA	Mouse	i.p.	20	(100)
DAM	Mouse	i.p.	51, 85, 900	(100, 151, 152)
2-Oximino-3-pentanone	Mouse	i.p.	350	(100)
2FMPOI(2-PAM)	Mouse	i.v.	140–178	(157, 158)
	Mouse	i.p.	136–260	(100, 150–154, 156, 159)
	Mouse	s.c.	290–340	(153, 159)
	Mouse	p.o.	1500–4000	(156, 160)
	Rat	i.p.	305	(150)
2FMPOCl	Mouse	i.v.	115	(158)
	Mouse	i.p.	205	(156)
	Mouse	p.o.	4100	(156)
	Rabbit	i.v.	95	(158)
2FMPOL	Mouse	i.v.	121	(158)
2FMPOMS(P2S)	Mouse	i.v.	118–122	(150)
	Mouse	i.p.	216	(150)
	Mouse	p.o.	3700	(161)
	Rat	i.v.	109	(150)
	Rat	i.p.	262	(150)
2FMPOMS(P2S)	Guinea pig	i.m.	305	(150)
	Rabbit	i.v.	147	(150)
	Monkey	i.m.	356	(150)
$4FPPOBr_2$	Mouse	i.p.	202	(154)
$B4FPBOBr_2$(TMB-4)	Mouse	i.v.	53–89	(157, 158)
	Mouse	i.p.	130	(154)
$B4FPBOCl_2$	Mouse	i.v.	57	(158)
	Rabbit	i.v.	44	(158)

[a] **2FMPOI** = 2-formyl *N*-methylpyridinium oxime iodide (2-PAM); **2FMPOCl** = 2-formyl *N*-methylpyridinium oxime chloride; **2FMPOL** = 2-formyl *N*-methylpyridinium oxime lactate; **2FMPOMS** = 2-formyl *N*-methylpyridinium oxime methanesulfonate; **4FPPOBr$_2$** = 1-(4-formylpyridinium)-3-pyridinium propane oxime dibromide; **B4FPBOBr$_2$** = 1,3-bis(4-formylpyridinium)-propane bisoxime dibromide (TMB-4); **B4FPBOCl$_2$** = 1,3-bis(4-formylpyridinium)-propane bisoxime dichloride.

oximes was between those of the two components and close to the value calculated from the toxicities of the individual components. This last finding suggests that there is no potentiation of the toxicity of $B4FPBOBr_2$ (TMB-4) by either the iodide, the lactate, or the methanesulfonate of 2-formyl *N*-methylpyridinium oxime and none of that of $B4FPBOCl_2$ by the chloride of the monoxime.

EFFECTIVENESS OF OXIMES

Table V summarizes available information about the effectiveness of the same oximes in antagonizing the toxic effects of organophosphorus and carbamate inhibitors of cholinesterases. This table shows several things: (*a*) the oxime with which the greatest volume of work has been done is 2FMPOI (or 2-PAM), (*b*) the only poisoning of humans in which an oxime has been used fairly extensively is that by parathion, (*c*) pyridinium oximes (either mono or bis oximes) are more active than the oxo-oximes, and (*d*) although the oximes are effective antagonists to many of the inhibitors of cholinesterase, there are some anticholinesterase compounds with effects that either are not antagonized by oximes or are made worse by administration of an oxime. In the latter group, Sevin and Diazinon seem to be particularly likely to have their toxic effects enhanced by administration of oximes. This enhancement of toxicity probably occurs through the formation of stable phosphorylated (60, 231) or carbamylated derivatives of the oximes.

The only recourse available today as an aid to atropine in the treatment of severe poisonings by such compounds as Sevin and Diazinon is artificial ventilation. In carrying out this form of therapy, a first requirement is that the airway be rendered patent by removal of secretions and other occlusive material from the pharynx. The maintenance of a patent airway is aided by tilting backwards the head of the supine patient. With a patent airway, one must next insure that the method of artificial respiration being used actually produces effective pulmonary ventilation.

Some of the papers from which the data in Table V are derived contain other information of considerable importance for most effective employment of the oximes in therapy of poisonings by inhibitors of cholinesterase. One such piece of information is that administration of atropine along with the oxime increases the antagonistic activity of MINA against sarin and tabun

TABLE V.—ACTIVITIES OF OXIMES AND SALTS OF OXIMES AS ANTAGONISTS OF LETHAL AND OTHER EFFECTS OF ANTICHOLINESTERASE COMPOUNDS

Oxime	Anti-ChE Compound[a]	Test Object[b]	Antagonism[c]	Ref.
Oximinoacetamide	Sarin	m, r	+	(100)
MINA	Sarin	m, r, gp	+	(59, 100, 102, 120, 144, 163–167)
MINA	Sarin	direct reaction	+	(120, 167)
MINA	Tabun	r	+	(168)
MINA	DFP	r, c	+	(59, 163, 166, 169)
MINA	DFP	rb eye	+	(167, 173, 174)
MINA	TEPP	m; r	−; +	(175; 59, 163, 166)
MINA	DMNP	r	+	(166)
MINA	OMPA; Parathion	m	−; +	(175)
MINA	CH_3-Parathion	m	0	(175)
MINA	Diazinon	m	−	(175)
MINA	EPN, Malathion	m	0	(175)
MINA	Dipterex	m	±	(175)
MINA	Physostigmine	rb eye	+	(171)
DINA	Sarin	m; r	±; +	(100; 59, 163)
DINA	DFP, TEPP	r	+	(59, 163)
DAM	Sarin	m, r, gp, rb, mk; man	+; ±	(59, 100, 102, 120, 144, 158, 164, 165, 171, 172; 218–220)
DAM	Sarin	direct reaction	+	(120)
DAM	Tabun	r	+	(168)
DAM	DFP	r, c	+	(59, 165, 169)
DAM	DFP	rb or human eye	+	(170, 174)
DAM	TEPP	m, r	+	(59, 118, 127, 165, 175)
DAM	OMPA	m, man	±	(175, 218–220)
DAM	Paraoxon; Parathion	m	0	(118; 175)
DAM	CH_3-Parathion	m	−	(175)
DAM	Diazinon; EPN	m	−; ±	(175)
DAM	Malathion; Dimefox	m	−; 0	(175; 118)
DAM	Dipterex	m	±	(175)
DAM	Physostigmine	human eye	+	(170)
DAM	Neostigmine	c; man	+; ±	(123; 218–220)
DAM	Bisneostigmine	man	+	(218–220)
DAM	Bispyridostigmine	man	±	(218–220)
DAM	Ambenonium	man	+	(218–220)
2-Oximino-3-pentanone	Sarin	m, r	+	(100)
2-Oximino-3-pentanone	DFP	rb eye	+	(174)
2FMPOI	Sarin	m, r	±	(100)
2FMPOI	Sarin	m, r, rb, c, d, man	+	(59, 102, 120, 124, 125, 144, 152, 158, 163, 176–181, 182, 218–220)
2FMPOI	Sarin	direct reaction	±	(60, 120)
2FMPOI	Soman	m, rb	0	(181, 182)
2FMPOI	Tabun	m, r, c, d	+	(124, 176)
2FMPOI	DFP	m, r, gp, c, man	+	(59, 81, 82, 116, 117, 154, 155, 163, 178, 183–185)
2FMPOI	DFP	m, rb or human eye	+	(116, 168)
2FMPOI	DFP	direct reaction	+	(185)
2FMPOI	TEPP	m, r, c	+	(59, 117, 123, 127, 154, 160, 163, 175, 176, 187)
2FMPOI	TEPP	direct reaction	+	(186)
2FMPOI	OMPA	m	0; +	(81, 82, 125; 155, 175)
2FMPOI	OMPA	man	±	(218–220)
2FMPOI	Paraoxon	m, r, gp, rb, c	+	(81, 82, 116–118, 121, 122, 151, 155, 178, 184, 188, 189)
2FMPOI	Paraoxon	m eye	+	(116)
2FMPOI	Paraoxon	direct reaction	+	(186)
2FMPOI	Parathion	r	0	(176, 188)
2FMPOI	Parathion	m, r, gp, rb, c, d, h, man	+	(122, 129–132, 151, 153, 175, 176, 178, 184, 189–209)
2FMPOI	CH_3-Parathion	m, r	+	(175, 191, 193, 210)
2FMPOI	Demeton	m, r	+	(191, 211, 213)
2FMPOI	CH_3-Demeton	m, rb	+	(190, 191)
2FMPOI	Isosystox	r	+	(161)
2FMPOI	Phenkaptone	m, r	+	(191, 193)
2FMPOI	CH_3-Phenkaptone	m	0	(191)
2FMPOI	Diazinon	m	−; +	(175; 191)
2FMPOI	Diazinon	r; ct	±; 0	(193; 212)

Oxime	Anti-CHE Compound[a]	Test Object[b]	Antagonism[c]	Ref.
2FMPOI	CH_3-Diazinon	m	0	(191)
2FMPOI	Guthion	r	−	(211)
2FMPOI	C_2H_5-Guthion	r	−	(211)
2FMPOI	EPN	m, man	+	(175)
2FMPOI	Malathion	m; ct; man	−; +; ±	(175; 212; 198)
2FMPOI	Morphothion; Dimethoate	r	±; 0	(211; 193, 211)
2FMPOI	DBD; Thimet	m; r	+	(160; 211)
2FMPOI	Endothion	m, rb	+	(190)
2FMPOI	D600	direct reaction	+	(186)
2FMPOI	I, II, III	m	+	(214)
2FMPOI	Phospholine	m, rb, c	+	(170, 216)
2FMPOI	Phospholine	rb or human eye	+	(170, 215)
2FMPOI	Phosdrin	r, man	+	(211, 217)
2FMPOI	$R_0$3-0340, $R_0$3-0351, $R_0$3-0422	m	+	(117, 154)
2FMPOI	$R_0$3-0340, $R_0$3-0422	direct reaction	+	(186)
2FMPOI	Dimefox	m; r	+; 0	(118; 193, 211)
2FMPOI	Phosphamidon	m, r	+	(211, 221, 222)
2FMPOI	Dipterex	m	+	(175)
2FMPOI	Physostigmine	gp	+	(184)
2FMPOI	Physostigmine	rb or human eye	+	(170)
2FMPOI	Neostigmine	m; man	0; +	(154, 217; 218–220)
2FMPOI	Bisneostigmine	man	+	(218–220)
2FMPOI	Mestinon	m	±	(217)
2FMPOI	Bispyridostigmine	man	±	(218–220)
2FMPOI	Dimetilan	m; r	+; ±	(191, 223; 211)
2FMPOI	Isolan	m; r	±; +	(223; 211)
2FMPOI	Sevin	r	−	(204, 211)
2FMPOI	Humorsol	m	±	(217)
2FMPOI	Pyramat; Pyrolan	m	+; ±	(223)
2FMPOI	G23091; Dimetan	m	+; 0	(223)
2FMPOI	Ambenonium	m; man	0; +	(216; 218–220)
2FMPOI	Edrophonium	m	0	(216)
2FMPOCl	Sarin, Tabun	rb	+	(158)
2FMPOL	Sarin	rb, d	+	(158, 225)
2FMPOMS	Sarin	m, r, gp, rb, d	+	(150, 158, 161, 164, 225, 226)
2FMPOMS	Tabun	m, r, gp, rb	+	(158, 161, 164)
2FMPOMS	DFP	m, r, gp, rb	+	(161)
2FMPOMS	DFP	rb eye	+	(174)
2FMPOMS	TEPP	m, r, gp, rb	+	(150, 161)
2FMPOMS	OMPA	m, r, gp, rb	+	(161)
2FMPOMS	Paraoxon	m, r, gp, rb	+	(161)
2FMPOMS	Demeton	r	+	(211)
2FMPOMS	Isosystox	m, r, gp, rb	+	(161)
2FMPOMS	Vinylphos	m, r, gp, rb	+	(161)
2FMPOMS	Guthion, C_2H_5-Guthion	r	+	(211)
2FMPOMS	Morphothion; Thimet	r	0; +	(211)
2FMPOMS	Phospholine	rb eye	+	(174)
2FMPOMS	Amiton	m, r, gp, rb	+	(161, 226)
2FMPOMS	S-l, Ch-l	r	+	(225)
2FMPOMS	Phosdrin, Phosphamidon	r	+	(211)
2FMPOMS	Physostigmine	m, r, gp, rb	+	(162)
2FMPOMS	Dimetilan	r	±	(211)
2FMPOMS	Isolan; Sevin	r	+; −	(211)
2FMPOMS	Humorsol	rb eye	+	(174)
4FPPOBr$_2$	Sarin, DFP	m	+	(154, 187)
4FPPOBr$_2$	TEPP, $R_0$3-0340	m	+	(154, 187)
B4FPBOBr$_2$	Sarin	m, r, rb, c, d	+	(89, 158, 164, 172, 177, 182, 187, 228)
B4FPBOBr$_2$	Soman	m, r	±	(182)
B4FPBOBr$_2$	Tabun; DFP	r; m	+	(168; 154, 172, 228)
B4FPBOBr$_2$	DFP	rb or human eye	+	(170)
B4FPBOBr$_2$	TEPP; OMPA	m	+; 0	(86, 127, 154, 187; 229)
B4FPBOBr$_2$	Paraoxon	m	+	(214)
B4FPBOBr$_2$	Parathion, CH_3-Parathion	r	+	(193)
B4FPBOBr$_2$	Armin, Phenkaptone	m, r	+	(193, 229)
B4FPBOBr$_2$	Diazinon; Dimethoate	r	±; 0	(193)
B4FPBOBr$_2$	I, II, III	m	+	(214)
B4FPBOBr$_2$	Phospholine, $R_0$3-0340	m	+	(154, 215)
B4FPBOBr$_2$	Dimefox; Phosphamidon	m	0; +	(193, 229; 221, 229)
B4FPBOBr$_2$	Dipterex	m	+	(229)
B4FPBOBr$_2$	Physostigmine	rb or human eye	+	(170)
B4FPBOBr$_2$	Neostigmine; Bisneostigmine	m	+; 0	(154, 229; 154)

Oxime	Anti-ChE Compound[a]	Test Object[b]	Antagonism[c]	Ref.
$B4FPBOBr_2$	Mestinon	m	0; +	(229; 216)
$B4FPBOBr_2$	Bispyridostigmine, Isolan	m	0	(154, 216, 229)
$B4FPBOBr_2$	Humorsol	m	0; ±	(229; 216)
$B4FPBOBr_2$	Dimetan; Ambenonium	m	0; −	(229; 216)
$B4FPBOBr_2$	Edrophonium	m	0	(216)
$B4FPBOCl_2$	Sarin; Soman	rb; c	+; ±	(158, 227; 230)
$B4FPBOCl_2$	Tabun	rb	+	(158)

[a] Symbols are: **I**: Diethyl-*p*-nitrophenyl phosphonate; **II**: Methyl-*O*-ethyl-*p*-nitrophenyl phosphonate; **III**: Methyl-*O*-isopropyl-*p*-nitrophenyl phosphonate; **Amiton**: *O,O*-Diethyl-S-(2-diethylaminoethyl) phosphonothioate; **Armin**: Ethyl-4-nitrophenyl ethylphosphonate; **Ch−1**: Isopropyl methyl phosphonothiocholinate; **D-600**: Diisopropyl-4-nitrophenylphosphate; **DBD**: *O,O*-Dimethyl-S-(4-oxo-3-H-1,2,3-benzotriazine-3-methyl) phosphorodithioate; **Demeton**: *O,O*-Diethyl-*O*-(2-ethylthioethyl) phosphorothioate; **DFP**: Diisopropyl phosphorofluoridate; **Diazinon**: *O,O*-Diethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidyl) phosphorothioate; **Dimefox**: Tetramethyl phosphorodiamidic fluoride; **Dimetan**: 5,5-Dimethyldihydroresorcinol dimethylcarbamate; **Dimethoate**: *O,O*-Dimethyl-S-(*N*-methyl-carbamylmethyl) phosphorodithioate; **Dimetilan**: 2-Dimethylcarbamyl-3-methylpyrazolyl-5-dimethylcarbamate; **Dipterex**: Dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate; **DMNP**: Dimethyl-4-nitrophenylphosphate; **Edrophonium**: *N*-Dimethyl-*N*-ethyl-*m*-hydroxyanilinium bromide; **Endothion**: 2-(Dimethoxyphosphinylthiomethyl)-5-methoxy-4-pyrone; **EPN**: *O*-Ethyl-*O*-(4-nitrophenyl) phenylphosphonothioate; **Ethylgluthion**: *O,O*-Diethyl-S-(4-oxo-3-H,1,2,3-benzotriazine-3-methyl) phosphorodithioate; **G23091**: 2,6-Dimethyl pyridyl-4-dimethylcarbamate; **Guthion**: *O,O*-Dimethyl-S-(4-oxo-3-H,1,2,3-benzotriazine-3-methyl) phosphorodithioate; **Humorsol**: Decamethylene bis [*N*-methylcarbamic acid *m*-dimethylaminophenyl ester] bromoethylate; **Isolan**: 1-Isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate; **Iso-Systox**: *O,O*-Diethyl-S-(2-ethylthioethyl)phosphorothioate; **Malathion**: *O,O*-Dimethyl-S-(1,2-dicarbethoxyethyl) thiothionophosphate; **Mestinon**: 1-Methyl-3-hydroxypyridinium bromide dimethylcarbamate; **Methyldemeton**: *O,O*-Dimethyl-*O*-(2-ethylthioethyl) phosphorothioate; **Methyldiazinon**: *O,O*-Dimethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidyl) phosphorothioate; **Methylparathion**: *O,O*-Dimethyl-*O*-(4-nitrophenyl) phosphorothioate; **Methylphenkaptone**: *O,O*-Dimethyl-S-(methylthio-2,5-dichlorophenyl) phosphorodithioate; **Morphothion**: *O,O*-Dimethyl-S-(*N*-morpholino carbamylmethyl) phosphorodithioate; **OMPA**: Tetramonoisopropyl pyrophosphortetramide; **Paraoxon**: Diethyl-4-nitrophenylphosphate; **Parathion**: *O,O*-Diethyl-*O*-(4-nitrophenyl) phosphorothioate; **Phenkaptone**: *O,O*-Diethyl-S-(methylthio-2,5-dichlorophenyl) phosphorodithioate; **Phosdrin**: Dimethyl-1-methyl-2-carbomethoxyvinyl phosphate; **Phosphamidon**: Dimethyl-1-methyl-2-chloro-2-*N,N*-diethylcarbamylvinyl phosphate; **Phospholine**: *O,O*-Diethyl-S-2-trimethylammonium-ethyl phosphonothiolate iodide; **Pyramat**: 2-Propyl-4-methylpyrimdyl-6-dimethylcarbamate; **Pyrolan**: 3-Methyl-1-phenyl-5-pyrazolyl dimethylcarbamate; **Ro3-0340**: 3-(Diethylphosphato)-*N*-trimethylanilinium-methyl sulfate; **Ro3-0351**: 3-(Diisopropylphosphato) pyridine; **Ro3-0422**: 3-(Diethylphosphato)-*N*-methyl-quinolinum methyl sulfate; **S-1**: Isopropylmethyl-S-dimethylaminoethyl phosphonothiolate; **Sarin**: Isopropyl methylphosphonofluoridate; **Sevin**: 1-Naphthyl-*N*-methylcarbamate; **Soman**: Pinacolyl-methylphosphonofluoridate; **Tabun**: Ethyl-*N*-dimethyl phosphoramido cyanidate; **TEPP**: Tetraethyl pyrophosphate; **Thimet**: *O,O*-Diethyl-S-ethylthio-methyl phosphorodithioate; **Vinylphos**: Diethyl-2-chlorovinylphosphate.

[b] Symbols are: m = mouse, r = rat, gp = guinea pig, rb = rabbit, c = cat, d = dog, mk = monkey, h = horse, and ct = cattle.

[c] Symbols are: − = administration of oxime increases severity of toxic or other effect of the anti-ChE compound; 0 = administration of oxime has no effect on the toxicity or other action of the anti-ChE compound; ± = administration of oxime may decrease toxicity or other effect of the anti-ChE compound; + = administration of oxime decreases toxicity or other action of the anti-ChE compound. The semicolon is used to separate entries including disparate or qualitatively different effects.

(167, 168), of DAM against sarin and tabun (144, 164, 168), of 2FMPOI or 2FMPOMS against sarin, tabun, DFP, TEPP, OMPA, paraoxon, parathion (−176, +190). CH_3-Demeton, endothion, S-1, CH-1, Ro3-0340, Ro3-0351, Ro3-0422 and phosphamidon (89, 116, 117, 124, 154, 164, 176, 177, 183, 184, 190, 222, 225), of $4FPPOBr_2$ against DFP and TEPP (154), and of $B4FPBOBr_2$ against sarin, tabun, DFP, TEPP and Ro3-0340 (89, 154, 164, 168, 177). On the other hand, the mixture of atropine and oxime has no significant advantage against guthion, C_2H_5-guthion, Demeton, and morphothion (211). Still, it appears that, as a general rule, the oxime should be used along with atropine sulfate. The necessity for large doses of atropine in treating poisoning by cholinesterase inhibitors has been pointed out many times (130, 131, 196, 218, 232–235).

The dosages for the only oximes used so far to treat intoxication by inhibitors of cholinesterase in man, DAM and 2FMPOI appear to be quite similar (218). Grob and Johns (218) recommend total intravenous doses of 2000 mg. of either oxime, given at rates not greater than 500 mg./minute for 2FMPOI or 200 mg./minute for DAM, in severe intoxication with cholinesterase inhibitors. This dose may be repeated if muscular weakness is not relieved or recurs. In less severe intoxication, total doses of 1000 mg. of either oxime may be used similarly. Durham and Hayes (236) recommend total intravenous doses of 1000 mg. of 2FMPOI in either severe or moderate poisoning by anticholinesterase compounds. Other salts of 2FMPO may be used similarly.

The finding of Crook *et al.* (224) that salts of 2FMPO administered orally prior to exposure to lethal concentrations of sarin vapor are effective in protecting dogs subsequently exposed to sarin and then treated by intramuscular injection of 5 mg./Kg. of atropine sulfate, suggests that prophylactic employment of such salts may be useful in cases of possible exposures to cholinesterase inhibitors. This possibility has not been reduced to practice, however.

Certain of the references in Table V compare $B4FPBOBr_2$ with 2FMPOI and conclude that the former compound is the more active and the more rapidly effective against poisoning by sarin, tabun, TEPP, paraoxon, I, II, III, and phosphamidon (86, 127, 154, 168, 177, 187, 214, 221, 228). Despite these apparently advantageous properties, no salt of B4FPBO has been used to treat intoxications of men by inhibitors of cholin-

esterase. The lack of interest in the bis-quaternary type of oxime for therapy may be due to the increased lethality of this type of oxime (Table IV) as well as to the toxic actions of the compound.

The principal toxic effects of B4FPBO are related to its being a bis-quaternary compound: they are a curariform block of neuromuscular transmission and a hexamethonium-like depression of blood pressure (237). This oxime recently has been found to be irritating to the human stomach when daily oral doses were given (238). 2FMPOMS has been reported (239) to produce fibrosis of the mucous membrane of the stomach in dogs given the material by mouth on 5 days a week for 17 weeks. The most striking toxic effects of 2FMPOI are (*a*) the production of pharyngeal pain and enlargement of the parotid glands in some men—effects that seem to be related to the iodide ion and that are absent from 2FMPOCl and 2FMPOMS—and (*b*) the production by large doses of block of neuromuscular transmission (123, 163, 218). Other toxic effects of DAM and 2FMPOI in man have been reported by Jager and Stagg (240). Daily doses of as much as 6 Gm. of 2FMPOCl have been given by mouth to humans for many weeks without signs of gastric discomfort or of any other sort of toxicity (238).

Salts of 2FMPO increase the pressor response to injections of catecholamines (238, 241). This pressor-sensitizing effect of salts of 2FMPO enables single doses of mixtures of these compounds and of salts of B4FPBO to be administered parenterally to experimental animals without marked deleterious effect on blood pressure and with excellent therapeutic activity against poisonings by sarin or tabun (158).

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Research Articles

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Spectrophotometric measurements have been made of systems of catalase and of lactic dehydrogenase containing either salicylic acid, 2-mercaptoethylamine, 2-mercaptoethylguanidine, or sodium diethyldithiocarbamate. Absorption differences indicated complex formation between the dithiocarbamate and lactic dehydrogenase and at least salt formation with catalase. No definite indication of complex formation between these enzymes and either salicylic acid or the basic mercaptans was given by the method employed, although salt or weak field complex formation is a possibility with the latter.

COMPLEXATION of the metal constituents of enzymes by drugs or biologically active molecules has been postulated as a possible mechanism of drug action. Enzyme inhibition by such molecules is often believed to involve complexation of the active catalytic site, which is frequently the metal. Little definite evidence has been provided to demonstrate such complex formation, however, although Vallee has been able to demonstrate enzyme-chelating agent complexations involving the metal constituent by means of both ultraviolet absorption spectral changes (1) and optical rotatory dispersion studies (2). Keilin and Hartree (3) have shown complexation of catalase with such inhibitors as cyanide, sulfide, fluoride, and azide by means of ultraviolet absorption, and Chance (4) has been able to demonstrate the existence of short-lived catalase-peroxide complexes spectrophotometrically using a rapid-flow technique. In these cases, significant differences in either absorption maxima or absorbance of the enzyme were observed.

No less than 30 enzymes are known to be affected by the presence of salicylates *in vivo*, *in vitro*, or both, and many of these enzymes are now recognized as metal-containing. Nitzescu and Cosma (5) reported a powerful depressing action on the succinodehydrogenases by salicylate; von Euler and Ahlstrom (6) stated that salicylate inhibits the activity of lactic dehydrogenase (LDH). Baker (7) later found that salicylate inhibited the conversion of lactate to pyruvate by means of LDH catalysis. Tomimura (8) has described the inhibition of a peroxide-catalase system by salicylate, and

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Hagihara (9) has shown that the rate of denaturation of catalase by salicylate differed from its rate of inhibition by salicylate.

Accordingly, experiments have been carried out to find whether complex formation between salicylate and either lactic dehydrogenase or catalase can be demonstrated with ultraviolet or visible spectrophotometry. In addition, similar experiments were carried out with several of the more powerful antiradiation agents, 2-mercaptoethylamine (MEA), 2-mercaptoethylguanidine (MEG), and diethyldithiocarbamate (DDC) and the same two enzymes, since inhibition of radiation damage in the cells is believed in some cases to result from inhibition of cellular oxidations (10).

EXPERIMENTAL

Materials.—The enzymes, catalase (from beef liver) and lactic dehydrogenase (from beef heart muscle), were obtained from L. Light and Co., Ltd., England. Catalase was stored until use at 10° and LDH at −5° as concentrated suspensions. Salicylic acid was obtained from Merck and Co., Inc., and was recrystallized to constant m.p. from 95% ethanol. 2-Mercaptoethylamine hydrochloride and 2-aminoethylisothiuronium bromide hydrobromide were purchased from Chemicals Procurement Labs., Inc.; 2-mercaptoethylguanidine was obtained from the latter compound by rearrangement in tris buffer at pH 7.2. Sigma 121 primary standard grade tris-(hydroxymethyl)aminomethane hydrochloride, Sigma Chemical Co., was used to prepare the tris buffer, pH 7.2.

Methods.—The Beckman DU quartz spectrophotometer equipped with a constant voltage power supply and a photomultiplier tube was used with 1-cm. cells. Enzyme solutions of the desired concentrations were prepared by dilution of the stock suspensions with the tris buffer solution. All experiments were run at 25°, except for one at 10°. They were all completed within 1 hour after the enzyme solutions were prepared.

For measurement of complex formation, four matched cells were employed. In a typical experiment, cell *1* contained buffer only and served as blank. Cell *2* contained the enzyme solution. Cell *3* contained the complexing agent, prepared in tris buffer solution. Cell *4* contained both complexing agent and enzyme in precisely the same concentrations as cells *2* and *3*. Immediately after mixing, measurements of the absorption spectra were made. The spectra obtained from cells *2* and *3* were added and compared with the spectrum from cell *4*. A difference in shape or degree of the added spectra from the spectrum of cell *4* was assumed to indicate complex formation.

A modification of the method of infinite dilution was also made. In this procedure, cell *1* contained buffer only and served as blank for cell *2*, which contained the enzyme solution. Cell *3* contained the complexing agent and served as blank for cell *4*, which contained both the enzyme and complexing agent in the same concentrations as they existed in cells *2* and *3*. Nonequality of the spectra from cells *2* and *4* was taken to indicate complex formation.

Varying ratios of complexing agent to enzyme were employed from large molar excesses of complexing agent to stoichiometric amounts. In the case of large excesses of complexing agent, however, the possibility existed that its absorption could minimize or swamp any change in absorbance due to complex formation; but in the case of stoichiometric amounts, the other possibility existed—that of swamping by the amount of enzyme absorbance.

RESULTS AND DISCUSSION

Two of the more common metals in enzymes affecting cellular oxidations are iron and zinc. Lactic dehydrogenase is a pyridine nucleotide-linked enzyme containing zinc, and catalase is an iron-porphyrin containing enzyme. Little is known regarding the bonding of the zinc in LDH, but it is assumed that mixed complex formation of the zinc is possible by an extraneous agent. Vallee (1) has already demonstrated mixed complex formation of the zinc in liver alcohol dehydrogenase with 1,10-phenanthroline. In regard to catalase, the iron is *strongly* bound to the porphyrin nucleus; in addition, only one bond is available for coordination, normally with peroxide (11). Chelation of the iron by an external agent is thus considered impossible, although complex or salt formation with such an agent should be possible. In this light, the same type of reaction between metalloenzyme and complexing agent would not occur with the two enzymes. As far as assessing this method for demonstrating complexation, one enzyme serves as control for the other.

For salicylic acid in tris buffer solution maximum absorption was at 296 mμ. Catalase, at a concentration of 100 mg./L., showed no true maximum in the ultraviolet but did show a plateau at 256–268 mμ. Higher concentrations of catalase absorbed maximally in the visible at 404 mμ. Although Keilin and Hartree (3) reported a maximum at about 630 mμ for horse liver catalase at pH 6.0, this maximum did not appear in our enzyme preparation at pH 7.2. The lactic dehydrogenase exhibited peak absorbance at 278–280 mμ.

The spectrophotometric measurements made with catalase and salicylic acid in both the ultraviolet and visible range did not indicate complex formation. Optical differences between the sum of the absorbances of cells *2* and *3* and the absorbance of cell *4* or between the absorbances of cells *2* and *4* in the method of infinite dilution did not exceed 0.010, which probably may be considered the limit of experimental error. The differences reported in Table I represent only the maximum deviations found; however, the average extent of difference over the absorption range observed generally was less than 0.004.[1] It is possible then that a difference of greater than 0.010 may represent salt[2]

[1] Beaven and Johnson (12) point out, however, that under favorable circumstances variations of 0.002 in absorbance are experimentally significant.

[2] A weak field complex, such as described by Eichhorn (11), may also be possible. In this case, the electron "pairing energy" necessary for complex formation is not affected much by changes in the ligand (13). This "pairing energy" is opposed by the "separation energy" between *d* orbitals in the case of iron, but not in the case of zinc. On this basis, the absorption spectrum of catalase would show less variation on complexation than that of lactic dehydrogenase, which is shown in the experiments described here.

TABLE I.—SPECTROPHOTOMETRIC MEASUREMENTS OF ENZYME SYSTEMS, 25°

Compd.	Concn., mg./L.	Enzyme	Concn., mg./L.	Optical Range, mμ	Absorbance Difference
Sal. acid	25	Catalase	100	240–340	0.005
Sal. acid[a]	20	Catalase	300	240–340	0.005
Sal. acid[a,b]	20	Catalase	300	240–340	0.008
Sal. acid	2.2	Catalase	300	240–340	0.003
Sal. acid	50	Catalase	2,000	340–700	0.004
Sal. acid[a]	500	Catalase	4,200	340–600	0.008
Sal. acid	100	Catalase	17,000	480–700	0.010
Sal. acid	12.5	LDH[f]	125	240–340	0.016
Sal. acid[a]	12.5	LDH	125	240–340	0.015
Sal. acid	0.26	LDH	125	240–340	0.018
MEA[c]	100	Catalase	400	240–350	0.014
MEA	1.82	Catalase	300	240–350	0.022
MEA	100	LDH	125	240–350	0.034
MEA	0.2	LDH	125	240–350	0.015
MEG[d]	100	Catalase	200	235–350	0.009
MEG	100	LDH	125	235–350	0.029
DDC[e]	6	Catalase	200	235–350	0.045
DDC	6	LDH	125	235–350	0.363

[a] Method of infinite dilution. [b] Run at 10°. [c] 2-Mercaptoethylamine hydrochloride. [d] 2-Mercaptoethylguanidine hydrobromide. [e] Sodium diethyldithiocarbamate. [f] Lactic dehydrogenase.

formation or ionic bonding between the complexing agent and the enzyme, the small increase in absorbance being due to electrostatic attraction with no significant change in electron densities in either complexing species. The over-all shape of the absorption curves remained the same for the mixture as for the added curves of the individual components in this case, however.

The optical differences recorded for the combination of salicylic acid and lactic dehydrogenase did not exceed 0.018. While this difference is probably not indicative of covalent complex formation, it may represent salt (ionic bonding) formation. It has been pointed out by Martell and Calvin (14), for instance, that if the bonding is ionic, the absorption bands for the complex and complexing agent may coincide fairly closely. Similarly, in the experiments involving 2-mercaptoethylamine and the same two enzymes, no definite complex formation is apparent from the extent of the optical differences measured, although salt formation is again a possibility. In the case of 2-mercaptoethylguanidine, complex formation appears not to have taken place, although the somewhat greater optical difference shown in the mixture with lactic dehydrogenase again suggests salt formation.

Sodium diethyldithiocarbamate showed maximum absorption at 257 and 281 mμ. Addition of this compound to catalase produced a very definite disparity between the spectra of the individual components and that of the mixture (see Fig. 1). A difference in absorbance of 0.040 remained approximately the same from 235 to 300 mμ, after which it became insignificant. An even more striking difference in spectra was noted on the addition of diethyldithiocarbamate to lactic dehydrogenase (see Fig. 2). Here a maximum difference of 0.363 was observed, and the minimal absorbance of the added spectra at about 245 mμ and the increase in absorbance to about 265 mμ are completely eliminated in the absorption spectrum of the mixture. The disparity between the curves remains significant throughout the ultraviolet region, but diminishes as the visible range is approached.

This marked difference in absorbance and the dramatic change in the shape of the absorption curves for the mixture of DDC and LDH, compared to the sum of the absorption curves of the components, may be considered evidence of complex formation, as noted previously for absorption of enzyme-inhibitor complexes (3). It appears quite possible that a cyclic complex, or chelate, has been formed with the zinc still bound in the lactic dehydrogenase, whereas a more loosely bound linear complex only is possible with catalase. That disul-

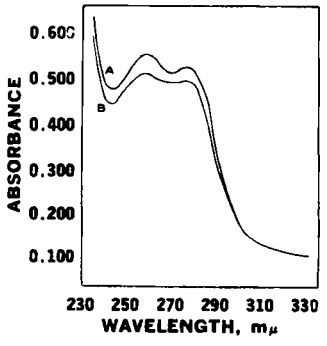


Fig. 1.—Absorption spectra at pH 7.2 of a mixture of sodium diethyldithiocarbamate and catalase (*A*) and of the sum of the spectra of the components (*B*).

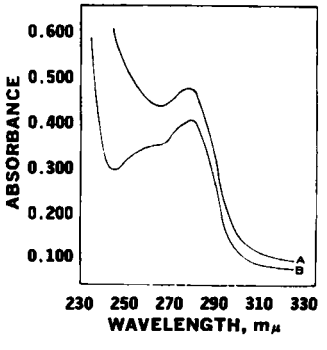


Fig. 2.—Absorption spectra at pH 7.2 of a mixture of sodium diethyldithiocarbamate and lactic dehydrogenase (*A*) and of the sum of the spectra of the components (*B*).

fide formation with protein mercaptan groups is not responsible for the change in absorption spectrum is indicated by the failure of the mercaptans MEA and MEG to cause a similar change.

However, it is again possible that salt formation has taken place with catalase since the over-all shape of the sum of the absorption curves has not been appreciably altered in the mixture, and the average difference in absorbance, 0.040, is not greatly different from those of the previous cases where salt formation is suspected. Moreover, it appears that complex formation of enzymes may be demonstrated by this method of absorbance differences in absorption spectra in certain cases at least.

CONCLUSIONS

Spectrophotometric evidence has been found that a known antiradiation agent, sodium diethyldithiocarbamate, undergoes complex formation with lactic dehydrogenase and either salt or weak field complex formation with catalase. No definite evidence for complex formation of these enzymes with either 2-mercaptoethylamine or 2-mercaptoethylguanidine was found, although salt or weak field complex formation is possible. Apparently, any complexation by the latter agents is more readily dissociable than that by the dithiocarbamate.

No spectrophotometric evidence of complex formation between salicylate and either lactic dehydrogenase or catalase was found, although both enzymes are known to be inhibited by salicylate. It is possible that any complex formed has dissociated too rapidly to be observed by this method.

Measurement of absorbance differences between the sum of the absorption spectra of the individual components and the spectrum of the mixture appears to be a possible method of demonstrating complex formation of enzymes in select cases. In these cases the binding is either irreversible or of sufficient strength to exist for a measurable period of time and thus be observable by the stationary, or nonflow, technique employed here.

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Assay of Iodine Content of Thyroid Extract by X-Ray Absorption Edge Analysis

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The assay of thyroid extract for iodine content by the X-ray absorption edge technique has been investigated. The method has been found to be applicable to the crude, U.S.P. powder, and uncoated Enseals. Excellent precision has been found for the assay of crude thyroid extract. It is also shown that because of the nature of the method the determination is specific for iodine. The instrumentation used is the same as that used for X-ray crystallography by the diffractometer method.

THE ASSAY of the iodine content of thyroid extract by X-ray absorption edge analysis was developed as a possible replacement for the chemical assay for this element.

The chemical assay that is used is prescribed by the "United States Pharmacopeia." The present procedure has undergone only minor improvements since the U.S.P. IX of 1916. Essentially, it is a potassium carbonate fusion to liberate the iodine from the organic matrix. After several intermediate steps, the determination ends with a sodium thiosulfate titration. It is about a 4-hour procedure with many opportunities for loss of sample.

X-ray absorption edge analysis has been applied in the analysis of molybdenum and zinc in hydrocarbons by Barieau (1). A recent review of the field has been given by Liebhafsky *et al.* (2). Because of the completeness of the description of the method given by Barieau, only a brief description will be given here.

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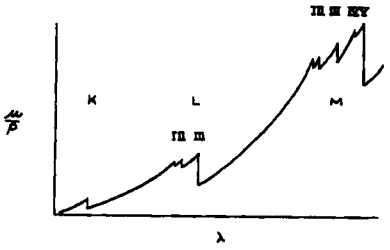


Fig. 1.—A plot of X ray absorption against wavelength. The ordinate shows the mass absorption coefficient which is the absorption from a beam of unit cross section by 1 Gm. of sample.

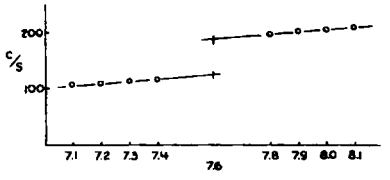


Fig. 2.—A graph showing the relation between transmitted X-ray intensity (ordinate) in terms of counts per second and the angular position of the detector (abscissa). The absorption edge of iodine is at 7.6° for the conditions used.

sample occurs by two mechanisms: (*a*) X-ray photons may be scattered by the sample; and (*b*) X-ray photons may be absorbed by using their energy to eject electrons from the atoms of the sample, that is, by ionizing the atoms.

The absorption due to scattering increases with increasing wavelength of the incident X-ray. Also, the absorption increases with increasing atomic number leading to the familiar fact that lead is a better X-ray shield than aluminum.

On the other hand, the absorption due to ionization is discontinuous with wavelength. In a generalized plot of absorption against increasing wavelength (Fig. 1), these discontinuities appear as definite, sharp decreases in absorption and are termed "absorption edges." The positions of the edges can be found in handbooks of chemical and physical data. An explanation of the physical basis of this effect including the meaning of the terms *K*, *L*, and *M* and the numerals can be found in any modern physics book—for example, the excellent text by Semat (3).

The over-all absorption of X-rays by a substance is defined by the equation

$$I = I_0 e^{-\mu t} \qquad \text{(Eq. 1)}$$

where I = intensity of transmitted beam, I_0 = intensity of incident beam, t = thickness of sample (X-ray path length), and μ = linear absorption coefficient. The linear absorption coefficient of a sample containing more than one element is the sum of the coefficients of each element times the fraction of the sample made up by that element.

Since the absorption of a given element changes at its absorption edge, the absorption coefficient of the sample containing that element will change at that absorption edge. By measuring the amount of X-rays transmitted by a sample containing a known amount of an element for wavelengths on each side of an absorption edge, the amount of change of the coefficient μ can then be measured. Conversely, if the amount of change for the coefficient is known, the measured change in the transmission can be used to evaluate the amount of the element present.

It is important to realize that the absorption edges of the elements occur at shorter wavelengths as the atomic number increases. Thus, for a sample containing several different elements, only that part of the absorption due to the element to be measured will change at its absorption edges.

Barieau (1) has shown that the relationship between X-ray intensities on each side of an absorption edge and the fraction of the element in question in the sample is given by

$$2.303 \log I''/I' = (\mu'_m - \mu''_m)\, WG \qquad \text{(Eq. 2)}$$

where I'' and I' = transmitted X-ray intensities on each side of an absorption edge, μ'_m and μ''_m = mass absorption coefficients at each side of an absorption edge, W = weight fraction of element in sample and G = pt. or mass thickness of the sample in Gm. cm.^{2}.

The term $(\mu'_m - \mu''_m)$ is characteristic for an element under any conditions. For this assay it was evaluated using solutions of potassium iodide. Reagent grade potassium iodide from Mallinckrodt was used. The test solutions were made up in concentrations to cover the range of iodine concentration expected in thyroid extract. The solutions were placed in a cell made of glass tubing with Mylar windows. By standardizing the method in

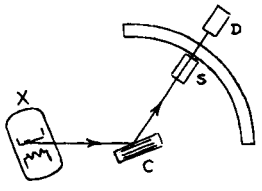


Fig. 3.—Schematic arrangement for X-ray absorption studies. The X-ray tube is at X, the analyzing crystal at C, the sample at S, and the detector at D.

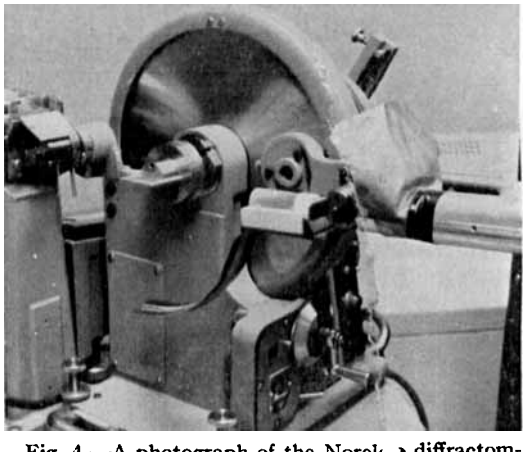


Fig. 4.—A photograph of the Norelco diffractometer set up for X-ray absorption work. (The ray scatter shield has been removed for this photograph. It is normally in place during operation.)

TABLE I.—RESULTS OF REPRODUCIBILITY STUDY OF X-RAY ABSORPTION EDGE ASSAY OF THYROID EXTRACT CRUDE[a]

Sample No.	Result, %	Deviation from Av.
1	0.79	−0.04
2	0.79	−0.04
3	0.82	−0.01
4	0.84	+0.01
5	0.87	+0.04
6	0.84	+0.01
7	0.84	+0.01
8	0.82	−0.01
9	0.82	−0.01
10	0.82	−0.01

[a] Same lot used throughout.

this fashion, the method becomes independent of the chemical assay.

Finally, a working equation for per cent iodine in thyroid extract can be derived from (2):

$$\% \text{ Iodine} = \frac{\log I''/I'}{\text{wt. sample}} \times 24.8 \quad \text{(Eq. 3)}$$

The full derivation of this equation is given in the paper by Barieau (1). The constant term 24.8 represents the $\mu'_m - \mu''_m$ term, a factor to convert logarithms from the natural base to the base 10, and a factor to convert to per cent of iodine.

The measurement of I'' and I' cannot be made directly at the absorption edge. It is necessary to measure intensities at some distance from the edge and extrapolate to the edge. This leads to a graph as shown in Fig. 2. In Fig. 2 the intensity of the X-rays is measured in counts per second (c/s) with a Geiger counter. The distance from the absorption edge is measured in degrees 2θ which is related to the wavelength of the X-rays by the familiar Bragg equation used in X-ray diffraction work.

INSTRUMENTATION

A Norelco X-ray diffractometer with a standard copper target X-ray tube was used for this work. The X-ray generator was operated at 45 kv. and 20 ma. This method requires the use of the "white radiation" which all X-ray tubes emit along with their characteristic radiation. A schematic diagram of the apparatus is given in Fig. 3. A photograph of the apparatus is shown in Fig. 4. The X-ray tube is at X. The diffracting crystal is at C. For this work a crystal of sodium chloride taken from a broken infrared absorption cell was used. The crystal was trimmed so that it fit in a holder of the type ordinarily used to hold the powder sample for crystallographic studies. This crystal "analyzes" the X-ray beam from the X-ray tube and provides a relatively monochromatic X-ray beam of the proper wavelength to pass through the sample. The sample, to be described below, is at S. The radiation detector is at D. In this work a Geiger tube was used. While a Geiger tube cannot count as fast as scintillation counters or flow counters, it has sufficient speed for the work presented here.

The essential point of the instrumentation is that it interferes as little as possible with the crystallographic use of the apparatus. The slit system used to define the X-ray beam for absorption studies is the same as that used for crystallographic work so that there is no time lost in changing from one mode of operation to the other.

The samples used in this work were disks of thyroid extract made by pressing the powder in a 0.75-in. die at about 10,000 p.s.i. The disks were weighed to determine the weight of sample. It was found that a 5-Gm. sample was adequate for the crude material; a 15-Gm. sample was used for the U.S.P. powder. Fortunately, neither preparation gives much trouble by sticking to the die. Because the samples used for determination of the crude were much smaller than those used for the U.S.P. powder, it was advisable to reduce the over-all transmitted intensity by placing a block of Lucite in the beam while running samples of crude. This does not change the absorption due to the iodine content of the sample.

The radiation counting was done on a fixed count basis. At wavelengths shorter than the absorption edge 32,000 counts were taken for each point, while at longer wavelengths 64,000 counts were taken.

RESULTS

The X-ray absorption edge assay has a peculiar advantage over other types of instrumental assays because it can be set up based on standards not of the substance itself but on elemental standards. That is, for the assay of iodine it would be possible to evaluate the $(\mu'_m - \mu''_m)$ term of Eq. 2 not on thyroid extract itself but on elemental iodine. In this work potassium iodide solutions were chosen for ease of handling. Because of this choice, the assay results of the X-ray absorption technique are independent of any systematic error which may be present in the chemical assay and which would be included if the X-ray absorption method had been based on a standard thyroid extract sample.

TABLE II.—COMPARATIVE RESULTS OF X-RAY ABSORPTION EDGE ASSAY AND CHEMICAL ASSAY OF THYROID EXTRACT

Lot No.	Product Type	Chemical	X-Ray
761476	Crude	0.86%	0.83%
761478	Crude	0.68%	0.68%
776573	Enseal	0.0728 mg. iodine/Enseal	0.0739 mg. iodine/Enseal
771318	U.S.P. powder	0.24%	0.22%
771320	U.S.P. powder	0.24%	0.21%
763648	U.S.P. powder	0.19%	0.18%
767265	Crude	0.705%	0.720%
773301	Crude	0.825%	0.845%
778288-1	U.S.P. powder	0.203%	0.205%
778288-3	U.S.P. powder	0.209%	0.187%
778288-4	U.S.P. powder	0.206%	0.206%
778288-5	U.S.P. powder	0.207%	0.217%
773302	Crude	0.859%	0.890%

Table I shows the precision of this assay method. The indicated 1Σ standard deviation calculated from these data is ±0.025. This amounts to about ±3% of the amount present.

Table II shows a comparison between the U.S.P. chemical assay and the X-ray absorption edge method. The X-ray values are all single assays, the chemical assays are the final resul. reported by the Lilly control division and are averages of several assays.

CONCLUSION

The X-ray absorption edge assay is a useful method. In the case of the iodine control of thyroid extract the values obtained by X-ray are comparable to those obtained by the chemical method.

The X-ray method requires, in our hands, about 1 hour. This allows about 0.5 hour for measurements and 0.5 hour for preparation and calculations.

It is important to realize that the method may be applied to other elements. Using the relatively simple apparatus described here, elements having an absorption edge in the region from about 0.3 Å. to about 2.0 Å., that is, from cerium to manganese in atomic number can be determined. With a more complicated apparatus, the list of elements can be expanded.

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(1) Barieau, R. E., *Anal. Chem*, 29, 348(1957).
(2) Liebhafsky, H. A., Winslow, E. H., and Pfeiffer, H. G., *ibid.*, 34, 282R(1962).
(3) Semat, H., "Introduction to Atomic and Nuclear Physics," 4th ed., Holt, Rinehart & Winston, New York, N. Y., 1962.

Kinetics and Mechanism of Isomerization and Hydrolysis of 4,6-Diamino-1-(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-triazine in Dilute Aqueous Solution

By D. H. SZULCZEWSKI, C. M. SHEARER, and A. J. AGUIAR

Rate measurements, concurrent with reaction product identification, indicate that there are three routes by which inactivation of the title compound can occur: (*a*) unimolecular isomerization of free triazine; (*b*) unimolecular isomerization of protonated triazine; (*c*) acid catalyzed hydrolysis of protonated triazine. The relative importance of these reactions on drug decomposition for various pH values is determined. Likewise, rate studies at critical pH values and several elevated temperatures enable accurate estimation of rate of degradation at any temperature. Equations are derived relating specific rate to both pH and temperature. Identification of reaction products by means of thin-layer chromatography shows that besides the 6-dichloroanilino isomer and 3,5-dichlorophenyl-biguanide hydrolysis product, *N*-3,5-dichlorophenyl-*N'*-guanylurea is formed as a secondary product of degradation.

In recent years a variety of substituted dihydrotriazines has been reported in several areas of chemotherapeutic research (1–3). Although many dihydrotriazines have been prepared and screened, there is little information available concerning their stability in aqueous systems. Likewise, information concerning the likely route and rate of degradation is meager.

In a communication by Carrington *et al.* (3) concerned with isolation, identification, and synthesis of the active metabolite of chlorguanide hydrochloride,[1] isomerization and hydrolysis of 1-phenyl-dihydrotriazines to nonactive products was noted. This suggests that loss of chemotherapeutic activity is possible and that relative rates of destruction need to be known to determine the feasibility of liquid formulations having a suitable shelf life.

The following study, concerned with the kinetics of isomerization and hydrolysis of 4,6-diamino - 1 - (3,5) - dichlorophenyl - 1,2 - dihydro-2,2 - dimethyl - 1,3,5 - triazine[2](I) in aqueous buffered solution, was carried out to determine these rates and to determine the chemistry of the degradative process.

RESULTS AND DISCUSSION

Order and Nature of Degradative Process.—Representative data obtained on the rate of degradation of I in aqueous buffered solution at 45° is shown in Fig. 1. This, together with studies at other temperatures, indicates that in aqueous buffered solution the decomposition of I is first order with respect to I over a broad pH range.

The rate of degradation was not influenced by changes in concentration of buffer (Table I). Likewise, the observed rate remained constant in

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extremely alkaline solutions despite a tenfold change in base concentration (NaOH) from 0.05 to 0.5 *N*. However, neutral salt did cause changes in the rate of reaction when carried out in the presence of strong acid (Fig. 2).

The nature of the product or products of the degradative reaction at various pH's was determined by thin-layer chromatography. This was necessary since the ultraviolet absorption spectra (Fig. 3) of both primary and secondary reaction products were too similar to permit spectrum analysis in mixtures. Chromatograms obtained on samples drawn from solutions held at 45° (Fig. 4) indicated that the isomer, 4-amino-6-(3,5)dichloroanilino-1,2-dihydro-2,2-dimethyl-1,3,5-triazine (II) is the only product of degradation over the broad pH range of 13 to 5. From pH 5 to 2 mixtures of 3,5-dichlorophenylbiguanide (III) and isomer (II) are found, whereas in extremely acid systems (pH 2 or less) only the biguandine or its hydrolysis product *N*-3,5-dichlorophenyl-*N'*-guanyl urea (IV) are observed.

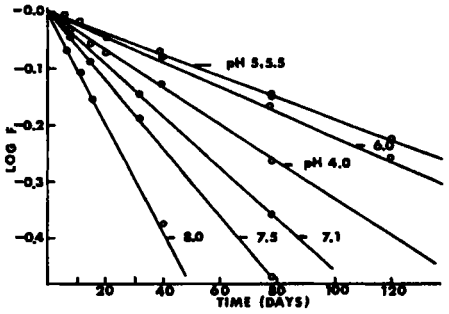


Fig. 1.—Degradation of I at 45° C. in aqueous solution at various pH's (F = fraction I remaining).

TABLE I.—EFFECT OF BUFFER CONCENTRATION ON OBSERVED RATE OF DEGRADATION AT 65° C.

Buffer Molarity	0.1	0.2
pH 8.8 (borate)	k_{obs} = 0.238 hr.$^{-1}$	k_{obs} = 0.236 hr.$^{-1}$
pH 7.0 (phosphate)	k_{obs} = 0.009 hr.$^{-1}$	k_{obs} = 0.009 hr.$^{-1}$

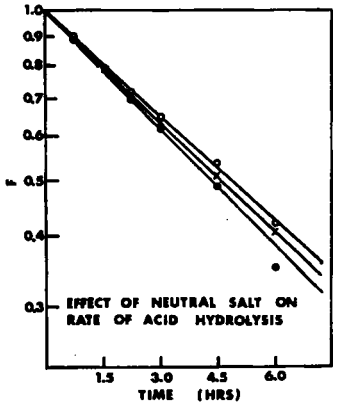


Fig. 2.—Rate of acid catalyzed hydrolysis at various ionic strengths but constant pH. pH = 1.4 maintained with $HClO_4$. Ionic strength varied by changing concentration of $NaClO_4$.

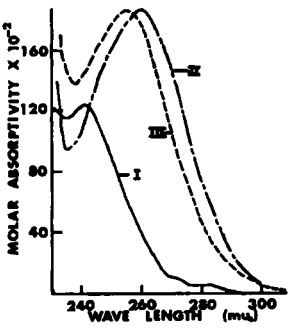


Fig. 3.—Ultraviolet spectra in pH 7 phosphate buffer. Key: I, 4,6-diamino - 1 - (3,5) - dichlorophenyl-1,2-dihydro - 2,2 - dimethyl - 1,3,5 - triazine; II, 4-amino-6 - (3,5) - dichloroanilino-1,2-dihydro-2,2 - dimethyl-1,3,5-triazine; and III, 3,5-dichlorophenylbiguanide.

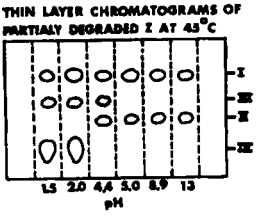
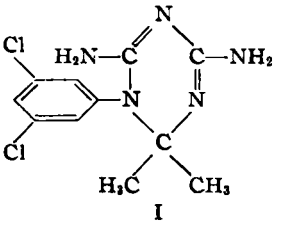
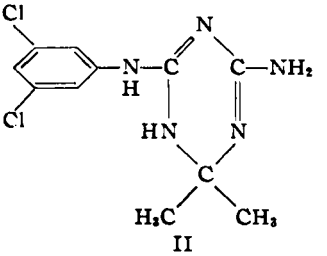


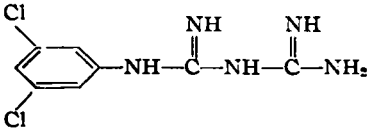
Fig. 4.—Thin-layer chromatograms obtained on solutions of partially degraded I at 45°. pH refers to pH of aqueous solution of I. Solution spotted directly after approximately two half-lives. Top boundary represents solvent front. Bottom boundary represents point of application.



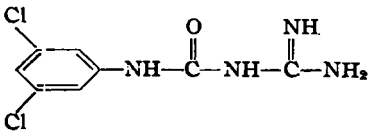
I



II



III



IV

Variation of Reaction Rate with pH.—Variation of the observed first-order rate constant with pH is shown in Fig. 5 for two temperatures. In brief, the pH profile is characterized by the following regions (at 65°): *A*, pH 1 to 2.5 straight line, slope −1; *B*, pH 4 to 5.5 plateau region; *C*, pH 8 to 9.5

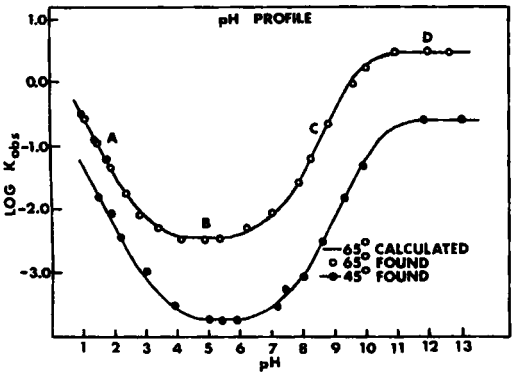


Fig. 5.—Plot of the logarithm of the observed specific rate *vs.* pH over the entire pH range. 65° solid line represents results of calculating observed specific rate by Eq. 13. Open circles represent experimentally determined values.

TABLE II.—k_1 VALUES CALCULATED AT 65° C. pKa′ = 10.0

pH	log k_{obs}		log k_1
9.6	0.00		0.40
8.8	−0.62		0.58
8.2	−1.19		0.61
7.9	−1.55		0.45
		Av.	0.51

straight line, slope +1; *D*, pH 10.5 to 13 plateau region.

Region D.—The independence of rate on concentration of alkali suggests two possibilities for the reaction occurring in region *D*: *a*, unimolecular isomerization or *b*, base-catalyzed isomerization of monoprotonated I.

The rate law governing the first premise would simply be

$$-d \ln I/dt = k_1 \qquad \text{(Eq. 1)}$$

while that for the second

$$-d \ln I/dt = k_{OH^-} \cdot \{(H^+)/[(H^+) + Ka']\} \cdot (OH^-) \qquad \text{(Eq. 2)}$$

where Ka′ = acid dissociation constant of IH^+, which under conditions where $(H^+) \ll Ka$ reduces to

$$-d \ln I/dt = k_{OH^-} \cdot K_w/Ka' = k^* \qquad \text{(Eq. 3)}$$

Both reactions would yield identical behavior with respect to the influence of pH on rate and would therefore be kinetically indistinguishable. However, the second possibility, involving attack of negatively charged hydroxyl ion on a protonated species bearing a positive charge, would be expected to show a primary salt effect (4). Since no salt effect is observed, Eq. 1 is favored.

The first possibility requires that pH affect the rate of degradation only by controlling the relative proportion of protonated and free I, not by fixing the concentration of a catalytic specie. For total drug the applicable rate law would be

$$-d \ln I_T/dt = k_1 \{Ka'/[(H^+) + Ka']\} \qquad \text{(Eq. 4)}$$

where I_T = total amount of *I* present regardless of ionic condition. From Eq. 4, in buffered solution

$$k_{obs} = k_1 \cdot Ka'/[(H^+) + Ka'] \qquad \text{(Eq. 5)}$$

where k_{obs} = observed first-order rate constant. At 65° where pKa′ = 10.0, if pH ≥ 11.5, Eq. 4 simplifies to

$$k_{obs} = k_1 \qquad \text{(Eq. 6)}$$

or

$$\log k_{obs} = \log k_1;$$

and if pH ≤9.5, Eq. 4 may be expressed as

$$\log k_{obs} = \log k_1 - pKa' + pH \qquad \text{(Eq. 7)}$$

Equation 6 would predict that at pH's equal to or greater than 11.5, k_{obs} should be essentially independent of hydroxyl ion concentration. Equation 7 predicts a straight line with slope +1 at pH's less than 9.5. As seen from Fig. 5 (regions *C* and *D*) these conditions are realized.

Equations 6 and 7 provide a check for consistency since k_1 can be independently calculated from two different regions of the pH profile. From the plateau in the extremely alkaline region k_1 at 65° is, $k_1 = 3.14$ hr.$^{-1}$ or $\log k_1 = 0.50$. Table II lists values of k_1, as calculated from Eq. 7, for the pH region 8 to 9.5 (region *C*, Fig. 5).

It is evident that the average value of k_1 calculated from Eq. 7 is in good agreement with the value of k_1 obtained by extrapolating values of k_1, determined directly at lower temperatures, to 65°.

Region B.—The plateau region, pH 4 to 6 at 65°, is apparently due to unimolecular isomerization of monoprotonated I or $IH^+ \xrightarrow{k_2} IIH^+$.

The rate law consistent with the data from pH 13 to 4 would become

$$-d \ln I_T/dt = k_1 \{Ka'/[Ka' + (H^+)]\} + k_2 \{(H^+)/[Ka' + (H^+)]\} \qquad \text{(Eq. 8)}$$

which reduces to

$$k_{obs} = k_2 \qquad \text{(Eq. 9)}$$

in the region of interest.

Region A.—Continuing to lower pH's (4 to 1) an increase in log k_{obs} is seen. The reaction responsible for this increase appears to be acid catalyzed hydrolysis of I to the biguanide $IH^+ + H_2O \xrightarrow[H^+]{k_3} IIIH^+ +$ acetone. If this were the case, the rate law applicable would be (pH 6 to 1) with water in great excess:

$$-d \ln I_T/dt = k_2 + k_3 (H^+) \qquad \text{(Eq. 10)}$$

so that $k_{obs} = k_2 + k_3 (H^+)$ and at sufficiently low pH so that $k_3 (H^+) \gg k_2$

$$\log k_{obs} = \log k_3 - pH \qquad \text{(Eq. 11)}$$

The above expression leads to the expectation of a region with a slope of −1 in the pH profile.

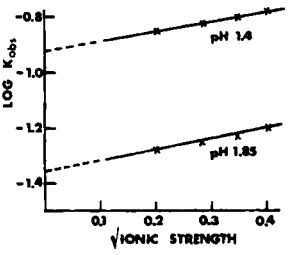


Fig. 6.—Log k_{obs} as a function of the square root of ionic strength at 65° C. at pH 1.85 and pH 1.40. pH maintained with $HClO_4$, ionic strength changed by varying concentration of $NaClO_4$.

TABLE III.—CALCULATED VALUES AT TWO TEMPERATURES

Temp., °C.	Ka′	k_1 (hr.$^{-1}$)	k_2 (hr.$^{-1}$)	k_3 (hr.$^{-1}$)
65	1×10^{-10}	3.16	5×10^{-3}	3.24
45	3.16×10^{-11}	2.18×10^{-1}	1.9×10^{-4}	5×10^{-1}

TABLE IV.—HEATS OF ACTIVATION

pH or pH Range	Log Frequency Factor	ΔH_a (Kcal. mole^{-1})
12 or greater	17.84	26.7
10	20.14	30.7
8.7	23.62	37.5
8.2	23.83	38.5
7.0	21.06	35.5
4.4	17.70	31.0
1.4	13.79	22.6

In this acidic region the reaction is reversible (3). However, under the experimental conditions of low concentration and great excess of water, the concentration of IH^+ at equilibrium is extremely low so that the reaction may be considered to be irreversible. Plots of log *F versus* time over a period of at least one half-life showed no deviation from linearity.

The rate of acid catalyzed hydrolysis would be expected to show a positive primary salt effect since two positively charged ions would be involved in the formation of the activated complex (4). As seen in Fig. 6, this is observed experimentally. Since region *A* of the pH profile is the only region in which a salt effect is observed, further evidence for the reaction as written is obtained.

The over-all relationship of k_{obs} as a function of pH for the range 1 to 14 would then be

$$k_{obs} = k_1 \{Ka'/[Ka' + (H^+)]\} + \{(H^+)/[Ka' + (H^+)][k_2 + k_3(H^+)]\} \quad \text{(Eq. 12)}$$

Values of Ka′, k_1, k_2, and k_3 for two temperatures are listed in Table III. In this equation, the effect of ionic strength on k_3 is ignored because although real, it is of minor importance compared to the large effect of pH on the over-all rate.

Substitution of the values from Table III in Eq. 12 gives a relationship which closely approximates the observed results over the whole pH range (Fig. 5).

Temperature Dependence.—Arrhenius plots at representative pH's were made and the information in Table IV obtained.

The assumption that the same reaction occurs over the pH range 14 to 6 or 7 requires that the heats of activation determined at various pH's over this range be the same. As is evident from Table IV, the observed activation energies at pH 8.2 and 8.7 are considerably higher than the values obtained for pH 12 or greater.

The equation for k_{obs} as a function of pH and pKa′ in region *C* (straight-line portion, slope +1) is shown by Eq. 7 while that in the alkaline plateau region *D* is shown by Eq. 6. Differentiating with respect to 1/T, holding pH constant,

$$\{\partial \log k_{obs}/[\partial(1/T)]\}_{pH} = \{\partial \log k_1/[\partial/(1/T)]\}_{pH} + \{\partial \text{ pKa}'/[\partial/(1/T)]\}_{pH} \quad \text{(Eq. 13)}$$

$$\{\partial \log k_{obs}/[\partial(1/T)]\}_{pH} = -\{\partial \log k_1/[\partial(1/T)]\}_{pH} \quad \text{(Eq. 14)}$$

Equations 13 and 14 indicate that the apparent heat, as determined in region *C*, should differ from that as determined in region *D* by the heat of ionization of IH^+, *i.e.*,

$$[\partial \text{ pKa}'/\partial(1/T)] = \Delta H_i/2.3\ R.T.$$

Attempts were made to determine the heat of ionization by an independent procedure. Unfortunately, conventional spectrophotometric determination of this quantity is very difficult. The instability of the drug at pH's near the pKa′ (10 to 11) precludes accurate spectrophotometric measurement at elevated temperatures. Also, there are inherent changes in absorptivity with temperature, not due to changes in sample composition, which further complicate the determination. In spite of these difficulties, an approximate value of 7 Kcal. mole^{-1} was obtained spectrophotometrically.

Although not providing an independent measurement, a better value is obtained from rate data: rearranging Eq. 5 gives

$$k_{obs}(H^+)/k_1/[1 - (k_{obs}/k_1)] = Ka' \quad \text{(Eq. 15)}$$

which under pH conditions such that $k_{obs}/k_1 \ll 1$ gives

$$\text{pKa}' = \text{pH} - \log k_{obs}/k_1 \quad \text{(Eq. 16)}$$

This provides for a check of consistency, because if Eq. 16 is valid, it should give the known pKa′ at room temperature from rate data obtained at elevated temperatures.

The values of k_1 and k_{obs} (pH 8 to 9) can be calculated at any temperature from the data in Table III: $k_1 = 17.84 - 26{,}700/2.3 \times 1.98 \times T$ at pH 8.2; $k_{obs} = 23.83 - 38{,}500/2.3 \times 1.98 \times T$ at pH 8.7; $k_{obs} = 23.62 - 37{,}500/2.3 \times 1.98 \times T$. Table V gives the values of pKa′ obtained by calculation at various temperatures.

The value obtained at 25° is in good agreement with that obtained spectrophotometrically (11.0) at room temperature. This agreement would strongly suggest that the 11 Kcal. difference between the heats of activation as determined in region *D*

TABLE V.—CALCULATED pKa′ VALUES

	log k_1 − log k_{obs}		pKa′ as Determined from Data at pH		
Temp., °C.	pH 8.2	pH 8.7	8.2	8.7	Av.
65	1.75	1.27	10.0	10.0	10.0
45	2.30	1.82	10.5	10.5	10.5
25	2.80	2.12	11.0	10.8	10.9

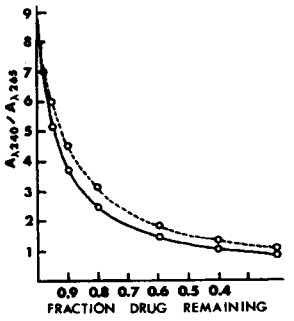


Fig. 7.—Absorbancy ratio ($A_{\lambda 240}/A_{\lambda 265}$) as a function of fraction drug remaining. Solvent: pH 7 phosphate buffer. Key: —O—, mixtures of I and II; ---O---, mixtures of I and III.

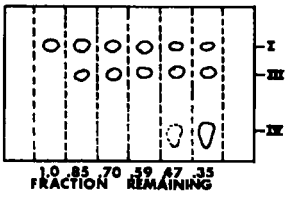


Fig. 8.—Thin-layer chromatograms obtained on acidic (0.04 N H_2SO_4) solutions of I concurrent with analysis.

and C is, in fact, due to the heat of ionization of IH^+ and that the true heat of activation is the same in both regions.

EXPERIMENTAL

Kinetic Procedure.—A standard solution (approx. 0.001 M with respect to I) was prepared. This was diluted tenfold into various buffers held at the temperature of the run. Samples of the thermostated solutions were withdrawn periodically and diluted tenfold into pH 7 phosphate buffer for spectrophotometric analysis of intact drug. The pH was maintained by using dilute $HClO_4$ at pH's below 2, glycine-HCl for pH 2 to 4, phosphate from 4 to 8, borate buffers in the region 8 to 10, carbonate for 10 to 11, and dilute NaOH (carbonate free) for pH's above 11. The actual pH of the buffered solution was measured at the temperature of the run with a Beckman model G pH meter. It did not vary more than 0.1 pH units during the course of a run.

Analysis.—The solution of partially decomposed drug was diluted tenfold into pH 7 phosphate buffer. These dilutions were scanned immediately using a Cary model 14 spectrophotometer. The ratio of absorbances at 240 mμ to 265 mμ was determined and the fraction of I remaining was read off a standard plot (Fig. 7). This procedure gives a sensitive measure of intact drug in the region 0 to 40% loss which is quite adequate for rate studies on pharmaceutical systems where the greatest emphasis is on an accurate estimate of 10% loss.

The similarity in ultraviolet spectra of the biguanide and its hydrolysis product makes a separate analytical procedure unnecessary. In addition, thin-layer chromatograms obtained concurrent with analysis (Fig. 8) demonstrated that the rate of conversion of biguanide to substituted urea was small compared to the rate of hydrolysis of I. Thus no N-3,5-dichlorophenyl-N'-guanylurea could be detected until approximately 50% of the drug was hydrolyzed.

Thin-Layer Chromatographic Analysis.—Solutions of I, 0.1% in various buffers were prepared and stored at 45° for approximately two half-life periods. Five microliters of these solutions was spotted directly on chromatographic plates prepared with aluminum oxide Fluka D5 containing a fluorescent indicator.

The plates were developed using pH 6 phosphate buffer until the solvent front had moved 6.5 cm. When viewed under a short wave ultraviolet lamp, the ultraviolet absorbing components appeared as dark spots on the yellow fluorescing background. The R_f values were not completely reproducible, but the various spots were identified by spotting knowns on the same plate.

Characterization of Secondary Hydrolysis Product.—One gram of I was dissolved to 20 ml. 1 N HCl and heated on the steam bath for 1 day. Likewise, 50 mg. of 3,5-dichlorophenylbiguanide was dissolved in 0.5 ml. 1 N HCl and treated similarly. In both cases, crystalline material was precipitated whose infrared spectra were identical. The precipitates had an R_f equal to that of the slow moving component observed during kinetic runs at pH's 3 or less. Physical data on the crystalline precipitate from I are as follows: U.V.: (in pH 7 phosphate buffer) λ 254 ε = 19,060; m.p. 189–191 (hot stage).

Anal.—Calcd. for $C_8H_8N_4OCl_2 \cdot HCl \cdot H_2O$: C, 32.0, H, 3.6; N, 18.6; Cl, 35.5; H_2O, 6.0. Found: C, 32.1; H, 3.4; N, 18.4; Cl, 36.6; H_2O, 5.0.

N-3,5-Dichlorophenyl-N'-guanylurea was synthesized by Henry's procedure (5) and found identical with the above precipitates.

SUMMARY AND CONCLUSIONS

The kinetics of degradation of 4,6-diamino-1-(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-triazine in dilute aqueous solutions have been investigated.

Variations of specific rate with pH gave a profile consistent with the expression

$$k_{obs} = k_1\{Ka'/[Ka' + (H^+)]\} + \{(H^+)/[Ka' + (H^+)]\ [k_2 + k_3(H^+)]\}$$

derived on the basis of: (*a*) unimolecular isomerization of both protonated and free drug, and (*b*) acid catalyzed hydrolysis of protonated drug.

Variation of specific rate with temperature was determined and found to comply with

pH	
11 or greater	$\log k = 17.84 - 26{,}700/2.3\ R.T.$
10	$\log k = 20.14 - 36{,}700/2.3\ R.T.$
8.7	$\log k = 23.62 - 37.500/2.3\ R.T.$
8.2	$\log k = 23.83 - 38{,}500/2.3\ R.T.$
7.0	$\log k = 21.06 - 35{,}500/2.3\ R.T.$
4.4	$\log k = 17.70 - 31{,}000/2.3\ R.T.$
1.4	$\log k = 13.79 - 22{,}600/2.3\ R.T.$

At 45° the isomer (4-amino-6-(3,5)-dichloroanilino-1,2-dihydro-2,2-dimethyl-1,3,5-triazine) was found to be produced in the pH range 13 to 5. Between pH's 5 and 3 both isomer and 3,5-dichlorophenyl-biguanide are products, while at more acid pH's only biguanide or its hydrolysis product N-3,5-dichlorophenyl-N'-guanylurea are observed.

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Solubilization and Stability of Phenobarbital by Some Aminoalcohols

By JAMES K. J. LIM, HERMAN O. THOMPSON, and CLAUDE PIANTADOSI

Four well-known surface-active aminoalcohols—namely, monoethanolamine, triethanolamine, *N,N*-dimethylethanolamine and *N*-methylglucamine—were found to be effective solubilizers for phenobarbital in water. Equimolar solubilization was possible only at extremely low concentrations. The relative stabilities of the resultant phenobarbital solutions at both autoclaving and room temperatures were generally comparable and in several cases even superior to aqueous solutions of the sodium salt of phenobarbital as stabilized by the inclusion of some polyhydric alcohols.

BOTH AQUEOUS and nonaqueous solutions of phenobarbital sodium have been stabilized by polyhydric alcohols, such as propylene glycol and the polyethylene glycols. The enhanced stability of these solutions has been ascribed to a possible complex formation (1). An attempt therefore was made to investigate further the possibilities for the successful use of some other nontoxic aminoalcohols for obtaining more stable phenobarbital solutions in addition to diethanolamine which previously had been used in conjunction with the solubilization and consequent stabilization of one of the derivatives of barbituric acid (2). In this study, the aminoalcohols selected were monoethanolamine, triethanolamine, *N,N*-dimethylethanolamine, *d*(+)glucosamine, and *N*-methylglucamine. However, glucosamine was discarded early during preliminary tests because of its poor solubilizing performance.

EXPERIMENTAL

Equipment.—A Beckman model DU spectrophotometer, Beckman model N pH meter, Fisher motor stirrers, Fisher mercury thermoregulators, Fisher magnetic stirrers (glass-covered), and Bausch & Lomb 100-w. illuminator were used.

Materials.—Phenobarbital U.S.P. and phenobarbital sodium U.S.P., Merck; triethanolamine and *N,N*-dimethylethanolamine, Eastman Kodak; monoethanolamine, Matheson; *N*-methylglucamine, Du Pont; *d*(+)glucosamine, Nutritional Biochemicals; and propylene glycol U.S.P., Fisher Scientific Co., were utilized.

Method of Analysis.—The ultraviolet spectrophotometric method of quantitative analysis for phenobarbital, employed successfully by a number of workers (3–5), was used wherever possible throughout this study. It was an accurate, simple, and rapid method of analysis and conformed to the Beer-Lambert law when spectrophotometric absorbance was plotted against phenobarbital concentrations within the concentration range of 2.5 to 30.0 mcg./ml. and at the wavelength range of 225–250 mμ. Maximum absorbance was maintained within the pH range 9.0 to 10.0 at wavelength 240 mμ (Fig. 1).

However, an argentometric method, similar to Budde's (6), was substituted in those cases where phenobarbital sodium solutions had stood in prolonged contact with aqueous solutions of polyhydric compounds. Briefly, this involved an accurate volumetric titration of the analytical samples with 0.1 *N* silver nitrate solution delivered from a microburet. Each sample which contained the equivalent of 100–200 mg. of phenobarbital, 50 ml. of freshly prepared 3% sodium carbonate solution, and 10 ml. of 95% ethyl alcohol was mixed continuously with the aid of a glass-covered magnetic stirrer throughout the process of each titration. All titrations were performed in a darkened room to facilitate the end-point determinations. The first permanent cloudiness, viewed at a right angle to the strong and narrow beam of light from an illuminator which was projected through the solution, marked the end point.

Procedure for Solubilization Study.—The various amounts of the aminoalcohols which were needed to prepare up to 10% (w/v) or approximately 0.4 *M* phenobarbital solutions were first computed, em-

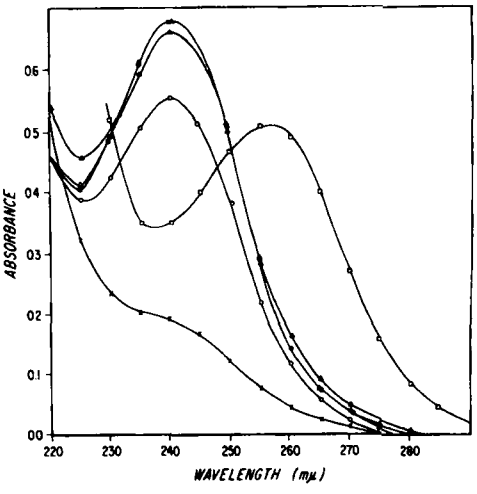


Fig. 1.—The ultraviolet spectrophotometric absorbance of phenobarbital as influenced by pH within the wavelength range of 220–290 mμ. Key: ×, pH 7.00; ○, pH 8.15; ●, pH 9.30; △, pH 9.85 ▲, pH 10.95; □, pH 12.00.

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TABLE I.—RELATIVE PROPORTIONS OF PHENOBARBITAL AND AMINOALCOHOLS FOR SOLUBILIZATION[a]

	Aminoalcohol		Phenobarbital		pH of Soln.
	Gm./100 ml.	Moles/L.	Gm./100 ml.	Moles/L.	
Monoethanolamine	0.40	0.0655	1.57	0.0676	8.10
	0.80	0.1310	2.93	0.1262	8.20
	1.20	0.1964	4.12	0.1774	8.20
	1.60	0.2619	5.21	0.2244	8.25
	1.80	0.2947	5.91	0.2545	8.25
	2.00	0.3274	6.46	0.2782	8.35
N,N-Dimethylethanolamine	1.00	0.1121	2.23	0.0960	8.40
	2.00	0.2244	3.95	0.1701	8.60
	3.00	0.3365	5.43	0.2338	8.70
	4.00	0.4487	6.94	0.2988	8.75
	5.00	0.5610	8.52	0.3668	8.90
	6.00	0.6732	9.61	0.4138	8.90
N-Methylglucamine	2.00	0.1024	2.22	0.0956	8.10
	3.00	0.1537	3.18	0.1370	8.20
	4.00	0.2049	4.17	0.1796	8.20
	5.00	0.2563	5.06	0.2179	8.30
	6.00	0.3075	6.03	0.2596	8.30
	7.00	0.3587	6.84	0.2950	8.45
Triethanolamine	3.00	0.2010	1.31	0.0564	8.30
	6.00	0.4022	2.06	0.0887	8.40
	12.00	0.8042	3.31	0.1425	8.60
	18.00	1.2060	4.44	0.1912	8.70
	24.00	1.6460	5.81	0.2502	8.80
	30.00	2.0100	6.45	0.2777	8.82

[a] Solubilization study was performed at room temperature of 25 ± 0.1°.

ploying the solubilization data from preliminary tests. Solutions of six different strengths of each of the aminoalcohols were then accurately prepared and placed in suitable conical flasks to be immersed in a constant-temperature water bath. They were allowed to attain equilibration of temperature at 25 ± 0.1° before the addition of an excess of phenobarbital. The solutions were subjected to gentle mechanical stirring and at regular intervals of 15 to 30 minutes, samples were withdrawn, filtered, suitably diluted, and their pH's adjusted to 9.0 to 10.5 with dilute sodium hydroxide solution for spectrophotometric analysis at 240 mμ. The concentrations of phenobarbital solubilized (Table I), as found at the point of equilibration of solution, were computed from the per cent transmittance readings when no further change in absorbance was noted. These values were plotted against the corresponding amounts of the aminoalcohols needed for the solubilization (Fig. 2) as well as against the respective pH's of the resultant solutions (Fig. 3). Interferences in analysis owing to absorbance by the solubilizing agents themselves were negligible, except for triethanolamine, in which case a correction factor was applied.

Procedure for Stability Study.—(*a*) *At Autoclaving Temperatures.*—Exactly 5% phenobarbital solutions were prepared under ordinary room temperature conditions by manual agitation of phenobarbital in aqueous solutions of 1.6% monoethanolamine, 3.0% *N,N*-dimethylethanolamine, 5.0% *N*-methylglucamine, and 24.0% triethanolamine. These were placed in air-tight containers and subjected to autoclaving at 10 p.s.i. pressure for 30 minutes. The solutions were gradually allowed to cool to room temperature and stored overnight. Any precipitate was filtered off. The calculated volumes for dilution and subsequent spectrophotometric analysis were pipeted from these filtrates as well as from the corresponding solutions before they were subjected to autoclaving. A 5.0% concentration of phenobarbital sodium in 10% dextrose solution and in 60% propylene glycol solution were studied under similar experimental conditions for purposes of comparison of stability.

(*b*) *At Room Temperatures.*—To simulate closely official elixir strength of 0.44% of phenobarbital sodium, exact 0.5% solutions were prepared employing proportionately lower concentrations of the aminoalcohols. These solutions together with a similar set of the above 5.0% phenobarbital solutions were stored in 120-ml. dispensing bottles

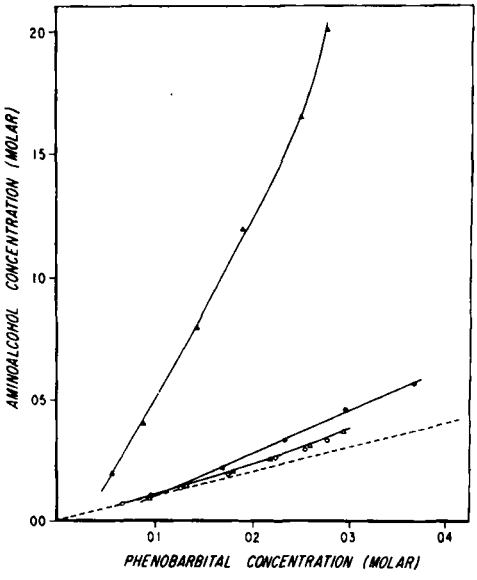


Fig. 2.—Curves for solubilization of less than 0.4 *M* phenobarbital by the following aminoalcohols at 25°. Key: - - -, hypothetical plot for equimolar solubilization; ▲, triethanolamine; ●, *N,N*-dimethylethanolamine; Δ, *N*-methylglucamine; O, monoethanolamine.

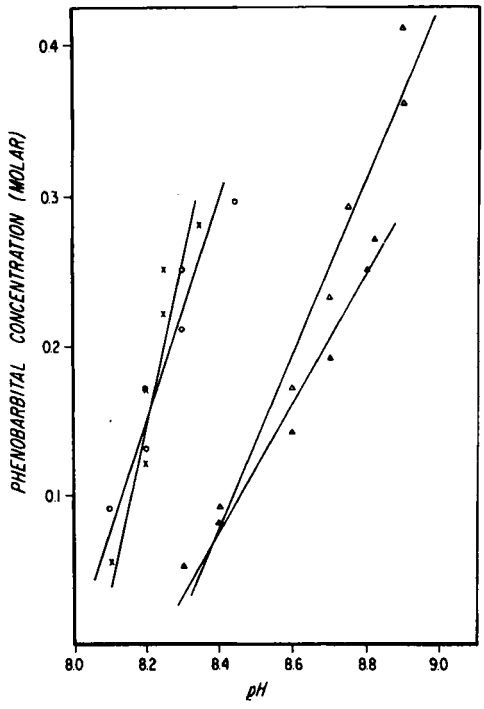


Fig. 3.—The effect of pH on the solubilization of phenobarbital by the following aminoalcohols. Key: ×, monoethanolamine; O, *N*-methylglucamine; △, *N*,*N*-dimethylethanolamine; ▲, triethanolamine.

away from direct light and at room temperatures of 23–25° for a total period of 2 weeks. At intervals of 5 days, samples were withdrawn and assayed spectrophotometrically as in (*a*), except for those aqueous solutions of phenobarbital sodium in pure distilled water, 10% dextrose, and 60% propylene glycol solutions. These aqueous solutions of phenobarbital sodium exhibited increased absorbance at 240 mμ on prolonged standing analogous to studies carried out by Kubota for the enhanced spectrophotometric absorbance of polyhydric alcohols or hydroxy acids in aqueous solutions owing to the effect of alkali metals. Kubota hypothesized this phenomenon to a possible metal-complex formation involving static electricity of the metal ion with hydroxyl groups of the polyhydric compounds (7, 8). The usual spectrophotometric method of analysis therefore was replaced by the argentometric titration method for the quantitative determination of phenobarbital in such solutions. Any attempt at spectrophotometric analysis of these solutions resulted in erratic and erroneous determinations (Table II), possibly because of the simultaneous effects of varying rates of increased spectrophotometric absorbance, due to metal-complex formation and the actual degradation of phenobarbital. The argentometric titration was considered unnecessary for the assay of those phenobarbital solutions where alkali metal ions were absent or where solutions of the phenobarbital salt had been in contact with some polyhydric compounds for only a relatively short period.

RESULTS AND DISCUSSION

The increased solubility of phenobarbital by the aminoalcohols may be attributed to the formation of either a salt or a "complex." The solubilization of phenobarbital caused by a slight increase in pH of the medium as a result of higher concentrations of the aminoalcohol followed a linear relationship for each series (Fig. 3) and indicated favorably a simple acid-base salt formation. In the case for the possible formation of a weak "complex" or an association compound, it is assumed that this occurs between the electronegative nitrogen of the

Table II.—Stability of 5.0 and 0.5% Phenobarbital Solutions on Storage at Room Temperature

Phenobarbital Soln.	pH	% Undecomposed Phenobarbital			
		5 Days[a]	10 Days[a]	15 Days[a]	15 Days[b]
Phenobarbital sodium in distilled water					
5.0% Concn.	8.80	100.00	99.65	98.15	92.41
0.5%	8.80	99.17	99.08	98.45	95.35
Phenobarbital sodium in 60% propylene glycol soln.					
5.0% Concn.	9.50	100.80	101.30	102.50	96.94
0.5%	9.25	102.10	103.70	100.80	98.15
Phenobarbital sodium in 10% dextrose soln.					
5.0% Concn.	8.00	100.20	99.38	92.51	91.49
0.5%	8.60	102.30	102.00	101.50	96.79
Phenobarbital with monoethanolamine					
5.0% Concn.	8.70	99.88	98.24	97.83	...
0.5%	8.50	99.93	99.51	98.97	...
Phenobarbital with *N*,*N*-dimethylethanolamine					
5.0% Concn.	8.80	99.42	98.63	97.90	...
0.5%	8.80	99.97	99.97	98.49	...
Phenobarbital with *N*-methylglucamine					
5.0% Concn.	8.75	99.19	98.63	97.16	...
0.5%	8.70	99.93	99.10	99.26	...

[a] Values for per cent undecomposed phenobarbital were obtained from spectrophotometric analysis at 240 mμ. [b] Corrected values for per cent undecomposed phenobarbital as obtained by argentometric titration.

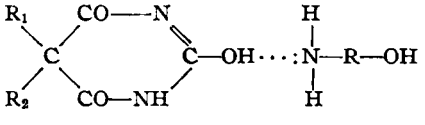
TABLE III.—RELATIVE PROPORTIONS OF PHENOBARBITAL AND AMINOALCOHOLS FOR APPROXIMATE EQUIMOLAR SOLUBILIZATION[a]

	Aminoalcohol		Phenobarbital	
	mg./100 ml.	moles/L. (moles × 10^{-3})	mg./100 ml.	moles/L. (moles × 10^{-3})
Monoethanolamine	10.0	1.6370	36.0	1.5500
	12.0	1.9640	44.2	1.9030
	14.0	2.2920	52.2	2.2480
	16.0	2.6190	57.8	2.4890
(Distilled water as blank)	18.0	2.9470	61.6	2.6530
	...	...	124.0	...
N,N-Dimethylethanolamine	5.0	0.5610	9.1	0.3876
	10.0	1.1220	21.7	0.9346
	15.0	1.6830	31.6	1.3610
	20.0	2.2440	42.3	1.8220
	25.0	2.8040	52.2	2.2480
	30.0	3.3660	65.4	2.8160
(Distilled water)	...	...	124.0	...
N-Methylglucamine	10.0	0.5124	10.7	0.6635
	20.0	1.0240	23.2	0.9990
	30.0	1.5370	35.1	1.5110
	40.0	2.0490	45.7	1.9680
	50.0	2.5630	56.8	2.4450
	60.0	3.0750	67.0	2.8850
	70.0	3.5870	72.8	3.1340
	80.0	4.1000	73.2	3.1520
	90.0	4.1000	73.2	3.1520
(Distilled water)	...	...	126.8	...
Triethanolamine	7.5	0.5030	9.7	0.4178
	15.0	1.0060	20.4	0.8784
	22.5	1.5090	30.9	1.3300
	30.0	2.0120	38.4	1.6540
	37.5	2.5150	45.9	1.9760
	45.0	3.0180	53.4	2.2990
(Distilled water)	...	...	127.8	...

[a] Solubilization study was carried out at room temperature of 25 ± 0.1°.

amine and the enolic hydrogen of phenobarbital, in analogy to the complex formed between the electron-donating oxygen of polyethylene glycol and the acidic hydrogen of phenobarbital described by Higuchi (1).

"COMPLEX" OF PHENOBARBITAL WITH AN AMINOALCOHOL



where $R_1 = C_6H_5$, $R_2 = C_2H_5$.

Such a complex will form hydrogen bonding with water molecules through the hydrophilic hydroxyl groups of the alcohol moiety to achieve water solubility. The inability to isolate these weak "complexes" from their aqueous solutions provided some indication toward their extreme instability.

The solubilization of phenobarbital by the aminoalcohols in approximately equimolar proportions was possible only with very low concentrations (Table III, Fig. 4); with increasing concentrations, however, especially with triethanolamine, more than the equimolar ratios were required. Results above indicate that a relatively much greater proportion of the aminoalcohols was required to solubilize and obtain the more concentrated phenobarbital solutions. This phenomenon can be attributed to a diminished activity of "solvent" where its rational

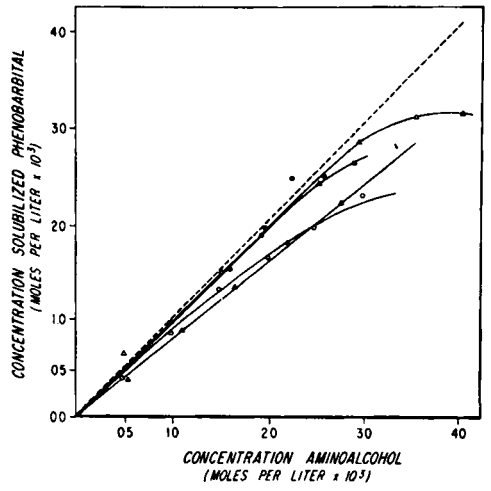


Fig. 4.—Curves for solubilization of less than 0.003 *M* phenobarbital by the following aminoalcohols at 25°. Key: - - -, hypothetical plot for equimolar solubilization; Δ, *N*-methylglucamine; ●, monoethanolamine; ▲, *N,N*-dimethylethanolamine; O, triethanolamine.

TABLE IV.—RELATIVE STABILITIES OF PHENOBARBITAL SOLUTIONS BASED ON ΔH_a OF THE ARRHENIUS EQUATION

Phenobarbital Soln.	pH	$k \times 10^4$ per Hr. 59.6°	69.6°	ΔH_a, Kcal./mole
0.10% Phenobarbital solution with monoethanolamine	8.25	17.08	28.77	11.80
0.44% Phenobarbital sodium in 60% propylene glycol	9.10	14.40	27.21	14.41
0.44% Phenobarbital sodium in 10% dextrose solution	8.45	11.90	28.78	20.00
0.10% Phenobarbital solution with triethanolamine	7.55	14.40	37.01	21.37
0.10% Phenobarbital solution with *N,N*-dimethylethanolamine	8.00	16.12	51.42	26.26
0.44% Phenobarbital sodium in distilled water	8.75	18.23	74.85	31.98
0.10% Phenobarbital solution with *N*-methylglucamine	8.40	12.21	59.06	35.69

activity coefficient becomes progressively less than 1 with increasing concentration of solution (9).

A prediction of the relative stabilities for the various phenobarbital solutions which was based upon the study of the energy of activation ΔH_a, for the decomposition of phenobarbital (Table IV), as directly derived from the Arrhenius equation $\log(k_2/k_1) = \Delta H_a/2.303\ R \cdot (T_2 - T_1)/T_2T_1$, at temperatures of 59.6 and 69.6° was unreliable for general application. Any evaluation of the relative stabilities from such a prediction differed significantly from the actual stabilities of phenobarbital solutions as determined on the basis of

per cent undecomposed drug by degradation at ordinary room temperature storage conditions and autoclaving temperatures (Tables II and V). This presumably may be due to several involved factors such as pH, dielectric constant of medium, and ionic strength of solution (10); each of these may considerably contribute toward the degradation of phenobarbital at varying levels of activity, depending upon temperature. Using data from Tables II and V, a final classification for the relative stabilities of phenobarbital solutions exposed to the above-mentioned experimental conditions is presented in Table VI.

SUMMARY AND CONCLUSIONS

1. A solubilization study for phenobarbital in water at 25° by means of mechanical stirring showed the following aminoalcohols as potential solubilizers for the drug; they are in decreasing order of solubilizing performance: monoethanolamine, *N,N*-dimethylethanolamine, and *N*-methylglucamine.

2. The formation of either a salt or a "complex" is postulated as a possible mechanism for the increased solubility of phenobarbital in water by the aminoalcohols.

3. The stability of aqueous solutions of phenobarbital as solubilized by these three aminoalcohols at both room and autoclaving temperatures compared favorably with that provided by 60% propylene glycol solution to phenobarbital sodium.

4. Optimum stability on a relative basis was indicated by the following solutions when studied under these conditions:

(*a*) At autoclaving temperatures—the 5.0% phenobarbital sodium in 60% propylene glycol closely followed by 5.0% phenobarbital as solubilized by *N*-methylglucamine.

(*b*) At room temperature—the 5.0 and 0.5% phenobarbital solutions as solubilized by *N,N* - dimethylethanolamine and *N*-methylglucamine, respectively.

5. The argentometric titration method was utilized as an alternative for the assay of aqueous solutions of phenobarbital sodium which had stood in prolonged contact with polyhydric compounds. Such solutions produced an increased spectrophotometric absorbance.

6. In conclusion, it is proposed that further investigations be performed on these solutions of phenobarbital which contain the aminoalcohols for their toxicological and pharmacological effects through biological testing.

TABLE V.—STABILITY OF 5% PHENOBARBITAL SOLUTIONS AT AUTOCLAVING TEMPERATURES

Phenobarbital Soln.	pH	Dec., %	Remarks: Precipitate	Remarks: Color
Phenobarbital sodium in 60% propylene glycol	9.50	7.83	...	...
Phenobarbital solution with 5.0% *N*-methylglucamine	8.80	8.83	*[a]	Straw-colored
Phenobarbital solution with 1.6% monoethanolamine	8.80	9.45	**	Yellowish tinge
Phenobarbital solution with 3.0% *N,N*-dimethylethanolamine	8.60	10.05	*	...
Phenobarbital sodium in distilled water	8.80	11.22	***	...
Phenobarbital sodium in 10% dextrose solution	8.80	18.69	***	Color and odor of caramel

[a] Quantitative representation of precipitate as observed visually.

TABLE VI.—STABILITY CLASSIFICATION FOR 5.0 AND 0.5% PHENOBARBITAL SOLUTIONS AT AUTOCLAVING AND ROOM TEMPERATURES

	Degradative Conditions: Autoclave Sterilization	Degradative Conditions: Room Temperature Storage
5.0% Phenobarbital Soln.		
Phenobarbital sodium in distilled water	V[a]	V
Phenobarbital sodium in 60% propylene glycol	I	IV
Phenobarbital sodium in 10% dextrose solution	VI	VI
Phenobarbital solution with monoethanolamine	III	III
Phenobarbital solution with *N,N*-dimethylethanolamine	IV	I
Phenobarbital solution with *N*-methylglucamine	II	III
5.0% Phenobarbital Soln		
Phenobarbital sodium in distilled water	...	VI
Phenobarbital sodium in 60% propylene glycol	...	IV
Phenobarbital sodium in 10% dextrose solution	...	V
Phenobarbital solution with monoethanolamine	...	II
Phenobarbital solution with *N,N*-dimethylethanolamine	...	III
Phenobarbital solution with *N*-methylglucamine	...	I

[a] Roman numericals I to VI show order of decreasing stability.

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Arylindolizines III

Methoxyl and Glyoxyl Derivatives of Several Substituted Phenylindolizines

By VINCENT S. VENTURELLA*

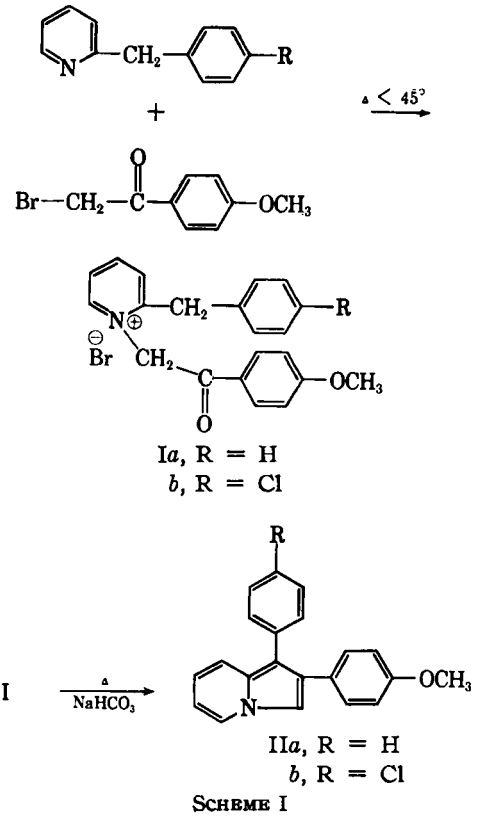
4-Methoxyphenacylbromide reacts in good yield with 2-benzylpyridine and 2-(4'-chlorobenzyl) pyridine in the Tschitschibabin synthesis to form the corresponding indolizines. The methyl ethers are cleaved with difficulty to the corresponding phenolic compounds which have anomalous solubility characteristics. Arylindolizines with an open 1 position react in good yield with ethoxalylchloride to form the corresponding ethyl glyoxylates.

As a continuation of the search for arylindolizines of possible psychotherapeutic activity (1, 2), it was desired to prepare compounds containing a nuclear methoxyl group and compounds containing the —COCOX moiety in the 1 position, where X = —OEt, —OH, or —N⟨ ⟩. Since previous efforts (1) to prepare II*a* by nuclear substitution of the corresponding bromo compound failed, it was decided to test the Tschitschibabin method (3) for the preparation of compounds II*a* and II*b* from the appropriate phenacyl compound[1] according to the reactions in Scheme I.

Compounds II*a* and II*b* formed in good yield in this procedure under mild conditions. In each case, 4-methoxphenacylbromide condensed with the 2-substituted pyridine with constant shaking at 35–45°. The condensation was accompanied by a small amount of decomposition inherent in the use of both starting materials. Cyclodehydration proceeded readily from this stage in refluxing $NaHCO_3$ solution. During the formation of I*b*, if the temperature was permitted to rise above 40°, spontaneous cyclodehydration occurred, preventing isolation of the intermediate. This phenomenon was experienced in previous work (1) employing 2-(4'-chlorobenzyl) pyridine; but the reaction was not temperature dependent, mostly because condensation could not be affected at below cyclodehydration temperatures.

Hydrolysis of II*a* and of II*b* to the corresponding phenols proceeded under vigorous treatment with 30% HBr/HOAc. Separation of the sodium salts of the phenols depended upon their insolubility in the aqueous basic media in the presence of ether. A portion of the phenol remained in the acidic form and was recovered from the ether extract. Both II*a* and II*b* gave distinct green when tested with anhydrous $FeCl_3$ in $CHCl_3$ (4).

Attempts to apply the reactions in Scheme I to 2-methoxyphenacylbromide resulted in failure to effect condensation when using either 2-benzylpyridine or 2-(4'-chlorobenzyl)pyridine. In each instance black intractable tars resulted at condensation temperatures ranging from 40 to 110°. Similar failures resulted during attempts at condensation at −5° for 2 weeks and 4 weeks and also at room temperature for 4 weeks. Failure of the condensation to occur presumably is due



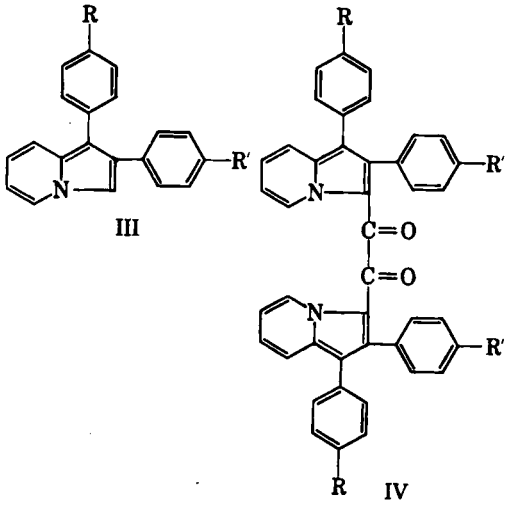
SCHEME I

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[1] Frinton Laboratories, Vineland, N. J.

to steric effects, since the electronic effect of an —OCH_3 in the 2 position is not markedly different from the 4 position.

The purity of the starting pyridines greatly affected the yield and purity of condensation product and ease of isolation of the subsequent indolizine during this study. Failure to purify the pyridine compounds resulted in as much as a 15–20% reduction in yield of the intermediate pyridinium bromide. In addition, the decreased yield was accompanied subsequently by the formation of a green indolizine, which showed a low elemental analysis and a qualitatively identical infrared spectrum.

The synthesis of glyoxylic derivatives of previously prepared arylindolizines (1) of general structure III was carried out to test the efficacy of preparing carbonyl derivatives which would



III *a*, R = H, R′ = Br
b, R = Cl, R′ = Br
c, R = H, R′ = NO_2
d, R = Cl, R′ = NO_2

lead easily to several derived functions. This approach was used to establish whether the arylindolizines would react easily with $ClCOCO_2Et$ (5) or if the longer less specific method of Galbraith (6) would be necessary. In the latter method, indolizine was reported to react with ClCOCOCl in a 2:1 ratio to form a diketone similar to IV (2 and 3 positions unsubstituted) in 15% yield. Evaporation of the reaction liquor followed by sublimation of the remaining solid gave an unreported yield of indolizylglyoxalylchloride. For the present study, the desired ethyl esters would have to be prepared from the corresponding indolizylglyoxalylchloride by ethanolysis. Thus, in addition to the formation of a small amount of by-product of type IV, the yield of the esters would presumably have been decreased by the longer procedure. Once the keto esters were prepared, it then seemed likely that the corresponding acids and amide derivatives could be formed easily.

The reaction with $ClCOCO_2Et$ took place in the manner anticipated. Unexpectedly, III*d* reacted to form the glyoxyl derivative in fair yield. The formation of this compound was not anticipated because previous attempts to form the corresponding benzoyl compound resulted in failure (1). The success of the reaction of $ClCOCO_2Et$ in this case must come from the increased strength of the ethylglyoxylium ion $(EtO_2C-\overset{\oplus}{C}=O)$ as an electrophile since the failure of C_6H_5COCl (1) and its *meta* amd *para* NO_2 derivatives (2) to form an acyl derivative was presumably due to the electronic effects of the *p*-Cl and *p*-NO_2 group on the formation of the necessary nucleophile of the indolizine.

The hydrolysis of the ethylglyoxylates of III was effected in aqueous NaOH suspension of the compounds; the resulting sodium salts of the acids were insoluble in the excess NaOH present. The products of these reactions remained as insoluble solids along with unreacted starting material and could be separated from the parent ethyl esters by the solubility of the acid salt in hot water.

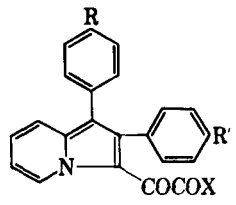
Recrystallization of the glyoxylic acids was accompanied by a large amount of decomposition if the primary solvent was too hot or if the acid was allowed to stand in the hot solvent for more than 5–10 minutes before cooling. In each case the product turned dark-green to brown. This was especially true of 1-glyoxyl-2-(4′-nitrophenyl)-3-phenylindolizine and its 3-(4″-chlorophenyl) analog.

In a limited number of experiments it was impossible to form the *N,N*-diethylglyoxamides, even in the absence of solvent. However, similar reactions employing piperidine resulted in fair to good yields of the corresponding piperidino compounds. In this procedure, the unreacted indolizine largely decomposed to gummy materials which were easily separated from the product.

The analytical data for the ethylglyoxylates, the corresponding acids, and piperidinoxalyl derivatives of III are given in Table I.

Several attempts at applying this general method of indolizine synthesis to substituted phenacylchlorides resulted in failure to effect the required condensation necessary to form the pyridinium intermediates. Representatives of this group of compounds include *m*-nitrophenacylchloride and 3,4-dihydroxyphenacylchloride.

TABLE I.—SUBSTITUTED ACYL DERIVATIVES OF ARYLINDOLIZINES OF THE TYPE



R	R'	X	M.p., ° C.	Yield, %	LS[a]	RS[b]	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
H	Br	OEt	190–192	71	...	*A*	64.43	4.03	3.13	64.42	3.97	3.06
Cl	NO_2	OEt	215.5–216.5	52	*A*	*A*	64.29	3.79	6.25	64.22	3.73	6.23
Cl	Br	OEt	179.5–180	62	*B*	*A*	60.00	3.52	2.90	60.28	3.40	2.87
H	NO_2	OEt	193–194	95	*B*	*A*	69.57	4.35	6.76	69.39	4.47	7.20
H	Br	OH	179–180	92	...	*B*	62.86	3.33	3.33	62.56	3.68	3.96
Cl	NO_2	OH	193–195	96	...	*C*	62.85	3.09	6.67	63.26	3.22	6.92
Cl	Br	OH	174.5–75.5	95	...	*D*	58.15	2.86	3.08	58.24	3.02	3.20
H	NO_2	OH	168–169	96	...	*E*	68.39	3.63	7.25	67.94	3.57	7.06
H	Br	$C_5H_{10}N$[c]	189–190	59	*C*	*A*	66.53	4.72	5.75	66.73	4.77	5.73
Cl	NO_2	$C_5H_{10}N$	174–75.5	91	*D*	*C*	66.53	4.52	8.62	66.31	4.74	8.23
Cl	Br	$C_5H_{10}N$	178.5–79.5	96	*E*	*A*	62.19	4.22	5.37	62.39	4.41	5.04
H	NO_2	$C_5H_{10}N$	186–187	91	*D*	*F*	71.25	5.08	9.27	71.45	5.11	8.85

[a] Leaching solvent: *A*, cold absolute EtOH; *B*, EtOH (−5° C.); *C*, Et_2O (0° C.); *D*, high boiling petroleum ether (at 50° C.); *E*, low boiling petroleum ether. [b] Recrystallization solvent: *A*, absolute EtOH; *B*, Et_2O-petroleum ether (30–60°); *C*, hot C_6H_6; *D*, warm C_6H_6-petroleum ether (30–60°) (1:1); *E*, 50% EtOH; *F*, warm C_6H_6, followed by Me_2CO-H_2O. [c] Piperidyl.

These failures are consistent with literature reports (7) which indicate an increased condensing power with decreasing electronegativity of the halide.

The pharmacological activity of the reported compounds is currently being investigated.

EXPERIMENTAL[2]

2-Benzyl-4′-methoxyphenacylpyridinium Bromide (Ia).—Fifty grams (0.22 mole) of 4-methoxyphenacylbromide was mixed with 75 Gm. (2 equivalents) of 2-benzylpyridine and heated at 40–45° with constant stirring for 40 minutes. The dark brown cake resulting was refrigerated for 3 days, broken up, and leached with 250 ml. of hot ethanol. The yellowish slurry was then filtered and washed with 50 ml. of ice-cold acetone to yield 55.5 Gm. (65%) of a yellowish-white solid, m.p. 227–230° dec. The melting point was raised to 241–242° dec., after recrystallization from hot acetone.

Anal.—Calcd. for $C_{21}H_{20}BrNO_2$: C, 63.47; H, 5.04; N, 3.53. Found: C, 63.40; H, 5.14; N, 3.76.

2-(4′-Methoxyphenyl)-3-phenylindolizine (IIa).—Forty grams of *Ia* was placed in a 3-L. three-necked flask equipped with an addition funnel, a stirrer, and a water condenser and mixed with 1.5 L. of saturated sodium bicarbonate solution. The mixture was stirred and heated under reflux for 6 hours. Any foaming that took place was controlled by the addition of ethanol as needed (volume of ethanol should not exceed 30 ml.). The mixture was allowed to stand at room temperature overnight. Filtration and washing of the solid with 500 ml. of hot water gave a greenish-yellow solid which was recrystallized from acetone-H_2O to give 23 Gm. of yellowish-white flakes, m.p. 97–98°. An additional 5 Gm. was obtained by overnight refrigeration of the recrystallization liquor. The total yield was 93%. If a green gum forms on standing, separate from the bulk of the solid, dry in air overnight, pulverize, and recrystallize from acetone-H_2O.

Anal.—Calcd. for $C_{21}H_{17}NO$: C, 84.28; H, 5.69; N, 4.68. Found: C, 84.18; H, 5.77; N, 4.95.

2-(4′-Chlorobenzyl)-4″-methoxyphenacylpyridinium Bromide (Ib).—Fifty grams (0.22 mole) of 4-methoxyphenacylbromide was mixed with 2 equivalents of 2-(4′-chlorobenzyl)pyridine as described above, warmed to 40° and stirred for 8 min. The light tan solid cake was cooled immediately in ice and, when the reaction subsided (as evidenced by the cessation of fuming), placed in the refrigerator and allowed to stand for 2 days. The solid was then fragmented and dissolved in 250 ml. of hot methanol. The solution was evaporated to 1/3 volume, placed at −10° for 3 hours, and filtered with suction to give 20.5 Gm. of product. The filtrate was diluted with an equal volume of ether and filtered to give an additional 41 Gm. The solids were combined washed with 100 ml. of ice-cold ether, dried, washed with 100 ml. of iced water, dried with suction, and then dried overnight over $CaCl_2$. Recrystallization from hot acetone gave 58.5 Gm. of snow white flakes, m.p. 231–232° dec. (64%).

Anal.—Calcd. for $C_{21}H_{19}ClBrNO_2$: C, 58.33; H, 4.63; N, 3.24. Found: C, 58.54; H, 4.51; N, 3.13.

2-(4′-Chlorophenyl)-3-(4″-methoxyphenyl)indolizine (IIb).—Forty-two grams (0.097 mole) of *Ib* was stirred and refluxed in the manner previously described with 2.5 L. of saturated sodium bicarbonate solution for 4 hours and the suspension allowed to stand overnight. Filtration, followed by washing with three 300-ml. portions of hot water and

[2] All melting points were taken on a Fisher-Johns apparatus and are uncorrected. Microanalyses by Swartzkopf Microanalytical Laboratories, Woodside, N. Y.

air drying for 60 hours, gave 30.5 Gm. (93%) of light tan solid. The product recrystallized from 50% ethanol in long gray needles, m.p. 118.5 to 119.5°.

Anal.—Calcd. for $C_{21}H_{16}ClNO$: C, 75.60; H, 4.81; N, 4.19. Found: C, 75.41; H, 4.76; N, 3.85.

2-(4′-Hydroxyphenyl)-3-phenylindolizine.—Twenty-five grams (0.084 mole) of II*a* was mixed with 100 ml. of 30% hydrobromic acid in acetic acid in a two-necked flask equipped with a stirrer and water condenser; the mixture was refluxed for 6 hours with constant stirring. The resultant dark green suspension was suction filtered and the precipitate pulverized and macerated 3 hours with water, then filtered to give a 30% recovery of starting material. The original acidic filtrate was poured slowly into 300 ml. of iced 5 *N* sodium hydroxide solution and mixed with 150 ml. of ether. The mixture was filtered, the precipitate mixed with enough cold water to effect solution, acidified with hydrochloric acid to give a solid which was dissolved in benzene. The benzene was dried over anhydrous sodium sulfate and evaporated to give a dark oil which changed to a brownish-yellow solid (solid *A*) when triturated with 50 ml. of petroleum ether (30–60°). The ether-sodium hydroxide mixture was separated. The ether layer dried and evaporated to give a brown oil, which changed to a brown gum when triturated with petroleum ether. The gum was allowed to dry in air overnight, pulverized, washed with petroleum ether, and air dried to give a brownish-yellow solid (*B*). Solid *A* weighed 6 Gm. and *B* weighed 6.2 Gm. (total 51% yield). Each was crystallized from hot benzene-petroleum ether to give dark yellow flakes, m.p. 157–158° dec. after drying at 85° for 1 hour. (Mixed melting point *A* and *B*, 156–157° dec.)

Anal.—Calcd. for $C_{20}H_{15}NO$: C, 84.30; H, 5.26; N, 4.92. Found: C, 84.44; H, 5.18; N, 4.71.

2-(4′-Hydroxyphenyl)-3-(4″-chlorophenyl)indolizine.—Twenty grams (0.062 mole) of II*b* was treated with 75 ml. of 30% hydrobromic acid in acetic acid as described above. After 6-hours reflux, the dark green solution was allowed to stand overnight. Filtration gave 1.8 Gm. of starting material. The filtrate was poured onto 300 ml. of iced sodium hydroxide solution (5 *N*), extracted with 150 ml. of ether, and processed as above. The ether layer was separated, dried, and evaporated to give a dark green gum. The gum was triturated with petroleum ether (30–60°), filtered, pulverized, and washed with 500 ml. of petroleum ether. Total yield was 13.2 Gm. of a brown powder which was carefully recrystallized from ethanol-water to give tan plates, m.p. 130–131° (mixed melting point with starting material 105–107°).

Anal.—Calcd. for $C_{20}H_{14}ClNO$: C, 75.23; H, 4.39; N, 4.39. Found: C, 75.08; H, 4.20; N, 4.09.

General Method for Preparation of the Ethylglyoxylates of III.—Into a 250-ml. three-necked flask equipped with a stirrer, water condenser, and dropping funnel was added 0.06 mole of the appropriate indolizine suspended in 100 ml. of benzene. The mixture was stirred and heated to reflux, at which time 1.05 *M* equivalents of ethoxalyl chloride was added all at once. The dark solution was refluxed for 90 minutes and allowed to cool and stand overnight. The mixture was processed by a modification of a previously described method (5). The dark solution was poured into 500 ml. of ice water and the mixture suction filtered to remove a portion of the impure product (with III*b*, this solid was unreacted starting material). The layers of the filtrate were separated, and the aqueous layer was washed with 10–30-ml. portions of chloroform. The organic phases were combined, washed with 200 ml. of water, dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give a solid product. The combined solid was then leached, dried in air, recrystallized, and dried under vacuum.

Hydrolysis of the Ethylglyoxylates of III.—One-hundred milliliters of 5 *N* sodium hydroxide was added to 2.5 Gm. of the appropriate ethylglyoxylate derivative contained in a two-necked 250-ml. flask equipped with a stirrer and water condenser, and the mixture was stirred at reflux for 10 hours (eight hours with ethyl 2-(4′-bromophenyl)-3-phenylindolizylglyoxylate). The suspension was cooled and filtered. The precipitate suction dried, then suspended in 500 ml. of water for 1 hour at 60°, then overnight at room temperature. The suspension was filtered, the filtrate treated with 6 *N* hydrochloric acid to pH 5 and allowed to cool in the refrigerator for 3–5 hours. The resulting suspension was suction filtered, the solid washed with 200 ml. of water, and thoroughly dried and recrystallized from the appropriate solvent.

Reaction of the Ethylglyoxylates of III with Piperidine.—Five grams of the ethylglyoxylate was mixed with 100 ml. of piperidine in a 150-ml. distilling flask and refluxed for 12 hours under a water condenser. The mixture was cooled and allowed to stand overnight. Any insoluble tars that formed were filtered out, and the filtrate was evaporated under reduced pressure to remove the excess piperidine. The oil which remained was triturated with low boiling petroleum ether until it was transformed into a thick gum or powdery solid. The partially purified solid was then allowed to dry in air, leached with 150 ml. of solvent, suction filtered until dry, and recrystallized to give the corresponding 2,3-disubstituted piperidinoxalylindolizine.

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Determination of the Major Component of Some U.S.P. Adrenocortical Steroids

By FRIEDA M. KUNZE and JOEL S. DAVIS

The determination of the total steroid content and the isolation and determination of the major steroid component in pharmaceuticals by continuous ascending chromatography and a blue tetrazolium colorimetric procedure are described. Isolation of the major steroid is achieved in 1.5 to 2 hours on 8 × 8 in. paper. The method has been applied to products containing prednisolone, hydrocortisone, prednisone, and the acetates of prednisolone, hydrocortisone, and cortisone. Accuracy within plus or minus 2 per cent was achieved.

SYNTHETIC adrenal corticoids are prepared from closely related intermediates. Since the finished product may contain a number of such intermediates in varying amounts, a method to determine the ratio of the major component to the total steroid content is desirable. Polarography, gas chromatography, and gradient elution techniques were considered; however, a paper chromatographic technique was selected for its specificity and the simplicity of the equipment required.

The method presented here is not intended for the detection of every possible intermediate or side reaction product obtained by all of the various methods of production, but it does measure with an accuracy of ±2% both the total amount of steroids present and also the major steroid fraction. Prednisolone, hydrocortisone, prednisone, and the acetates of prednisolone, hydrocortisone, and cortisone in tablets have been analyzed successfully.

The procedure is divided into three steps: (*a*) extraction of the total steroid fraction of the tablets, (*b*) paper chromatographic isolation of the major component and its quantitative removal from the paper, and (*c*) colorimetric measurement of both the total steroid content and the paper fractionated component by a blue tetrazolium procedure previously described (1).

EXPERIMENTAL

Materials

(Reagent grade materials are used except as noted.) Materials used include alcohol U.S.P.; methylcyclohexane, b.p. 100–101°; formamide (redistilled reagent grade solvent); blue tetrazolium solution[1]—dissolve 50 mg. of the salt in 10 ml. of alcohol; and dilute tetramethylammonium hydroxide—dilute 1 ml. of tetramethylammonium hydroxide T.S. to 20 ml. with alcohol.

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[1] Dajac, B. T., The Borden Co., Philadelphia, Pa., has been found satisfactory and was used for these studies.

Apparatus

Chromatography Chamber.—Use a rectangular tank, about 22.5 cm. in height and 22.5 × 8.75 cm. in its other dimensions, equipped with a support for holding a 4 × 20 cm. chromatographic tray about 4 cm. from the tank bottom. Fit the tank with a lid having a slot approximately 22 cm. long and 0.6 mm. wide. Slot width is important. The lid is available from the Arthur H. Thomas Co., Philadelphia, Pa., or it can be made with glass strips separated by stainless steel shims. The strips are taped together with 0.75-in. plastic tape and may be used repeatedly without retaping.

Source of Ultraviolet Light and Viewing Apparatus.—Use a light source having an intensity of approximately 1.4 amp. at 2537 Å. units, suspended no less than 9 in. above the paper. [A Chromato-Vue model 3-C Black Light (Eastern Corp., Bayside, N. Y.) apparatus is convenient.]

Standard Preparations

Using an accurately weighed sample of U.S.P. reference standard of the steroid under test, prepare a chloroform solution containing approximately 1 mg./ml. Solution of the standard may be facilitated by the prior addition of 0.2 ml. of alcohol to the volumetric flask for each 10 ml. of chloroform to be used.

(*Caution:* These compounds are unstable in some lots of chloroform and may break down to a number of fractions in 1 to 7 days.)

Test Preparations

Bulk Steroids.—Transfer about 10 mg. of steroid, accurately weighed, to a 10-ml. volumetric flask containing 0.2 ml. of alcohol. After it is dissolved, add chloroform to volume and mix.

Steroids in Tablets.—Weigh and finely powder not less than 10 tablets. Weigh accurately a portion of powder equivalent to about 10 mg. of the active ingredient and transfer to a 125-ml. separator with the aid of 15 ml. of 8% sodium bicarbonate solution. Extract the steroid using four 25-ml. portions of chloroform, filtering each portion through chloroform-washed glass wool into a 150-ml. beaker.

Evaporate the chloroform to a volume of about 2 to 3 ml. on a steam bath with a gentle current of air. Avoid evaporating to dryness. Transfer the solution quantitatively to a 10-ml. volumetric flask with several small washings of chloroform and make to volume.

TABLE I.—CHROMATOGRAPHIC SOLUTIONS

Steroid	Mobile Solvent	Development Time, hr.
Prednisolone	Chloroform	2
Hydrocortisone	Chloroform	1.5
Prednisone	Chloroform:methylcyclohexane (30:7)	2
Prednisolone acetate	Chloroform:methylcyclohexane (30:15)	2
Hydrocortisone acetate	Chloroform:methylcyclohexane (30:15)	1.5
Cortisone acetate	Chloroform:methylcyclohexane (30:20)	1.5

Paper Chromatography

Prepare one paper for the test preparation and one for the corresponding reference standard. Select sheets of Whatman No. 1 filter paper approximately 20 cm. square and draw a line across each 1.25 cm. from the top and a starting line 2.5 cm. from the bottom. Mark margins on the latter at points 4 cm. from the left edge and 5 cm. from the right edge of the paper. The steroid to be determined will be streaked on the starting line between these margins. At a point 2.5 cm. from the right side, place another dot for spotting the reference standard. The reference standard used with the test preparation serves to identify the major steroid component.

Immerse the papers in the immobile solvent (formamide 40:methanol 60) for 20 seconds and remove the excess solvent by blotting lightly and uniformly between sheets of filter paper (Whatman No. 1 or the equivalent). Place the uniformly impregnated paper on a fresh piece of filter paper and allow it to dry for 10 minutes before spotting. With a micropipet, streak 0.200 ml. of the *Test Preparation* across the starting line superimposing successive portions as closely as possible. During the streaking, evaporate the solvent with a gentle current of air. Carefully blow out the pipet and wash the tip with 1 or 2 drops of chloroform. The formamide is eluted from the area by the streaking process and should be replaced with about 0.025 ml. of immobile solvent. Apply 0.010 ml. of the reference standard solution to the starting line at the designated point and again replace the eluted formamide with about 0.005 ml. of immobile solvent.

In a similar fashion, streak the second paper with 0.200 ml. of the appropriate reference standard, omitting the identity spot. The same pipet should be used for streaking the solutions on all papers.

Place the appropriate mobile solvent (Table I) in the tray of the tank about 10 minutes before beginning development. The sample and standard papers are developed in separate tanks. Insert the paper through the slot in the tank lid so that 1.25 cm. project about the lid and secure it with stainless steel pins. Set the cover with the suspended paper on the tank, insuring that the paper will dip into the mobile solvent. Seal the cover to the tank with masking tape and allow the chromatogram to develop for the length of time indicated in Table I.

Remove the chromatogram and air dry it for about 2 or 3 minutes. Place it, spotted side up, on a clean glass plate at least 9 in. from the light source equipped with a 2537 Å. unit filter. Rapidly identify the major steroid component by comparing it with the corresponding reference standard and outline the area covered by it. During this procedure, minimum exposure to ultraviolet light is imperative, but with reasonable caution, no loss is incurred. With experience, the marking can be accomplished within 10 to 15 seconds.

Carefully cut the encircled major component area from the paper. To facilitate extraction, cut it into about 10 pieces and transfer them to a 125-ml. separator. Avoid the use of Teflon-stoppered separators as the paper pulp damages the stopcock. Add 10 ml. of a 1% alcohol solution and extract with four 20-ml. portions of chloroform shaking vigorously each time for 1 minute. The paper should be pulplike when the extraction is completed. Filter the chloroform extracts through chloroform-washed glass wool into a 125-ml. glass-stoppered conical flask and evaporate the combined extracts to complete dryness on a steam bath with the aid of a gentle stream of air. Treat the developed chromatogram of the reference standard in a similar manner.

Colorimetric Procedure

Into separate glass-stoppered 50-ml. conical flasks pipet 0.200 ml. of the appropriate standard preparation and 0.200 ml. of the sample preparation (use the same pipet for measuring both samples). Evaporate the solvent to dryness on a steam bath with gentle current of air.

To each of these flasks and to the flasks containing the dried residues of the chromatographed sample and standard, add 20.0 ml. of alcohol. Stopper and swirl the four flasks to effect solution. To a fifth flask add 20.0 ml. of alcohol to serve as a reagent blank. To each flask, add 2.0 ml. of blue tetrazolium solution, mix, then add 2.0 ml. of dilute tetramethylammonium hydroxide solution to each flask and mix. Allow to stand in the dark for 90 minutes; with a suitable spectrophotometer, determine the absorbance of the solutions at the absorption peak near 525 mμ relative to the reagent blank.

DISCUSSION AND RESULTS

The U.S.P. extraction procedure was modified by use of the 8% sodium bicarbonate solution since the current official procedure permitted the extraction of excipients which interfered with the resolutions of the steroids on the chromatograms.

The paper chromatographic method used is a modified form of the ascending continuous-flow technique described by Fischbach and Levine (2), and Mitchell (3). Using this technique, the mobile solvent can ascend the paper for an indefinite period, since the portion of the paper that emerges from the tank through the slit in the cover acts as a wick for the evaporation of the mobile solvent. The major advantage of this technique is that it makes possible the use of a short paper for the rapid resolution of closely related compounds while maintaining discrete, sharply defined zones. Conventional R_f values are meaningless here, but the systems are designed to move the major component from the line of origin to a point midway on the paper in 1.5 to 2 hours. Because of the small chamber volume

and short development time, only a short chamber equilibration period and minimum temperature control are required.

Initially, a series of control samples was carried through the paper chromatographic separation, elution, and the color development stages. These control samples were prepared mixtures of prednisolone containing hydrocortisone and various other steroids likely to be present in commercial preparations of these products. The samples contained approximately 85% prednisolone, 10% hydrocortisone, 3% epihydrocortisone, and 2% epiprednisolone. Colors were developed by one or more of the reactions using triphenyltetrazolium, phenylhydrazine, or isonicotinic acid hydrazide. The average recovery of prednisolone from 54 determinations was 98.2% with a maximum deviation of plus or minus 5%.

The colorimetric techniques presented certain problems. For the phenylhydrazine or isonicotinic acid hydrazide reactions, additional steps were necessary to remove traces of paper and formamide residue which interfered with color development. The oxygen sensitivity of triphenyltetrazolium chloride and the precise timing required by that procedure are disadvantages when a number of samples are to be assayed simultaneously. The simplicity of the blue tetrazolium technique recommended it as the method of choice.

The feasibility of allowing colors to develop overnight was investigated. While the precision of standards read at 90 minutes and again at 18 hours did not change, erratic values were obtained after 18 hours for samples exposed to ultraviolet light during the paper scanning procedure. Hamlin *et al.* (4) have pointed out the sensitivity of these steroids to ultraviolet light and the particular susceptibility of $\Delta^{1,4}$-3-keto steroids to photolytic degradation.

A study was made by comparing the absorbance of untreated standard solutions and of standards which, after streaking on impregnated papers, were exposed to short-wave light (2537 Å.) for periods of 0, 15, 30, and 60 seconds. The papers were extracted without chromatographic development. After 90 minutes of color development time with blue tetrazolium reagent, the maximum deviation of absorbance between standards and extracts of papers exposed 0, 15, and 30 seconds was less than 1.0%, while the absorbance of the extract of the paper exposed for 60 seconds was 3.0% below the unexposed standard. Checked again after 18 hours of development, the absorbance of the standards and of the papers exposed for 0 and 15 seconds was identical, while the absorbance of the papers exposed for 30 and 60 seconds was 4 and 9% lower than the standard. The above results indicate the necessity for viewing and marking the chromatograms rapidly, preferably in less than 30 seconds.

Table II.—Analysis of Simulated Tablet Formulations

U.S.P. Ref. Std.	Prior to Chromatography, % Recovery	After Chromatography, % Recovery
Hydrocortisone	99.5	101.4
	99.5	99.5
	100.6	100.0
	Av. 99.9	Av. 100.3
Prednisolone	99.3	100.5
	100.1	99.8
	99.3	98.2
	Av. 99.6	99.1
		Av. 99.9
Prednisone	99.5	101.0
	99.3	100.5
	99.3	100.1
	Av. 99.4	Av. 100.6
Hydrocortisone acetate	100.0	98.1
	99.8	99.2
	99.8	Av. 98.7
	Av. 99.9	

Table III.—Analysis of Commercial Powders

	Prior to Chromatography, % Claim	After Chromatography % Claim
Prednisolone	98.5	95.4
	98.5	96.5
	98.5	96.7
	Av. 98.5	Av. 96.2
Prednisone	99.4	96.2
	99.4	95.2
	99.4	93.6
	Av. 99.4	Av. 95.0
Prednisolone acetate	101.4	100.8
	101.4	100.4
	101.4	99.5
	Av. 101.4	Av. 100.3

A mixture comprising 19 parts of prednisolone to 1 part of hydrocortisone was subjected to extraction, paper fractionation, and blue tetrazolium color development. In 12 determinations, recovery of the major steroid fraction was 97.4 ± 1.0%, when compared directly with the standard solution. When the standard was subjected to chromatographic separation and extraction before comparison with the sample, the recovery of prednisolone averaged 99.5%.

Failure to achieve complete recovery from the papergrams is not due to pipet error, paper residue, effect of light, incomplete extraction, loss during evaporation, or reagent contaminants. Since recently distributed U.S.P. reference standards were used in the control studies, it is assumed that the loss is due to tailing on the papergrams rather than to impurity of the standards. While the dangers inherent in the use of procedural standards are well recognized, they are minimized in this case by the relatively complete recoveries found by direct comparison with the standard. Since the factors contributing to incomplete recoveries appear to be constant, the improved results obtained with procedural standards justify their use.

Approximately 10 mg. of accurately weighed U.S.P. reference standard was added to 200 mg. of a simulated tablet mixture and carried through the extraction, paper chromatography, and blue tetrazolium color development steps. The excipient mixture contained 80% starch, 15% lactose, 3% talc, 0.6% stearic acid, 0.5% magnesium stearate, 0.6% tragacanth, and 0.3% sodium alginate. Triplicate aliquots of standard solutions and tablet extracts were used for direct color development prior to chromatography, and triplicate papers of both standard solutions and tablet extracts were developed and determined by the colori-

metric procedure. From inspection of Table II, it is seen that quantitative recovery was achieved by the extraction and chromatographic techniques. The analyses reported in Table III were carried out in triplicate by the assay method described.

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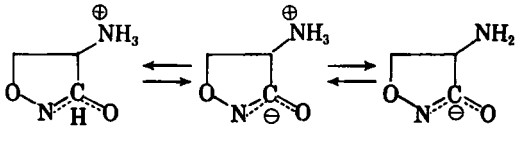
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Stability of Cycloserine in Buffered Aqueous Solutions

By LOUIS MALSPEIS and DAVID GOLD

The pH rate profile of the hydrolysis of cycloserine in aqueous buffers is reported. Apparent pseudo first-order plots of the hydrolytic degradation are observed over the entire pH range. Minimal over-all velocity occurs in the vicinity of pH 12. A linear log k-pH plot is found for the protonated molecule and the dipolar ion in the pH ranges 0.4 to 2.3 and 3.0 to 7.0. The hydrolysis is catalyzed by undissociated acetic acid, and the acetic acid-catalyzed reaction appears to be first order with respect to cycloserine, regardless of whether the protonated molecule or the dipolar ion is present in the solution. Primary kinetic salt effects were not detected in hydrolyses involving the protonated, dipolar, or anionic molecules. The kinetics suggest that in the acid-catalyzed hydrolysis the substrate is the protonated cycloserine molecule. An intermolecular electrophilic-nucleophilic mechanism involving this substrate is considered.

THE STRUCTURE of the antibiotic cycloserine[1] has been shown to be D-4-amino-3-isoxazolidone (1, 2); the dipolar ion structure, as deduced from its infrared absorption spectrum and from potentiometric titration in aqueous solution, is II.



I II III

Reports of qualitative observations of the degradation of cycloserine in aqueous solution indicate that the compound is relatively stable in alkaline solution, whereas it is readily hydrolyzed in acid solution. Kuehl *et al.* (1) noted that cycloserine was facilely degraded to serine and hydroxylamine upon acid hydrolysis and that it was relatively stable to alkali. Hidy *et al.* (2) reported that mild acid hydrolysis yielded D-serine and hydroxylamine, while prolonged hydrolysis gave DL-serine and hydroxylamine. In aqueous solution at pH 6, the compound was 63% degraded in 20 hours at room temperature (3). In 15% sodium hydroxide solution at 100°, Ratouis and Behar observed that the compound was 38% degraded in 6 hours (4). Felder and Tiepolo reported that cycloserine is about 2% degraded in 24 hours at physiological conditions (5). The decomposition products were stated to be hydroxylamine, serine, and 3-aminoxyalanine in acidic solution and 2,5-bis(aminoxymethyl)-3,6-dioxopiperazine and 2,5-bismethylene-3,6-dioxopiperazine in neutral solution (5).

Analogous results were reported for 4-benzamido-3-isoxazolidone. This amide was stable to boiling 2 *N* sodium hydroxide, while acid hydrolysis produced ring opening in addition to amide cleavage (6). Ratouis and Behar (4) identified serine and serine amide as the products of the acid-catalyzed hydrolysis.

Inconsistent with these observations is the report of Nishimura and Shimohima (7), who found that the stability point of cycloserine in aqueous solution is at pH 6.0.

Structurally, cycloserine may be viewed as a cyclic *O*-alkyl hydroxamic acid. The observed stability of cycloserine to alkaline hydrolysis indicates that *N*-amide substitution with an oxygen atom results in a compound which is much less susceptible to nucleophilic attack. Simple amides are approximately equally susceptible to nucleophilic and to electrophilic attack (8); the pH rate profile of the hydrolysis of simple amides exhibits a minimal over-all velocity

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The authors are grateful to Dr. S. N. Gellis, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind., for generous supplies of cycloserine.

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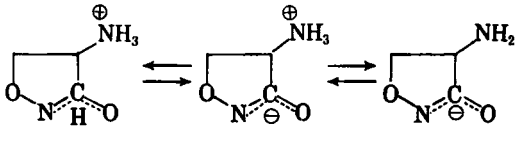
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TABLE I.—BUFFER SOLUTIONS

Buffer	Buffer Compn.	Apparent pH[a] 25°	70°	80°	95°
A	0.50 *M* HCl	0.38[b]	...	...	...
B	0.095 *M* HCl 0.005 *M* KCl	1.07	1.14	1.30	1.30
C	0.01 *M* HCl 0.09 *M* KCl	2.10	2.15	2.30	2.40
D	0.106 *M* phthalic acid 0.159 *M* KHphthalate 0.041 *M* NaCl	2.72	2.96	3.11	3.12
E	0.160 *M* CH_3COOH 0.020 *M* CH_3COONa 0.160 *M* NaCl	3.60	3.65	3.77	3.88
F	0.060 *M* CH_3COOH 0.180 *M* CH_3COONa 0.020 *M* NaCl	5.00	5.20	5.35	5.49
G	0.204 *M* H_3BO_3 0.002 *M* $Na_2B_4O_7$	7.13	7.16	7.17	6.95
H	0.04 *M* H_3BO_3 0.09 *M* $Na_2B_4O_7$	9.08	8.90	8.94	8.91
I	0.546 *M* NaOH 0.023 *M* $Na_2B_4O_7$	11.10	11.14	11.10	11.17
J	0.10 *M* NaOH 0.90 *M* NaCl	12.86[c]	...	...	...
K	1.00 *M* NaOH	13.81[c]	...	...	...

[a] pH Values determined at temperature cited with glass calomel electrodes. [b] pH Values calculated from mean activity coefficients (26). [c] pH Values calculated from mean activity coefficients (27).

in the pH range 3 to 4 (9). Accordingly, with the limitation that each region of the pH rate profile must be considered in terms of the degree of protonation of the substrate present in solution, minimal over-all hydrolysis of cycloserine is expected at high pH values.

Relatively few examples of intermolecular general acid catalysis of the hydrolysis of carboxylic acid derivatives are known (10).[2] Recently, the only example of intermolecular general acid catalysis of the hydrolysis of amides was reported by Wyness (11), who demonstrated the acetic acid-catalyzed hydrolysis of *N-n*-butylacetamide. It was observed by Garrett (12, 13) that structural modification of esters which resulted in decreased hydrogen ion-catalyzed hydrolysis promoted intermolecular general base catalysis by acetate ion. Similarly, intermolecular general acid catalysis should be favored in carboxylic acid derivatives exhibiting decreased susceptibility to hydroxyl ion-catalyzed hydrolysis. According to this reasoning, cycloserine appeared to be a favorable system for demonstrating intermolecular general acid catalysis.

With this view, the pH rate profile of the hydrolysis of cycloserine was investigated, and preliminary experiments to obtain evidence for intermolecular general acid-catalyzed hydrolysis were conducted.

EXPERIMENTAL

Materials.—Cycloserine (Lilly, lot No. 501041) recrystallized according to the procedure of Stammer *et al.* (6) and dried *in vacuo*, m.p. 154 to 154.5° dec The reported melting points of this compound include 154–155° (1), 156° (2), and 153–154° (6). All compounds used in the preparation of buffer solutions and in the assay procedure were reagent grade.

Buffer Solutions, pH Measurement, and pK Determination.—The rates of the decomposition of cycloserine as a function of pH and the concentrations of general acids and bases were studied using buffers. The buffer systems employed in the kinetic studies to elucidate the pH rate profile are listed in Table I. The concentrations of the buffer components cited are the values at room temperature.

The Beckman model G pH meter was used to determine the pH of the buffer solutions at room temperature. The instrument was standardized with standard buffer solutions having pH values within two pH units of the solutions measured. At elevated temperatures the pH measurements were performed with the Beckman zeromatic pH meter. The instrument was standardized at the temperature of the solution which was measured with standard phosphate buffer. The pH values of the standard buffer at elevated temperatures were those cited by Britton (14). In all kinetic studies, pH measurements were taken prior and subsequent to the kinetic runs; no change in pH was observed.

The dissociation constants of cycloserine were essentially determined by the method described by Stern (15). The total concentration of dipolar cycloserine and its conjugate acid or base was 0.010 *M*, and the ionic strength of the solution was adjusted to 0.200 with sodium chloride. The pH of the solutions was determined at all temperatures with the Beckman model G pH meter; appropriate temperature compensation corrections were applied.

Kinetic Procedure.—The kinetic measurements of the degradation of cycloserine were conducted at 0.001 *M* in the given buffer solution. Aliquots were sealed in U.S.P. XVI type I ampuls of 10-ml. capacity; all the ampuls for a run were immersed in a liquid constant temperature bath. Time was per-

[2] For an excellent discussion and pertinent references see Bender, M. L., *Chem. Rev.*, **60**, 53(1960).

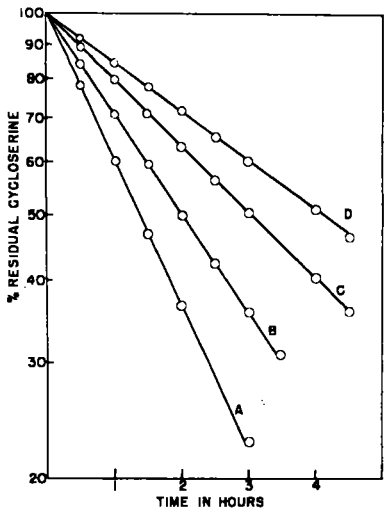


Fig. 1.—Apparent pseudo first-order plots of the hydrolysis of cycloserine at 80° at pH 5.2 in the presence of varying acetic acid concentration. Ionic strength is adjusted to 1.6 with sodium chloride. The following values are given for [CH_3COOH] and [CH_3COONa], respectively. Key: A, 0.064 and 0.136; B, 0.122 and 0.278; C, 0.220 and 0.580; D, 0.385 and 1.215.

mitted for temperature equilibration before removal of the zero-time sample which was considered to contain 100% cycloserine. Individual ampuls were removed at recorded times and immediately immersed in an ice-water bath. The ampuls were stored in the ice-water mixture until completion of the run. The ampuls were then opened, the solutions allowed to warm to room temperature, and the concentration of cycloserine was determined by the colorimetric procedure of Jones (16). The absorbance of each sample was determined against a blank at 625 mμ in a Beckman model DU spectrophotometer. Plots of log (A_t/A_∞) against time, as illustrated by the typical plots shown in Fig. 1, gave good straight lines. The pseudo first-order rate constants, k_1', were obtained by multiplying the slope by 2.303.

For certain runs in the pH region 2 to 0.4, the samples were quenched by chilling, followed by the addition of standard sodium hydroxide to give a pH of 7. The samples were then stored in a refrigerator until the solutions were analyzed.

Thermostats were maintained with ±0.03° at temperatures of 80° or below and within a ±0.06° range at 95°.

Table II.—Observed Rate Constants for the Degradation of 0.001 *M* Cycloserine in Various Buffer Solutions

Buffer	$10^5k_1'$, Sec.$^{-1}$ 50°	60°	70°	80°	95°
A	...	88.8	174	357	...
B	36.9	67.8	136	269	...
C	...	55.0	103	216	502
D	...	44.3	86.9	167	330
E	20.6	...	86.2	169	398
F	...	...	29.6	48.9	103
G	...	...	4.21	9.79	33.5
H	...	...	2.44	6.75	24.1
I	...	...	1.63	3.93	16.0
J	...	...	1.57	4.43	14.7
K	...	...	2.08	5.35	18.0

RESULTS

Dependence of Rate Upon Cycloserine Concentration.—The kinetics of the disappearance of cycloserine from solutions of constant hydronium ion concentration showed the reaction to be first order with respect to the total cycloserine concentration. The observed pseudo first-order rate constants, k_1', were estimated from the semilog plots of the fraction of undegraded cycloserine as a function of time and are listed in Table II. In each run, the reaction was followed to 30% residual cycloserine, and each plot included 7 to 10 points. Typical plots are shown in Fig. 1. At all pH values and irrespective of the buffer components, $-d[C]/dt = k_1'[C]$, where C = cycloserine.

Absence of Salt Effects.—The effect of varying ionic strength on the observed first-order rate constants of the hydronium ion-catalyzed hydrolyses was studied in solutions in which the predominant species of cycloserine is protonated, zwitterionic, or anionic. The results are shown in Table III. No significant change in the rate constant was noted when cycloserine was hydrolyzed at 50° in 0.1 *M* hydrochloric acid solutions adjusted to different ionic strengths with potassium chloride. A salt effect was not observed in the hydrolyses at 80° in boric acid–sodium borate buffer, pH 8.94, over the ionic strength range 0.27 to 0.81. The slight decrease in observed rate with increasing ionic strength seen in the hydrolyses conducted at 80° in biphthalate-phthalate buffer, pH 5.18, over the ionic strength range 0.20 to 0.80, is assumed to be within

Table III.—Hydrolysis of Cycloserine at Constant pH and Varying Ionic Strength

[HCl][a]		[KCl][a]	μ	pH[b]	10^5k_1, Sec.$^{-1}$	Temp., ° C.
0.095		0.005	0.100	1.14	2.69	50
0.095		0.105	0.200		2.79	
0.095		0.305	0.400		2.75	
[KH Phthalate][a]	[K_2 Phthalate][a]	[NaCl][a]				
0.0527	0.0473	...	0.195	4.18	4.19	80
0.0527	0.0473	0.205	0.400		4.11	
0.0527	0.0473	0.605	0.800		4.04	
[H_3BO_3][a]	[$Na_2B_4O_7$][a]	[KCl][a]				
0.04	0.09	...	0.27	8.94	6.75	80
0.04	0.09	0.27	0.54		6.70	
0.04	0.09	0.54	0.81		6.79	

[a] Concentration at 25°. [b] pH Values determined at reaction temperature.

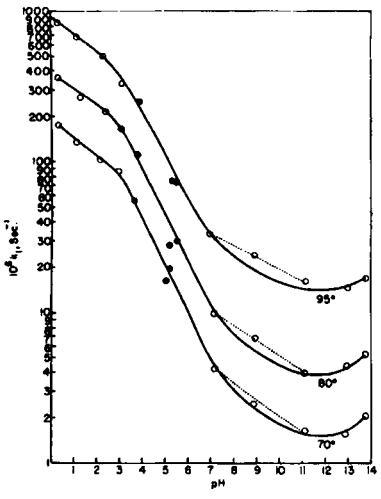


Fig. 2.—The pH rate profile of the hydrolytic degradation of cycloserine. Key: O, observed rates; ●, rates corrected for acetic acid catalysis.

the estimated reproducibility of the first-order constants. It appears that primary kinetic salt effects are not significant, irrespective of the degree of protonation of the substrate. Accordingly, the compositions of the buffer solutions were not maintained at constant ionic strength. It is observed, however, that the ionic strength range of the buffer solutions employed in the acid-catalyzed regions of the pH rate profile is 0.1 to 0.3. It is interesting that primary salt effects were absent in the hydronium ion-catalyzed hydrolysis of L-asparaginylglycine under conditions where the substrate molecule is protonated (17).

Dependence of Rate Upon Hydronium Ion Concentration.—The pH rate profiles of the hydrolysis of cycloserine at 70, 80, and 95° are shown in Fig. 2. It is convenient to examine four regions of the profile. In the pH range 2.3 to 0.4, an apparent linear increase of log k_1' with increasing pH is seen. In the pH range 7.0 to 3.0, the apparently linear dependence of log k_1' on pH exhibits a greater slope than is found in the pH 2.3 to 0.3 region. Between pH 11 and 7 log k_1' decreases with pH; above pH 12, the limited data indicate an increase of log k_1' with pH. The dashed lines in Fig. 2 show linear dependence of log k_1' on pH in the pH range 11 to 7, and the continuous line approximates a catalytic catenary with a minimum in the neighborhood of pH 12. Since the present investigation is concerned with demonstrating general acid-catalyzed hydrolysis, study of the alkaline region of the pH rate profile is deferred. If alkaline degradation of cycloserine above pH 12 is significant, then both acid and base-catalyzed reactions occur simultaneously between pH 12 and 10.

Both linear segments of the pH rate profile in the pH range 7 to 0.4 exhibit apparent fractional slopes. In a study of the degradation of streptozotocin, Garrett (18) attributed such apparent fractional slopes of the log k-pH plot in the acid region to the observed rate due to hydronium ion attack on protonated and nonprotonated substrate molecules in ionic equilibrium with each other. Perhaps the pH rate profile of cycloserine in the acid region presents a comparable situation.

The apparent pKa′ values of cycloserine ±0.01, where the error is the average deviation of three determinations, are given in Table IV. Because of the lability of cycloserine to hydronium ion-catalyzed hydrolysis at elevated temperatures, the experimentally determined pKa′ values are uncertain. However, the pKa′ values do afford a reasonable estimate of the concentrations of protonated, zwitterionic, and anionic cycloserine molecules which are present in each region of the pH rate profile. The classical kinetic expression (12) for the hydronium ion-catalyzed hydrolysis of cycloserine is

$$-dC/dt = -d([C^+] + [C^\pm] + [C^-])/dt \quad \text{(Eq. 1)}$$

$$= -k_{c^+}[H^+][C^+] - k_{c^\pm}[H^+][C^\pm] - k_c[H^+][C^-] - k_{H_2O}[H_2O][C] \quad \text{(Eq. 2)}$$

$$= -k_{H^+}[H^+][C] - k_{H_2O}[H_2O][C] \quad \text{(Eq. 3)}$$

$$= -k_1'[C] \quad \text{(Eq. 4)}$$

Neglecting the contribution of $k_{H_2O}[H_2O]$, where $[H^+]$ = hydronium ion activity, $[C] = [C^+] + [C^\pm] + [C^-]$ = total cycloserine concentration, C^+ = protonated cycloserine, $C^\pm$ = dipolar ion cycloserine, C^- = anion cycloserine.

Inasmuch as the observed rates are correlated with the experimentally measured hydronium ion activity at elevated temperatures, there is considerable uncertainty regarding the 95° pH rate profile. Nevertheless, the large differences in the slopes of the pH rate profile permit interpretation of the hydronium ion dependence. It can be estimated that the second-order rate constant in the region where the molecule is predominantly the dipolar ion is larger than the constant in the region where the principal species is the protonated molecule by a factor of 10^4. One interpretation is the $k_{c^\pm}$ in the above equations is much larger than k_{c^+}, denoting that the hydrolytic lability of the dipolar ion far exceeds that of the protonated molecule, a conclusion which is in accord with the view that the decreased rate is a consequence of the coulombic repulsion of the hydronium ion attack on the protonated molecule. This interpretation of the observed apparent fractional slopes of the pH dependency curve is based upon the assumption that the order of the reaction with respect to hydronium ion is the same in the reactions involving C^+, $C^\pm$, and C^-.

An alternate explanation is required if the order of the reaction with respect to hydronium ion is not the same for the differently protonated substrate molecules. The reaction order with respect to hydronium ion is generally deduced from a linear logarithmic increase of $(k_1' - k_o)$ with pH. The

Table IV.—Apparent Dissociation Constants of Cycloserine at μ = 0.2

Temp., ° C.	pKa₁′	pKa₂′
20.0[a]	4.39	7.39
70.0	4.23	6.83
80.0	4.08	6.76
95.0	3.99	6.64

[a] Reported values at room temperature; pKa₁′, 4.4; pKa₂′, 7.3 (2); pKa₁′, 4.4; pKa₂′, 7.4 (1); pKa₁′, 4.57; pKa₂′, 7.40; $\mu \sim 0.4$ (28).

TABLE V.—HYDROLYSIS OF CYCLOSERINE IN ACETIC ACID–SODIUM ACETATE BUFFERED SOLUTIONS

Part A

[CH_3COOH][a]	[CH_3COONa][a]	[NaCl][a]	pH[b]	Temp., °C.	[CH_3COONa]/[CH_3COOH]	$10^5 k_{obs}$, Sec.$^{-1}$	$10^4 k_{HA}$, L. Mole^{-1} Sec.$^{-1}$
0.048	0.102	0.098	4.04	70	2.13	2.36	
0.064	0.136	0.064				2.63	
0.070	0.149	0.051				2.75	
0.086	0.183	0.017				3.04	1.75[d]
0.032	0.032	0.168	4.67	70	1.00	3.04	
0.064	0.064	0.136				3.59	
0.096	0.096	0.104				4.20	
0.128	0.128	0.072				4.80	1.79[e]

Part B

[CH_3COOH][a]	[CH_3COONa][a]	[NaCl][a]	pH[b]	Temp., °C.	$10^5 k_{obs}$, Sec.$^{-1}$	$10^5 k_1'$,[c] Sec.$^{-1}$	$10^4 k_{HA}$, L. Mole^{-1} Sec.$^{-1}$
0.064	0.136	1.464	5.04	70	2.64	2.64	
0.122	0.278	1.322	5.08		3.64	3.65	
0.220	0.580	1.020	5.01		5.13	5.13	
0.385	1.215	0.385	5.23		7.66	7.68	1.64[f]
0.064	0.136	1.464	5.20	80	4.68	4.68	
0.122	0.278	1.322	5.23		6.31	6.32	
0.220	0.580	1.020	5.29		9.55	9.57	
0.385	1.215	0.385	5.50		14.08	14.14	2.97[g]
0.064	0.136	1.464	5.32	95	11.77	11.77	
0.122	0.278	1.322	5.35		16.04	16.05	
0.220	0.580	1.020	5.41		23.57	23.60	
0.385	1.215	0.385	5.63		35.54	35.63	7.47[h]

[a] Concentration at 25°. [b] pH Values measured with glass calomel electrodes at the temperature cited. [c] Pseudo first-order rates corrected to pH 5.04 at 70°, pH 5.20 at 80°, and pH 5.32 at 95°. [d] $k_o = 1.54 \times 10^{-5}$ sec.$^{-1}$. [e] $k_o = 2.48 \times 10^{-5}$ sec.$^{-1}$. [f] $k_o = 1.59 \times 10^{-5}$ sec.$^{-1}$. [g] $k_o = 2.81 \times 10^{-5}$ sec.$^{-1}$. [h] $k_o = 7.03 \times 10^{-5}$ sec.$^{-1}$.

spontaneous reaction rate, k_o, commonly is small compared to the observed apparent first-order rate constant. If substrate molecules having differing degrees of protonation are present in solution and the slope of the log k_1' *versus* pH plot is not integral, the order of the reaction with respect to hydronium ion activity is equivocal. Moreover, if a plot of $-\log k_1'$ *versus* pH is linear with nonintegral slope, then a plot of $-\log k_1'$ *versus* n pH will also be linear with nonintegral slope. From the reaction mechanism viewpoint, the reaction order with respect to hydronium ion is expected to be an integer, probably 1 or 2, corresponding to a linear plot of $-\log k_1'$ against pH or 2 pH, respectively. Should $-\log k_1'$ for the hydrolysis of C^+ be a linear function of n pH and $-\log k_1'$ for the hydrolysis of $C^\pm$ be a linear function of $2n$ pH, then the observed slopes of the respective plots would prove to be similar. The estimated slope of the pH rate profile (Fig. 2) in the pH range 2.0 to 0.4 is -0.122, and in the pH range 7.0 to 3.0 is -0.313, expressing the observed rate dependence on pH of the hydrolysis of C^+ and $C^\pm$, respectively. A plot of $-\log k_1'$ against 2 pH in the pH range 7.0 to 3.0 is linear with slope -0.157, which indicates that the order with respect to hydronium ion of the hydrolysis rate of $C^\pm$ is approximately twice that of C^+.

Dependence of Rate Upon Acetic Acid Concentration.—Catalysis by buffer components was studied by determining the pseudo first-order hydrolysis rate as a function of the buffer concentration of a series of acetic acid–sodium acetate buffers of constant pH and ionic strength. In accord with the traditional treatment of general acid–general base catalysis (12), at constant pH where both C^+ and $C^\pm$ are present in solution the observed rate constant, k_1', is given by

$$k_1' = k_o + \frac{k_{HA}'[HA] + k_{A^-}'[A^-]}{1 + \frac{Ka_1'}{[H^+]}} + \frac{K_{HA}''[HA] + K_{A^-}''[A^-]}{1 + \frac{[H^+]}{Ka_1'}} \quad \text{(Eq. 5)}$$

$$= k_o + [HA]\left\{\frac{k_{HA}'[H^+] + k_{HA}''Ka_1'}{[H^+] + Ka_1'} + \frac{[A^-]}{[HA]}\left(\frac{k_{A^-}'[H^+] + k_{A^-}'Ka_1'}{[H^+] + Ka_1'}\right)\right\} \quad \text{(Eq. 6)}$$

so that a plot of k_1' *versus* the acetic acid concentration, $[HA]$, is linear. As observed by Wyness (11), constancy of slope for different ratios $[A^-]/[HA]$ indicates that k_{A^-}' and k_{A^-}'' are negligibly small or zero and that catalysis is due to undissociated acetic acid.

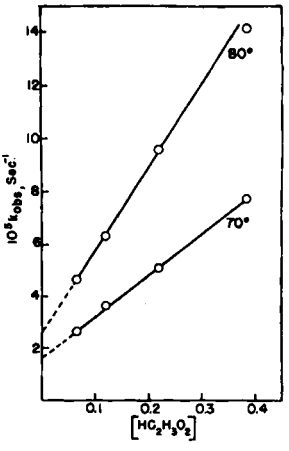


Fig. 3.—Plots showing the catalysis of the hydrolysis of cycloserine by acetic acid at constant pH and ionic strength. Key: 70°, pH 5.04, $\mu = 1.6$; 80°, pH 5.20, $\mu = 1.6$.

Typical plots of the observed rate constants at elevated temperatures as a function of the acetic acid concentration at room temperature (Table V, *Part B*) are shown in Fig. 3. At constant pH and ionic strength, the rates vary linearly with the acetic acid concentration. The apparent second-order rate coefficients, $k_{HA} = k_{obs}/[CH_3COOH]$, for different salt/acid ratios (Table V, *Part A*) are sensibly the same. Hence, catalysis appears to be due to undissociated acetic acid or to both hydronium and acetate ions, but not due to the acetate ion concentration alone. Slight curvature is seen as the acetic acid concentration becomes large.

The fact that the second-order rate coefficients, estimated at different hydronium ion concentrations, are similar indicates that the acetic acid catalytic coefficients k_{HA}' and k_{HA}'' are identical. This conclusion is inconsistent with the generally accepted formulation that k_{HA}' and k_{HA}'' are the catalytic coefficients which apply to C^+ and $C^\pm$, respectively. On the other hand, if the acetic acid-catalyzed hydrolysis of $C^\pm$ depends on the hydronium ion concentration as well as the concentrations of $C^\pm$ and HA, then $k_{HA}' = k_{HA}''$ and $k_{HA} = k_{HA}'(1 + [H^+])$. Accordingly, it would appear that the acetic acid-catalyzed reaction is first order with respect to cycloserine, regardless of whether the protonated molecule or the dipolar ion is present in the solution.

The intercept of the k_1' *versus* CH_3COOH plot, k_o, is a measure of all catalytic effects except acetic acid. Therefore, the intercept rates should fall on the graph of the pH rate profile. Representative intercept values are indicated by the solid circles in Fig. 2, and reasonable fit is observed. The intercept values are tabulated as a footnote in Table V.

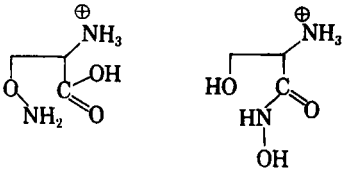
The rate of hydrolysis of cycloserine in phthalic acid–potassium biphthalate buffer solutions of similar pH and ionic strength was studied (Table VI). A fivefold variation in the phthalic acid concentration produced no significant change in rate.

Table VI.—Hydrolysis of Cycloserine in Phthalic Acid–Potassium Biphthalate Buffered Solutions at 80°

[Phthalic Acid]	[KH Phthalate]	[NaCl]	Apparent pH	$10^4k_1'$, Sec.$^{-1}$
0.1060	0.1590	0.0410	3.11	1.67
0.0530	0.0795	0.1205	3.11	1.63
0.0265	0.0398	0.1602	3.09	1.71

DISCUSSION

Intermediates in the Acid-Catalyzed Hydrolysis Reactions.—The end products of the acid-catalyzed hydrolysis of cycloserine have been found to be serine and hydroxylamine (1, 2, 5). In one report, 3-aminoxyalanine was observed to be a product of the acid-catalyzed degradation (5). Inasmuch as carbon-oxygen cleavage is considered unlikely, cleavage of either the carbon-nitrogen bond or the nitrogen-oxygen bond must occur initially during the course of the hydrolysis reaction. Therefore,



the two possible intermediates in the reaction are 3-aminoxy-D-alanine and D-serine hydroxamic acid. The hydroxamate was not identified as an intermediate in the hydrolysis mixture in this laboratory. When ferric ion was included in the hydrolysis mixture, the characteristic red of the ferric hydroxamic acid chelate was not noted. Failure to detect serine hydroxamic acid does not preclude the possibility that it is also an intermediate and that two competitive reactions are taking place, inasmuch as the acid-catalyzed hydrolysis of the hydroxamic acid may occur rapidly.

Justification for the expectation that 3-aminoxyalanine is an intermediate is based upon analogous reactions. The treatment of cycloserine with methanolic hydrogen chloride yields 3-aminoxy-D-alanine methyl ester dihydrochloride (1, 2, 6). Refluxing DL-4-benzamido-3-isoxazolidone in absolute ethanol saturated with hydrogen chloride gives the hydrochloride of 3-aminoxy-DL-alanine ethyl ester (6). The hydrochloride of 5-aminoxymethyl-3-phenylhydantoin is formed from the hydrochloric acid treatment of the reaction product of cycloserine and phenyl isocyanate. In addition, good yields of aminoxyalkanes are obtained from the aqueous acid or alkaline hydrolysis of *O*-alkyl hydroxamic acids and *O*-alkyl urethanes. Thus, heating *O*-butyl benzohydroxamic acid with hydrobromic acid gives aminoxybutane hydrobromide and benzoic acid (19); refluxing ethyl *O*-butylcarbamate with concentrated potassium hydroxide solution yields aminoxybutane (20). Moreover, it is probable that 3-aminoxyalanine is an intermediate in the formation of 2,5-bis(aminoxymethyl)-3,6-dioxopiperazine from cycloserine (21, 22). Therefore, it is reasonable to expect that the carbon-nitrogen bond is preferentially cleaved.

The apparent pKa values of 3-aminoxy-D-alanine methyl ester are 2.3 and 6.9 (1), the former value applying to the aminoxy group. Therefore, in dilute acid solution, the aminoxy group is incompletely protonated. It is possible that serine and hydroxylamine are formed from the aminoxy amino acid by solvent or nucleophilic displacement at either the aminoxy nitrogen or the β carbon atom.

Consideration of the Mechanism of the Hydrolysis of Cycloserine.—The kinetics of the hydrolysis of cycloserine have shown that both the protonated molecule and the dipolar ion are subject to hydronium ion-catalyzed hydrolysis, since the observed rates increase with decreasing pH. The question arises of whether the mechanisms of C^+ and $C^\pm$ differ.

It is not feasible that the rate-determining step in the hydrolysis of the dipolar ion involves proton transfer to the substrate

$$C^\pm + H_3O^+ \rightleftarrows (M^*) \rightarrow C^+ + H_2O \qquad \text{(slow)}$$

$$C^+ + H_2O \rightarrow \text{products} \qquad \text{(fast)}$$

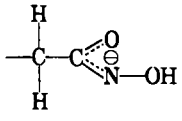
where M^* is the transition state complex in the theory of absolute reaction rates, inasmuch as the hydrolysis of the protonated molecule was not rapid. Pre-equilibration of the dipolar ion and

H_3O^+, followed by the bimolecular reaction between the protonated cycloserine and water

$$C^{\pm} + H_3O^+ \rightleftarrows C^+ + H_2O \quad \text{(fast)}$$

$$C^+ + H_2O \rightleftarrows (M^*) \rightarrow \text{products} \quad \text{(slow)}$$

is similar to the generally accepted mechanism for amide hydrolysis and in this instance corresponds to the solvent-catalyzed reaction of C^+. This mechanism implies that there is present in the C^+ molecule a center which is sufficiently electrophilic to make the water-catalyzed reaction appreciable. Essentially, the mechanism is analogous to the water-catalyzed hydrolysis of a nonprotonated amide, and the rates of such spontaneous reactions are extremely low. If the intermediate product of the hydrolysis reaction is the 3-aminoxy amino acid, the water molecule must attack the carbonyl-carbon; if the product is the hydroxamic acid, the reaction probably occurs at the ring nitrogen. Stammer and co-workers (6) have presented evidence that in hydroxamic acid salts the negative charge resides near the carbonyl-carbon, and it seems likely that there is also a relatively high electron density on the carbonyl-carbon of dipolar cycloserine.



Accordingly, the structure of C^+ is represented as I. It appears unlikely that either the carbonyl-carbon or the ring nitrogen in this molecule have sufficient electrophilic character to make the water-catalyzed hydrolysis of C^+ significant. Therefore, it is more probable that the dipolar molecule must be doubly protonated and that the hydronium ion-catalyzed hydrolysis of $C^{\pm}$ and C^+ proceed *via* the same transition state complex. The resemblance of the energies and entropies of activation in both regions of the pH rate profile (Table VII) supports this contention.

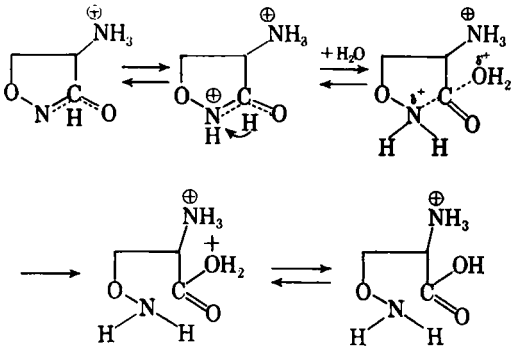
Therefore, in the region of the pH rate profile where the dipolar ion is the principal species, an increase in the hydronium ion concentration causes an increase in the hydrolysis rate as a consequence of the increase in the concentration of diprotonated substrate and $k_1' = k_o + k_{H^+}[H_3O^+]^2$, so that $-\log (k_1' - k_o) = -\log k_{H^+} + 2$ pH. As noted previously, a plot of $-\log k_1'$ *versus* 2 pH in the pH range 7 to 3 is linear, and the slope of this plot approximates the slope of the $-\log k_1'$ *versus* pH plot in the region where the hydronium ion-catalyzed hydrolysis of C^+ takes place. Hence, the steep slope in the pH rate profile is not attributed to the greater hydrolytic lability of $C^{\pm}$ but instead to the premise that the rate increases with the square of the hydronium ion concentration.

TABLE VII.—ACTIVATION ENERGIES AND ENTROPIES FOR THE HYDROLYTIC DEGRADATION OF CYCLOSERINE AT VARIOUS HYDRONIUM ION ACTIVITIES

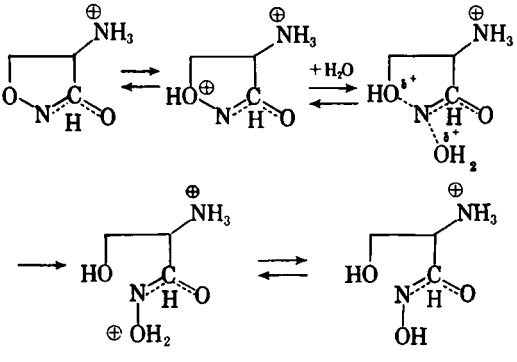
Apparent pH	ΔEa Kcal./Mole	ΔS^+ 80°, e.u.
0.4	16.3	−30.7
1.3	16.1	−31.6
2.3	16.0	−32.3
3.1	15.5	−34.2
3.8	15.9[a]	−33.8[a]
7.2	20.8	−24.9
8.9	23.1	−19.1
11.1	22.8	−21.0
12.9	22.7	−21.1
13.8	21.8	−23.2

[a] Values exclude contribution due to acetic acid catalysis.

A plausible mechanism for the hydrolysis of C^+ is the classical mechanism cited above, in which the transition state results from the attack of a molecule of water on the conjugate acid of the substrate. If the aminoxy amino acid is the intermediate product, the mechanism could be formulated as



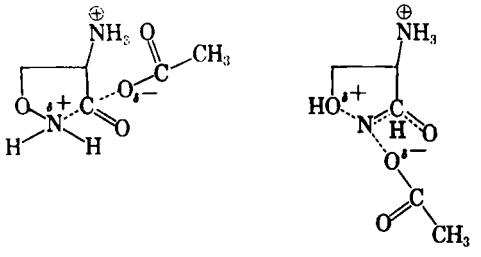
and based upon the hydroxamic acid as the intermediate product, the reaction would proceed by reaction of H_3O on the ring nitrogen. Thus



Before the latter mechanism can be accepted, it will be necessary to preclude the possibility of nucleophilic displacement at the β carbon atom and the possibility of carbonium ion formation at the β carbon atom, in addition to establishing that the hydroxamic acid is an intermediate product.

The kinetics of the hydrolysis of cycloserine in acetic acid–sodium acetate buffers indicates the apparent catalytic dependence of the hydrolysis on undissociated acetic acid. This does not appear to be a case of general acid catalysis, since catalysis by undissociated phthalic acid was not observed. As noted by Wyness (11), Bender's electrophilic-nucleophilic mechanism (23, 24) applied to intermolecular catalysis accommodates the observed kinetic dependence. In essence, the mechanism involves protonation of the substrate by acetic acid, followed by nucleophilic attack by acetate ion on an electrophilic center with concerted anhydride formation and bond cleavage. The postulated transi-

tion states for aminoxyalanine and hydroxamic acid formation are



and the resulting anhydride is then hydrolyzed rapidly. As in the instance of the hydronium ion-catalyzed reaction, it is concluded that the substrate molecule is probably C^+ rather than $C^{\pm}$, since neither the dipolar ion nor the protonated molecule appears to be susceptible to nucleophilic attack by hydroxide ion. The acetic acid-catalyzed hydrolysis of the dipolar ion requires the reaction of acetate ion with a nonprotonated center as the rate-determining step. Based upon the fact that the simple amide is not subject to acetate ion-catalyzed hydrolysis and the premise that the amide has substantially more electrophilic character than is present in the isoxazolidone ring system, such a rate-determining step is highly improbable.

The alternate mechanism (11) for the acetic acid-catalyzed reaction which involves a transition state wherein H_3O attacks the electrophilic center of a hydrogen-bonded complex between acetic acid and cycloserine is not presently considered. Wyness (11) found that the effect of D_2O on the acetic acid-catalyzed hydrolysis of *N-n*-butylacetamide does not support this mechanism.

Above pH 9, the anionic cycloserine molecule III is the only species present in solution. Moreover, at pH values greater than 9, the pH dependency curve has appearance of a hydrolytic catenary, with a minimum in the vicinity of pH 12. The increase in the rate above pH 12 signifies that the degradation of the anionic molecule is base-catalyzed. Considering the broad base of the pH dependency curve, it is probable that the base-catalyzed degradation contributes to the observed rate of the degradation of cycloserine in the pH range 10 to 12. The increase in the observed rate with decreasing pH in the pH range 11 to 9 is indicative of a hydronium ion-catalyzed reaction involving the C^-. Thus, it appears likely that in this region of the pH rate profile the degradation is proceeding by both base and acid-catalyzed reactions. The comparability of the values of the activation parameters in this region with those determined for the base-catalyzed reaction (Table VII) is probably fortuitous.

The extremely low susceptibility of cycloserine to hydroxide ion-catalyzed hydrolysis is noteworthy. Based solely upon inductive effects, it is expected that *N*-amide substitution with an electrophilic oxygen atom should result in enhanced electrophilic character of the carbonyl-carbon and a compound which is much more susceptible to nucleophilic attack. It is evident that the inductive effect does not operate in this way in cycloserine. To a large extent the negligible base-catalyzed hydrolysis in the pH range 6 to 12 is due to the coulombic repulsion of the hydroxide ion by the formal negative charge on the N—C—O system of the zwitterionic and anionic substrate. However, the hydrolysis kinetics in the pH range 4 to 5, a region where the hydroxide ion-catalyzed hydrolysis of simple amides is considerable, indicates that the base-catalyzed hydrolysis of C^+ is unimportant. Thus, the low susceptibility of cycloserine is due to the inherent character of the isoxazolidone ring system, in which the carbonyl-carbon has decreased electrophilic character compared with the carbonyl-carbon of an amide. It is suggested that the *p* orbital on the ring oxygen interacts with those on the adjacent N–C–O system to form a streamer molecular orbital of greater stability than the streamer orbital occurring on the N—C—O system alone. A crystallographic study of cycloserine (25) reveals that the five-membered ring system is nearly planar, so that the steric requirement for such streamer molecular orbital formation is present. A consequence of the delocalization of the π electrons is the apparent decreased electrophilic character of the carbonyl-carbon, so that the isoxazolidone ring is relatively resistant to nucleophilic attack but readily undergoes acid-catalyzed hydrolytic cleavage.

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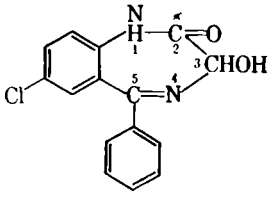
Absorption, Metabolism, and Excretion of Oxazepam and Its Succinate Half-Ester

By SIDNEY S. WALKENSTEIN, ROBERT WISER, CATHRYN H. GUDMUNDSEN, HAZEL B. KIMMEL, and ROBERT A. CORRADINO

C^{14}-Oxazepam was administered orally to dogs, rats, and pigs. Radioassay of blood, various tissues, and excreta indicated that the drug was well absorbed. A single dose was largely eliminated within 2 days, mostly *via* urine in dogs and pigs and *via* feces in rats. One metabolite, a glucuronide, accounted for more than 95 per cent of the radioactivity of dog and pig urine. The radioactivity of rat urine was distributed among at least seven metabolites. C^{14}-Labeled-Wy-4426 (a succinate half-ester of oxazepam) was found to disappear rapidly from an i.m. injection site in rats and to be distributed uniformly throughout the organs. Exceptions were the gastrointestinal tract, which appeared to be the route favored for excretion in this species, and the liver and kidney, in which somewhat higher than average levels were attained shortly after injection. About two-thirds of the dose was excreted in feces and about one-fifth in urine by the forty-eighth hour after injection. The drug appeared to undergo the same extensive metabolic alteration in rats as oxazepam—at least seven distinct metabolites appearing in urine. After i.v. injection of Wy-4426 in dogs, blood levels peaked within 15 minutes but declined slowly, so that even at 48 hours appreciable levels were found. The blood values from the fourth hour compared closely with those obtained following oral administration of oxazepam. Dogs excreted about two-thirds of the dose in urine and one-third in feces within 72 hours. Three radioactive spots in chromatographed urine of dogs injected with Wy-4426 were identified as unchanged drug, oxazepam, and the glucuronide of oxazepam. Similar spots were obtained when Wy-4426 was administered to pigs. The fate of oxazepam in humans was similar to that in dogs and pigs.

OXAZEPAM,[1] 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepine-2-one, has been characterized pharmacologically in our laboratories as an anticonvulsant and mild central depressant and is currently under clinical investigation as an antianxiety agent.

For oral use in this study we synthesized the drug with C^{14} at the 2-position. Wy-4426, the water-soluble sodium salt of the succinate half-ester, was prepared from C^{14}-oxazepam for parenteral use.



Oxazepam

EXPERIMENTAL

Synthesis of C^{14}-Oxazepam.—2-Amino-5-chlorobenzophenone oxime in acetic acid was treated with a solution of 2-chloroacetyl-1-C^{14} chloride in hexane. The mixture was allowed to stand at room temperature for 48 hours. Methylene chloride and water were then added with mixing, the methylene chloride layer was separated, washed with sodium carbonate solution, and concentrated in a stream of nitrogen. Without isolation the crude 2-chloromethyl-4-phenyl-6-chloroquinazoline-3-oxide was treated with sodium hydroxide in aqueous ethanol, stirred 1 hour, diluted with water, and acidified with HCl. The product, 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepine-2-one-4-oxide was separated and recrystallized from aqueous ethanol. Treatment of this material with acetic anhydride in acetic acid at 90–100° afforded crystals of 7-chloro-5-phenyl-3-acetoxy - 1,3 - dihydro - 2*H* - 1,4 - benzodiazepine-2-one after cooling. This solid was then hydrolyzed with sodium hydroxide in aqueous ethanol and oxazepan isolated by dilution with water and acidification with acetic acid. Recrystallization from hot dimethyl formamide gave a product, m.p. 204.5 to 205.5°, specific activity 2970 disintegrations/minute/mcg.

Synthesis of Wy-4426.—C^{14}-Oxazepam and succinic anhydride were heated in pyridine at 95° for 1.5 hours. The mixture was cooled and diluted with ethanol. Addition of water precipitated the pyridinium salt of the succinate half-ester. The pyridinium salt was dissolved in ethanol and treated with sodium hydroxide solution to yield the sodium salt of the half-ester. The Wy-4426 thus obtained was chromatographed and found by radioautography to have a trace of oxazepam.

Colorimetric Assay of Oxazepam and Its Glucuronide.—The following method takes advantage of the fact that 2-amino-5-chlorobenzophenone, the product of acid hydrolysis of oxazepam, is quantitatively extracted from aqueous acid by organic solvent and thereby effectively separated from substances which may interfere in the Bratton-Marshall reaction. The method would appear to be ap-

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[1] Formerly coded Wy-3498.

plicable to the determination of any benzodiazepine, such as chlordiazepoxide, as well as for the metabolites of these drugs, which yield 2-amino-5-chlorobenzophenone on acid hydrolysis.

Oxazepam.—Two milliliters of plasma, urine, or homogenates of feces or tissue was shaken in glass or polyethylene-stoppered centrifuge tubes with 10 ml. of ethylene dichloride for 30 minutes on a mechanical shaker. One milliliter of aqueous phase (A) was removed for subsequent determination of oxazepam glucuronide. Eight milliliters of the organic phase was transferred to a clean 40-ml. centrifuge tube containing 4 ml. 6 *N* HCl and agitated for 1 minute on a Vortex, Jr. mixer. The tube was centrifuged; 3 ml. of the aqueous phase was transferred to a clean 40-ml. centrifuge tube which was then heated for 1 hour in a boiling water bath. The tube was stoppered after temperature equilibration. After removal from the bath and cooling, 15 ml. of H_2O was added, followed by 10 ml. of CCl_4. The tube was stoppered and shaken mechanically for 30 minutes. Following centrifugation and removal of the aqueous phase, 8 ml. of the organic layer was transferred to a clean 40-ml. tube and extracted with a mixture of 2 ml. 1 *N* HCl and 1 ml. freshly prepared 0.1% $NaNO_2$ by agitation for 2 minutes on a Vortex, Jr. mixer. Two milliliters of the aqueous phase was transferred to a test tube containing 0.5 ml. of 0.5% ammonium sulfamate. After mixing and allowing 2 minutes for complete destruction of nitrite, 0.5 ml. of 0.1% *N*-(1-naphthyl)ethylenediamine·2HCl was added, and the contents of the tube mixed. After standing for 10 minutes, the sample was read at 550 mμ in a Bausch & Lomb spectronic 20 colorimeter.

Standards were prepared by shaking 2-ml. portions of appropriate biological control samples with 10-ml. ethylene dichloride solutions containing known amounts of oxazepam. When assayed, a 0.5 mcg./ml. ethylene dichloride solution of oxazepam gave a reading of 0.05 absorbance units.

Oxazepam Glucuronide.—Since no sample of feces from species investigated—dog, rat, pig, or man—contained this metabolite, this part of the assay of feces is unnecessary. To 1 ml. of aqueous phase A (from which oxazepam was removed by extraction with ethylene dichloride) in a clean 40-ml. centrifuge tube was added 1 ml. concentrated HCl. The tube was heated for 1 hour in a boiling water bath (stoppered after temperature equilibration). Following the addition of 10 ml. of H_2O to the cooled tube, the procedure employed was the same as that for oxazepam following its hydrolysis.

Standards were prepared by addition of 1-ml. solutions of oxazepam in 6 *N* HCl to 1 ml. ethylene dichloride-extracted biological samples from untreated animals. After addition of 1 ml. concentrated HCl to this mixture, hydrolysis and subsequent treatment followed the procedure for oxazepam. Treated in this manner, a 5-mcg. solution of oxazepam gave a reading of 0.08 absorbance units, which was 70% of the value obtained by direct coupling of an equivalent amount of 2-amino-5-chlorobenzophenone. The absorbance-concentration curve was linear up to 30 mcg. of oxazepam, and replicate samples agreed within 5%. Because of negligible blanks, it was possible to detect as little as 0.25 mcg. of oxazepam in the assay for glucuronide. Lower concentrations were determined by increasing the sample size; there was no concomitant increase in the blank.

Fluorometric Assay of Oxazepam in Urine and Serum.—Three milliliters of urine or serum was added to 0.5 ml. of capryl alcohol in a 12-ml. centrifuge tube equipped with a standard taper joint and polyethylene stopper. The liquids were mixed thoroughly by a Vortex mixer. Six milliliters of ethylene dichloride was added, and the phases were thoroughly mixed by shaking. The tube was centrifuged and the aqueous layer removed by aspiration. Four milliliters of the organic layer was transferred to a 40-ml. centrifuge tube containing 20 ml. of 0.1 *M* KH_2PO_4; the mixture was shaken. After centrifugation, the aqueous layer was removed by aspiration, 20 ml. of fresh phosphate solution was added, and again the mixture was shaken, centrifuged, and the aqueous layer removed. Three milliliters of the ethylene dichloride layer was transferred to another tube containing 3.0 ml. 3 *N* HCl. The mixture was then shaken and centrifuged and the acid layer transferred to a quartz cell.

The fluorescence was measured on a Photovolt fluorometer equipped with a high-pressure mercury lamp, a 200–400 mμ broad-band primary filter, and a B 540 (520–660 mμ) broad-band secondary filter.

Standards were prepared by substituting measured volumes of a standard solution of oxazepam in ethylene dichloride for an equivalent volume of the pure solvent. This preparation was placed in a tube containing control urine or serum and was carried through the procedure.

The method described will detect as little as 0.5 mcg. oxazepam/ml. urine or serum. As in all fluorescence analysis, standards used should be fairly close in concentration to that of the sample being determined. Low blank values were obtained in urine and serum, and none of the metabolites interfered with the test for unchanged drug. Duplicate samples agreed within 5%.

Paper Chromatography.—Samples of urine spotted directly and extracts of feces were chromatographed on Whatman No. 1 paper in butanol 17, ethanol 3, water 20 (upper phase) and butanol 6, pyridine 4, water 3.

After development and drying of the chromatograms, they were either placed in contact with Kodak No Screen X-Ray Film for radioautography or were sectioned for radioanalysis in a thin-window gas flow counter (Nuclear-Chicago Ultrascaler).

Radioassay of Biological Samples.—Feces—cecum and small intestine—were homogenized and an aliquot was taken for Schoeniger combustion; a 10% ethanolamine solution was used as absorbent. An aliquot of this solution was added to scintillator fluid in a counting vial. Urine was added directly to counting vials containing scintillator made up in dioxane-naphthalene-ethanol. Tissue samples, other than small intestine and cecum, were dissolved in hyamine hydroxide prior to addition of scintillator fluid. The samples were then assayed in a Packard TriCarb scintillation spectrometer.

Administration of Drug.—C^{14}-Oxazepam was made up in gelatin capsules for oral administration to three fasted female dogs at a dose of approximately 2 mg./Kg. Plasma samples and urine (by catheterization) were taken at various time intervals for 120 hours following administration of the drug. A suspension of the drug (finely ground) in 1% poly-

TABLE I.—DOG PLASMA LEVELS[a] OF C^{14} AFTER A SINGLE ORAL DOSE OF C^{14}-OXAZEPAM

	A	B	C	Av.
Dog Wt., Kg.	11.0	11.8	9.5	
Dose, mg.	23.0	25.0	20.0	
30 min.	0	0	0.20	0.07
1 hr.	0	0	0.67	0.22
2 hr.	0.28	0.07	2.34	0.90
4 hr.	1.62	0.79	2.2	1.50
6 hr.	1.53	1.43	1.15	1.37
24 hr.	0.12	0.54	0.14	0.27
48 hr.	0.06	0.06	0.01	0.04
120 hr.	0.01	0.04	0	0.02

[a] Expressed in mcg./ml. of equivalent oxazepam.

TABLE II.—DOG PLASMA LEVELS[a] OF C^{14} AFTER A SINGLE INTRAVENOUS DOSE[b] OF C^{14}-WY-4426

	A	B	C	Av.
Dog Wt., Kg.	8.8	11.7	10.0	
15 min.	21.4	10.5	16.1	16.0
30 min.	19.1	8.15	11.9	13.1
1 hr.	11.4	5.05	9.25	8.57
2 hr.	3.09	2.76	5.00	3.62
4 hr.	1.23	1.35	1.99	1.52
6 hr.	1.14	1.09	1.50	1.24
24 hr.	0.14	0.20	0.18	0.17
48 hr.	0.08	0.27	0.09	0.14
72 hr.	0	0.10	0.10	0.07

[a] Expressed in mcg./ml. of equivalent oxazepam. [b] Dose was equivalent to 1.92 mg./Kg. of oxazepam.

sorbate 80[2] was prepared containing 0.4 mg. oxazepam/ml. This suspension was administered by stomach tube at a dose of 2 mg./Kg. to fasted white male rats weighing between 250 and 370 Gm.

For parenteral administration Wy-4426 was made up in physiological saline so that 1 ml. of solution contained the equivalent of 3.84 mg. of oxazepam. The dose was such that animals received an amount equivalent to a dose of 1.92 mg./Kg. oxazepam. The solution of Wy-4426 was given i.v. to three fasted female dogs. Urine (by catheterization) and plasma samples were drawn at intervals up to 72 hours. The solution was also injected i.m. into fasted male white rats at a dose of 1.92 mg./Kg. For the collection of feces and urine, all rats were kept in metabolism cages until sacrificed in groups of three at intervals up to 48 hours.

A similar dose of oxazepam was given p.o. to an 8.7-Kg. pig and Wy-4426 was given i.m. to a 6.7-Kg. pig.

A 60-mg. dose of oxazepam was given p.o. to each of two humans—one a healthy male (V. M.) and the other a female with sickle cell anemia, rheumatic heart disease, and rheumatic fever (A. R.).

RESULTS

A preliminary study was carried out in which unlabeled oxazepam, at doses of 5 to 10 mg./Kg., was given orally and parenterally to female dogs. Only after administration by the i.v. route could appreciable levels of unchanged drug be demonstrated in plasma. Examination of urine bore out these results; only 1 to 2% of metabolically unaltered drug could be found. Accordingly the C^{14}-labeled drug was employed in further studies.

[2] Marketed as Tween 80 by the Atlas Powder Co., Wilmington, Del.

Plasma Levels in Dogs.—Table I gives plasma levels of C^{14} in three dogs after an oral dose of C^{14}-oxazepam. Dog C showed the most rapid uptake of drug and also peaked earlier than the other two dogs. Peak levels were reached at from 4 to 6 hours in all three dogs. Radioactivity persisted in the blood of all three dogs for 48 hours and in two of the dogs for 120 hours, although the levels present at these times were insignificant.

Table II gives C^{14} plasma levels in dogs following an intravenous dose of Wy-4426. Peak levels were reached within the first 15 minutes after injection; radioactivity, although not at appreciable levels, persisted in the plasma of all three dogs for 48 hours and in two of the dogs for 72 hours. It should be noted that from the fourth hour the data closely corresponded with that obtained with oxazepam.

Urinary Excretion in Dogs.—Following an oral dose of C^{14}-oxazepam, amounts of C^{14} excreted at varying time intervals in urine corresponded roughly with its blood level (Table III). By the twenty-fourth hour each dog had excreted about three-fifths of the dose. All the dogs continued to excrete detectable amounts of C^{14} up to 96 hours, but virtually none beyond this time.

After i.v. administration of C^{14}-labeled-Wy-4426, a peak rate of urinary excretion of about 15%/hour occurred for the first 30 minutes (Table IV). Sixty per cent of the drug was excreted by the twenty-

TABLE III.—EXCRETION[a] OF C^{14} AFTER A SINGLE ORAL DOSE OF C^{14}-OXAZEPAM TO DOGS

Hr.	A	B	C	Av.
		Urine		
0.5	0	0	0	0
1	0.01	0	0.50	0.17
2	4.25	0.11	6.65	3.67
4	11.20	2.03	24.95	12.73
6	27.10	8.75	32.86	22.90
24	62.50	57.15	57.61	59.09
48	69.03	68.85	61.35	66.41
96	70.33	70.65	61.85	67.61
120	70.41	70.65	61.86	67.64
		Feces		
24		8.68	38.10	23.39
48	31.50[b]	19.93	41.66	31.03
96	34.58	24.81	42.90	34.90
144	35.54	27.21	44.08	35.61

[a] In cumulative per cent of dose. [b] 0–48 hours.

TABLE IV.—EXCRETION[a] OF C^{14} AFTER A SINGLE INTRAVENOUS DOSE OF C^{14}-WY-4426 TO DOGS

Hr.	A	B	C	Av.
		Urine		
0.25	...	4.36	1.71	3.02
0.5	5.36	7.48	8.91	7.25
1	9.21	12.23	13.15	11.53
2	14.01	20.98	18.50	17.83
4	24.71	32.08	33.36	30.05
6	31.30	42.98	42.16	32.15
24	44.01	65.41	72.90	60.77
48	45.06	78.08	77.03	66.72
72	45.06	79.03	78.12	67.40
		Feces		
24	39.0	0	0.1	13.0
48	44.5	11.2	18.8	24.5
72	44.5	23.5	22.4	30.4

[a] In cumulative per cent of dose.

fourth hour and an additional 6% by the forty-eighth hour. When the experiment was terminated at 72 hours, somewhat over two-thirds of the dose had been excreted in urine.

Fecal Excretion in Dogs.—That part of the dose not accounted for by urinary excretion appeared in feces (Tables III and IV). There appeared to be considerable individual variation in the amounts excreted in feces with both forms of the drug; animals receiving oxazepam ranged from about 20 to 42% of the dose in feces in 48 hours, and those on Wy-4426 ranged from 11 to 45% in the same period.

Rat Tissue Levels.—Table V summarizes the levels of C^{14} in tissues and excreta of rats dosed orally with C^{14}-oxazepam. A more rapid uptake can be noted than was apparent in the dogs (each evidenced C^{14} in all tissues in the first 30 minutes). Contributing to this more rapid uptake might be the effect of liquid volume on increasing the rate of gastric emptying, and of polysorbate 80 and fine particle size on absorption. Values for liver exceeded those for all other organs outside the gastrointestinal tract for up to 6 hours. Liver, however, showed no tendency to accumulate drug, as indicated by the rapid decline in radioactivity after the 30-minute peak and the virtual clearance of C^{14} from this organ by the twenty-fourth hour.

There was apparently good uptake by all organs examined and fairly rapid clearance from many by the sixth hour. Continued appearance of low levels of activity in kidney and several other organs may be accounted for by reabsorption from gut of drug or metabolites.

In contrast to the dog, the rat appeared to favor the fecal route for excretion of drug and metabolites, since about 1.5 to four times as much radioactivity appeared in the feces of the rat as in the urine by 48 hours. At the end of the same period the dog had excreted twice as much in urine as in feces (Table III).

Within 30 minutes of injection of C^{14}-labeled-Wy-4426, half the dose was absorbed from the intramuscular injection site (Table VI). By the twenty-fourth hour, no drug remained at the injection site in the three animals of that group.

Peak levels occurred in plasma within 30 minutes of injection. Except for the gastrointestinal tract, only liver and kidney exhibited higher values than the general average for all organs. These higher levels did not persist beyond the first hour, and at 24 and 48 hours, liver and kidney contained amounts of C^{14} comparable to over-all organ averages. Small intestine remained high for the first 6 hours, but by 48 hours, small intestine was free of drug and metabolites.

Excretion of Wy-4426 and metabolites was accomplished largely *via* the fecal route, which accounted for over two-thirds of the dose in 48 hours. During this period, less than one-fifth of the dose was excreted in urine. This urine to feces ratio, similar to that in the oxazepam rats, was the reverse of the ratio obtained in dogs.

Metabolism.—Virtually all of the radioactivity in the urine of dogs dosed with C^{14}-oxazepam was found in a single metabolite (Fig. 1). Treatment of this urine with propionic anhydride in pyridine resulted in the quantitative conversion of this metabolite to a new substance. On the chromatograms this detoxi-

TABLE V.—TISSUE LEVELS IN MCG./GM. IN RATS AFTER A SINGLE ORAL DOSE[a] OF C^{14}-OXAZEPAM

	30 Min.		1 Hr.		2 Hr.		4 Hr.		6 Hr.		24 Hr.		48 Hr.	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Urine[b]	0.09	0.38	2.23	0.74	0.66	3.18	1.63	40.6	1.45	0.54	12.55	10.42	10.70	9.80
Plasma[c]	0.18	0.32	0.24	0.18	0.15	0.30	0.11	0.10	0.15	0.08	0	0	0	0
Lung	0.88	1.00	0.48	0.31	0.12	0.59	0.14	0.19	0.06	0	0	0	0	0
Liver	3.58	5.55	4.86	1.85	2.89	4.55	0.53	0.83	0.82	0.60	0.08	0	0.08	0.05
Brain	0.28	0.76	1.15	0.25	0.65	0.39	0.09	0.14	0.05	0.34	0	0	0.19	0
Heart	0.54	0.95	0.61	0.32	0.98	0.44	0.26	0.17	0	0	0	0	0	0
Spleen	0.50	0.34	0.27	0.12	0.12	0.23	0.08	0.12	0.26	0	0	0	0	0
Kidney	1.23	2.19	1.79	0.89	0.71	1.97	0.34	0.61	0.80	0.60	0.15	0	0.21	0.21
Fat	0.21	0.74	0.21	0.17	0.47	0.12	0.07	0.11	0.10	0	0	0	0	0.18
Muscle	0.24	0.43	0.23	0.14	0.14	0.26	0.13	0.13	0.19	0.08	0.09	0	0	0
Skin	0.17	0.51	0.13	0.05	0.53	0.29	0.12	0.14	0.35	0	0	0	0	0
Bladder	0.20	0.84	0.31	0.09	0.89	0.53	0.10	0.17	0.06	0.06	0.07	0	0	0
Stomach and contents	131	0.39	0.15	0.16	0.89	0.13	0.49	0.18	0.06	0.06	2.3	0	0	0
Small intestine	17.2	49	220	72	18.5	39	5.16	21.9	3.5	2.4	0	0	0	3.35
Caecum	0.97	71.5	176	17.0	19.1	0.25	29.8	15.1	24.8	16.0	9.45	0	0.24	0.33
Feces[b]	...	28.7	32.1	14.1	0.35	6.50	0.42	3.90	37.0	22.6	49	3.25	4.85	27.2
		9.60	0.69	0.55	0.65	...	...	7.65	6.90	0.03	0.02	3.96	16.0	12.2
		0	...	...	...	...	...	...	1.57	0.50	30.0	24.5	18.2	40.6
		0.32	...	...	...	...	...	...	...	...	...	...	...	...
		...	...	...	...	...	...	...	...	...	...	...	...	...

	48 Hr.	
	A	C
Urine[b]	10.70	24.55
Plasma[c]	0	0
Lung	0	0.19
Liver	0.08	0
Brain	0.19	0
Heart	0	0
Spleen	0	0.31
Kidney	0.21	0.14
Fat	0.18	0.08
Muscle	0	0
Skin	0	0
Bladder	0	0.75
Stomach and contents	0.33	3.87
Small intestine	1.90	17.5
Caecum	12.2	45.6
Feces[b]	40.6	

[a] 2 mg./Kg. [b] Per cent of dose. [c] mcg./ml.

TABLE VI.—C^{14} TISSUE LEVELS[a] IN RATS FOLLOWING A SINGLE INTRAMUSCULAR DOSE[b] OF C^{14}-LABELED-WY-4426

	30 Min.		1 Hr.		2 Hr.		4 Hr.		6 Hr.		24 Hr.		48 Hr.	
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Injection site (% of dose)	44.6	55.5	51.5	45.2	34.9	35.7	3.04	7.24	27.0	5.15	1.91	2.41	3.52	10.2	7.2	0	0	0.19	0.2	0	0.22
Plasma[c]	1.65	1.51	1.20	0.92	1.19	1.29	0.76	0.69	0.52	0.34	0.24	0.34	0.20	0.25	0.24	0.22	0.22	0.81	0.32	0.2	0.78
Lung	1.32	1.57	2.20	0.85	1.67	1.34	1.21	1.87	1.06	0.98	0.56	0.56	0.45	0.38	0.54	0.17	0.55	0.29	0.41	0.79	0.38
Liver	7.32	6.95	4.75	4.39	7.61	8.7	3.73	2.92	3.84	1.5	1.21	1.77	1.15	1.37	0.81	0.25	0.26	0.20	0.20	0.28	0.24
Brain	0.48	0.78	0.82	0.36	0.71	0.64	0.75	0.71	0.59	0.31	0.33	0.29	0.28	0.29	0.18	0.17	0.20	0.22	0.23	0.22	0.41
Heart	1.25	1.91	1.78	0.85	1.91	1.50	1.17	1.16	1.05	0.42	0.35	0.47	0.33	0.36	0.36	0.29	0.44	0.40	0.31	0.25	0.52
Spleen	0.44	2.56	1.28	0.54	1.00	1.0	0.82	1.32	0.68	0.39	0.24	0.48	0.24	0.21	0.21	0.16	0.49	0.31	0.31	0.29	0.46
Kidney	5.60	5.9	5.3	3.40	4.39	5.57	2.47	3.14	2.36	1.35	1.1	1.62	1.39	1.08	1.23	0.38	0.50	0.46	0.29	0.46	0.46
Fat	0.30	0.93	0.46	0.17	0.44	0.33	0.74	0.89	0.47	0.07	0.16	0.41	0.11	0.09	0.13	0	0.12	0.04	0.05	0.15	0.19
Muscle	0.20	1.17	0.79	0.35	0.50	0.42	0.54	0.61	0.53	0.32	0.14	0.26	0.14	0.15	0.16	0.12	0.09	0.12	0.09	0.21	0.15
Skin	0.44	0.87	0.8	0.18	0.28	0.42	0.56	0.61	0.63	0.40	0.14	0.26	0.26	0.12	0.29	0.15	0.34	0.28	0.08	0.30	0.11
Bladder	0.65	1.21	1.93	1.41	1.98	1.28	3.28	9.33	2.95	0.60	0.4	1.52	0.36	0.29	2.41	0.38	0.44	0.42	0.20	0.25	0.23
Stomach and contents	0.60	0	2.8	2.0	0	0.5	1.2	8.0	1.8	1	0	1.1	0	0	0	0	0.9	0.7	0	0	0
Small intestine	13.0	14.4	11.0	17	27.4	20.4	57.5	33	37.4	46.1	56	24.2	8.42	33.2	7.64	0.2	1.2	12.7	0	0.45	0
Caecum	0.38	0.3	0.73	0.15	0.47	0.2	0.56	0.42	0.57	1.74	0	17.2	53.0	12.8	35.6	8	20	16.7	0.28	20.9	18.75
Urine[d]	0.16	...	...	...	0.91	1.23	5.2	...	2.35	12.1	8.14	11.9	30.0	18.9	10.2	21.6	14.0	16.7	17.2	68.7	76.5
Feces[d]	...	...	...	...	...	...	...	...	...	...	...	...	...	0	...	65.9	46.9	72.5	72.9		

[a] Values are given in mcg./Gm. [b] Equivalent to 1.92 mg./Kg. oxazepam. [c] mcg./ml. [d] Per cent of dose.

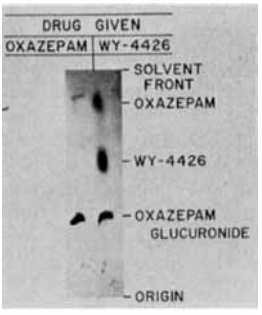


Fig. 1.—Metabolites of oxazepam and Wy-4426 found in dog urine. Developing solvent: butanol 17, ethanol 3, water 20.

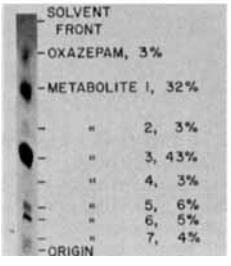


FIG. 2.—Metabolites of oxazepam found in rat urine. Developing solvent: butanol 17, ethanol 3, water 20.

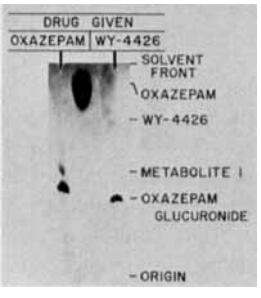


Fig. 3.—Metabolites of oxazepam and Wy-4426 found in pig urine. Developing solvent: butanol 17, ethanol 3, water 20.

TABLE VII.—DISPOSITION OF OXAZEPAM IN HUMANS

Time, Hr.	Plasma Oxazepam, mcg./ml.		Urine Oxazepam Glucuronide[b]		Feces Oxazepam[c]
	A. R.	V. M.	A. R.	V. M.	V. M.
1	...	0	...	0	...
1.5	0.39	...	...	...	...
2	1.11	0	0.18	0.12	...
4	2.08	0.68	...	2.02	...
8	1.49	0.62	...	7.30	...
10	...	...	1.56	...	...
11	...	...	...	23.06	...
24	1.19	0.47	9.51	24.81	...
28	0.88	...	...	26.50	...
31	...	...	...	...	10.9
32	0.63	...	...	29.20	...
35	...	...	...	30.93	...
48	0.36[a]	0.23	17.86	35.44	...
60	...	...	...	...	20.9

[a] This sample also contained oxazepam glucuronide equivalent to 0.75 mcg./ml. [b] Values are equivalent amounts of oxazepam expressed as cumulative per cent of dose. [c] Values are cumulative per cent of dose.

cation product ran near the solvent front, lending support to the view that it is a glucuronide. Treatment of the urine with β-glucuronidase liberated oxazepam, thus establishing the structure of the metabolite as a β-glucuronide. That this glucuronide is of the "ether" type was indicated by its failure to form a hydroxamate, its relative stability to mild acid hydrolysis, and its failure to reduce alkaline copper reagent.

Intramuscular injection of C^{14}-labeled-Wy-4426 in dogs gave rise to three radioactive substances in

urine (Fig. 1). Two of these were identified as unchanged Wy-4426 and oxazepam. When this urine was subjected to mild acid hydrolysis, Wy-4426 was converted to oxazepam, but the glucuronide was relatively unaffected. When the urine was treated with glucuronidase instead of acid, only Wy-4426 and oxazepam remained. Chromatograms of rat urine (Fig. 2) indicated more complex biotransformation of both drugs in this species; at least seven distinct metabolites were evident. Pigs appeared to metabolize oxazepam and Wy-4426 like dogs (Fig. 3); almost all the radioactivity appearing in oxazepam glucuronide. Traces of radioactivity were distributed among four or five additional metabolites.

Disposition of Oxazepam in Humans.—As seen in Table VII, plasma levels peaked at 4 hours and persisted for at least 48 hours in both subjects following a single oral 60-mg. dose. Only one plasma sample, 48 hour of A. R., contained glucuronide in addition to unchanged drug. As in dog and pig, the urine of both subjects contained only glucuronide, and the feces (V. M.) only unchanged drug.

Pharmacology and Toxicology of Lutetium Chloride

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The pharmacological and toxicological properties of lutetium chloride have been investigated. The intraperitoneal and oral LD_{50}'s were 315 and 7.1 mg./Kg., respectively. Studies of chronic toxicity showed no effect on growth and the hemogram or any internal organ changes at autopsy. Transient ocular irritation was observed, and the chemical produced extensive scar formation when applied to abraded skin. Nodules were formed at the site of intradermal injection. Pharmacological studies showed the chemical to be a depressant on all systems studied. Death resulted from cardiovascular collapse coupled with respiratory paralysis. Such effects could not be counteracted by atropinization or epinephrine injection.

RECENTLY, there has been considerable interest in the chemistry of the rare earths and their employment as alloying agents (1–3). Studies of their biological effects have not been comparable, particularly concerning the heaviest member of the series, lutetium. Spode (4) employed Lu^{177} for interstitial radiotherapy in guinea pigs. Snyder *et al.* (5) showed that intravenous injection of lutetium chloride did not increase liver lipids like cerium did. Durbin *et al.* (6) reported that 50 to 65% of an injected dose of lutetium deposited in the skeleton and the extraskeletal burden was excreted in the urine within 2 weeks. Schepers *et al.* (7–9) studied the effects of mixtures of rare earth oxides or fluorides on the lungs; although damage did occur, the exact element involved could not be pinpointed because a mixture was used. Bruce *et al.* (10) reported that the intraperitoneal LD_{50}'s for lutetium nitrate in female mice and rats were 290(259–325) and 335(294–382) mg./Kg., respectively. Inasmuch as the above reports constitute the bulk of the known effects of lutetium, a more extensive investigation of the pharmacology and toxicology of this element has been undertaken.

METHODS AND MATERIALS

The intraperitoneal LD_{50} was obtained with 60 male CFl mice and the oral LD_{50} with 50 male CFl mice. Chronic toxic effects of the element were studied by including 0.01, 0.1, and 1.0% of the compound in the diet and feeding it over a period of 90 days to three groups of CRW rats. Each group contained six males and six females. Observations were made every 2 weeks of total erythrocytes, total leukocytes, differential cell count, platelets, hemoglobin, hematocrit, and body weight. Upon completion of the study, histopathological examination was made of the heart, lung, liver, kidney, spleen, pancreas, adrenal, and small intestine. The method of Draize *et al.* (11) was used to study ocular and skin irritation in rabbits and intradermal irritation in guinea pigs. In the ocular studies two rabbits were used; each animal had one eye exposed to 0.1 ml. of a 1:1 aqueous solution of compound, while the other eye served as a control. Rabbit skin irritation studies used six animals according to the design of Draize *et al.* (11). Three guinea pigs were used for the compound in the intradermal series; the concentrations were 1:10, 1:100, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$. Histopathological examination was made of the skin areas injected with the $1:10^6$ concentration. Effects of the chemical on guinea pig ileal strips bathed in Locke-Ringers solution were studied in a thermostatically regulated 25-ml. bath using the Trendelenburg method (12). Studies were also made on the isolated rabbit ileum in the presence of either 2.5 mcg. of acetylcholine or 0.5 mcg. of nicotine. Ten cats of both sexes, weighing 2.3–4.18 Kg., were anesthetized with 0.5 ml./Kg. of Dial-urethane intraperitoneally. A six-channel Offner Dynagraph with Statham transducers was used to record carotid arterial pressure, respiration, nictitating membrane contraction, ECG lead II, femoral arterial pressure, and femoral arterial flow. The latter was obtained with a 25-ml. Shipley-Wilson flowmeter (13). Preganglionic stimulation of the cervical sympathetic fibers and the contralateral vagus fibers was accomplished with a Grass model S-4 stimulator at

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8 v./10 seconds. Two hours were allowed to elapse prior to drug administration. Intravenous doses of the drug used were: lutetium, 1 to 40 mg./Kg.; epinephrine, 5 mcg./Kg.; acetylcholine, 5 mcg./Kg.; histamine, 2 mcg./Kg.; and atropine, 2 mg./Kg. All injections were made at a constant volume of 1 ml. Where appropriate, the results were analyzed statistically by the Litchfield-Wilcoxon method (14), or standard errors were calculated.

Acute Toxicity.—The symptoms of acute toxicity were writhing, ataxia, labored respiration, walking on toes with back arched, and sedation. The first deaths occurred within 24 hours, and the peak was reached at 48 hours. Throughout the 7-day period of observation, the animals were unthrifty. The intraperitoneal LD_{50}/7 days for lutetium chloride was 315 (267–372) mg./Kg. with a slope value of 1.54 (1.11–2.13). The oral LD_{50}/7 days was 7100 (6630–7590) mg./Kg. with a slope value of 1.19 (1.03–1.38).

Chronic Toxicity.—Male and female rats ingesting various levels of lutetium chloride in the diet showed no differences in growth rate from the controls (Fig. 1). Moreover, the chemical had no significant effect on the hematology of the animals. A normal growth pattern would account for the increases in both the cellular elements and the hemoglobin. In fact, the values given in Table I are in good agreement with those listed for the rat by Gardner (15). No data are given for the differential cell counts because there was no significant difference between the medicated and the control groups. At autopsy, the internal organs of all groups of rats appeared normal; there were no outward signs of damage from ingestion of lutetium chloride for 12 weeks. Histopathological examination of the heart, lung, liver, kidney, spleen, pancreas, adrenal, and small intestine revealed no changes that could be attributed to ingestion of lutetium chloride.

Ocular Irritation.—Instillation of 0.1 ml. of a 1:1 solution of lutetium chloride into the eyes of two albino rabbits produced no effect upon the cornea. However, within 1 hour the conjunctiva was a diffuse beefy red, chemosis had caused swelling of the lids closing the eyes, and a profuse discharge had moistened the lids and a considerable area surrounding the eyes. At 24 hours the maximum irritation index of 20 was still present, and minute ulcers appeared. The irritation index was reduced to 6 in 1 week, but complete healing required 2 weeks. There was no evidence of residual damage.

Skin Irritation.—No reaction was observed when 0.5 Gm. of crystalline lutetium chloride was applied to intact rabbit skin. The response of abraded skin was very severe, showing a maximum irritation index of 8 at 24 hours. No changes occurred in the irritation index up to 14 days. However, healing with the formation of scars 25 to 30 mm. in diameter occurred at 35 days. Intradermal injection of lutetium chloride at concentrations of 1:10, 1:100, and 1:1000 produced necrosis within 1 hour and an irritation index (erythema plus edema) of 8 to 3, respectively. These reactions persisted for 7 days; complete healing with epilation and scar formation did not occur for 5 weeks. The concentrations of $1:10^4$ to $1:10^6$ gave a 24-hour irritation index of 2 and healing with nodule formation at 46 days. Histopathological examination of these nodules revealed the presence of crystalline

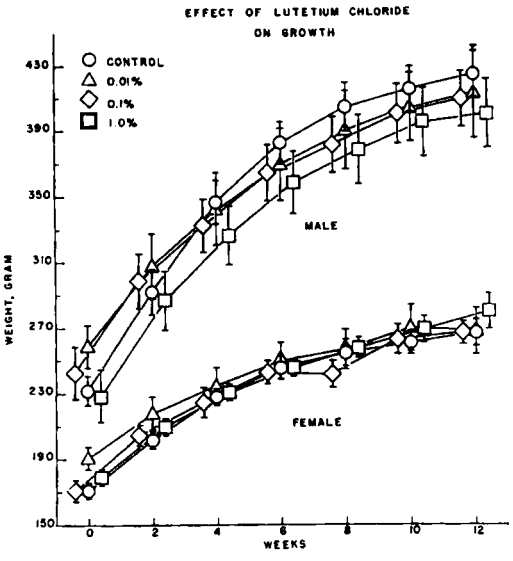


Fig. 1.—Growth of male and female CRW rats on feeding various levels of lutetium chloride in the diet for 90 days. Bars signify the standard errors. Each group contained six animals for each dosage level.

deposits of unknown composition. Foreign body giant cells and histocytes surrounded the crystals, while fibroblasts and granulation tissue extended into the deposits. Such cellular changes were not found in control skin sections from the same animals. Inasmuch as necrosis was not observed at these concentrations, the response does not appear to be related to the hydrogen ion concentration of the injected solutions. The pH values were 5.61 to 6.04. It seems more likely that there was a reaction between the lutetium and tissue phosphate to produce insoluble lutetium phosphate; however, the small number and size of the crystals prevented a more quantitative analysis.

Effects on Isolated Ileum.—In the dosage range of 10 to 40 mg., lutetium chloride produced an increasing depression of intestinal tonus and contractility of the rabbit ileum ending in a nonreversible paralysis. This depressant effect counteracted the spasmogenic effect of both acetylcholine and nicotine. The antispasmodic ED_{50}'s were 0.6(0.5–0.7) mg./ml. and 0.5(0.3–0.8) mg./ml., respectively, with slope values of 1.33(0.91–1.94) and 2.22 (0.96–5.13). A similar depression also occurred with the Trendelenburg guinea pig enteric ganglia preparation, where the ED_{50} figures for blocking the circular and longitudinal muscular contractions were 0.5(0.4–0.7) mg./ml. and 0.3(0.2–0.4) mg./ml., respectively. Although this would seem to indicate that lutetium chloride had ganglionic blocking properties, such activity appears unlikely because the compound failed to block the cat superior cervical ganglion preparation.

Pharmacological Effects.—No pharmacological effects were observed on the cat in the dosage range of 1 to 10 mg./Kg. When death did not occur at 20 mg./Kg., there was a transient hypotension of 10 to 15 mm. Hg with a concomitant decrease in peripheral blood flow. Complete cardiovascular collapse, coupled with respiratory paralysis, was

TABLE I.—HEMATOLOGIC EFFECTS OF LUTETIUM

Dosage	Sex	Erythrocytes, mm.³ × 10⁶ 0 Wks.	Erythrocytes, mm.³ × 10⁶ 12 Wks.	Leukocytes, mm.³ × 10³ 0 Wks.	Leukocytes, mm.³ × 10³ 12 Wks.	Hematocrit, Vol. % 0 Wks.	Hematocrit, Vol. % 12 Wks.	Hemoglobin, Gm. % 0 Wks.	Hemoglobin, Gm. % 12 Wks.	Platelets, mm.³ × 10³ 0 Wks.	Platelets, mm.³ × 10³ 12 Wks.
Control	♂	7.19 ± 0.22[a]	8.37 ± 0.28	25.43 ± 0.22	20.89 ± 0.24	41 ± 0.88	46 ± 0.88	10.9 ± 0.77	13.7 ± 0.50	4.11 ± 0.009	4.35 ± 0.004
	♂♂	5.98 – 8.14[b]	7.45 – 9.80	16.8 – 36.4	12.8 – 32.2	37 – 45	42 – 50	10.0 – 11.9	11.1 – 15.4	3.04 – 5.20	3.80 – 4.86
	♀	6.88 ± 0.35	8.02 ± 0.30	25.71 ± 0.37	19.93 ± 0.21	43 ± 0.21	46 ± 1.07	11.9 ± 1.20	13.5 ± 0.39	4.66 ± 0.012	4.18 ± 0.008
	♀	5.62 – 8.32	6.30 – 9.55	13.6 – 45.4	13.2 – 30.8	39 – 46	38 – 48	9.7 – 13.7	10.7 – 14.4	3.40 – 7.00	3.04 – 5.44
Lutetium 0.01%	♂	7.54 ± 0.45	9.39 ± 0.48	24.33 ± 0.32	19.57 ± 0.23	43 ± 1.28	50 ± 0.92	12.0 ± 0.69	15.2 ± 0.25	5.09 ± 0.023	5.27 ± 0.016
	♂♂	6.18 – 8.82	7.10 – 10.35	12.0 – 36.6	13.8 – 29.8	39 – 48	47 – 52	10.0 – 14.3	14.4 – 16.1	3.56 – 7.80	4.00 – 6.80
	♀	6.95 ± 0.36	8.86 ± 0.48	18.20 ± 0.20	13.12 ± 0.11	42 ± 1.00	46 ± 0.49	12.1 ± 0.40	14.5 ± 0.22	3.93 ± 0.009	5.18 ± 0.006
	♀	6.18 – 8.48	7.45 – 10.10	10.0 – 24.4	10.0 – 16.6	38 – 45	45 – 48	11.0 – 13.6	13.9 – 15.1	3.05 – 4.92	4.68 – 5.80
Lutetium 0.1%	♂	6.87 ± 0.40	9.36 ± 0.33	23.43 ± 0.42	22.33 ± 0.30	43 ± 0.99	47 ± 0.88	12.2 ± 0.27	14.4 ± 0.27	3.70 ± 0.006	4.37 ± 0.008
	♂♂	5.08 – 7.74	8.40 – 10.40	12.6 – 42.2	15.8 – 36.2	38 – 45	44 – 50	11.0 – 12.8	13.2 – 14.9	3.16 – 4.48	3.44 – 5.00
	♀	6.97 ± 0.39	8.81 ± 0.37	23.20 ± 0.23	23.68 ± 0.66	43 ± 1.09	47 ± 1.81	12.9 ± 0.60	14.8 ± 0.51	3.96 ± 0.004	4.87 ± 0.015
	♀	5.16 – 7.80	8.20 – 10.25	18.8 – 34.4	9.8 – 48.6	40 – 48	43 – 54	10.8 – 15.0	13.7 – 16.4	3.44 – 4.44	3.42 – 5.88
Lutetium 1.0%	♂	6.43 ± 0.10	8.46 ± 0.25	30.40 ± 0.50	25.00 ± 0.28	41 ± 1.14	44 ± 0.60	11.5 ± 0.25	12.8 ± 0.45	4.43 ± 0.014	4.56 ± 0.014
	♂♂	6.18 – 6.84	7.80 – 9.30	14.2 – 43.8	16.4 – 33.4	37 – 45	42 – 46	10.4 – 12.0	11.3 – 14.5	3.40 – 6.44	3.40 – 6.36
	♀	6.99 ± 0.46	9.07 ± 0.36	19.67 ± 0.16	13.33 ± 0.16	42 ± 1.18	45 ± 2.55	11.7 ± 0.50	13.5 ± 0.92	5.30 ± 0.022	5.37 ± 0.017
	♀	5.44 – 8.44	7.40 – 9.70	10.7 – 34.4	9.8 – 19.8	38 – 46	33 – 50	9.9 – 13.3	9.0 – 14.8	3.64 – 8.48	4.04 – 7.04

[a] Mean ± standard error. [b] Range.

produced by 20 mg./Kg. in five animals and by 40 mg./Kg. in the other five. The respiration was not affected prior to exitus. Terminal electrocardiographic changes included inversion of the QRS complex, transient ventricular fibrillation, 2:1 and 3:1 heart block, diphasic T-waves, inverted T-waves, increase in height of T-wave, and high takeoff of T-waves. Injection of epinephrine caused QRS complexes of low amplitude and an absence of the T-wave. In the presence of atropine, epinephrine induced a fatal ventricular fibrillation. Within the dosage range employed, lutetium chloride had no effect on the pharmacological responses to acetylcholine, histamine, or vagal stimulation. Moreover, it had no effect on transmission in the superior cervical ganglion or on contraction of the nictitating membrane. None of the above effects of lutetium chloride could be modified by atropinization, and the cardiovascular collapse was not counteracted by epinephrine.

DISCUSSION

Lutetium chloride is less toxic—both acutely and chronically—than gadolinium and samarium (16) or niobium (17) or hafnium (18) and about equal in toxicity to scandium (19), terbium, thulium, and ytterbium (20), and praseodymium and neodymium (21). The ocular irritation produced by lutetium was similar to and of the same duration as that produced by the other rare earths studied (16, 19–21). The same was true for its ability to produce skin irritation and nodule formation in the dermal layers (22). The depressant effects on other biological systems was similar to the observations of Mines (23) concerning the frog heart. The mode of death—respiratory paralysis coupled with cardiovascular collapse—was identical to that observed with the other rare elements (16–21). Precautionary measures to be instituted in handling lutetium chloride should include respirators and protective clothing.

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Factors Influencing Stability of Prednisolone in Aqueous Solution

By THOMAS O. OESTERLING and DAVID E. GUTTMAN*

An investigation was made of some of the factors which influence the rate of disappearance of prednisolone from aqueous solution. The rate exhibited a marked dependency on buffer concentration. Incorporation of a sequestering agent into the solutions eliminated this dependency and indicated that trace-metal impurities which were present in the buffer reagents were catalytically involved in the degradation. Rate constants were determined over a wide range of pH in borate and phosphate buffers and in the presence and absence of disodium ethylenediaminetetraacetic acid. The rate of the apparent metal-catalyzed reaction was pH independent above pH 7 and below pH 5 and exhibited a first-order dependency on hydroxide-ion in the intermediate range. In the presence of sequestering agent, the rate of reaction was strongly dependent on hydroxide-ion concentration above pH 8 but exhibited only slight dependency at lower pH values.

A NUMBER OF studies has demonstrated that prednisolone and related corticosteroids are susceptible to degradative reactions which involve the 17-dihydroxyacetone side-chain (1–4). Transformations and elimination of the side-chain have been shown to occur both in the presence and absence of oxygen. Autoxidation, however, appears to be the mode of destruction which is most likely to be responsible for stability problems in drug products. The involvement of trace metals in catalyzing the autoxidation is an obvious possibility and has been reported in the literature. For example, Caspi *et al.* (5) effected an oxidative cleavage of the dihydroxyacetone moiety of cortisone with lead tetraacetate to therapeutically inactive products. Similarly, trace amounts of copper are actively involved in the reactivity of the C-17 function. Lewbart and Mattox (6) reported that trace amounts of copper, present in glassware, caused destruction of cortisone and related steroids. The steroidal destruction was prevented by the addition of ethylenediaminetetraacetic acid. Other workers have shown that 20-keto-21-hydroxy steroids, such as prednisolone, are readily oxidized to the corresponding glyoxals (20-keto-21-aldehydes) in the presence of cupric acetate (7–10).

The objectives of this investigation were to quantitate an observation that the trace metal content of chemicals commonly employed to formulate buffers was sufficient to accelerate markedly the destruction of prednisolone in solution and to investigate stability characteristics of the steroid in the presence and absence of a sequestering agent.

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EXPERIMENTAL

Materials.—Prednisolone was crystallized from a hydro-alcoholic solution several times and dried at 110°, m.p. 233–234°, uncorrected. Sequestrene Na_2 [disodium salt of ethylenediaminetetraacetic acid (EDTA)] was obtained from the Alrose Chemical Co. Assay reagents were 2,3,5-triphenyltetrazolium chloride (Mann Research Laboratories, Inc.) and tetramethylammonium hydroxide (Eastman Organic Chemicals) as a 10% aqueous solution. Buffers were prepared with reagent grade chemicals and double-distilled deionized water and were adjusted to the desired pH using a Beckman model G pH meter. Solutions containing sequestering agent were adjusted to the desired pH after the concentration had been made 0.1% with respect to sequesterene Na_2. A constant ionic strength of 0.2 was maintained by adding KCl to the borate systems utilized in studying the effect of buffer concentration on rate of degradation.

Procedure.—A 100-ml. low-actinium volumetric flask containing approximately 98 ml. of appropriate buffer was placed in a Sargent Thermonitor constant temperature bath. After thermal equilibrium had been attained, 25 mg. of prednisolone in 1 ml. of absolute methanol was added, and the contents of the flask were mixed. The solution was made to volume with buffer and again rendered homogeneous. At appropriate intervals 5-ml. samples were withdrawn and added to 5 ml. of 0.2 *N* acetic acid to quench the reaction. Four-milliliter aliquots of each sample were extracted with four successive 10-ml. portions of chloroform. The chloroform was removed *in vacuo*, and the residue was reconstituted with 20 ml. of absolute ethanol. The concentration of undergraded prednisolone in the sample was determined by subjecting 10 ml. of the reconstituted ethanolic solution to a tetrazolium assay (11).

RESULTS AND DISCUSSION

This study of factors which influence the stability of corticosteroids was motivated primarily by an observation that the rate of disappearance of pred-

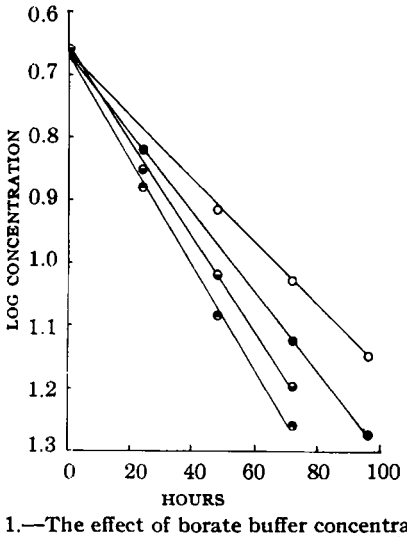


Fig. 1.—The effect of borate buffer concentration on the rate of prednisolone degradation at 35° and ionic strength 0.2. Key: ○, 0.05 *M*; ●, 0.01 *M*; ◒, 0.15 *M*; ◓, 0.20 *M*.

nisolone from buffered aqueous solutions, prepared with double-distilled deionized water, exhibited a dependency on the concentration of buffer, in spite of the fact that pH and ionic strength were maintained constant. This effect is illustrated in Fig. 1, where some representative results are plotted and show that the first-order disappearance of prednisolone occurred much more rapidly from solutions which contained a relatively high concentration of borate buffer than from less concentrated solutions. This effect was noted at a number of different pH values and suggested, at least initially, the possibility that general acid and/or general base catalysis was responsible. A more definitive study, which is illustrated in Fig. 2, ruled out this possibility. This plot summarizes the results of a study in which first-order rate constants were determined as a function of buffer concentration in two sets of systems, which differed only in that one contained 0.1% of the disodium salt of EDTA, while the other contained no sequestering agent. It may be seen that in the presence of EDTA, the observed rate was independent of the concentration of buffer. In its absence the rates were significautly faster, dependent on the concentration of buffer, and the rate constant appeared to be approaching an asymptotic value at high concentrations of buffer. On the basis of this evidence, it was concluded that components of the buffer had no catalytic influence *per se*, but that they served as carriers of trace metal contaminants which accelerated the decomposition.

The influence of pH on the stability of prednisolone in borate buffer in the presence and absence of EDTA is graphically summarized in Fig. 3, where rate constant is plotted as a function of pH. In reference to this plot and Fig. 4, it should be pointed out that there is some uncertainty about the accuracy of the rate constants for systems which did not contain EDTA. In such systems the disappearance of steroid occurred quite rapidly, and the scatter of experimental points in the semilog plots precluded precise graphical determination of rate constants. In contrast, excellent fits were obtained in equivalent plots for systems which contained EDTA.

The shape of the curves indicates that, both in the presence and in the absence of EDTA, the rate of disappearance of prednisolone was first order with respect to hydroxide-ion at higher pH values but less than first order at the lower values studied. A comparison of the two curves shows that the sequestering agent had little effect on rate in systems maintained at the lower pH values. At higher pH values the disappearance of prednisolone occurred much more rapidly from systems which were not protected by sequestering agent. The degree of protection, reflected by the vertical distance between the two curves, increases with an increase in pH. The nature of the curves suggests that a constant difference is approached at higher pH values.

The pH profile was extended to a lower pH region by investigating the stability in phosphate buffers. A higher temperature (70°) was utilized here to increase the rate for the sake of expediency. The results are shown in Fig. 4. The observed influence of pH is rather unusual and indicates interesting changes in the nature of the involvement of hydroxide-ion in the degradative reactions. In the EDTA-containing system, the rate exhibited only a relatively small pH dependency over a fairly wide range of pH 5–8. Above pH 8, the rate is much more dependent on pH; the curve suggests that in this pH region the order of the degradation with respect to hydroxide-ion changes from zero order

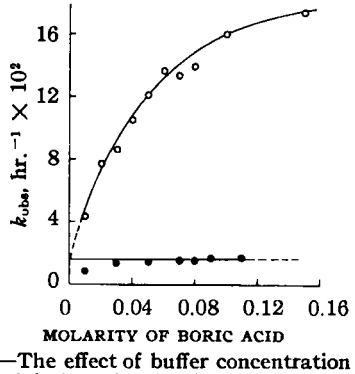


Fig. 2.—The effect of buffer concentration on the rate of prednisolone degradation in the presence and absence of sequestrene Na_2 at 30°, pH 10, and ionic strength 0.2. Key: ○, no EDTA; ●, 0.1% EDTA.

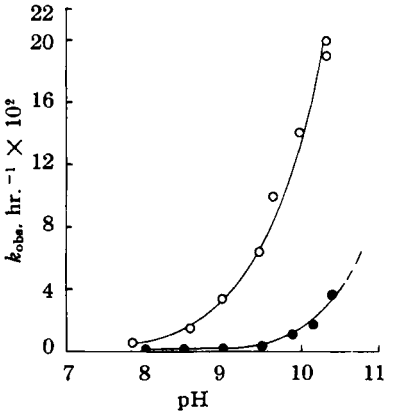


Fig. 3.—The effect of pH on the rate of degradation of prednisolone in the presence and absence of sequestrene Na_2 at 35° in 0.1 *M* borate buffers. Key: ○, no EDTA; ●, 0.1% EDTA.

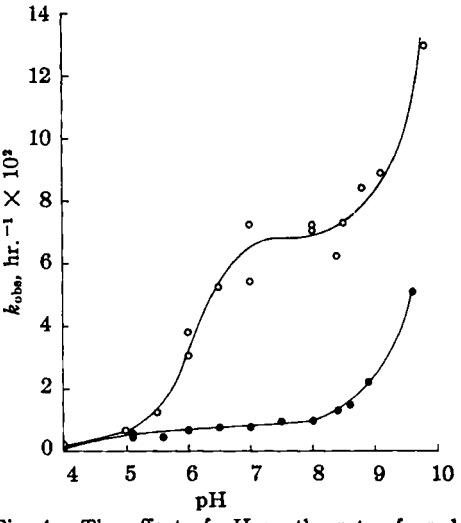


Fig. 4.—The effect of pH on the rate of prednisolone degradation in the presence and absence of sequestrene Na_2 at 70° in 0.1 *M* phosphate buffers. Key: ○, no EDTA; ●, 0.1% EDTA.

to first order. In the absence of EDTA, the order with respect to hydroxide-ion appears to be first at lower pH values, zero at intermediate values, and first at the higher extreme.

An insight into the nature of the metal-catalyzed reaction was obtained by a treatment of the data which are illustrated in Fig. 5. Here rate constants for the metal-catalyzed degradation were plotted as a function of pH. These constants were obtained by taking the difference between rate constants determined in the absence (k_0) and presence (k_s) of EDTA at the same pH. The differences were interpolated from the curves of Fig. 4.

Below pH 5, incorporation of EDTA into the solutions had little effect on the velocity of disappearance of prednisolone. This might have resulted from a reduction in the efficiency of the sequestering agent at lower pH values. Thus, metal catalysis might have been operant in systems which contained EDTA. Alternatively, and possibly more likely, the observed instability in both systems in this pH region was primarily due to reactions which were not dependent on metal catalysis. As the pH was increased, the velocity of the metal-catalyzed reaction increased in a manner which suggested first-order dependency on hydroxide-ion. With subsequent increases in pH, the order changed from first to zero, so that at higher pH values the rate of the metal-catalyzed reaction was essentially independent of pH.

It is interesting to note that the influence of pH on the metal-catalyzed reaction in phosphate buffer was somewhat different from that in borate buffer. At the lower pH values investigated in borate buffer (*i.e.*, below approximately 8.5), only small differences in rate constants were obtained between solutions which contained EDTA and those which did not. In contrast, the differences were quite large in phosphate systems throughout this pH range. No clear-cut explanation can be offered to account for this effect. The different behavior might be due to the difference in temperature between the two studies. Differences in the nature and concentration of trace metals also might be a causative factor. Further experimentation is required to elucidate this observation.

It has been reported that at least three products are formed by at least three parallel pseudo first-order reactions when 17-dihydroxyacetone steroids are subjected to an aqueous alkaline environment (11). The presence of trace metals in such systems increases the complexity of the degradation, and the precise role of the metals is unknown. The metals may function by catalyzing any number of the previously reported reactions, or they may increase the observed rate constant by providing a new degradative route for the steroid. The results of this study lend evidence to the postulation that trace metals are involved primarily in the oxygen-dependent degradative route of the steroid. This evidence manifests itself in that the hydroxide-ion dependency of the metal-catalyzed reaction of this study is similar to the hydroxide-ion dependency of the oxygen-dependent reaction described by Guttman and Meister (11). On the other hand, the fact that products of both the oxygen-dependent and oxygen-independent reactions have been isolated from the metal-catalyzed degradation of 17-dihydroxyacetone steroids (5, 12) suggests that catalytic amounts of metals are involved in more than one of the degradative reactions. It is possible that the metals function as catalysts for all of the reactions or are involved in an initial step resulting in the formation of an intermediate which then decomposes to the reported products.

Lewbart and Mattox (13) reported that the glyoxals obtained by cupric acetate oxidation of 17-dihydroxyacetone steroids are unstable and undergo rearrangement both in the presence of alcoholic cupric acetate and aqueous alkali. The cupric acetate catalyzed rearrangements in alcoholic media are many times slower than those in basic aqueous systems and are futher retarded by the addition of water. Our study is in agreement with these results. The nonmetal-catalyzed reaction exhibited a strong hydroxide-ion dependency at high pH values (Fig. 4), whereas the metal-catalyzed reaction was pH independent in this range (Fig. 5). Thus, at a very high concentration of hydroxide ion, the contribution of the metal-catalyzed reaction to the ob-

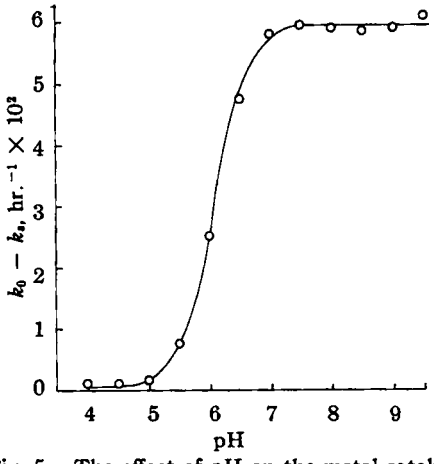


Fig. 5.—The effect of pH on the metal-catalyzed degradation of prednisolone at 70° in 0.1 *M* phosphate buffers.

served rate constant would be small. It should be pointed out, however, that trace metals markedly accelerate the degradation of corticosteroids in the pharmaceutically important pH range. The presence of these impurities should be a prime factor in steroid stability considerations.

SUMMARY

1. The decomposition of prednisolone in aqueous solution was markedly accelerated by contaminants present in buffer reagents. Evidence has been presented which strongly indicates that the contaminants were trace metals. However, no attempt was made to identify the metals or to determine the nature of their influence on the degradative reactions.

2. Addition of a sequestering agent provided a method to isolate and quantitate the rate of the apparent metal-catalyzed reaction.

3. The rate of the apparent metal-catalyzed reaction was pH independent above pH 7 and below pH 5 and exhibited a first-order dependency on hydroxide-ion in the intermediate range.

4. In the presence of sequestering agent, the reaction rate was strongly dependent on hydroxide-ion concentration above pH 8 and only slightly dependent at lower pH values.

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Ultraviolet Spectrophotometric Estimation of Arylglycolate Drugs

By LESTER CHAFETZ*

Oxidation of arylglycolic acids to carbonyl compounds affords ultraviolet chromophores which make possible the spectrophotometric estimation of the acids with greatly increased sensitivity and selectivity. Oxidative spectrophotometric methods for representative arylglycolic acids were studied; the procedures were applied to the estimation of some arylglycolate drugs in dosage forms.

SALTS OF mandelic acid have been employed as urinary antiseptics, and several arylglycolate esters are used in medicine for antispasmodic, mydriatic, psychotropic, and other pharmacological activities. A study of spectrophotometric procedures was carried out using mandelic acid, benzilic acid, α-cyclopentylphenylglycolic acid, and α-cyclopentyl-2-thiopheneglycolic acid. The application of the procedures to the assay of representative arylglycolate drugs is reported.

Phenylglycolic acids and their esters have benzenoid ultraviolet spectra which are neither sufficiently intense to afford sensitivity in determination nor sufficiently characteristic to serve for identification (1). The majority of the analytical methods reported for arylglycolate drugs are based on the presence of a tertiary amine or quaternary ammonium function in the alcohol moiety, and few of the methods are selective for unhydrolyzed drug (2–8).

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A number of workers have described procedures for oxidation of arylglycolic acids with decarboxylation to carbonyl compounds. The reagents used included "zinc manganite" (9–11), sodium bismuthate (12), *N*-bromosuccinimide (13), lead tetraacetate (14), periodate (14–16), and ceric sulfate (17–19). Some of these oxidative procedures were made the basis of titrimetic assay methods.

Spectrophotometry of the carbonyl compound products of the oxidation of arylglycolic acids appeared to offer a promising means for analysis of arylglycolate drugs. The increase in ultraviolet absorption effected by thus obtaining a carbonyl group in conjugation with an aromatic ring is evident in a comparison of the molar absorptivities, ϵ, of the acids with values reported in the literature for the carbonyl compounds. Mandelic acid has $\epsilon_{max.}$ 212 and benzaldehyde $\epsilon_{max.}$ 14,000 (20); benzilic acid has $\epsilon_{max.}$ 538 and benzophenone $\epsilon_{max.}$ 19,500 (21); and α-cyclopentylphenylglycolic acid has $\epsilon_{max.}$ 276 compared with $\epsilon_{max.}$ of 12,900 (22) for cyclopentyl phenyl ketone.

served rate constant would be small. It should be pointed out, however, that trace metals markedly accelerate the degradation of corticosteroids in the pharmaceutically important pH range. The presence of these impurities should be a prime factor in steroid stability considerations.

SUMMARY

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Ultraviolet Spectrophotometric Estimation of Arylglycolate Drugs

By LESTER CHAFETZ*

Oxidation of arylglycolic acids to carbonyl compounds affords ultraviolet chromophores which make possible the spectrophotometric estimation of the acids with greatly increased sensitivity and selectivity. Oxidative spectrophotometric methods for representative arylglycolic acids were studied; the procedures were applied to the estimation of some arylglycolate drugs in dosage forms.

SALTS OF mandelic acid have been employed as urinary antiseptics, and several arylglycolate esters are used in medicine for antispasmodic, mydriatic, psychotropic, and other pharmacological activities. A study of spectrophotometric procedures was carried out using mandelic acid, benzilic acid, α-cyclopentylphenylglycolic acid, and α-cyclopentyl-2-thiopheneglycolic acid. The application of the procedures to the assay of representative arylglycolate drugs is reported.

Phenylglycolic acids and their esters have benzenoid ultraviolet spectra which are neither sufficiently intense to afford sensitivity in determination nor sufficiently characteristic to serve for identification (1). The majority of the analytical methods reported for arylglycolate drugs are based on the presence of a tertiary amine or quaternary ammonium function in the alcohol moiety, and few of the methods are selective for unhydrolyzed drug (2–8).

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A number of workers have described procedures for oxidation of arylglycolic acids with decarboxylation to carbonyl compounds. The reagents used included "zinc manganite" (9–11), sodium bismuthate (12), *N*-bromosuccinimide (13), lead tetraacetate (14), periodate (14–16), and ceric sulfate (17–19). Some of these oxidative procedures were made the basis of titrimetic assay methods.

Spectrophotometry of the carbonyl compound products of the oxidation of arylglycolic acids appeared to offer a promising means for analysis of arylglycolate drugs. The increase in ultraviolet absorption effected by thus obtaining a carbonyl group in conjugation with an aromatic ring is evident in a comparison of the molar absorptivities, ϵ, of the acids with values reported in the literature for the carbonyl compounds. Mandelic acid has $\epsilon_{max.}$ 212 and benzaldehyde $\epsilon_{max.}$ 14,000 (20); benzilic acid has $\epsilon_{max.}$ 538 and benzophenone $\epsilon_{max.}$ 19,500 (21); and α-cyclopentylphenylglycolic acid has $\epsilon_{max.}$ 276 compared with $\epsilon_{max.}$ of 12,900 (22) for cyclopentyl phenyl ketone.

EXPERIMENTAL

A study of the oxidation of arylglycolic acids was carried out using sodium metaperiodate and ceric sulfate solutions. The spectra of the carbonyl compounds were measured in hydrocarbon solvents to eliminate interferences from polar compounds and to provide the most characteristic spectra. The extent of formation of carbonyl compounds was estimated from the relation % carbonyl product = 100 ϵ_A/ϵ_C, where ϵ_A = molar absorptivity calculated from the concentration of the arylglycolic acid and its absorbance after oxidation, and ϵ_C = molar absorptivity, literature value, of carbonyl compound which results from the oxidation.

Reagents and Supplies.—A 2% solution of sodium metaperiodate (Matheson, Coleman & Bell No. 879) and a 0.2 *M* ceric sulfate solution in *N* sulfuric acid, prepared by dissolving 12.6 Gm. of ceric ammonium sulfate (Fisher C-249) in 50 ml. of distilled water and 3 ml. of concentrated sulfuric acid and diluting to 100.0 ml., were used as oxidants. Hexane (Phillips high purity grade) and isooctane (Phillips pure grade 2, 2,4-trimethylpentane) were satisfactory as spectrophotometric solvents. Mandelic acid (Matheson, Coleman & Bell No. 5474), benzilic acid (Eastman No. 36), α-cyclopentylphenylglycolic acid,[1] and α-cyclopentyl-2-thiopheneglycolic acid[2] were used as test compounds. Homatropine methylbromide U.S.P., poldine methylsulfate,[3] oxyphencyclimine hydrochloride,[4] and penthienate bromide[2] were used as standards for tablet analysis. Homatropine methylbromide tablets[5] and locally purchased tablets of cyclandelate, poldine methylsulfate, oxyphencyclimine hydrochloride, and penthienate bromide N.F. were used to test the method.

Equipment.—A Bausch & Lomb spectronic 505 ratio recording spectrophotometer and a Beckman DU spectrophotometer were used in this study. The wavelength calibration was the same for both instruments, and absorbance measurements agreed within 1%. All absorbance measurements were made using 1-cm. silica cells. Other equipment included a Burrell model BB wrist-action mechanical shaker, a Thelco model 16 oven, and standard laboratory glassware.

Development of Periodate Oxidation Procedure.—Preliminary trials were conducted using mandelic acid as the test compound. The observation of Courtois (16) that elevated temperature was necessary to effect measurable oxidation of the acid to benzaldehyde was confirmed. The acidity of the reaction mixture was an important variable; no yield of benzaldehyde could be detected when saturated sodium bicarbonate was added, but addition of 3 *N* hydrochloric acid resulted in good yields. Heating mixtures of 2.0 ml. of 5 mg./100 ml. mandelic acid, 2.0 ml. of periodate, and 0.1 ml. of 3 *N* acid in glass-stoppered tubes in a boiling water bath for varying periods of time, cooling, and extracting with 20.0 ml. of hexane afforded an apparent 94% yield of benzaldehyde in 15 minutes and 85% in 45 minutes. It was concluded that the aldehyde was further oxidized. This problem was circumvented by including 20.0 ml. of isooctane in the reaction mixture to extract the product as it formed. Isooctane boils at boiling water temperature, and spectra in isooctane are identical with those in hexane. The procedure was applicable to benzilic acid, α-cyclopentylphenylglycolic acid, and α-cyclopentyl-2-thiopheneglycolic acid and to their ester drugs after saponification. Low or negligible amounts of carbonyl product were obtained by reaction with unhydrolyzed ester drugs.

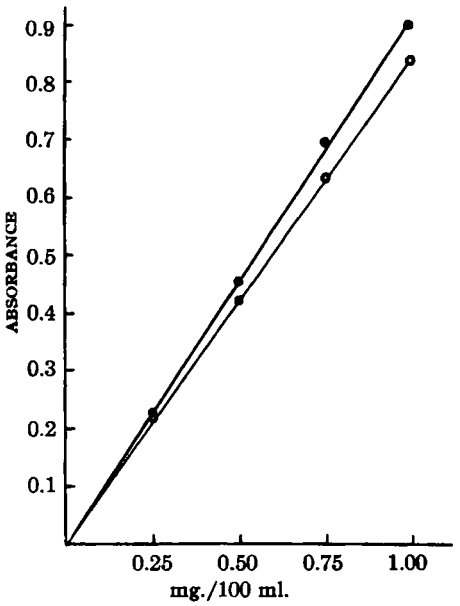


Fig. 1.—Beer's law adherence of mandelic acid oxidized to benzaldehyde. Key: ●, by 0.1 *M* ceric sulfate; ○, by 0.078 *M* ceric sulfate.

Development of Ceric Sulfate Oxidation Procedure.—Initial trials were based on the findings of Mathur and Rao (19) that oxidation of mandelic acid is activated by light. A mixture of 2.0 ml. of 5 mg./100 ml. mandelic acid, 2.0 ml. of 0.1 *M* ceric sulfate titrant (23), and 20.0 ml. of hexane was shaken 15 minutes in the light supplied by a 200-w. tungsten filament bulb at a distance of 20 cm. Benzaldehyde was evident in the spectrum of the filtered hexane phase in quantitative yield. The yield was not diminished, however, when light was totally excluded by wrapping the reaction flask in aluminum foil. Subsequent experiments were conducted in room light.

The effect of extracting the carbonyl product as it formed was determined by oxidizing benzilic acid in the presence and in the absence of hydrocarbon solvent. Unlike benzaldehyde, benzophenone would be expected to be stable under oxidizing conditions. The yield of benzophenone in the absence of extractant was 77% of that obtained when extractant was included in the reaction mixture.

Use of a 0.08 *M* ceric sulfate reagent gave 90% of the benzaldehyde obtained when mandelic acid was oxidized with 0.1 *M* reagent, but Beer's law was followed with both concentrations as shown in Fig. 1. Similarly, a quantitative amount of benzophenone was produced when benzilic acid was oxidized with 0.1 *M* reagent, but 94% of that amount was evident with an 0.08 *M* concentration of

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TABLE I.—COMPARISON OF SPECTRAL DATA FOR OXIDIZED ACIDS WITH LITERATURE DATA FOR THE CARBONYL PRODUCTS

Oxidized Acid	Solvent	$\lambda_{max.}$, mμ	ε	Carbonyl Compd.	Solvent	$\lambda_{max.}$, mμ	ε	Ref.
Mandelic	Hexane	242	13,200	Benzaldehyde	Hexane	242	14,000	(20)
		248	11,100			248	12,500	
		280	810			280	1,400	
		289	610			289	1,200	
Benzilic	Isooctane	248	19,800	Benzophenone	Isooctane	248	19,500	(21)
						276s	2,460	
α-Cyclopentyl-phenylglycolic	Isooctane	239	15,500	Cyclopentyl phenyl ketone	Isooctane	238	12,900	(22)
		278	1,000			278	930	
		286	830			286	760	
α-Cyclohexyl-phenylglycolic[a]	Isooctane	239	13,100	Cyclohexyl phenyl ketone	Isooctane	239	12,000	(22)
		279	870			278	915	
		287	630			287	710	
α-Cyclopentyl-2-thiopheneglycolic	Isooctane	258	9,450	Methyl[b] 2-thienyl ketone	Isooctane	256.5	9,800	(24)
		275	7,700			273	7,250	

[a] Saponified oxyphencyclimine HCl was used. [b] Data for cyclopentyl 2-thienyl ketone were not available.

oxidant. To assure maximum sensitivity and to obviate standardizing the reagent, a 0.2 *M* ceric reagent was employed.

The procedure was applicable to all of the arylglycolic acids used in the study. Approximately quantitative yields were obtained.

Selectivity of the Oxidative Spectrophotometric Procedure.—Because ceric sulfate is a reagent with a high oxidation potential, it was of some interest to try the oxidative spectrophotometric procedure on a few aromatic compounds with low absorptivity. Oxidation of amphetamine provided no hydrocarbon-soluble chromophore. Benzaldehyde was identified by its characteristic spectrum after oxidation of phenylacetic acid in an amount equivalent to 11% of theoretical. Phenethyl alcohol gave 2.4%, and tropic acid about 15% of the calculated amount of benzaldehyde after oxidation and extraction. The extent of benzaldehyde formation from these compounds, unlike the arylglycolic acids, was enhanced by light; production of benzaldehyde was completely inhibited in the dark.

RESULTS AND DISCUSSION

Identity of Oxidation Products.—Ultraviolet spectra provide two criteria for identification, the position of the absorption maxima, $\lambda_{max.}$ and the intensity of absorption or absorptivity. A close correspondence of both criteria with literature values for the carbonyl compounds was found for the ceric oxidation products of phenylglycolic acids. Although no spectra data for α-cyclopentyl-2-thienyl ketone were found in the literature, the absorption curve obtained on oxidation of α-cyclopentyl-2-thiopheneglycolic acid corresponds well with data for an analog, methyl 2-thienyl ketone. Data obtained for oxidation products of the arylglycolic acids are compared with the literature values in Table I. Molar absorptivity was calculated from the concentration of acid oxidized in replicated experiments.

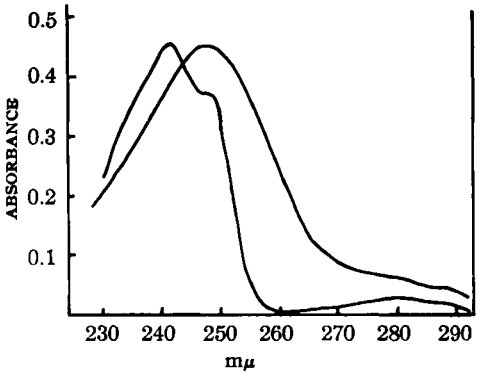


Fig. 2.—Ultraviolet absorption spectra of benzaldehyde from oxidation of mandelic acid, 0.5 mg./100 ml. (curve on left) and benzophenone from oxidation of benzilic acid, 0.5 mg./100 ml. (curve on right).

TABLE II.—SPECTRAL DATA FOR PERIODATE OXIDATION PRODUCTS OF ARYLGLYCOLIC ACIDS IN ISOOCTANE

Oxidized Acid	Oxidized, Min.	$\lambda_{max.}$, mμ	ε
Mandelic	15	242	14,080
	30		14,800
	60		15,150
Benzilic	15	248	19,380
	30		19,250
	60		20,450
α-Cyclopentylphenylglycolic	15	239	9,940
	30		11,500
	60		13,700
α-Cyclopentyl-2-thiopheneglycolic	15	258	8,020
		275	6,370

The molar absorptivities derived by ceric sulfate oxidation showed practically quantitative conversion to carbonyl compounds in 15 minutes. The periodate procedure gave yields which varied with reaction time and the structure of the glycolic acid. These data are presented in Table II.

The absorption curves for the carbonyl compounds are sufficiently characteristic to serve for identification of the arylglycolic acids, except for the α-cyclopentyl- and α-cyclohexylphenylglycolic acids. The spectra are shown in Figs. 2–4.

Oxidation of Arylglycolate Drugs.—Ester hydrolysis is catalyzed by both acid and base; thus, some degree of hydrolysis might be expected under the acid oxidative conditions employed. However, the ester drugs studied were hydrolyzed only to a

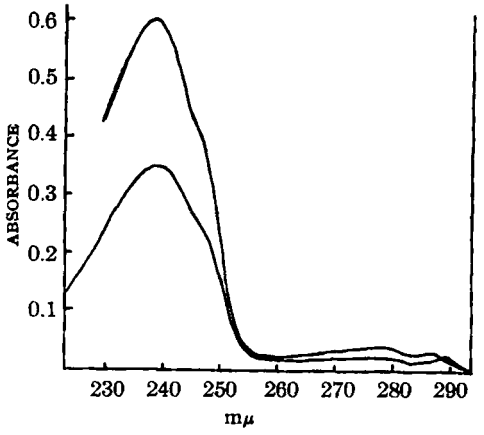


Fig. 3.—Ultraviolet absorption spectra of cyclohexyl phenyl ketone from oxidation of α-cyclohexylphenylglycolic acid, 1.165 mg./100 ml. (upper curve); and cyclopentyl phenyl ketone from oxidation of α-cyclopentylphenylglycolic acid, 0.505 mg./100 ml. (lower curve).

small extent evidenced by very low absorbances obtained when their solutions were oxidized. Heating 2.0 ml. of approximately 10 mg./100 ml. aqueous phenylglycolate solution or approximately 25 mg./100 ml. penthienate bromide with 0.1 ml. of 2.5 *N* sodium hydroxide at 80° for 15 minutes accomplished saponification efficiently. Shaking the cooled mixtures 15 minutes with 2.0 ml. of 0.2 *M* ceric sulfate and 20.0 ml. isooctane provided the data in Table III.

The precision obtained using this procedure was excellent. Measurement of the absorptivity of poldine methylsulfate had a standard deviation (S.D.) of 0.55% in five trials. Standard deviations for penthienate bromide in six trials were 0.62% at 258 mμ and 0.79% at 275 mμ.

Determination of Drugs in Tablets.—The procedure which follows was applicable to the determination of homatropine methylbromide tablets U.S.P. and commercial tablets of poldine methylsulfate and oxyphencyclimine hydrochloride.

Determine the average weight of not less than 20 tablets, and shake weighed amounts of pulverized tablet equivalent to 8–10 mg. of drug with exactly 100.0 ml. of distilled water for 15 minutes. Filter the mixture, and treat duplicate 2.0-ml. aliquots

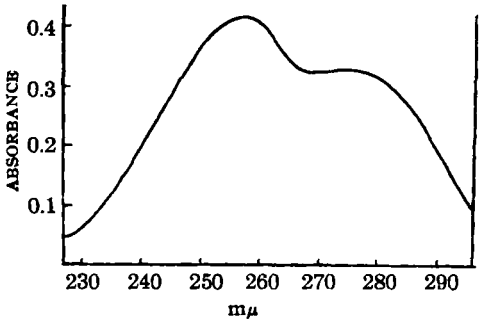


Fig. 4.—Ultraviolet absorption spectra of cyclopentyl 2-thienyl ketone from oxidation of α-cyclopentyl-2-thiopheneglycolic acid, 1.0 mg./100 ml.

with 0.1 ml. of 2.5 *N* sodium hydroxide for 15 minutes at 80° in a glass-stoppered 40-ml. centrifuge tube or 50-ml. volumetric flask. Cool to room temperature, and add 2.0 ml. of 0.2 *M* ceric sulfate and exactly 20.0 ml. of isooctane or hexane. Shake 15 minutes, and filter the solvent layer through paper. Similarly, saponify and oxidize a solution of reference drug of known concentration. Concomitantly, determine the absorbance of the sample and reference solutions in 1-cm. silica cells at the $\lambda_{max.}$ Calculate the amount of drug per tablet from the equation

$$\text{drug, mg./tablet} = \frac{A_{sample}}{A_{ref.}} \times \text{mg. std.} \times \frac{\text{av. tablet wt.}}{\text{sample wt.}}$$

(See Table IV.)

Cyclandelate.—The drug, the only ester of the group not an amine salt, has a low water solubility, but it is soluble in alcohol and ether. It was extracted from tablets with 100.0 ml. of methanol, shaking for 10 minutes. A 2.0-ml. aliquot of the filtered extract was treated with 2 ml. of water and 5 ml. of 0.05 *N* sodium hydroxide. The solution was evaporated to about 2 ml. on the steam bath.

TABLE III.—SPECTROPHOTOMETRIC DATA CALCULATED FOR ARYLGLYCOLATE DRUGS AFTER SAPONIFICATION AND CERIC OXIDATION

Oxidized Drug	$\lambda_{max.}$, mμ	ε
Homatropine methylbromide	242	13,200
Poldine methylsulfate	248	17,700
Oxyphencyclimine hydrochloride	239	13,100
Penthienate bromide	258	9,890
	275	7,750

It was then oxidized by the ceric procedure given above and by the periodate procedure, using 0.2 ml. of 3 *N* sulfuric acid to neutralize the excess alkali and insure an acid medium. Since no pure cyclandelate was available as a reference standard, an equivalent concentration of mandelic acid was determined concomitantly, using the same dilutions and conditions. The hydrocarbon extracts were filtered and read at 242 mμ, and the amount of drug was calculated from the equation

$$\text{Cyclandelate, mg./tablet} = \frac{A_{sample}}{A_{M.A.}} \times \frac{276.36}{152.14} \times \text{mg. M.A.} \times \frac{\text{av. tablet wt.}}{\text{sample wt.}}$$

where 276.36 is the formula weight of cyclandelate and 152.14 the formula weight of mandelic acid (M.A.).

The periodate and ceric sulfate procedures yielded results which corresponded closely. The absorbances for sample and standard obtained in the periodate procedure, however, were 90% of the values obtained by ceric oxidation. (See Table IV.)

Penthienate Bromide.—Determination of the drug in tablets by the general procedure for arylglycolates presented above gave an apparent 80% of label declaration, but direct spectrophotometry (2) showed 98% was present. It was determined that penthienate bromide was incompletely ex-

TABLE IV.—ASSAY OF ARYLGLYCOLATE DRUGS IN TABLETS

Drug	mg./Tablet Declared	mg./Tablet Found	Method
Homatropine methylbromide	2.5	2.4	Ceric sulfate
Cyclandelate	100.0	99.5	Ceric sulfate
		99.2	Periodate
Poldine methylsulfate	4.0	4.0	Ceric sulfate
Oxyphencyclimine hydrochloride	10.0	10.1	Ceric sulfate
Penthienate bromide	5.0	5.0	Ceric sulfate

TABLE V.—PER CENT ARYLGLYCOLATE DRUG HYDROLYZED IN TABLET EXTRACTS

Drug	% Hydrolyzed	Procedure
Homatropine methylbromide	2.3	Ceric
Cyclandelate	17.6	Ceric
	11.6	Periodate
Poldine methylsulfate	13.8	Ceric
Oxyphencyclimine hydrochloride	4.3	Ceric
Penthienate bromide	2.1	Ceric

tracted from the tablets by shaking with 100 ml. of water. Direct spectrophotometry at the 239 mμ maximum showed the drug was completely extracted with methanol. A quantity of pulverized tablet equivalent to 5 mg. of penthienate bromide was shaken 15 minutes with 100.0 ml. of methanol. A 2.0-ml. aliquot of methanol filtrate was treated with 2.0 ml. of water and 0.1 ml. of 2.5 *N* sodium hydroxide. The solution was evaporated to 2 ml. on the steam bath, then heated 15 minutes at 80°. A reference solution of penthienate bromide treated similarly was run concomitantly. Only 92% of claim was obtained when the ceric procedure was used, and the isooctane layer after periodate oxidation was violet. Quite stable and reproducible absorbance values for the reference solution were obtained; no trace of color was seen after periodate oxidation of reference penthienate bromide or α-cyclopentyl-2-thiopheneglycolic acid. It was concluded that an excipient with strong reducing properties was present in the tablets. (The violet obtained in the periodate oxidation of the tablet extract may have been due to iodine, but it was not positively identified.) Use of 4.0 ml. of 0.2 *M* ceric reagent, instead of 2.0 ml., increased the absorbance obtained by oxidation of the tablet extract without, however, affecting the absorbance of the standard. (See Table IV.)

Stability of Arylglycolate Drugs.—Although no stability studies were performed, a semiquantitative indication of the stability of the drugs to the acid oxidative conditions was obtained by running the procedure on the tablet extracts without prior saponification. The results in Table V were derived from the ratio of the absorbance of the unsaponified to the saponified tablet extract after oxidation. Some indication was obtained that these values may be partly due to the extraction of alkaline substances from the tablet, for the extract of poldine methylsulfate tablets had pH 7.5 compared with a pH of 6.3 for an equivalent concentration of reference drug in water. A comparison of the absorbances obtained in the oxidative spectrophotometric determination on extracts from drug dosage forms and reference arylglycolate drugs, omiting the saponification step of the procedure, may provide useful stability information for pharmaceutical formulators of these compounds.

SUMMARY

Oxidation of arylglycolic acids to carbonyl compounds greatly increases the sensitivity of their determination by ultraviolet spectrophotometry and affords a means for identification. Periodate and ceric sulfate oxidation procedures are described which are general for arylglycolic acids.

The oxidative spectrophotometry procedure is shown to be applicable to mandelic acid, benzilic acid, α-cyclopentylphenylglycolic acid, and α-cyclopentyl-2-thiopheneglycolic acid. Assay procedures are presented for the arylglycolate drugs, homatropine methylbromide, cyclandelate, poldine methylsulfate, oxyphencyclimine hydrochloride, and penthienate bromide in tablets. The possible application of the procedure in stability studies is suggested.

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Stress-Relaxation Studies on Hydrated Callus Strips II

Effects of Some Electrolytes and Nonelectrolytes in Aqueous Solutions

By WILLIAM J. TILLMAN* and TAKERU HIGUCHI

Mechanical responses of stretched, hydrated callus strips to several electrolytes and nonelectrolytes in dilute aqueous solutions have been determined. At equal concentrations of cations (of the order of 0.1 *M*), sodium chloride, potassium chloride, lithium chloride, and sodium sulfate produced marked decreases in tension essentially indistinguishable from each other. Magnesium chloride elicited a similar response but at much lower concentrations. The limiting values at higher concentration were, however, roughly the same as those produced by the monovalent cations. The astringency of aluminum ion was clearly reflected in the behavior of the cornified strip to its salts. Responses to urea and dextrose solutions have also been studied.

RESULTS OF further investigations on the mechanoelastic behavior of cornified epithelium elicited by changing solution environments are presented. In an earlier publication (1) the solvating and softening effects of several hydroxylic solvents were reported. The present communication is concerned with the results of similar experiments designed to determine the influence of certain nonelectrolytes and electrolytes on the tendency of stretched callus strips in aqueous media to shrink or relax. Information of this nature is presumed to be of value in the rational formulation of preparations applied to the skin for either cosmetic or medicinal purposes. The degree of solvation and hydration of the cornified layer, the primary factor in these measurements, is becoming increasingly recognized as a major concern in maintenance of the health and appearance of the outer epidermal layer.

This report is concerned specifically with results obtained on hydrated callus strips when exposed to aqueous solutions of sodium chloride, potassium chloride, lithium chloride, magnesium chloride, aluminum chloride, several sulfates, dextrose, and urea.

EXPERIMENTAL

The technique and strain-gauge apparatus employed in this study have been previously described by Tillman and Higuchi (1). The only significant modification for this study was an insertion of a preamplifier in the circuit before the recorder to increase the sensitivity of the method.

Materials.—The chemicals used in this study were reagent grade unless otherwise noted. The callus strips used in this study were prepared as previously described (1).

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General Procedure for Obtaining Stress Relaxation and Modulus Data.—Six to 12 hours prior to a run a dehydrated callus strip was removed from a desiccator. The strip was extracted twice for 30-minute periods in fresh anhydrous ether and stored in distilled water at 5°. Just prior to use, the strip was wiped dry, measured, and quickly weighed. The strip was then clamped between the two stainless steel clips of the apparatus (with the ridges and grooves of the callus running parallel to the applied force) and immersed in distilled water at 24 ± 1°.

The continuity of the strip was tested by applying a tension of approximately 20 Gm. (10×10^6 dynes/cm^2). If no breaks, tears, or holes appeared, the strip was considered suitable for further experimentation. A load of approximately 2.5 Gm. (12.5×10^5 dynes/cm.^{2}) as indicated by the recorder next was applied sharply to the strip by extending it, and the relaxation of this force at constant extension was followed on the recorder. After equilibration (about 45 minutes), the additional force required to extend the fiber 1% of its length (the 1% index) was determined; the length of the strip between the two clips was measured with a cathetometer. The fiber was stretched and immediately returned to its original length, the force response being automatically followed on the recorder chart.

After allowing a short time for the reattainment of equilibrium, the distilled water in the chamber was replaced by the solution under study, the force reading being recorded continuously. After a 15-minute exposure to this solution, the above readings were repeated. In most cases the strip was then removed from the solution and again immersed in distilled water to observe whether the effect was reversible. Each callus strip was used once.

RESULTS AND DISCUSSION

Effects of Extraction on Callus Strips.—When stress-relaxation studies were carried out in aqueous solutions on several unextracted hydrated callus strips, responses were variable. However, successive extraction with ether and water yielded specimens which behaved reproducibly. This phenomenon may be explicable at least in part on the basis of a study by Blank (2), who reported that the extraction of strips of cornified epithelium with lipid solvents, *e.g.*, ether, was necessary for the subsequent extraction with distilled water of hydro-

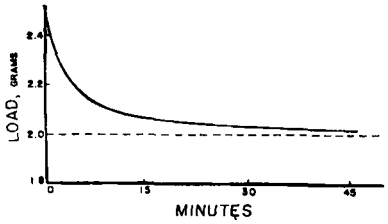


Fig. 1.—Typical stress-relaxation phenomenon observed when a hydrated, extracted callus strip was placed under an initial load while immersed in distilled water.

philic material normally present in the strips. Varying concentrations of this component in the unextracted callus strips may be responsible for the differences observed.

Before ether extraction, a dehydrated strip appeared translucent and straw-yellow in color. After ether extraction, the strip became opaque-white, similar in appearance to a hydrated unextracted strip. The ether-extracted strip was less flexible than the unextracted strip and appeared to imbibe significantly less water on rehydration. Extraction with water, followed by dehydration, left the strip translucent and off-white in color. Upon hydration, the ether-water extracted sample appeared opaque along the groove areas and translucent to slightly cloudy in the ridge areas. The unextracted strips, on the other hand, became completely opaque-white following hydration.

Stress-Relaxation Behavior of Extracted Callus Strips in Distilled Water.—Figure 1 depicts a typical stress-relaxation phenomenon observed when a hydrated callus strip, subjected to ether-water extraction as described, was immersed in distilled water and placed sharply under a 2.5-Gm. load. The relaxation under constant extension appeared to involve two parallel processes. The relatively fast exponential decrease in tension observed during the early phase may be attributed to changes in the amount of water held by the protein structure. This initial rapid decrease, which seemed to be completed within about 10 minutes, appeared to be followed by a very slow continuing decrease in tension as indicated in the figure. A similar relaxation effect noted with wool fibers has been ascribed to the slipping of polypeptide chains over one another, a result of scission of side-chain bonds (3).

A typical chart recording obtained during the course of these studies is presented in Fig. 2. This particular record depicts the response elicited by a 0.1 *M* magnesium chloride solution. The modulus (1% index) measurements are recorded as perpendicular lines and are labeled 1%. All subsequent figures depicting load *versus* time curves are tracings of the actual recordings.

Effect of Dextrose.—The response of extracted callus strips, initially equilibrated with distilled water and under load, to an isotonic solution of dextrose (0.30 molal) is shown in Fig. 3. The behavior depicted in the recorded plot and those given in the succeeding sections for other systems were at least qualitatively reproducible even when using strips from various sources. Quantitative reproducibility was unusually good in experiments in which the strips used were from a single callus source and best when the strips were from a single layer. Relative reproducibility of such systems is discussed in greater detail under *Effect of Sodium and Magnesium Chloride.*

As can be seen in the plot, the switch from distilled water to dextrose solution resulted in a slight increase in tension, followed by a relaxation of equal magnitude. The sharp initial rise in stress can probably be ascribed to a change in the degree of hydration of the strip (osmotic effect); the subsequent relaxation may be the result of either a minor molecular reorientation or, more probably, a diffusion of dextrose into the interior of the hydrated strip thereby restoring the system to its initial osmotic state.

The reversibility of the observed phenomenon was demonstrated by reimmersing the callus strip in distilled water. As can be seen in Fig. 3, a slight decrease in tension, followed by a shrinkage of equal magnitude, was recorded. The callus strip apparently had returned to its original state. These observations were further substantiated by 1% index measurements which showed no apparent change in magnitude both before and 15 minutes after immersion of the strip in the dextrose solution and distilled water.

Effect of Urea.—Small decreases in tension and a small decrease in the 1% index were observed when a stretched callus strip was immersed in an isotonic (0.30 molal) solution of urea. These responses were reversed by replacing the strip in distilled water. No significant change in the appearance of the strip was noticeable.

With a more concentrated 6 *M* urea solution, a very large relaxation was recorded, even though the strip became completely translucent upon immersion in the solution. There was also a very large decrease, amounting to approximately 60%, in the 1% index measurements. The loss in opacity of the strip

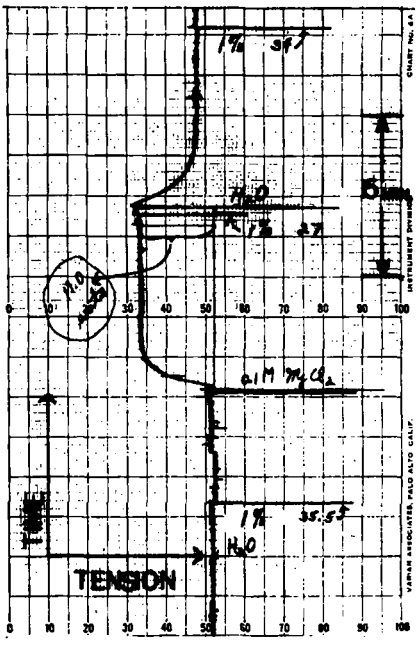


Fig. 2.—Photograph of actual chart recording obtained during the study of the effect of 0.1 *M* magnesium chloride on a hydrated, extracted callus strip. 1% ≈ 1% index (in chart units).

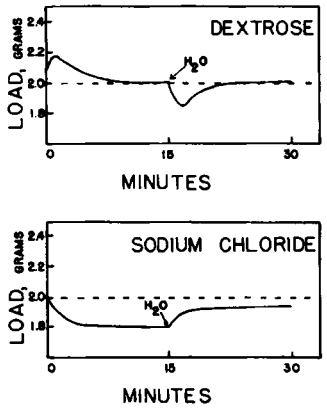


Fig. 3.—Change in load maintaining hydrated, extracted callus strips at a fixed extension upon the immersion of the strips into isotonic dextrose and isotonic sodium chloride solutions. Response obtained upon reimmersion of the treated strip into distilled water is also presented.

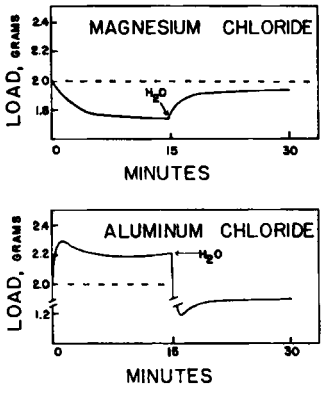


Fig. 4.—Same as Fig. 3, but for isotonic magnesium chloride and isotonic aluminum chloride.

was most probably due to the dehydration of the strip caused by the osmotic properties of the highly concentrated urea solution. This effect, if it were the only thing happening in the system, would have caused a significant increase in tension. But this response was probably obscured by the effect of the urea on the protein, resulting in relaxation of the forces maintaining this strip at constant length.

It is obvious from these results that urea does not behave as a simple nonelectrolyte (like dextrose) but does, in some manner, affect the structural components of the system. Possibly, the reported ability of urea in high concentrations to alter protein hydrogen bonding may be involved. Contrary to these observations were those reported by Matoltsy and Balsamo (4), who reported that epidermal keratin was not visibly affected by neutral solutions of urea (10 to 50%), whereas it was readily dissociated in alkaline urea, such as 50% urea in 0.02 *N* sodium hydroxide. Their conclusions were based on double refraction data.

Effect of Sodium and Magnesium Chloride.—Immersion of a hydrated extracted callus strip, under load, into either an isotonic solution of sodium chloride (0.163 molal) or magnesium chloride (0.113 molal) usually produced an immediate and lasting decrease in tension as shown in Figs. 3 and 4; the extent of relaxation was always somewhat greater for magnesium chloride. In some instances a very slight initial increase in tension was noted before the relaxation occurred; this was thought to be an osmotic effect. This effect was small and usually obscured by the tension decrease caused by the salt. The effect of the salt ions on the ionic linkages existing between the protein chains of the callus may be responsible for the relaxation effect. The forces between the ionized parts of the protein system would be expected to be substantially reduced by any increase in the ionic strength of the environment. One per cent index measurements made before and after immersion of the strip in the salt solution also decreased in magnitude.

Reimmersion of the treated strips in distilled water did not appear to reverse the effect of sodium or magnesium chloride completely. This failure to return, within 15 minutes, to the initial equilibium tension was reflected in the 1% index measurements. They, too, did not return to their original values. It is possible, however, that a more extended soaking period might have reversed completely the effect of these salts.

Additional experiments performed with sodium and magnesium chloride indicated that the absolute change in force was dependent upon thickness of the strip and the concentrations of the salt solution employed. As is evident in Fig. 5 a curious sort of a straight-line relationship seems to exist between the change in the residual tension and the thickness of the specimens. The initial load applied to each strip in these measurements was approximately 2 Gm. Because of the variation in the thickness of the strips (ranging from 40 to 100 μ), force per unit area (tension) varied from about 10 to 25 $\times$ 10^5 dynes/cm.^{2}.

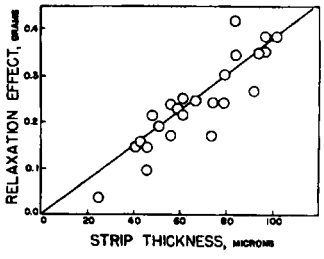


Fig. 5.—Dependency of the relaxation response elicited by isotonic sodium chloride solution on the thickness of the hydrated, extracted callus strips.

TABLE I.—AVERAGE REDUCTION IN FORCE PER UNIT AREA PRODUCED BY SODIUM CHLORIDE SOLUTION ON HYDRATED CALLUS STRIPS CUT FROM VARIOUS SPECIMENS

Specimen	N[a]	$\bar{x}$,[b] (dynes/cm.^{2}) $\times 10^{-5}$	S.E.,[d] (dynes/cm.^{2}) $\times 10^{-5}$
1	13	1.99	0.06
2	10	1.91	0.08
3	4	1.34	0.31
4	4	1.65	0.11
5	2	1.96	0.15
		Av.[c] = 1.77	0.14

[a] N = Number of runs per sample. [b] $\bar{x} = \Sigma x/N$, where x equals the reduction in tension for each run per sample. [c] Av. = $\Sigma\bar{x}/5$. [d] S.E. = Standard error = standard deviation/$\sqrt{N}$.

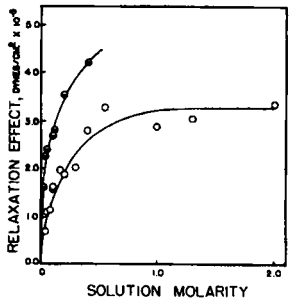


Fig. 6.—Dependency of the relaxation response on the concentrations of sodium chloride and magnesium chloride. Key: Upper curve $MgCl_2$; lower curve NaCl.

The reduction in tension at constant extension produced by the salt solutions was quite reproducible when expressed as changes in force per unit area (see Table I). An average decrease in tension of 1.77×10^5 dynes/cm.^{2} was elicited by the sodium chloride solution on 33 individual strips from five different callus sources (the mean of the average values from each source). The average decrease in tension obtained for isotonic magnesium chloride was of the order of 2.8×10^5 dynes/cm.^{2}, which appears to be significantly larger than the value obtained for sodium chloride. The changes in modulus (1% index measurements) produced by the salt solutions appeared to parallel the reduction in tension.

The relationship observed between the concentration of salt present and the decrease in tension elicited is evident in the results presented in Fig. 6. It can be seen from the plot that for both the sodium and magnesium chloride systems the response was fairly proportional to concentration in the low concentration range; but at higher concentrations it appears to level off, suggesting a limiting effect. An equation representing such a situation is: $1/\text{response} = (1/k_1C) + (k_2/k_1)$. Plots of the reciprocal response *versus* reciprocal concentration as shown in Fig. 7 appear to yield straight lines for both salt systems. The near identity of the intercept values strongly suggest that the limiting effects of the two cationic species are the same. It would appear that the response is produced by a process similar to an ion exchange process and that the full effect is produced when the system is saturated, irrespective of the nature of the particular cation.

Effect of Aluminum Chloride.—Transfer of a hydrated extracted strip from distilled water to isotonic (0.098 molal) aluminum chloride solution resulted in a rather large initial increase in tension (astringency) which reached a maximum within approximately 1 minute. This increase was followed by a momentary relaxation, then by a very gradual increase in tension. At the end of 15 minutes, a very slight decrease in the 1% index was recorded. The reimmersion of the strip in distilled water resulted in a large relaxation as shown in Fig. 4. This large change was considered to be due to the alteration in protein structure caused by both the effect of the aluminum ion and the acidic pH of its solution.

A significant factor in the effect of aluminum chloride solution was the pH of the solution. An isotonic aluminum chloride solution had a pH of 3.17, while isotonic concentrations of sodium and magnesium chlorides did not alter the pH of distilled water significantly. A more detailed study of the effect of pH on the effect of the various salt ions will be described in a subsequent report.

The binding of aluminum ion to powdered callus has been reported by Lyon and Klotz (5). Their investigation also included studies of Al^{3+} binding by human skin (surface scrapings) and hair. They found that aluminum ion was bound by each and in the following decreasing order: callus > skin > hair cuticle. It was suggested that the primary sites of Al^{3+} [and $Al(OH)^{2+}$ and $Al(OH)_2^+$] sorption were the carboxylate groups of the protein. The extent of binding was found to be dependent upon pH.

The reason for the initial increase in tension observed with aluminum chloride, as opposed to the decrease noted with sodium and magnesium chlorides, is probably the ability of the tripositive aluminum ion to react with more than one carboxylate group. The Al^{3+} could possibly bind with carboxylate groups from more than one chain causing a tightening of the structure.

Effect of Other Inorganic Ions.—Studies performed with lithium chloride and potassium chloride indicated that the lithium and potassium ions produced effects essentially the same as those of the sodium ion. No differences could be detected within the sensitivity of the method employed.

Runs were also made in which the sulfate salts of sodium, magnesium, and aluminum were employed. In unbuffered aqueous solutions the concentration of the cation was the important variable. Equivalent relaxation factors were obtained with solutions containing 0.1 *M* sodium chloride and 0.05 *M* sodium sulfate and similarly with 0.1 *M* magnesium chloride and 0.1 *M* magnesium sulfate. In the case of aluminum chloride and aluminum sulfate no quantitative data were collected because of the type of response elicited by these salts, but qualitatively these substances yielded similar responses. The major difference between the two salts was that the rather pronounced relaxation following the initial increase in tension obtained with aluminum chloride was almost completely missing in the aluminum sulfate response. It was noted also that in the case of aluminum sulfate the change in the 1% index reading was greater than 100% of the original value.

GENERAL DISCUSSION AND CONCLUSIONS

Data presented in the preceding section suggest that stress-relaxation measurements of this type can yield information of significant interest to those

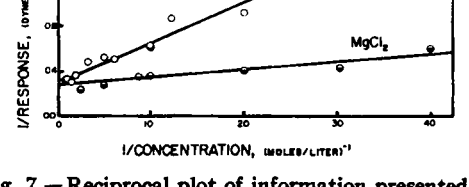


Fig. 7.—Reciprocal plot of information presented in Fig. 6 on solution molarity.

concerned with influences of various solutes and vehicles on the physical states of cornified epithelial tissues. The presence of a natural hydrating factor which can be removed by the ether-water treatment appears to be clearly established by these studies. It is further evident that very dilute aqueous solutions of various salts and nonelectrolytes have pronounced effects on the configuration of the protein structure.

Some of these observations may be pertinent in formulation of skin preparations. Although the astringency of aluminum ion is well known, observation of the effect of sodium, potassium, lithium, and magnesium ion in producing relaxation of stressed tissue, even in dilute solutions, appears to be new. This behavior seems to be in opposition to the dehydrating effect normally produced by the presence of solutes in water.

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Procedure for Assay and Stability Determination of Idoxuridine

By CHARLES A. SIMPSON and ALFRED F. ZAPPALA

In acid solution, idoxuridine (5-iodo-2′-deoxyuridine) is decomposed by light to form 2′-deoxyuridine and material whose U.V. absorption has been destroyed. Idoxuridine is hydrolyzed by heat to form 5-iodouracil and 2-deoxyribose in nearly quantitative yield; a small amount of 2′-deoxyuridine is also formed. A procedure for assay of these mixtures is given. The method involves a chromatographic separation on a partition column with a standing phase of 0.1 *N* HCl and a moving phase of chloroform plus *n*-butanol (5 : 1 by volume). That portion of the eluate containing the idoxuridine is analyzed by its U.V. absorption. Data are presented to show the accuracy and precision of the method.

IDOXURIDINE (5-iodo-2′-deoxyuridine) was first synthesized in 1959 by Prusoff (1). Following its synthesis, several papers were published in connection with its effect on transplantable neoplasms (1–3). In addition, it was studied in its role as a bacterial inhibitor (1, 4). More recently, Kaufman has shown that it is an agent of proven value in treatment of herpetic keratitis in man (5). This has been hailed as the first chemotherapeutic agent effective against a specific virus. Because of this growing biochemical and medical interest in idoxuridine, we undertook to study its decomposition and find a method for stability determination. This report describes the results of that effort.

EXPERIMENTAL

Study of Light-Initiated Decomposition.—Unbuffered aqueous solutions of idoxuridine (1 mg./ml., pH = 5–6) were placed in quartz containers and irradiated with a mercury vapor arc lamp. Figure 1 shows that the U.V. absorption spectra of these solutions decrease in intensity with an increase in time of exposure. The nature of this decomposition product was not studied, since it appeared to offer no interference with an ultraviolet method of determination. Moreover, the photolytic decomposition of related compounds such as uridine and uracil has been studied by Sinsheimer and Hastings and many others (6). These studies showed that the product formed is the 4-hydroxyhydro derivative.

Another considerably less severe irradiation experiment was conducted with a Sylvania type RS sunlamp, where the spectrum includes portions of the I.R. and visible as well as the U.V. ranges. As previously, the solutions irradiated were unbuffered and aqueous (1 mg./ml., pH = 5–6). After 3 months of exposure, the solution was spotted on paper along with a known solution of some suspected decomposition products, and a chromatogram was developed in a butanol–3 *N* ammonia system. The results of this study are shown in Table I.

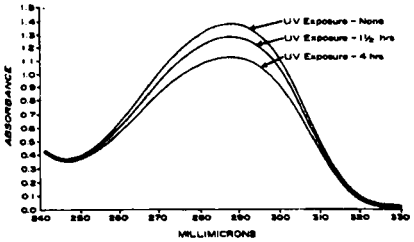


Fig. 1.—U.V. absorption spectra of aqueous idoxuridine exposed to U.V. light.

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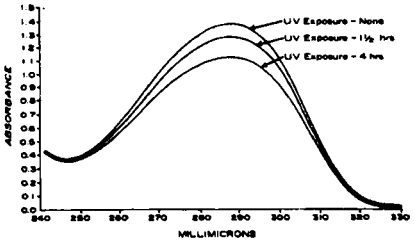


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TABLE I.—COMPARISON OF R_f VALUES OBTAINED FOR SOME POSSIBLE DECOMPOSITION PRODUCTS AND A SUNLAMP-DECOMPOSED AQUEOUS SOLUTION OF IDOXURIDINE

Material	R_f Values: Known Soln.	R_f Values: Sunlamp Decomposed Soln.
Unknown	...	0.00 (Significant)
2'-Deoxyuridine	0.23	0.22 (Significant)
Idoxuridine	0.31	0.31 (Large)
2-Deoxyribose	0.37	0.37 (Small)
5-Iodouracil	0.50	0.47 (Small)

The formation of a small amount of deoxyribose and iodouracil was expected, since the writers showed in an earlier note that these are the principal thermal decomposition products (7). The major breakdown is to deoxyuridine and material giving no absorbance or fluorescence in U.V. light, yet still giving the characteristic deoxyribose pink color with cysteine and sulfuric acid. As before, this may indicate the hydration of the 4,5 double bond or the destruction of the ring.

Study of Heat-Initiated Decomposition.—In the solid state, idoxuridine shows considerable stability toward heat. After storage for 9 months at 60°, its paper chromatogram and U.V. assay were unchanged from the original. This is not the case with aqueous solutions, however. Unbuffered aqueous solutions of idoxuridine (1 mg./ml., pH = 5–6) were stored for a few months at temperatures ranging from room temperature to 45°. They were then spotted on paper along with a solution of suspected breakdown products, and chromatograms were developed in a butanol–3 *N* ammonia system. The results of this study are shown in Table II, which shows that the initial breakdown is to deoxyuridine. However, its rate of formation is relatively low; the main decomposition results in the nearly quantitative formation of iodouracil and deoxyribose. This rise in iodouracil content, coincidental with the decline in idoxuridine, was determined using the chromatographic procedure to be described later. These data are shown in Table III.

Since uracil is not well separated in the paper system described above, those same solutions were also chromatographed in a 2-methyl-3-butyn-2-ol, pH 8 buffer system. No uracil was found in these samples. Subsequent studies have shown that uracil can only be found in badly decomposed solutions (50% or more degraded). In fact, uracil was the only pyrimidine compound still present after treatment of an aqueous unbuffered solution for 17 hours at 100°.

Some decomposition studies were also conducted at pH's as high as 10. This aspect was not investigated to a great degree, except to observe that the ionized form of idoxuridine (pKa ~ 8) is considerably less stable than the acid form.

Method Development.—The determination of idoxuridine can be accomplished in a number of ways. Its deoxyribose moiety will react with cysteine in 75% v/v H_2SO_4 to form a pink color (8, 9). A direct method of assay for the pyrimidine base is based upon its ability to absorb four equivalents of iodine from basic solution (10, 11). There is a colorimetric method which uses the ability of the base to reduce arsenotungstic acid (12). Finally, idoxuridine can be determined from its U.V. absorption spectrum ($\epsilon_{max.}$ = 7763).

Unfortunately, in each of these methods one or more of the breakdown products interferes. The complex nature of this interference is illustrated by the U.V. absorption spectra in Fig. 2. Consequently, it was desirable to devise a relatively simple but versatile system of separation, one which would be capable of the stability determination of idoxuridine in research systems where it would be mixed with a variety of drug substances or where the pH would be varied through a considerable part of the aqueous range. After separation, any suitable property could be selected for measurement. The separation technique chosen was liquid-liquid partition chromatography on a Celite 545 support; the property chosen for measurement was U.V. absorption.

Partition Column.—Because the great majority of situations under study involved aqueous solutions,

TABLE II.—COMPARISON OF R_f VALUES OBTAINED FOR SOME POSSIBLE DECOMPOSITION PRODUCTS AND HEAT-DECOMPOSED AQUEOUS SOLUTIONS OF IDOXURIDINE

Material	R_f Values: Known Soln.	R_f Values: 3 Mo. at Room Temp.	R_f Values: 3 Mo. at 45°
2'-Deoxyridine	0.23	0.24 (Trace)	0.23 (Trace)
Uracil	0.28	...	...
Idoxuridine	0.31	0.32 (Large)	0.31 (Large)
2-Deoxyribose	0.37	...	0.39 (Significant)
5-Iodouracil	0.50	...	0.48 (Significant)
Idoxuridine assay, % of original		98	69

TABLE III.—MATERIAL BALANCE OF IDOXURIDINE AND 5-IODOURACIL IN THERMAL DEGRADATION

Sample	Storage Condition	Mole % of Original: Idoxuridine	Mole % of Original: 5-Iodouracil	Mole % of Original: Total
Aqueous, 1 mg./ml., pH 5.0, buffered	1 wk. at 60°	79	21	100
Aqueous, 1 mg./ml., pH 6.2, buffered	1 wk. at 60°	76	22	98
Aqueous, 1 mg./ml., pH 5–6, unbuffered	3 mo. at 45°	74	28	102

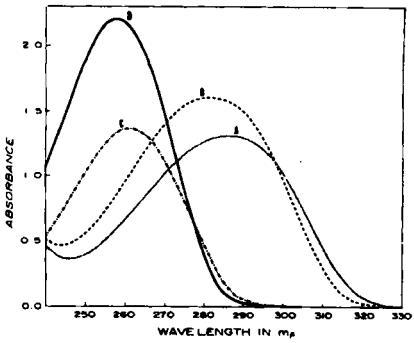


Fig. 2.—The U.V. absorption spectra of A, idoxuridine (0.06 mg./ml.); B, 5-iodouracil (0.06 mg./ml.); C, 2′-deoxyuridine (0.025 mg./ml.); D, uracil (0.025 mg./ml.) in 0.1 *N* hydrochloric acid, 1-cm. cells.

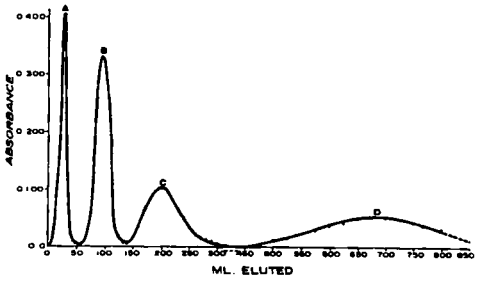


Fig. 3.—Partition column chromatogram of a mixture approximately 0.003 *M* in A, 5-iodouracil; B, idoxuridine; C, uracil; D, 2′-deoxyuridine. Column diameter = 26 mm.

only aqueous standing phases were considered. Idoxuridine is not ionized appreciably in acid solution and therefore is more readily extracted by organic solvents. This fact and its decreased stability in basic solutions led to the choice of 0.1 *N* HCl as the standing phase.

For use as the moving phase, carbon tetrachloride, chloroform, *n*-butylacetate, isooctane, and *n*-butanol were tried. None of these served well alone. Of the mixtures tried, the best separation for the lowest elution volume was given by a solvent consisting of 5 vol. of chloroform and 1 vol. of *n*-butanol.

For convenience and ease of handling, a 30-cm.-long column packed with a bed of Celite about 10 cm. long was chosen. One such column which gave a complete separation from all decomposition had a diameter of 26 mm. and a chromatographic packing of 8 ml. of 0.1 *N* HCl adsorbed on 8 Gm. of Celite 545. A typical chromatogram of an approximately equimolar synthetic mixture is shown in Fig. 3. It will be seen that these constituents are well separated and that determination of each one is accomplished readily by simple U.V. absorption spectrophotometric measurements.

The above method suffers from requiring a high elution volume (and time) and a relatively large amount of Celite. A satisfactory compromise between these factors and the degree of separation was obtained by employing a smaller column (19-mm. diameter) packed with 4 ml. of 0.1 *N* HCl adsorbed on 4 Gm. of Celite 545. Figure 4 shows typical chromatograms obtained with this setup for a synthetic mixture and for a partly decomposed sample. It is apparent from Curve A that the separation from uracil may not always be complete. However, idoxuridine can still be determined by U.V. absorption, provided a properly selected two-point calculation method is used. The U.V. absorption spectra of the eluted species are shown in Fig. 5. The method reported in detail below is the one that was used in many assays.

PROPOSED METHOD

Reagents.—Hydrochloric acid, 0.1 *N*; chloroform, Merck reagent; *n*-butanol, Merck reagent; and Celite 545, acid-washed, Johns-Manville Co., were used. Eluting solvent: mix 5 vol. of chloroform with 1 vol. of *n*-butanol.

Apparatus.—A Cary model 11 M recording spectrophotometer was used. The chromatographic column was approximately 19 mm. in diameter × 30 cm. high with a stopcock for controlling the flow rate.

Idoxuridine Standard.—Idoxuridine (California Corp. for Biochemical Research) was recrystallized twice from an aqueous methanol system. This material was shown to be 99.3% pure by nonaqueous titration, 99.6% by iodine determination, and 99.6% by phase solubility analysis. In addition, its

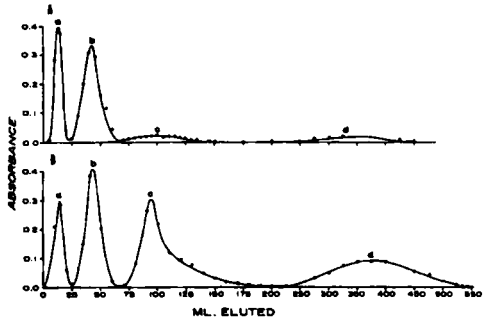


Fig. 4.—Partition column chromatograms with column diameter = 19 mm. Curve A, a heat-decomposed solution of idoxuridine; curve B, a synthetic mixture approximately 0.003 *M*. Key: *a*, 5-iodouracil; *b*, idoxuridine; *c*, uracil; *d*, 2′-deoxyuridine.

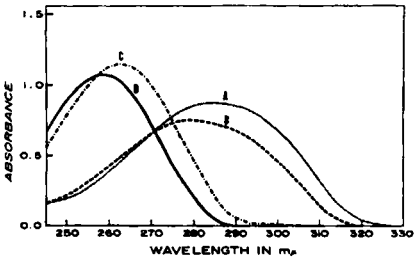


Fig. 5.—The U.V. absorption spectra of A, idoxuridine; B, 5-iodouracil; C, 2′-deoxyuridine; D, uracil, all 0.6 × 10^{-4} *M* in chloroform–*n*-butanol (5:1 by volume), 2-cm. cells.

TABLE IV.—CHROMATOGRAPHIC RESULTS FOR A 0.1% AQUEOUS SOLUTION OF IDOXURIDINE

Analyst	Assay, % of Theory
A	100.4, 97.6
B	98.2, 100.1
C	99.4, 99.8
D	100.5, 99.7

specific rotation $[\alpha]_D^{26}$, was +28.6° (C = 1, *N* NaOH). Its U.V. absorption spectrum exhibited a maximum at 288 mμ (ϵ = 7763) and a minimum at 247 mμ (ϵ = 2093).

Assay Procedure.—Add 4 ml. of 0.1 *N* HCl to 4 Gm. of Celite in a 4-oz. mortar and mix well using a small plastic rod or spatula. The mixture has been worked sufficiently when it appears light, fluffy, and uniform. Transfer this mixture to a chromatographic tube which contains a small pledget of glass wool, and tamp down with a rod of slightly smaller diameter using only sufficient force to give the packing a uniform appearance. Add a 2.00-ml. aliquot of an aqueous sample (pH < 7, concentration 0.5–1 mg. idoxuridine/ml.) to 2 Gm. of Celite in a 4-oz. mortar, mix as before, and transfer to the same tube; do not tamp it down yet. Add 2 ml. of 0.1 *N* HCl to 2 Gm. of Celite in the same mortar used for the sample and mix well. Transfer about half of this to the same tube and tamp as before until the packing appears to be uniform. Then transfer the remaining portion to the tube, pack it in place, and top with a small pledget of glass wool.

Pass chloroform-butanol eluting solvent through the column at a flow rate of approximately 1 ml. per minute. Since the exact nature of the elution profile may vary slightly from lot to lot of Celite, it will be necessary to run a known idoxuridine solution (0.5–1.0 mg./ml.) through the procedure to determine exactly what part of the eluate contains idoxuridine. Collect and save for spectrophotometric examination that portion of the eluate containing all of the idoxuridine. For most lots of Celite, collect a fore-run of 15–20 ml. and discard. Then place a 100-ml. volumetric flask under the column and collect the next 80 ml. Dilute to volume with the same solvent. Record the U.V. absorption spectrum *versus* this solvent in 2-cm. cells from 320 to 260 mμ.

$$\text{Idoxuridine, mg./original sample, ml.} = \frac{(\text{Å}_{.292} - \text{Å}_{.310})\text{sample}}{(\text{Å}_{.292} - \text{Å}_{.310})\text{std.}} \times \text{concn. of std. (mg./ml.)}$$

RESULTS

Some typical results obtained with the chromatographic method are shown in Table IV. The standard deviation for these values is 1.0%, although experience has shown that a more realistic value is 1.3%.

That this method is a stability method of assay is amply demonstrated by all of the chromatographic evidence and the results given in Table III. This can also be demonstrated by assaying known solutions of idoxuridine where the breakdown products have been added; *e.g.*, a mixture of 95.3 mg. idoxuridine, 14.8 mg. uracil, and 13.5 mg. iodouracil was assayed, and the average result was 95.2 mg. idoxuridine (99.9% recovery). Similarly, deoxyuridine was shown to cause no interference.

DISCUSSION

The method presented above appears to have no especially critical steps. No care is taken to store Celite protected from the atmosphere; acid-washing will remove a small background absorbance, but this is usually unnecessary. The absorptivity for idoxuridine dissolved directly in chloroform-butanol eluting solvent is identical with that obtained by putting a known aqueous solution through the chromatographic procedure; this indicates that there is no retention or destruction on the column. However, if the mixing of Celite with standing phase is accomplished with vigorous grinding, a material of pastelike consistency is obtained. This results in considerably lower recoveries.

With minor modification, the chromatographic method has been applied to other pharmaceutical forms and mixtures containing other drug substances. For example, in the presence of hydrocortisone, prednisolone, or dimethisoquin, which would normally interfere, the loaded column is given a prewash of chloroform prior to normal chromatographic development. Some materials such as neomycin sulfate are so soluble in the standing phase that they normally do not interfere.

All of the data indicate that this procedure is sufficiently accurate and precise for assaying freshly prepared, aged, or decomposed solutions, or mixtures of idoxuridine in a wide variety of situations.

SUMMARY

Ultraviolet light reacts with aqueous solutions of idoxuridine in a way which destroys its characteristic U.V. absorption spectrum. This suggests that the pyrimidine ring has been hydrated or opened.

Light from a sunlamp reacts with aqueous idoxuridine solutions to produce two major products: 2′-deoxyuridine and material whose pyrimidine ring has been altered.

The thermally induced hydrolysis products of idoxuridine are principally 5-iodouracil and 2-deoxyribose, with a trace to 2′-deoxyuridine. In time these will lead to the formation of uracil.

A column partition chromatographic procedure has been developed for the assay of idoxuridine in the presence of its decomposition products.

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Analysis of Drug Mixtures Containing Antihistamines by Quantitative Thin-Layer Chromatography

By J. C. MORRISON and L. G. CHATTEN

A method is described which utilizes the application of thin-layer chromatographic techniques to the analysis of antihistamines in multicomponent pharmaceuticals. The procedure is relatively rapid and convenient and requires no expensive equipment. Tablet and capsule excipients do not interfere. Quantitative measurements are accomplished by spraying the developed plate and measuring the area of the spot.

THE WIDE-SPREAD USE of antihistaminic preparations has resulted in the development of several analytical techniques for the assay of these compounds in pharmaceutical preparations. The "United States Pharmacopeia" (1) utilizes nonaqueous titrimetry for the quantitative determination of the crystalline antihistamines, but prefers analyses by ultraviolet spectrophotometry for the commercial products. The 1963 edition of "The British Pharmacopoeia" (2) also employs nonaqueous techniques for the crystalline materials, but methods for the products vary widely from spectrophotometry to the rather obsolete Kjeldahl procedure. None of the procedures in either compendium are of value in the analysis of multicomponent pharmaceuticals.

A report by Clair and Chatten (3) presented a nonaqueous technique which is an accurate and convenient means of assaying single-component antihistaminic products, but is of no value where the antihistamines are combined with other nitrogenous bases.

Since its introduction by Stahl (4), thin-layer chromatography has found an increasing number of applications in the separation and estimation of inorganic, organic, and biological mixtures. Within the past few years, several papers have been published which describe quantitative thin-layer techniques (5–7). Usually these have involved either the elution of the substance from the adsorbant and its subsequent estimation by chemical analyses (8) or the careful measure of the area of the spot from which the concentration of the substance can be calculated. By this latter technique Purdy and Truter (9) have shown in thin-layer chromatography that the square root of the area of the spot is directly proportional to the logarithm of the weight of the material present. These workers developed a procedure which separated and quantitatively determined mixtures of organic compounds and inorganic cations.

Since antihistamines are combined with a wide variety of other medicinal agents in multicomponent products, their isolation and estimation from such mixtures frequently is time consuming; the results leave much to be desired. One of the great advantages of thin-layer chromatography is its rapidity. Hence, the purpose of this present investigation was to determine whether this technique could be utilized in the analysis of multicomponent antihistaminics.

EXPERIMENTAL

Materials and Apparatus.—Glass plates (200 × 200 mm.) and glass developing tanks (12-in. diameter, 12 in. deep) lined with solvent saturated filter paper were used.

Adsorbant.—The adsorbant was silica gel G (Cave & Co.).

Solvent.—Glacial acetic acid, A.C.S.: distilled water (20:80) served as the solvent.

Spray Reagent.—Five grams of ceric sulfate was dissolved in 10 ml. of concentrated sulfuric acid. Distilled water was added to 100 ml.

Preparation of Plates.—The plates were coated with a layer of adsorbant 250 mμ thick according to the method of Stahl (4). The slurry was prepared by mixing 25 Gm. of silica gel G with 50 ml. of 20% 1.2 dimethoxyethane in distilled water. This gave a smooth, even dispersion of silica gel which was not liable to flake or crack.

Standards.—One-hundred milligrams of the drug was dissolved in 25 ml. of water. All standards were freshly prepared. Compounds used were methapyrilene hydrochloride (I), chlorpheniramine maleate (II), diphenhydramine hydrochloride (III), promethazine hydrochloride (IV), pyrilamine maleate (V), and *l*-phenylephrine hydrochloride (VI). All drugs were supplied by manufacturers and used without further purifications.

Application of Drugs.—Three solutions of each drug were prepared: a standard solution, a dilution

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TABLE I.—CHROMATOGRAPHIC DATA ON SELECTED DRUGS

Drug	Spray	Spot, Color	Spot, Shape	Min. Detectable Quantity, mcg.	R_f in 20% Acetic Acid
Methapyrilene hydrochloride	Ceric sulfate	Grey	Elliptical	15	0.38
Chlorpheniramine maleate	Ceric sulfate	White	Elliptical	25	0.27
Diphenhydramine hydrochloride	Ceric sulfate	Yellow	Circular	15	0.55
Promethazine hydrochloride	Ceric sulfate	Pink	Circular	10	0.51
Pyrilamine maleate	Ceric sulfate	Purple	Elliptical	15	0.35
l-Phenylephrine hydrochloride	Ceric sulfate	Brown	Circular	15	0.83
Ephedrine hydrochloride	Ninhydrin	Pink	Circular	15	0.59
Phenindamine tartrate	Ceric sulfate	Grey	Elliptical	15	0.35

TABLE II.—QUANTITATIVE RESULTS ON SINGLE DRUGS AND BINARY MIXTURES

	Amount Added, mcg.	Recovered, ±	Recovered, %	Determinations, No.	Area, mm.²
Individual Drugs					
Thenylene HCl	25	25.18 ± 2.17	100.72	23	164
	50	48.86 ± 3.57	97.72	23	247
Chlorpheniramine maleate	25	24.93 ± 2.27	99.72	35	136
	50	50.13 ± 3.57	100.26	26	271
Diphenhydramine HCl	25	25.78 ± 1.76	103.12	23	170
	50	52.46 ± 2.47	104.92	29	179
Promethazine HCl	25	25.20 ± 1.60	100.80	29	160
	50	50.29 ± 4.01	100.58	24	175
Pyrilamine maleate	25	25.43 ± 1.69	101.72	25	184
	50	51.71 ± 4.61	103.42	21	216
l-Phenylephrine HCl	25	25.48 ± 1.34	101.92	28	161
	50	50.76 ± 4.59	101.52	27	180
Binary Mixtures					
Thenylene HCl	25	24.99 ± 1.44	99.96	25	164
l-Phenylephrine HCl	25	25.61 ± 1.59	102.44	25	161
Chlorpheniramine maleate	25	25.04 ± 1.58	100.16	26	136
l-Phenylephrine HCl	25	25.46 ± 1.41	101.84	26	161
Diphenhydramine HCl	25	24.61 ± 1.60	98.44	28	170
l-Phenylephrine HCl	25	25.51 ± 1.81	102.04	28	161
Promethazine HCl	25	25.32 ± 1.83	101.28	25	160
l-Phenylephrine HCl	25	25.89 ± 1.88	103.56	25	161
Mepyramine maleate	25	24.94 ± 1.50	99.76	29	184
l-Phenylephrine HCl	25	25.54 ± 1.71	102.16	29	161

prepared from the standard, and the unknown. Fifteen microliters (3 mcg./ml.) of the diluted standard, 20 μl. (4 mcg./ml.) of the standard, and 25 μl. (5 mcg./ml.) of the unknown were applied to the plates from self-filling lambda pipets calibrated to deliver accurately known volumes. The size of the spot of application of these solutions was about 10 mm. or less. The spots were applied 20 mm. from the edge of the plates and were allowed to develop over 150 mm. from the starting line after the tanks had equilibrated overnight.

Detection of Spot Area.—When the solvent reached the finishing line, the plates were removed, dried for 30 minutes at room temperature, and sprayed with ceric sulfate solution. They were developed at 120° for 2 hours.

Measurement of Spot Area.—The area of the spot was outlined carefully with a dissecting needle. A sheet of transparent paper was superimposed on the spot and the area traced. Sheets of acetate viewfoil, on which circles and ellipses of known area had been imprinted, were superimposed on the paper and the area calculated. Areas could be added or subtracted from the circles or ellipses by placing a sheet of millimeter graph paper under the viewfoil and calculating the specific area to be determined.

RESULTS AND DISCUSSION

The investigation fell into three parts: (*a*) to ascertain whether the concentration of single drugs could be related directly to their spot area and to determine the maximum and minimum quantities of the drugs that could be employed; (*b*) to separate and estimate the concentration of individual drugs in synthetic binary mixtures; and (*c*) to apply this technique to the analysis of antihistamines in various pharmaceutical preparations.

When the developed plates were sprayed with the ceric sulfate solution, each drug gave a characteristic color on a faint yellow background. The lower limits of detection, in micrograms, varied with each drug. The values quoted in Table I are the minimum quantities necessary for quantitative area measurement. All areas were marked out following the removal of the plate from the oven because the spots faded slightly with time. In general, the smaller the initial area of application of the solutions, the more uniform the area of the developed spot. In practice, the area of application should not exceed 10 mm. It was also advantageous to insure that the unknown solution had a slightly higher concentration than the reference standard.

Table II shows that most of the drugs when applied in concentrations of 25 or 50 mcg. can be estimated with an accuracy of approximately 3%. Diphenhydramine hydrochloride appears to be an exception to this when applied alone. However, when this antihistamine was applied in combination with *l*-phenylephrine, the percentage recovery was improved considerably. In binary mixtures, the recoveries of all other antihistamines were highly satisfactory. Figure 1 depicts a classical plate which has been developed and sprayed in the manner described in this paper.

The formula employed to calculate the areas was as follows (9):

$$\log W = \log W + \left(\frac{\sqrt{A} - \sqrt{A_s}}{\sqrt{A_d} - \sqrt{A}}\right) \log d$$

where W = weight of the unknown, W_s = weight of the drug in the standard solution, d = the factor involved in diluting the standard to the weaker standard solution, A = area, in mm.^{2}, of the "unknown" spot, A_s = area, in mm.^{2}, of the "standard" spot, and A_d = area, in mm.^{2}, of the "diluted standard" spot.

The pharmaceutical preparations were assayed conveniently by the proposed technique, and the results are reported in Table III. The recoveries are within the limits of experimental error for thin-layer chromatography. However, it was essential to vary the concentration of the solution applied to the plates from one drug to another in a way such that the solution of a particular drug being estimated contained at least the minimum detectable quantity. A classical example would be Histadrina capsules which contain 8 mg. of ephedrine hydrochloride and 25 mg. of methapyrilene hydrochloride. In determining the methapyrilene content, five capsules were dissolved in water, filtered, and made up to 25 ml. Five microliters of this solution and 5 μl. of a 3–5 times dilution of it were applied to the plate. Twenty micrograms in 5 μl. of methapyrilene hydrochloride was applied as the standard. Similar adjustments were made to estimate the ephedrine content.

Attempts were made to obtain comparative analysis of the commercial samples by the procedure reported by Clair and Chatten (3). In every instance, no satisfactory visual or potentiometric end point was observed; undoubtedly, the complexity of the products was the major contributing cause. The nonaqueous technique can only be considered to be satisfactory for certain single-component products, as previously reported (3). The present method has the advantage of being applicable to single or multicomponent products.

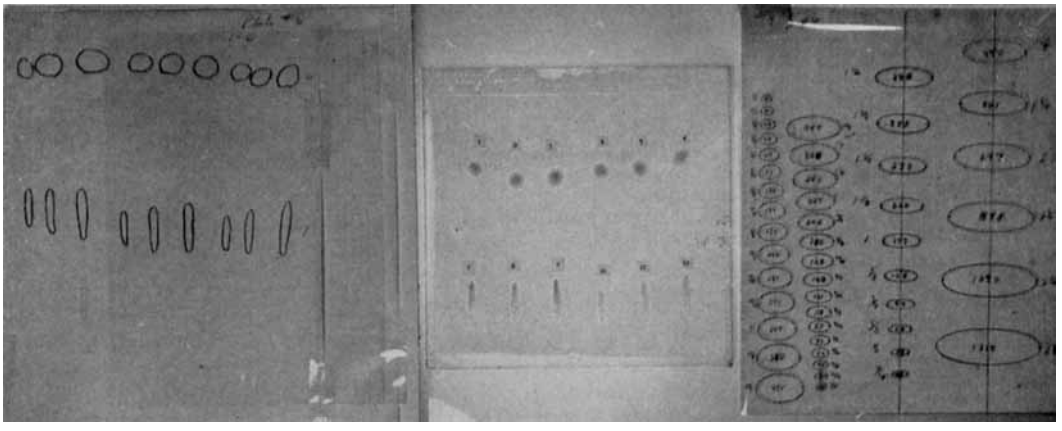


Fig. 1.—Developed chromatoplate with acetate viewfoil used in area measurement. Spots 1–6, *l*-phenylephrine hydrochloride; spots 7–9, thenylene hydrochloride; spots 10–12, pyrilamine maleate.

TABLE III.—QUANTITATIVE ANALYSIS OF PHARMACEUTICALS BY THIN-LAYER CHROMATOGRAPHY

Trade Prepn.	Amount per Tablet Labelled mg.	Amount per Tablet Recovered, ±	Recovered, %	Determinations, No.
Histadrina (capsules)				
Ephedrine HCl	8.00	7.79 ± 0.46	97.42	30
Methapyrilene HCl	25.00	26.12 ± 2.75	104.48	30
Romilar CF (capsules)				
Phenindamine tartrate	6.50	6.64 ± 0.73	102.15	30
l-Phenylephrine HCl	5.00	4.90 ± 0.37	98.96	30
Thenylene A.P.C. (capsules)				
Methapyrilene HCl	25.00	25.05 ± 2.87	100.20	30
Chlor-tripolon (tablets)				
Chlorpheniramine maleate	4.00	3.86 ± 0.30	96.42	29
Histadyl (capsules)				
Methapyrilene hydroxybenzoyl benzoate	25.00	23.63 ± 2.48	94.52	29
Romilar CF full strength (capsules)				
Chlorpheniramine maleate	1.00	0.94 ± 0.09	94.32	30
l-Phenylephrine HCl	5.00	5.09 ± 0.36	101.73	30

provided the respective components separate sufficiently sharp to permit measurement on the plate.

Determining a satisfactory solvent system can be done qualitatively by finding the R_f values of each component on a silica gel coated glass microscope slide. A 250-ml. beaker covered with aluminum foil makes a satisfactory developing chamber. Development takes about 20 minutes and, in this way, a large number of solvents can be examined rapidly.

In their report, Purdy and Truter (9) estimated all area measurements by using millimeter graph paper. This method is tedious and can result in eye strain. Using viewfoil on which circles and ellipses of known areas are imprinted is much more rapid and convenient. When standard areas were checked by both methods, the resulting differences were 3% or less.

The proposed method is conveniently applicable to pharmaceutical preparations. For example, tablet excipients and diluents do not interfere with the separation of the active constituents or their delineation. In addition, several assays can be performed on multicomponent dosage forms in a normal 8-hour day.

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Novel Decarboxylative *N*-Alkylation Reaction Resulting from Controlled Pyrolysis of Procaine

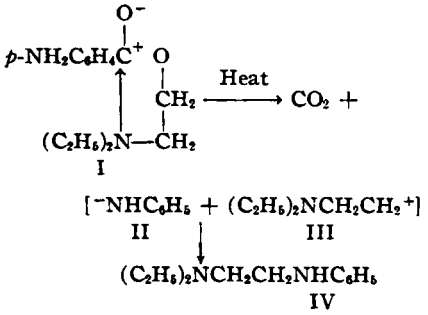
By NATHANIEL GRIER

2-Diethylaminoethyl *p*-aminobenzoate is unstable at elevated temperatures and evolves carbon dioxide, 2-diethylaminoethanol, and *N,N*-diethyl-*N'*-phenylethylenediamine. The pharmacological properties of the diamine correlate with some of the actions of systemic procaine.

AN INCREASED interest has been evidenced in the pharmacological properties of systemic procaine. One active pursuit has been the resolution of conflicting clinical observations reported by various investigators in studies of its long-term administration to humans. Another aspect centers upon the inability of correlating useful clinical effects with the drug's rapid *in vivo* metabolism by hydrolysis to *p*-aminobenzoic acid and 2-diethylaminoethanol. Intravenous procaine has been found effective for the relief of herpetic pain and postherpetic neuralgia (1). Efforts to solve some of the uncertainties include searches for active metabolites and the identification of significant impurities which may be present initially in commercial drug preparations.

With this in mind, a novel decarboxylative *N*-alkylation reaction of procaine which yields carbon dioxide and *N,N*-diethyl-*N'*-phenylethylenediamine is reported. The process was first encountered upon subjecting the ester to moderately elevated temperatures in a study of its manufacture. At temperatures above 200°, preferably in the range 225–235° and under pressures of 20–25 mm., the compound evolves carbon dioxide and easily condensable vapors consisting of a mixture of the diamine and 2-diethylaminoethanol. The aminoalcohol is formed by intermolecular amine-ester self-condensation and is separated readily from the diamine by fractional distillation.

Production of the diamine appears to be initiated intramolecularly by attack of the nucleophilic ternary ester nitrogen atom upon the procaine acyl carbonium ion (I). After loss of carbon dioxide, the aniline anion (II), the result of a proton shift to the ring, and the diethylaminomethylcarbonium ion (III) combine to give *N,N*-diethyl-*N'*-phenylethylenediamine (IV).



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provided the respective components separate sufficiently sharp to permit measurement on the plate.

Determining a satisfactory solvent system can be done qualitatively by finding the R_f values of each component on a silica gel coated glass microscope slide. A 250-ml. beaker covered with aluminum foil makes a satisfactory developing chamber. Development takes about 20 minutes and, in this way, a large number of solvents can be examined rapidly.

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The proposed method is conveniently applicable to pharmaceutical preparations. For example, tablet excipients and diluents do not interfere with the separation of the active constituents or their delineation. In addition, several assays can be performed on multicomponent dosage forms in a normal 8-hour day.

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Novel Decarboxylative *N*-Alkylation Reaction Resulting from Controlled Pyrolysis of Procaine

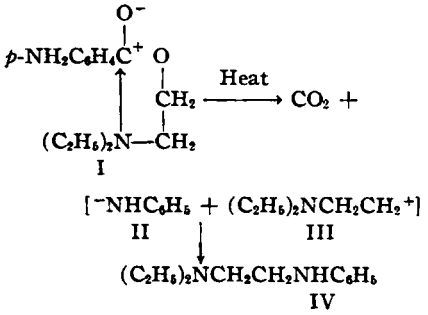
By NATHANIEL GRIER

2-Diethylaminoethyl *p*-aminobenzoate is unstable at elevated temperatures and evolves carbon dioxide, 2-diethylaminoethanol, and *N,N*-diethyl-*N'*-phenylethylenediamine. The pharmacological properties of the diamine correlate with some of the actions of systemic procaine.

AN INCREASED interest has been evidenced in the pharmacological properties of systemic procaine. One active pursuit has been the resolution of conflicting clinical observations reported by various investigators in studies of its long-term administration to humans. Another aspect centers upon the inability of correlating useful clinical effects with the drug's rapid *in vivo* metabolism by hydrolysis to *p*-aminobenzoic acid and 2-diethylaminoethanol. Intravenous procaine has been found effective for the relief of herpetic pain and postherpetic neuralgia (1). Efforts to solve some of the uncertainties include searches for active metabolites and the identification of significant impurities which may be present initially in commercial drug preparations.

With this in mind, a novel decarboxylative *N*-alkylation reaction of procaine which yields carbon dioxide and *N,N*-diethyl-*N'*-phenylethylenediamine is reported. The process was first encountered upon subjecting the ester to moderately elevated temperatures in a study of its manufacture. At temperatures above 200°, preferably in the range 225–235° and under pressures of 20–25 mm., the compound evolves carbon dioxide and easily condensable vapors consisting of a mixture of the diamine and 2-diethylaminoethanol. The aminoalcohol is formed by intermolecular amine-ester self-condensation and is separated readily from the diamine by fractional distillation.

Production of the diamine appears to be initiated intramolecularly by attack of the nucleophilic ternary ester nitrogen atom upon the procaine acyl carbonium ion (I). After loss of carbon dioxide, the aniline anion (II), the result of a proton shift to the ring, and the diethylaminomethylcarbonium ion (III) combine to give *N,N*-diethyl-*N'*-phenylethylenediamine (IV).



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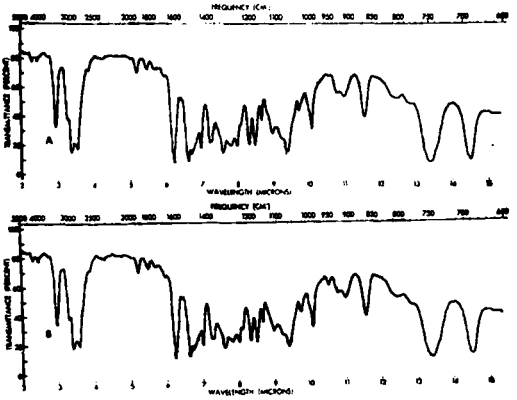


Fig. 1.—Infrared absorption spectra of (top) *N,N*-diethyl-*N'*-phenylethylenediamine, synthetic; (bottom) procaine pyrolysis isolate.

A possible ring alkylate, *p*-amino *N,N*-diethylphenethylamine, $p\text{-}NH_2C_6H_4CH_2CH_2N(C_2H_5)_2$, which could form by interaction of the carbonium ion (III) and *p*-aminophenyl anion, was not detected.

An authentic sample of the aniline *N*-alkylate was prepared by reacting aniline with 2-diethylaminoethyl chloride (2). It was identical to the pyrolysis isolate in boiling point, 131–133° (6 mm.); melting point as the monohydrochloride, 135° (3); melting point as the salicylic acid salt of the *N*-benzoyl derivative (4), 112–113°; and by comparison of chromatograms and infrared spectra (Fig. 1).

Ethyl *p*-aminobenzoate (benzocaine) was unaltered when heated at 235° over a 4-hour period. Cruickshank and Sheehan noted the essential role of intramolecular interactions between functional groups to facilitate decarboxylative acylation of γ- or δ- dialkylamino acids with anhydrides (5).

The pyrolysis of benzoic esters at temperatures above 400° yields benzoic acid and olefins corresponding to the alcohol moiety; as a secondary process the acid decomposes losing carbon dioxide. No recombination of the pyrolytic fragments was reported. Esters which contained at least one β-hydrogen atom were thought to pyrolyze *via* a cyclic transition state in which some carbonium ion character is attributed to the α-carbon atom (6). Dialkylaminopropyl and dialkylaminoisopropyl benzoates decompose at temperatures of 440–450°, forming benzoic acid and the olefinic amines, allydimethylamine and 2-dimethylamino-1-propene; no reference was made to carbon dioxide as a secondary product (7).

A consideration of the pharmacological properties of systemic procaine with those of *N,N*-diethyl-*N'*-phenylethylenediamine reveals areas of similar actions. One of the puzzling effects of intravenous procaine is systemic analgesia.[1] Administration of its water-soluble salts leads to liberation of the active ester base, which in turn is destroyed rapidly by the liver and plasma in humans (8). Essentially, the mechanism is enzymatic hydrolysis; procaine in human plasma completely disappears within 2 minutes in the absence of esterase inhibitors (9).

[1] This property of systemic procaine has been questioned in Keats, A. S., D'Alessandro, G. L., and Beecher, H. K., *J. Am. Med. Assoc.*, **147**, 1761(1951).

The kinetics of hydrolysis were studied in detail by Kalow (10). It is apparent that neither the short-lived ester nor its hydrolytic end products can be the agents responsible for the useful effects; the concentration of nonhydrolyzed drug capable of being achieved in extracellular fluid is too low for peripheral nerve block, and the hydrolysis products are without anaesthetic action. The analgesic properties of the diamine indicate it may be a significant metabolite of procaine, a possible enzymatic decarboxylation product formed in varying amount. A series of potent synthetic analgesics has been described in which the basic structure included *N,N*-dialkyl-*N'*-phenylethylenediamines (11).

In another area, some of the clinical properties ascribed to procaine parallel those of compounds classified as monoamine oxidase inhibitors (12). One of the significant effects, *i.e.*, increased psychomotor activity (13), is of particular interest because it is associated in part with tryptamines, compounds formed *in vivo* by the enzymatic decarboxylation of tryptophanes. These amines may be considered cyclic vinylogs of the *N*-alkylated aniline; a most important member of the group is serotonin or 5-hydroxytryptamine.

N,N-Diethyl-*N'*-phenylethylenediamine is an effective inhibitor of monoamine oxidase in mice measured by the accumulation of serotonin in the brain.[2] A single dose of 50 mg./Kg. body weight was injected intraperitoneally for bioassay, and a second dose was given 16 hours later. The animals were sacrificed 1 hour after and the brain serotonin content determined by the usual analytical fluorescent procedure. The serotonin content of treated animals was compared to that of control animals receiving no compound. In two separate determinations using five animals in each test, brain serotonin ratios of 1.79 and 1.69 compared to controls were obtained, a potency equal to iproniazid[3] in this method. Intact procaine, on the other hand, appears to be a direct antagonist of serotonin (14).

The behavioral stimulant capacity of the diamine was tested in rats and squirrel monkeys at oral dosages up to 90 mg./Kg. body weight. In a conflict schedule involving trained rats, who pressed a lever for food and simultaneously received electric shocks, no significant effects were noted. Similarly, using an avoidance schedule wherein squirrel monkeys had to operate a lever to avoid receiving electric shock, the compound was without promise.

EXPERIMENTAL

Melting points in capillary tubes were determined using an oil bath with Anschütz thermometers. Elemental analyses were performed by Schwarzkopf Laboratories, New York, N. Y. Infrared spectra were obtained with a 0.0025 in. thickness of compound *versus* air in a Perkin-Elmer model 21 spectrophotometer.

Procaine Pyrolysis.—Pure procaine base was prepared by precipitation from an aqueous solution of its hydrochloride using dilute ammonium hydroxide solution. After drying at 40° for 16 hours, it was

[2] Animal bioassays and tests were obtained through the cooperation of Dr. J. M. Sprague and his associates, Merck Sharp and Dohme Research Laboratories, Division of Merck and Co., Inc., West Point, Pa.

[3] Marketed as Marsilid by Hoffmann-LaRoche, Inc. Nutley, N. J.

twice recrystallized from petroleum ether, b.p. 30–60°; m.p. 61–62°.

In a 100-ml. Claisen flask set for vacuum distillation 59 Gm. (0.25 mole) procaine base was held at an internal temperature range of 225–235°, while a pressure of 19 mm. was maintained using a controlled air leak at the vacuum pump. Within a 1-hour period under these conditions 17.8 Gm. of practically colorless liquid was collected in a brine-cooled receiver; the residue in the distillation flask was a gummy yellow solid. Evolution of carbon dioxide throughout the distillation was observed by passing the noncondensed vapor through a Drierite U-tube to a soda-lime trap (indicator type, 4–8 mesh, Fisher Scientific Co.).

Fractionation of the distillate through a 10-cm. Vigreux column at 5 mm. pressure yielded two liquids in nearly equal amounts; the first was collected until an upper vapor temperature of 80° and the second at 133–135°. The lower boiling fraction was identified as 2-diethylaminoethanol and converted to its hydrochloride, m.p. 133–135° (15).

The second liquid was submitted to a more detailed study. Treatment of the residue, a resinous polyamide, with boiling aqueous 5% sodium hydroxide led to the recovery of *p*-aminobenzoic acid on adjustment of pH to 4.0–4.5.

Proof of Structure of Pyrolysate II as N,N-Diethyl-N′-phenylethylenediamine.—The second pyrolysis fraction was converted to a monohydrochloride salt by treatment with dry hydrogen chloride in ether or 99% isopropyl alcohol. The salt so obtained was recrystallized from 99% isopropyl alcohol, m.p. 133–135°.

Anal.—Calcd. for $C_{12}H_{21}ClN_2$: C, 63.02; H, 9.18; Cl, 15.53; N, 12.25. Found: C, 63.15; H, 9.02; Cl, 15.25; N, 11.98.

For comparison, the free base was synthesized by the alkylation of aniline with 2-diethylaminoethyl chloride in absolute alcohol containing excess sodium carbonate (2). On reaction with dry hydrogen chloride it formed a monohydrochloride salt which melted at 133–135° (3). A mixed melting point with the hydrochloride salt of pyrolysate II showed no depression.

The second pyrolysis fraction was characterized further by benzoylation and isolation as a crystalline salicylate.

Pyrolysate II was heated with the calculated quantity of benzoyl chloride at 100° for 24 hours (4).

One equivalent of salicylic acid was added to a 30% solution of the liquid benzoyl derivative in warm benzene. Addition of 0.5 vol. of petroleum ether (b.p. 30–60°) and gradual cooling deposited the colorless crystalline salicylate, which was recrystallized from the same solvent system, m.p. 112–113°.

Anal.—Calcd. for $C_{26}H_{30}N_2O_4$: C, 71.85; H, 6.95; N, 6.45. Found: C, 72.11; H, 6.88; N, 6.50.

N,N - Diethyl - *N′* - benzoyl - *N′* - phenylethylenediamine salicylate was prepared similarly from the synthesized base. This salt melted at 112–113°, a mixed melting point of the two salicylates showed no depression.

p-Amino-N,N-diethylphenethylamine.—Catalytic hydrogenation of *p*-aminobenzyl cyanide in the presence of diethylamine using 10% palladium on barium sulphate yielded the product after removal of the catalyst and fractional distillation, b.p. 115–116° (0.5 mm.) (16).

Chromatographic Comparisons.—Chromatograms were obtained using strips of Whatman No. 1 filter paper in an ascending system. Development included the use of a mixed solvent prepared from 2 vol. *n*-butanol and 1 vol. each glacial acetic acid and water; the time required was 16 hours at 20°. For location of compounds the paper was air-dried, sprayed with a 0.2% ninhydrin in acetone solution, then heated at 110° for 10 minutes.

The synthetically prepared *N,N*-diethyl-*N′*-phenylethylenediamine and *p*-amino-*N,N*-diethylphenethylamine were compared to the second fraction of the pyrolysate. The *p*-substituted aniline, a colorless liquid stored under nitrogen, gave an immediate yellow color on contact with the paper. The other two products remained colorless. Identical R_f values were obtained—0.80—for the ethylenediamine and the pyrolysate, with bluish-green spots on reaction with ninhydrin. An R_f of 0.69 was noted for the *p*-substituted aniline and a yellowish-brown color with ninhydrin. Repeated chromatography trials failed to show its presence in the pyrolysate. The closeness in boiling point of the diamine, 154–158° (17 mm.) (17), and the *p*-alkylated aniline, 154–157° (13 mm.) (16), prevented seperation by distillation.

Ethyl p-Aminobenzoate Pyrolysis Attempt.—The ester was twice recrystallized from ethyl alcohol, m.p. 87.5–88.8°. It was heated at the same internal temperature range (225–235°) as procaine, but a pressure of 65 mm. was selected to maintain reflux of the ester in the Claisen flask without condensation of any formed aniline or *N*-ethylaniline. There was no detectable carbon dioxide evolution over a 4-hour period of heating; the pressure was reduced to 0.8 mm., and the ester recovered quantitatively at a boiling point of 141–143°. The distillate rapidly solidified on cooling and showed no change in melting point.

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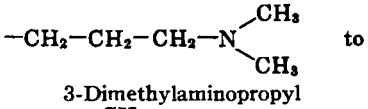
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Hepatic Secretion and Urinary Excretion of Three S^{35}-Labeled Phenothiazines in the Dog

By EDWARD J. VAN LOON, THOMAS L. FLANAGAN, WILLIAM J. NOVICK, and ALFRED R. MAASS

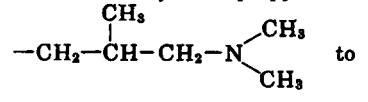
The present studies indicate that in the dog chlorpromazine, prochlorperazine, and trifluoperazine are rapidly absorbed from the intestine and that there is a rapid hepatic clearance of the phenothiazines. The biliary and urinary excretions of these compounds are greatly affected by the side chain of the phenothiazine nucleus. The "methylpiperazinylpropyl" side chain (prochlorperazine and trifluoperazine) markedly increases the rate of biliary excretion and reduces the urinary excretion of the parent compounds and/or their metabolites compared with the excretion of chlorpromazine and/or its metabolites (dimethylaminopropyl side chain). Moreover, chlorpromazine and/or its metabolites excreted in bile are reabsorbed from the intestine, resulting in an entero-hepatic recirculation.

FYODOROV REPORTED (1–3) a high degree of radioactivity in the bile of dogs and rabbits following the administration of chlorpromazine-S^{35} [1] and stated that, following oral administration (2), a large part of the chlorpromazine-S^{35} does not enter into the general blood stream but circulates in a closed circuit—intestine, portal vein, liver, bile, and back to the intestine, gradually being eliminated in the feces. Flanagan *et al.* (4) have reported on the biliary and urinary excretion patterns of chlorpromazine in the dog following intraduodenal administration. In excretion studies in rats using five S^{35}-labeled phenothiazines (5), the effect of change in structure of the side chain upon the mode of S^{35} excretion has been reported. The urinary S^{35} excretion decreased, and the fecal S^{35} excretion correspondingly increased as the side chain was changed from



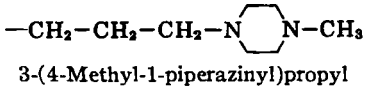
to

3-Dimethylaminopropyl



to

3-Dimethylamino-2-methylpropyl



3-(4-Methyl-1-piperazinyl)propyl

Three S^{35}-labeled phenothiazines were used in the present studies to show: (*a*) that phenothiazine drugs are readily absorbed from the intestinal tract, (*b*) that the side chain structure attached to the phenothiazine nucleus has a definite effect upon the route of excretion, and (*c*) that there is a rapid biliary recirculation of chlorpromazine and/or its metabolites.

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[1] Aminazine-S^{35}.

EXPERIMENTAL

Preparation of S^{35} Phenothiazines.—The three phenothiazine-S^{35} compounds used in the present studies were prepared by Dr. D. W. Blackburn (6). The structures of these compounds are shown in Table I.

Operative Procedure.—Mongrel female dogs, weighing 10 ± 0.2 Kg., which had been fasted for 16 hours were used in these studies. The operative procedure to insure collection of hepatic bile only was described previously (4). Urine specimens were obtained by catheterization.

Each drug was administered at a dosage level known to produce in dogs pharmacological responses, such as depression, sedation, and antiemetic activity.

In the chlorpromazine-S^{35} experiments, three dogs received only the operative intraduodenal dose—200 mg. of the labeled drug contained in 20 ml. of isotonic saline. To discover if priming had an effect upon excretion, a fourth dog was treated daily for 9 days preoperatively with 100 mg. of unlabeled chlorpromazine administered orally. On the tenth day, this dog received 200 mg. of labeled drug at the the time of operation. Bile and urine specimens were collected at 2-hour intervals for 10 hours throughout the course of each experiment.

Venous blood samples were withdrawn periodically from two unprimed dogs throughout each experiment. In the third study with an unprimed dog, periodic samples of blood were obtained from the portal, hepatic, and jugular veins in an attempt to show absorption (portal), liver clearance (hepatic), and circulation (jugular) of chlorpromazine. Cannulae were inserted into the three veins, and simultaneous blood samples were withdrawn at designated time periods. Blood specimens were not obtained from the primed dog. All bile, urine, and blood samples were analyzed for total S^{35} activity.

In another experiment, the bile collected from two of the operated dogs which had received 200 mg. of chlorpromazine-S^{35} intraduodenally was combined and made to 25 ml. volume with saline. An aliquot was assayed for S^{35} content. The remainder (20 ml.) was instilled into the duodenum of a third dog which had received no previous phenothiazine but

TABLE I.—STRUCTURES OF THE THREE COMPOUNDS

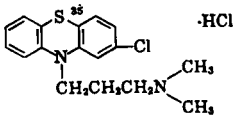
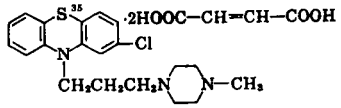
Compd.	Name	Structure
2-Chloro-10-(3-dimethylaminopropyl)phenothiazine-S^{35} hydrochloride	Chlorpromazine	
2-Chloro-10-[3-(4-methyl-1-piperazinyl)propyl]phenothiazine-S^{35} dimaleate	Prochlorperazine	
10-[3-(4-Methyl-1-piperazinyl)propyl]-2-trifluoromethylphenothiazine-S^{35} dihydrochloride	Trifluoperazine	

TABLE II.—BILIARY AND URINARY EXCRETION OF CHLORPROMAZINE-S^{35} EXPRESSED AS PER CENT OF ADMINISTERED DRUG (OPERATIVE DOSE, 200 mg.)

Collection Periods, Hr.	Dog 1[a]		Dog 2[a]		Dog 3[a]		Dog 4[b]	
	Bile	Urine	Bile	Urine	Bile	Urine	Bile	Urine
0–2	3.9	2.0	2.1	1.1	10.1	2.3	1.8	1.7
2–4	6.1	3.7	3.0	3.4	7.7	5.7	5.9	4.3
4–6	3.8	4.6	3.2	5.0	6.0	4.5	5.7	4.9
6–8	3.2	4.5	3.3	4.8	3.7	2.6	5.0	3.8
8–10	3.1	3.4	2.9	2.7	3.6	2.0	4.9	4.4
Total	20.1	18.2	14.5	17.0	31.1	17.1	23.3	19.1

[a] Single operative dose. [b] Primed for 9 days with "cold" chlorpromazine (100 mg./day) prior to receiving operative dose.

which had been prepared for quantitative collection of hepatic bile and urine. Bile and urine were then collected from this animal for a 10-hour period and assayed for S^{35} activity. Any S^{35} appearing in the collected bile or urine would necessarily arise from the intestinal reabsorption of the administered labeled chlorpromazine and/or its metabolites.

In the experiments with prochlorperazine-S^{35}, 100 mg. of the labeled drug dissolved in saline was administered intraduodenally to three dogs. Bile and urine specimens were obtained from each animal. Portal, hepatic, and venous blood specimens were obtained simultaneously from one dog. Blood specimens were not taken from the other animals.

TABLE III.—HEPATIC RECIRCULATION OF CHLORPROMAZINE-S^{35}. PER CENT OF RECOVERY OF S^{35} BILE COLLECTED FROM A CHLORPROMAZINE-S^{35}-TREATED DOG

Time, Hr.	Bile	Urine
0–2	4.1	0.9
2–4	8.8	4.1
4–6	6.6	3.6
6–8	6.5	2.9
8–10	3.4	2.7
Total	29.9	14.2

In the studies with trifluoperazine-S^{35}, 25 mg. of the labeled drug dissolved in saline was administered to a single dog. Only bile and urine specimens were collected from this animal.

Oxidation and Counting Procedures.—In the chlorpromazine-S^{35} studies, each specimen was subject to a nitric acid and a nitric-perchloric acid oxidation to convert any S^{35} material to $S^{35}O_4^-$. The $S^{35}O_4^-$ was then precipitated as $BaS^{35}O_4$, filtered, dried, weighed, and counted on a gas flow counter having a micromil window (Nuclear-Chicago 192). The samples were corrected to infinite thinness. A known amount of the original chlorpromazine-S^{35} was oxidized and counted in a similar manner tc obtain the specific activity of the administered drug.

In the prochlorperazine-S^{35} and trifluoperazine-S^{35} studies, the bile and urine specimens were made to volume; suitable aliquots were counted in a Packard Tricarb liquid scintillation spectrometer, model 314-X, using a phosphor system which contained 80 Gm. of naphthalene and 5 Gm. of 2,5-diphenyloxazole dissolved in a mixture of toluene, 1,4-dioxane, and absolute alcohol (1:1:0.6).

The blood samples from the prochlorperazine-S^{35} dogs were oxidized and counted in the same manner as the specimens from the chlorpromazine-S^{35} dogs.

TABLE IV.—BILIARY AND URINARY EXCRETION OF PROCHLORPERAZINE-S^{35} AND TRIFLUOPERAZINE-S^{35} EXPRESSED AS PER CENT OF ADMINISTERED DRUG

Collection Periods, Hr.	Prochlorperazine-S^{35} Operative Dose, 100 mg.						Trifluoperazine-S^{35} Operative Dose, 25 mg.	
	Dog 5		Dog 6		Dog 7		Dog 8	
	Bile	Urine	Bile	Urine	Bile	Urine	Bile	Urine
0–2	16.7	1.4	18.7	1.4	15.6	1.9	24.5	0.4
2–4	17.8	1.9	22.3	1.8	18.3	2.5	19.4	0.8
4–6	11.8	1.6	13.6	1.6	12.2	1.9	10.4	0.5
6–8	7.8	1.1	7.6	1.1	7.8	1.3	13.3	0.5
8–10	6.1	1.0	5.7	0.9	7.3	0.8	3.9	0.1
Total	60.2	7.0	67.9	6.8	61.2	8.4	71.5	2.3

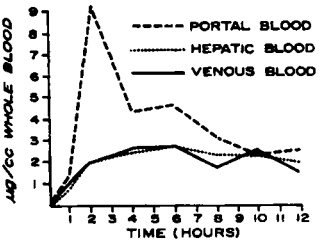


Fig. 1.—Blood levels of chlorpromazine following intraduodenal administration (200 mg.).

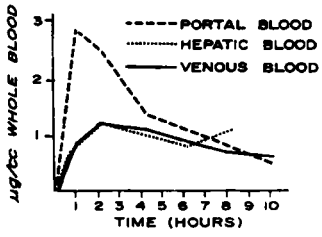


Fig. 2.—Blood levels of prochlorperazine following intraduodenal administration (100 mg.).

RESULTS AND DISCUSSION

The S^{35} concentrations of the bile and urine specimens, expressed as per cent of the administered dose for the chlorpromazine-S^{35}-treated dogs are shown in Table II.

The 10-hour recovery of S^{35} in the urine of all four animals was remarkably constant: 18.2, 17.0, 17.1, and 19.1%. Priming of a dog for 9 days with cold chlorpromazine did not appear to alter the urinary excretion of the labeled drug on the tenth day.

The 10-hour biliary excretion of chlorpromazine-S^{35} was also fairly consistent for all animals: 20.1, 14.5, 31.1, and 23.3%. The average total biliary excretion for the unprimed animals was 21.9%. The primed animal (Dog 4) excreted 23.3% in 10 hours, again suggesting that the 9 days' priming with cold chlorpromazine did not alter the rate at which the dose on the tenth day was excreted in the bile.

The collection of bile and urine from Dog 3 was continued for 24 hours. During the 22–24 hour collection period, 1.4% of the dose was recovered in the bile, while 0.7% was recovered from the urine. The total excretion during the 24-hour collection was 46.7% in bile and 24.3% in urine, amounting to a 71% total recovery of the administered dose in bile and in urine.

In the experiment where bile labeled with chlorpromazine and/or its metabolites was instilled in the duodenum, 30% of the radioactivity appeared in the bile during the 10-hour collection period, while 14% appeared in the urine (Table III). This compares with 22% for bile and 17% for urine when only chlorpromazine-S^{35} was administered. These data support the theory of the entero-hepatic circulation of chlorpromazine and/or its metabolites following its secretion into bile as postulated by Fyodorov (2).

The data obtained from bile and urine specimens following the intraduodenal administration of prochlorperazine to three dogs and trifluoperazine to one dog are shown in Table IV. The results are expressed as per cent of administered dose of drug. The biliary and urinary excretions of S^{35} in all three animals following prochlorperazine administration were consistent: 60.2, 67.9, and 61.2% for bile and 7.0, 6.8, and 8.4% for urine. The biliary excretion exceeded the urinary excretion eightfold; the average biliary excretion was 63.1%, compared with an average of 7.4% recovery from urine. The initial rate of biliary excretion of prochlorperazine-S^{35} and metabolites was high, and S^{35} activity was detected in bile within 5 minutes after intraduodenal administration of this compound.

Because of a limited supply of the radiochemical, only one animal was tested with trifluoperazine. As was observed with prochlorperazine-S^{35}, trifluoperazine-S^{35} was excreted predominantly by the biliary route. The 10-hour biliary excretion of S^{35} was 71.5% of the dose, while the urinary S^{35} excretion amounted to only 2.3%; thus, the biliary excretion was thirtyfold greater than the urinary excretion.

It would appear from these studies that prochlorperazine and trifluoperazine are excreted predominantly by the biliary route in the dog. The biliary to urinary excretion ratio was 30/1 for trifluoperazine, 8/1 for prochlorperazine, and about 1/1 for chlorpromazine. These data, together with previous excretion data in rats (5), indicate that the 3-(4-methyl-1-piperazinyl)propyl side chain affects the excretion of the phenothiazine nucleus, resulting in an increased biliary and decreased urinary excretion of the parent compound and/or its metabolites.

Systemic venous blood levels of the three chlorpromazine-S^{35}-treated dogs ranged from 3 to 7 mcg. of chlorpromazine per milliliter of whole blood, based upon S^{35} activity, with the peak concentration occurring at 4 to 6 hours post drug administration.

To determine the hepatic clearance of phenothiazines in the dog, the S^{35} content was determined on blood samples obtained simultaneously from the portal, hepatic, and jugular veins following intraduodenal administration of 200 mg. of chlorpromazine-S^{35} (Fig. 1). There was a marked difference between the concentration of S^{35} in the portal blood and that of the hepatic and jugular blood. At 2 hours, this difference was fourfold, but by 10 hours all three blood samples contained essentially the same concentration of S^{35}. This study demonstrates the rapid absorption of chlorpromazine from the intestine and its equally rapid clearance from the blood by the liver.

A similar study using 100 mg. of prochlorperazine-S^{35} administered intraduodenally again demonstrated rapid absorption, as there was a rapid increase in the portal concentration of S^{35} which reached its peak about 1 hour post drug administration. Hepatic clearance of prochlorperazine was again demonstrated by the marked difference between portal and hepatic blood concentrations of S^{35} (Fig. 2). The rapid peak of portal S^{35} concentration is in good agreement with the high level of S^{35} obtained in the bile during the first few hours after prochlorperazine-S^{35} administration.

It is fully realized that an intact entero-hepatic circulation would alter the results obtained in this study for blood levels and urinary excretion. However, it is felt that the general pattern would be the same and only the absolute values altered.

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Relationship of Surfactant Properties of Some Synthetic Steroids to Bactericidal Action

By RODNEY F. SMITH, DONALD E. SHAY, and NORMAN J. DOORENBOS

Properties of seven azasteroids and a steroidal acid, which have been found to possess antibacterial activity, were compared with benzalkonium chloride (BAC). Bactericidal action against *Gaffkya tetragena*, lecithin inactivation, hemolysis of sheep erythrocytes, and the lowering of surface tension were studied. The positive relationship between surface-active properties and antibacterial activity observed with many surfactants, such as BAC, was found with only two of these steroids. Certain of the bactericidal steroids may be mechanistically similar to BAC, but the properties of the steroids studied suggest different modes of action.

SEVERAL STEROIDS have been found to possess high antimicrobial activity (1, 2). Most of these steroids were nitrogen-containing bases or their quaternary salts (quats). A recent study (3) demonstrated that the activity of these steroidal bases and quaternary salts was decreased by serum, blood, lecithin, tweens, soaps, and bile salts. Similar observations have been made on the quaternary salt antiseptics (4). The lethal action of the quaternary salt antiseptics has been attributed to their surface activity and to their ability to adsorb to negatively charged sites (5). This study was undertaken to determine whether a relationship could be established between the surface-active properties of these steroids and their antimicrobial activity. This report includes a comparison of benzalkonium chloride[1] (BAC) with eight steroids previously shown to possess antibacterial activity. The antibacterial activity of ND-15 and ND-307 has been reported (1).

EXPERIMENTAL

Test Compounds.—The structures and names of the steroids used in this study are illustrated in Fig. 1.[2]

A 100-mg. quantity of each steroid was placed into a 150-ml. bottle. Steroids 307 and 497 were dissolved by adding 5 ml. of distilled water to form a slurry. To this was added 0.1 ml. of concentrated HCl. The bottles were heated gently in a boiling water bath, with shaking, until the two compounds were completely dissolved. The total volume was then brought to 100 ml. with distilled water. Solutions of 498, 495, 436, 477, and 271 were prepared by dissolving 100-mg. samples in 5 ml. of 95% ethanol and diluting to 100 ml. with distilled water. Compound 15 was dissolved in distilled water made slightly basic with 1 *N* NaOH. A 1-mg./ml. stock solution of BAC was prepared with distilled water.

Test Culture.—The Gram-positive bacterium, *Gaffkya tetragena* ATCC 10875,[3] was used for the bactericidal experiments. The microorganism was maintained in C.T.A. medium,[4] and grown and tested in brain heart infusion broth.[4]

Antibacterial Test Conditions.—The minimal inhibitory concentrations (M.I.C.) for the steroids and BAC were determined by the conventional twofold serial dilution method. The test systems were incubated in a 37° water bath for 48 hours. The lethal studies involved the inoculation of a 24-hour culture of *G. tetragena* into 50-ml. flasks. Each flask contained broth with the test compound in a concentration of 100 mcg./ml. A second series of flasks was inoculated. They contained the test compounds in broth, to which had been added 0.05% lecithin.[5] The flasks, equilibrated at 37°, were shaken in a Dubnoff metabolic shaking water bath at 50 c.p.m. At the termination of the experiment, the flasks were transferred to an ice bath at 4°. Aliquots of the reaction broths were removed, and total plate counts were made using brain heart infusion agar.[4] Plates were incubated at 37° for 48 hours in a water-jacketed incubator and counted.

Hemolysis.—Suspensions of unhemolyzed 10% sheep erythrocytes (R.B.C.'s) were diluted with sterile saline. Test compounds and R.B.C.'s were mixed to give final concentrations of 100 mcg./ml. of each compound in 1% R.B.C.'s. The tubes and controls were incubated at 37° and observed for hemolysis for up to 16 hours.

Surface Tension Measurements.—Surface tension measurements of the test compounds were determined with the DuNouy tensimeter. All measurements were made at 25° with triplicate readings.

RESULTS

The minimal inhibitory concentration measurements (M.I.C.) shown in Table I revealed that the five aza-cholestanes, 307, 497, 498, 495, and 271, were active. The aza-pregnane derivative 477 was active also, while the aza-androstenol compound 436 and the keto acid 15 were not inhibitory.

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[1] Marketed as Zephiran chloride by Winthrop Laboratories, Inc., New York, N. Y.

[2] The steroids represent eight of a group prepared by Norman J. Doorenbos, Department of Pharmaceutical Chemistry, School of Pharmacy, University of Maryland. In previous publications the steroid code numbers have been prefixed with ND.

[3] American Type Culture Collection, Washington, D. C.

[4] Baltimore Biological Laboratories, Inc., Baltimore, Md.

[5] Nutritional Biochemical Corp., Cleveland, Ohio.

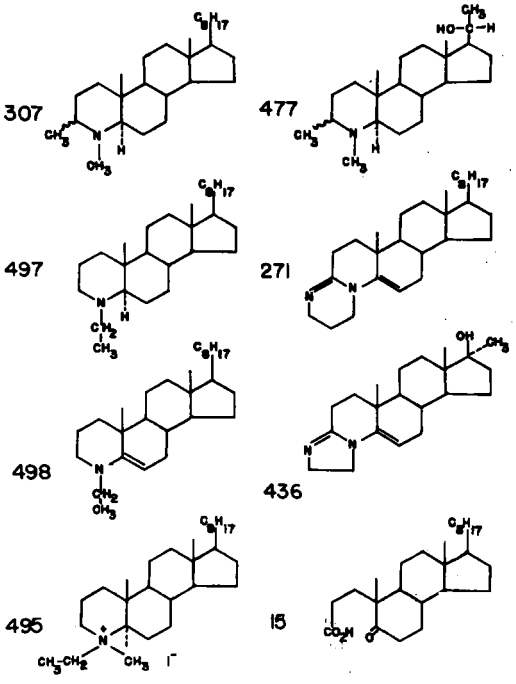


Fig. 1.—Structures of the eight steroids evaluated.

307, 3xi,4-Dimethyl-4-aza-5α-cholestane (6).
497, 4-Ethyl-4-aza-5-α-cholestane (6).
498, 4-Ethyl-4-aza-5-cholestene (6).
495, 4-Ethyl-4-aza-5α-cholestane methiodide (6).
477, 3xi,4-Dimethyl-4-aza-5α-pregnan-20β-ol (6).
271, 1′,4′,5′,6′,-Tetrahydropyrimidino[a-4,3]-4-aza-5-cholestene (7).
436, 2′-Imidazolino[a-4,3]-17α-methyl-4-4-aza-5-androsten-17β-ol (7).
15, 3,5-Seco-4-nor-5-cholestanon-3-oic acid (8).

The lethal action of the steroids and BAC is shown in Table II. Generally, there was agreement between the M.I.C. values (Table I) and the relative killing action of the active steroids.

The reversal effects of the lecithin (0.05%) on the lethal action of the steroids suggest the formation of lecithin-steroid complexes. BAC, which is known to be inactivated by phospholipids (5), presumably was used in a concentration too high to be totally inactivated by 0.05% lecithin. The appearance of turbidity in the lecithin flasks, which were incubated for 48 hours, revealed that 307, 477, and 271 were completely inactivated. The absence of turbidity in the flasks containing 497, 498, and 495 showed that these compounds were only partially inactivated, demonstrating that a substantial concentration of free steroid inhibited or killed the remaining viable cell population.

The relationship of lethal action to other properties regarded as important in the action of the quats is shown in Table III. None of the active steroids caused complete disruption of sheep R.B.C.'s. The 2+ reaction demonstrated marked extrusion of hemoglobin; but approximately 50% of the cells were still intact after 16 hours. This was observed by measuring the volume of packed cells that settled out of suspension. The weakest of the lethal steroids, 477, produced only a trace of hemolysis. The compound 436 was not hemolytic, while 15 caused complete hemolysis spontaneously, as was previously reported (3). Neither 436 nor 15 was inhibitory or lethal to the organism.

It should be noted that all surface tension values for steroids were higher in broth than in distilled water. Perhaps materials in the broth removed portions of the steroids from solution by complex formation. The results given in Table III indicate no consistency among lethal action, hemolysis, and lowering of surface tension by the steroids.

A summary of the results obtained in this study can be seen in Table IV. BAC, 307, and 497, representing Type 1, are positive in all categories studied. Compounds 498, 495, and 271 (Type 2) resemble the BAC group but deviate in surface

Table I.—Minimal Concentration of Test Compounds Inhibitory to *G. tetragena*

Test Compd.		mcg./ml.
Steroids	307	0.0185
	497	3.10
	498	0.185
	495	0.185
	271	0.75
	477	25.00
	436	...
	15	...
BAC		0.01

Table II.—Comparative Bactericidal Action of Test Compounds with Growing Cells of *G. tetragena*[a]

Test Compd.		Viable Cell Count/ml. Plain Broth	Viable Cell Count/ml. With 0.05% Lecithin	Growth After 48 Hr.[c]
Steroids[b]	307	600	246,000	+
	497	100	101,000	−
	498	10	115,000	−
	495	10	186,000	−
	477	10,000	575,000	+
	271	100	920,000	+
BAC		10	100	−

[a] Cells were exposed to compounds (100 mcg./ml.) for 3 hours at 37° C. Initial viable cell concentration was 810,000/ml. [b] 436 and 15 had no inhibitory or lethal effects. [c] Lecithin flasks were incubated 48 hours to observe for recovery of viable cells.

Table III.—Comparison of Lethal Steroid Action to Hemolytic and Surface Tension Reduction

Test Compd. (100 mcg./ml.)		Lethal Action	Hemolysis[a] 1 Hr.	Hemolysis[a] 16 Hrs.	Surface Tension (dynes/cm.)[b] Distilled Water	Surface Tension (dynes/cm.)[b] Broth
Steroids	307	+	2+	2+	39	44
	497	+	1+	2+	38	44
	498	+	2+	2+	51	52
	495	+	2+	2+	44	46
	477	+	0	1	58	61
	271	+	2+	2+	44	61
	436	−	0	0	62	70
	15	−	4+	4+	37	42
BAC		+	2+	2+	38	38

[a] Results are given as relative values compared to control systems. Hemolysis: 4+, complete; 3+ and 2+, partial; 1+, trace; 0, negative: no hemolysis detected. [b] Distilled water control, 70 dynes/cm.; broth medium control, 60 dynes/cm.

TABLE IV.—SUMMARY OF EXPERIMENTAL DATA

Type	Compd.	Lethal Action	Hemolysis	Surface Action	Lecithin Inactivation
1	BAC	+	+	+	±[a]
	307	+	+	+	+
	497	+	+	+	+
2	498	+	+	±	+
	495	+	+	±	+
	271	+	+	±	+
3	15	−	+	+	−
4	477	+	−	−	+
5	436	−	−	−	−

[a] ±, Weakly positive.

action. Further dissimilarities to BAC can be noted in the categories of lethal action and lecithin inactivation, as exhibited by 15 (Type 3), and in hemolytic and surface action, demonstrated by 477 (Type 4). In none of the categories does 436 (Type 5) compare with the qualities of BAC.

DISCUSSION

Both naturally occurring and synthetic steroids have been known to exert inhibitory effects on microorganisms. Arnaudi (9) suggested that lowering of surface tension by cholesterol might induce antibacterial action. It is generally assumed that the inhibitory effects of fatty acids are due to their surface activity, which causes nonspecific alterations in cell permeability (10). The relationship of surface action and permeability damage to the lethal action of the quats has been recognized by Hotchkiss (11).

Hemolytic action on erythrocytes is considered to be indicative of permeability damage (12), and lowering of surface tension usually suggests the probability that hemolysis will occur (13). The complex physiochemical changes that surround membrane alterations have been studied with many detergent substances.

The steroids described in this report exhibited one or more properties characteristic of most surfactant-type compounds, suggesting that they may be mechanistically related to lethal compounds that act by altering membranes.

Partial or complete nullification of lethal action by lecithin is indicative of cationic quats (5) and is shared by all of the lethal steroids studied here. Evidence points to phospholipids as the membranous binding sites for quats (14). The inactivation of lethal agents by exogenous substances may be looked upon as a complex formation which mimics the true cell receptor sites for the lethal agent. This concept has held true for nystatin-ergosterol interactions (15, 16). Other systems of lethal agents, such as the steroids studied here and an exogenous inactivator (lecithin), may be quite nonspecific and misleading.

While some surface-membrane action might appear to be the most obvious mechanism, each steroid-bacterial cell union is highly specific. Not all of the steroids active against *G. tetragena* will inhibit or destroy other related Gram-positive bacteria (17). Moreover, some quaternary ammonium steroids have been completely inactive against bacteria in screening tests (18).

More detailed studies on steroids will be required to delineate a physiochemical-biochemical mechanism of action.

SUMMARY

Eight synthetic steroids were compared with BAC for their bactericidal action against *G. tetragena*. The hemolysis of sheep erythrocytes, lowering of surface tension, and inactivation by lecithin were studied. The properties of two aza-cholestanes were similar to those of BAC. The remaining steroids were classified into four types, depending upon their properties. A positive relationship between antimicrobial activity and surface or hemolytic activities was not found. Since the lethal action of the quats is attributed to these properties, it is doubtful that these steroids and quats act by an identical mechanism. Nevertheless, since these steroids are surface-active agents, their lethal effects may be assisted by this property.

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Critical Micelle Concentration of Nonionic Surfactants in Water and Carbon Tetrachloride

By MARIA ROSARIO VIDAL-PARUTA* and LOUIS D. KING

The critical micelle concentration (CMC) of two nonionic surface-active agents was studied. The micellization properties of polysorbate 80 and sorbitan monooleate were investigated in water and chloroform utilizing iodine as the indicator. Mixtures of polysorbates and sorbitans in various proportions were also tested relative to the CMC for mixed micelles in both solvents. A spectrophotometric method was used to determine the CMC in both solvents; the aqueous systems were also studied by a surface-tension method.

CERTAIN SUBSTANCES have the distinct characteristic of altering the surface properties of liquids and are referred to as surface-active agents or surfactants. Most of these agents exhibit the ability to aggregate in solution into what are called "micelles."

Many theories have been advanced on the size and shape of micelles. Notable among these are the McBain lamellar micelle, the Hartley spherical micelle, and the Debye rod micelle (1–3). These models serve to explain much of the published data on micelle formation, but no one model is sufficient in scope to be generally applicable.

Nonionic surfactants offer an unusual opportunity to study micelle formation. The absence of charged species in solution may facilitate the aggregation phenomenon because charge repulsion is minimized. Generally, their structure may be described as having both a hydrophilic and a hydrophobic group in these molecules. It is the relative balance of these groups which determines their characteristic behavior.

A number of the properties of solutions of surface-active agents exhibit an abrupt discontinuity at the concentration where aggregates or micelles start to form in appreciable numbers. One property that shows a marked change is the ability of the surfactant to solubilize certain dye materials. Harkins *et al.* (4) studied cationic and anionic surface-active agents utilizing this method. At the critical concentration, the dye is thought to be solubilized in the micelle and is in equilibrium with the dye in the bulk solution. This results in a spectral change. Additional work on this method was reported by Corrin (5), Nichols (6), and Sata (7).

Another spectrophotometric technique has been advanced by the work of Ross and Olivier (8). Their investigations dealt with nonionic surfactants in aqueous and nonaqueous solvents. The method is based on the use of iodine as an indicator; the equilibrium of iodine in the micelle and in solution lent itself well to spectrophotometric analysis.

This method has several absorption advantages. The major one is that the absorption maximum at 360 mμ does not vary from one solvent to another; the small molecular size of iodine compared with surface-active agent or dye molecules probably does not affect micelle formation.

The study reported here dealt with the physical properties of two nonionic surfactants in an aqueous and nonaqueous solvent. The nonionic surfactants selected were polysorbate 80[1] U.S.P. and sorbitan monooleate,[2] and these compounds were studied as single solutes and mixed in various proportions. Spectrophotometric absorbance and static surface-tension measurements were examined in an attempt to correlate the properties of these nonionic compounds. Measurements were made after various periods of aging to determine any variation in these properties. Specifically, the CMC for these systems was determined so that micelle formation under the conditions of this study might be further elucidated.

EXPERIMENTAL

Materials.—Sorbitan monooleate, lots 585 and 621; polysorbate 80, lots 360 and 678; carbon tetrachloride, analytical grade; iodine crystals, C.P., lot 472610 (Fisher Scientific Co.); and distilled water were used.

Equipment.—Beckman DU and DK-2 spectrophotometers, silica cells, and a Christian Becker laboratory balance, model AB-4, modified for surface-tension measurements were employed.

Methods.—Stock solutions (w/v) of surface-active agents were prepared in water and carbon tetrachloride. These solutions were subsequently

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* Recipient of a Lunsford Richardson Award.

[1] Marketed as Tween 80 by the Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Marketed as Span 80 by the Atlas Chemical Industries, Inc., Wilmington, Del.

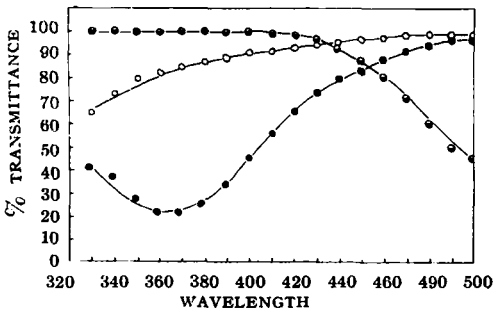


Fig. 1.—The development of the iodine-micelle complex maximum at 360 mμ and the corresponding isosbestic point at 452 mμ. One per cent polysorbate 80 in carbon tetrachloride (O); iodine in carbon tetrachloride (◒); iodine–polysorbate 80–carbon tetrachloride (●).

TABLE I.—ABSORPTION PEAKS AND CONCURRENT ISOSBESTIC POINT OF VARIOUS SYSTEMS EXPRESSED IN MILLIMICRONS

System	Peak	Isosbestic Point
Iodine in water	450	...
Iodine with sorbitan monooleate in water	350[a]	390
Iodine with polysorbate 80 U.S.P. in water	360	428
Iodine in carbon tetrachloride	510	...
Iodine in sorbitan monooleate and carbon tetrachloride	360	440
Iodine in polysorbate 80 U.S.P. and carbon tetrachloride	360	452

[a] Broad peak.

bureted into a solution of iodine in the solvents studied; a sufficient amount was added so that the surfactant concentration with respect to the final volume was incrementally increased. The solvents used were saturated with iodine, and a 1:10 dilution with the appropriate solvent was made just prior to use. These samples were assayed spectrophotometrically at the appropriate wavelength (360 mμ) for a given system.

Surface-tension measurements were carried out on the samples described above, the determinations were made at 5-minute intervals for 30 minutes. These results were averaged and used directly in the determination of the CMC.

Samples prepared for both methods were carried out at ambient temperatures (25°) and stored for a 1-week period and a 30-day period at the same temperature. At the end of each aging period, the samples were analyzed by the previously indicated methods.

RESULTS AND DISCUSSION

The absorption spectra of iodine in water and iodine in carbon tetrachloride were obtained on the Beckman DK-2 recording spectrophotometer. Peaks for these systems occurred at 450 mμ for iodine in water and at 510 mμ for iodine in carbon tetrachloride. The spectra of 1% solutions of sorbitan monooleate and polysorbate 80 were determined by the DK-2; they have relatively low absorption values in the 500 to 330-mμ range. The spectrum of polysorbate 80 in carbon tetrachloride is illustrated in Fig. 1. Since the concentration of most sample solutions was about 1% or less in the water and carbon tetrachloride systems, it was felt that a correction for the colored constituents of these surfactants need not be applied.

When the absorption spectra of solutions containing iodine alone and solutions containing the surfactants and iodine were plotted, an isosbestic point was found between the two absorption maxima. This was done for both solvents studied in this work. The results are recorded in Table I. Figure 1 shows the iodine-carbon tetrachloride peak and the corresponding isosbestic point for iodine–polysorbate 80–carbon tetrachloride system.

The isosbestic point which developed between the two absorption maxima of the solutions containing iodine alone and solutions containing surfactants and iodine may indicate that a simple equilibrium exists between the iodine molecules in the solution and the iodine in the micelles. It may also indicate that one type of iodine-micelle complex existed in the solution which has an absorption maximum at 360 mμ.

Figure 2 shows that the iodine–polysorbate 80–water system had an increased absorption up to the CMC, followed by a change in slope. After the CMC had been reached, the absorption of the iodine-micelle complex decreased upon the addition of polysorbate 80. Although the concentrations of polysorbate 80 were low, the concentration of the polyglycol contaminant (approximately 5% of the total surfactant) became sig-

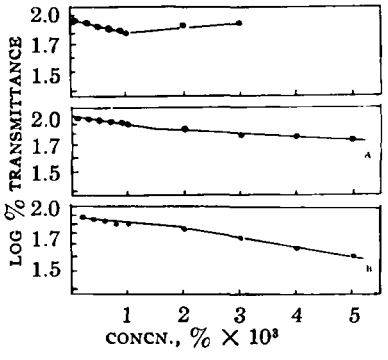


Fig. 2.—Log per cent transmittance *versus* concentration of polysorbate 80 in iodine-water system. *A*, Log per cent transmittance *versus* concentration of 1:1 mixture of sorbitan monooleate–polysorbate 80 in iodine-water system. *B*, Log per cent transmittance *versus* concentration of 2:1 mixture of sorbitan monooleate–polysorbate 80 in iodine-water system.

TABLE II.—CMC OF NONIONICS EXPRESSED AS PER CENT w/v IN WATER-IODINE SYSTEMS

Surfactant	Spectrophotometric Data, %	Surface-Tension Data, %
Sorbitan monooleate	0.0020	0.002
Sorbitan monooleate, 2/3 Polysorbate 80 U.S.P., 1/3	0.0020	0.001
Sorbitan monooleate, 1/2 Polysorbate 80 U.S.P., 1/2	0.0015	0.001
Sorbitan monooleate, 1/3 Polysorbate 80 U.S.P., 2/3	0.0013	0.001
Polysorbate 80 U.S.P.	0.0010	0.001

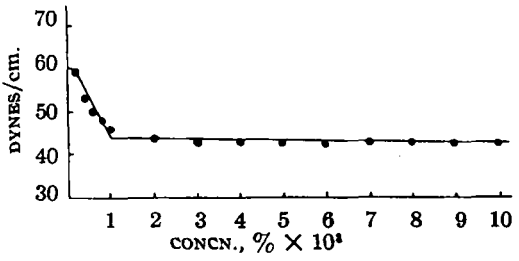


Fig. 3.—Surface tension *versus* concentration of a 1:1 mixture of sorbitan monooleate–polysorbate 80 in water.

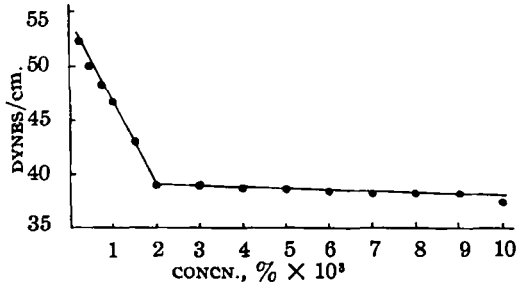


Fig. 4.—Surface tension *versus* concentration of sorbitan monooleate in water.

nificant in relation to the iodine concentration which remained constant. Therefore, it may be postulated that the polyglycol-iodine complex formed, disturbing the equilibrium and showing an apparent micelle decrease at the higher concentrations of polysorbate 80.

The data obtained from the spectrophotometric determinations were plotted in several fashions relative to the values of the abscissa. A plot of the log per cent transmittance *versus* concentration illustrated the CMC "break" in the clearest fashion. Thus, all the representative plots utilizing spectrophotometric data were illustrated in this manner.

To determine micelle formation for mixed surfactants, polysorbate 80 and sorbitan monooleate were mixed in various proportions and tested. The ratios of polysorbate 80 to sorbitan monooleate tested were 2:1, 1:1, and 1:2. The results for two of these systems were plotted and are illustrated in Fig. 2*A* and *B*. The CMC's for the aqueous systems are recorded in Table II. The data showed values of the CMC for the mixed solutes intermediate between the CMC value of the single solutes.

It was then felt necessary to attempt the possible correlation of the determined CMC's using a surface-tension method. This was done for single and mixed solutes. Data for two of these systems were plotted and are illustrated in Figs. 3 and 4. The CMC's determined by the surface-tension method for these systems are also included in Table II.

It is interesting to note that the CMC's for the mixed solutes showed no apparent correlation with composition for the surface-tension method. The CMC's for the spectrophotometric method were approximately proportional to composition. This is illustrated in Fig. 5.

Aging for 30 days showed the same CMC's but with a slight decrease in the absorption of the iodine-micelle complex. This may be explained by the reaction of the iodine with the double bond of oleate in the polysorbate 80 and the formation of the polyglycol-iodine complex (12). These two effects may have disturbed the iodine–iodine-micelle complex equilibrium, and a consequent decrease in absorption would be expected. Experimentally, this was the case.

The CMC's in carbon tetrachloride were also determined for the single and mixed solutes. The CMC's for polysorbate 80 and sorbitan monooleate resulted in values of 0.07% and 0.025% in the iodine-carbon tetrachloride mixtures. The data for polysorbate 80 have been plotted and are illustrated in Fig. 6. Again, the absorption increased up to the CMC; after this point, the absorption remained fairly constant, an implication of virtually complete micelle formation at the break.

It is interesting that the shapes of the curves of systems containing single solutes were different from the mixtures, but the CMC's were in the same range.

As shown in Fig. 6*A* and *B*, the carbon tetrachloride systems showed the same shaped curves in all cases. It is proposed that the general shape of the curve was due to the following. The absorption increased up to the CMC; a subsequent decrease in absorption was due to the polyglycol-iodine and the oleate-iodine reactions for a certain concentration

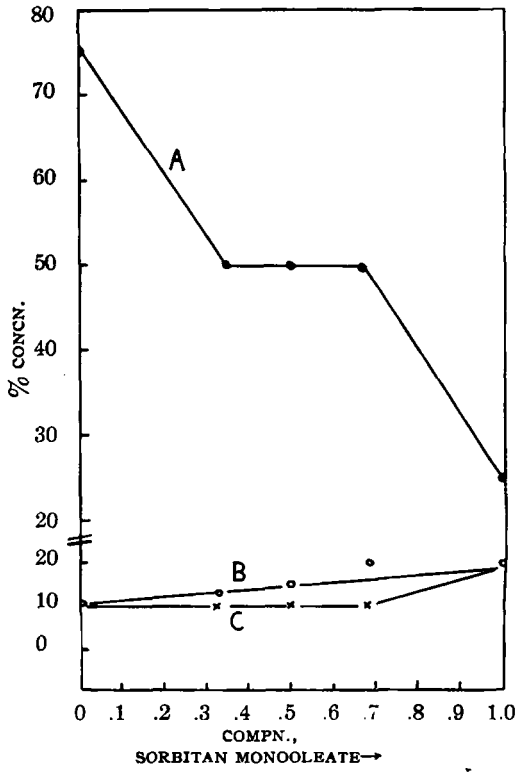


Fig. 5.—Variation of the CMC as a function of composition. Curve A is for carbon tetrachloride systems (10^{-3}%). Curves B and C are the spectrophotometric and surface-tension data for the aqueous systems (10^{-4}%).

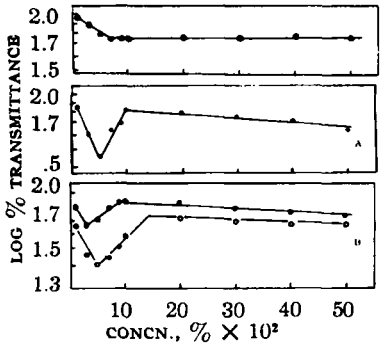


Fig. 6.—Log per cent transmittance *versus* concentration of polysorbate 80 in iodine-carbon tetrachloride system. *A*, Log per cent transmittance *versus* concentration of 2:1 mixture of sorbitan monooleate–polysorbate 80 in iodine-carbon tetrachloride systems. *B*, Log per cent transmittance *versus* concentration of 1:1 mixture of sorbitan monooleate–polysorbate 80 in iodine-carbon tetrachloride systems. Data shown for 1 week (●) and for 30 days (○).

TABLE III.—CMC OF NONIONICS EXPRESSED AS PER CENT w/v IN CARBON TETRACHLORIDE-IODINE SYSTEM

Surfactant	Spectrophotometric Data— 1 Hr., %	1 Wk., %	30 Days, %
Sorbitan monooleate	0.025	0.020	0.020
Sorbitan monooleate, 2/3 Polysorbate 80 U.S.P., 1/3	0.050	0.050	0.050
Sorbitan monooleate, 1/2 Polysorbate 80 U.S.P., 1/2	0.050	0.050	0.030
Sorbitan monooleate, 1/3 Polysorbate 80 U.S.P., 2/3	0.050	0.040	0.040
Polysorbate 80 U.S.P.	0.075	0.065	0.060

range. After this point, due to the relatively high concentrations of surfactants and the partial consumption of iodine in the system, the absorption was essentially due to the surfactants.

The samples for a given run were stored at 25° in a dark place for 1-week and a 30-day period. Upon aging, the above reactions would tend to be favored, and a general decrease in absorption would be expected; but the shape of the curve should remain unchanged. Experimentally, this was the case, and the absorption essentially due to the surfactant remained relatively constant.

However, it was also found that the CMC's in these systems decreased upon aging to various degrees. This observation is illustrated in Fig. 6*A*. All the results have been summarized in Table III.

The CMC's in the iodine-carbon tetrachloride systems showed higher values than the CMC's in the aqueous systems. Approximately a tenfold increase was noted for sorbitan monooleate in carbon tetrachloride relative to water, whereas polysorbate 80 gave a thirty-fivefold increase. These values may be explained on the basis of decreased solubility in carbon tetrachloride requiring higher concentrations for micelle formation. The CMC's for single solutes and mixed solutes have been plotted and are also illustrated in Fig. 5 as a function of composition. From the values for the mixed solutes it seems that polysorbate 80 had a more pronounced contribution to the formation of micelles than sorbitan monooleate.

The nonionic surfactants used in this study are known to be heterogenous mixtures of compounds of differing physical properties. It is important to note, however, that batch variation for both single and mixed solutes showed no apparent differences in the CMC's for the systems studied. Therefore, it can be assumed that the average physical properties of these heterogenous mixtures are relatively reproducible.

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Relationships Between the Surface Activity and Cholinesterase Inhibition of Mono- and bis[3-(*N*,*N*-Diethylcarbamoyl)piperidino]alkanes

By RONALD P. QUINTANA

The static surface tension of equimolar aqueous solutions of a series of mono- and bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]alkanes and of two mono[3-(*N*,*N*-diethylcarbamoyl)pyridinium]alkanes has been determined. The extent of relationships existing between the surface activity of these compounds and their inhibitory effect upon human plasma pseudo-cholinesterase is reported.

During the course of synthetic work on carbamoylpiperidine derivatives (1), it became apparent that some compounds among the derivatives possessed surface-active properties. This observation, coupled with (*a*) an awareness of the work of Coleman and Eley (2) and that of Hoffmann and co-workers (3), from which one may expect a possible correlation between enzyme inhibition and surface activity, and (*b*) the suggestions of Long and Schueler (4) and Thomas and Marlow (5) that cholinesterase inhibition may in part be due to the surface activity of inhibitors, prompted us to explore relationships between the surface-active and inhibitory properties of our series of cholinesterase inhibitors.

While the literature records studies of relationships between surface activity and effect upon enzymes (*e.g.*, 6–10), we believe that this is the first published expression of relationships between observed and actually measured surface activity, and inhibition of an isolated cholinesterase system.

EXPERIMENTAL

Materials.—The chemistry and properties of the mono- and bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-alkanes and of the mono[3-(*N*,*N*-diethylcarbamoyl)-pyridinium]alkanes utilized in this investigation were described elsewhere (11, 12). All of the compounds employed were of analytically pure grade.

Solutions.—Measurements of surface tension were made on 0.01 and 0.005 *M* solutions in redistilled water obtained by distillation through a 45-cm. Vigreaux column from aqueous permanganate. The solvent water obtained by this method had a corrected surface tension of 70.26 to 70.87 dynes/cm.

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The author acknowledges valuable discussions with Dr. Andrew Lassio and his generosity in furnishing analytical samples of the compounds employed in this investigation. The technical assistance of Miss Linda F. Lorenzen, who performed all of the surface tension measurements, is also gratefully acknowledged.

Instrumentation.—We employed the model TE03 du Nouy interfacial tensiometer (Kahlsico, Calif.) equipped with an insulated water-jacketed holder accommodating a 70-mm. diameter sample dish. The use of a Thermoboy model KTS5 circulating bath (Kahlsico, Calif.) in conjunction with the water-jacketed holder permitted rigid control of sample temperature. Measurements were made at 25.00 ± 0.02°.

Platinum-iridium rings having the following characteristics were employed: ring No. 1, mean circumference 5.950 cm., $R/r = 51.5$; ring No. 2, mean circumference 5.978 cm., $R/r = 51.5$.

The operation of the calibrated tensiometer was evaluated by determining the surface tension of spectro grade benzene. The corrected surface tension obtained (28.21 dynes/cm.) was in excellent agreement with the reported value (28.22 dynes/cm.) (13).

Methods.—Measurement of surface tension was performed by the ring method, in accordance with those directives described in the "Standard Methods of Test for Surface and Interfacial Tension of Solutions of Surface-Active Agents" (ASTM designation: D 1331-56) (14) and the "Standard Method of Test for Interfacial Tension of Oil against Water by the Ring Method" (ASTM designation: D 971-50) (15).

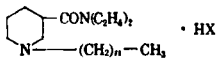
Measurements were made on 50-ml. samples at each concentration; in each case at least five determinations were employed in calculating an average value for the static surface tension (16). To determine the static value, the surface tension of each solution was checked periodically; in some instances measurements were made on solutions approximately 1.5 to 12.5 hours old. With each determination providing the static value, an equivalent determination was made on a control sample of redistilled water. Appropriate correction factors (13) were applied to the values obtained, and the surface pressure was then determined by subtracting the value for the surface tension of the solution from that of the redistilled water control.

Measurements of pH were made with a Beckman model 72 pH meter. Specific gravities, required in calculating correction factors, were determined by use of a Westphal balance (Fisher Scientific Co.).

RESULTS AND DISCUSSION

Values for the corrected, static surface tension of 0.01 and 0.005 *M* solutions of the compounds investigated, together with the corresponding surface

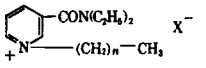
TABLE I.—INHIBITION OF ISOLATED HUMAN PLASMA PSEUDO-CHOLINESTERASE AND SURFACE-ACTIVE PROPERTIES OF MONO[3-(*N,N*-DIETHYLCARBAMOYL)PIPERIDINO]ALKANES



Compd.	*n*	Acid Component	Human Plasma Cholinesterase Inhibition (I_{50} ± S.E.)[a]	Surface Pressure,[b] dynes/cm. at 0.01 *M*	Surface Pressure,[b] dynes/cm. at 0.005 *M*	Surface Tension,[c] dynes/cm. ± S.E.[d] at 0.01 *M*	Surface Tension,[c] dynes/cm. ± S.E.[d] at 0.005 *M*
I	0	HCl	$(63.5 \pm 1.0) \times 10^{-5}$ *M*	1.98	0.58	68.59 ± 0.08	69.99 ± 0.04
II	1	HBr	$(118.5 \pm 0.5) \times 10^{-5}$ *M*	1.23	0.50	69.34 ± 0.05	70.07 ± 0.08
III	2	HBr	$(101.0 \pm 1.0) \times 10^{-5}$ *M*	3.12	2.41	67.36 ± 0.03	68.07 ± 0.04
IV	3	HBr	$(78.0 \pm 2.0) \times 10^{-5}$ *M*	5.60	2.75	65.05 ± 0.11	67.90 ± 0.09
V	4	HBr	$(26.1 \pm 1.1) \times 10^{-5}$ *M*	4.43	3.69	66.10 ± 0.02	66.84 ± 0.07
VI	5	HBr	$(8.13 \pm 0.33) \times 10^{-5}$ *M*	5.82	3.65	64.64 ± 0.05	66.81 ± 0.06
VII	9	HBr	$(0.527 \pm 0.011) \times 10^{-5}$ *M*	26.15	20.68	44.49 ± 0.06	49.96 ± 0.04

[a] Summarized from a paper by Lasslo and co-workers (17). [b] The surface pressure is the corrected surface tension of a redistilled water control minus the corrected surface tension of the solution. [c] The static surface tension is reported. [d] Standard error (18).

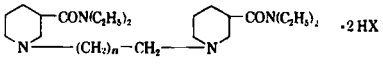
TABLE II.—INHIBITION OF ISOLATED HUMAN PLASMA PSEUDO-CHOLINESTERASE AND SURFACE-ACTIVE PROPERTIES OF MONO[3-(*N,N*-DIETHYLCARBAMOYL)PYRIDINIUM]ALKANES



Compd.	*n*	Halide	Human Plasma Cholinesterase Inhibition (I_{50} ± S.E.)[a]	Surface Pressure,[b] dynes/cm. at 0.01 *M*	Surface Pressure,[b] dynes/cm. at 0.005 *M*	Surface Tension,[c] dynes/cm. ± S.E.[d] at 0.01 *M*	Surface Tension,[c] dynes/cm. ± S.E.[d] at 0.005 *M*
VIII	0	I	Insignificant inhibition at at 100 × 10^{-5} *M* concn.	0.53	0.18	69.98 ± 0.08[e]	70.33 ± 0.05
IX	9	Br	$(0.365 \pm 0.000) \times 10^{-5}$ *M*	17.95	12.29	52.87 ± 0.05[e]	58.53 ± 0.05

[a] Summarized from a paper by Lasslo and co-workers (17). [b] The surface pressure is the corrected surface tension of a redistilled water control minus the corrected surface tension of the solution. [c] The static surface tension is reported. [d] Standard error (18). [e] Ciusa (19) has reported the surface tension of the corresponding chlorides.

TABLE III.—INHIBITION OF ISOLATED HUMAN PLASMA PSEUDO-CHOLINESTERASE AND SURFACE-ACTIVE PROPERTIES OF BIS[3-(*N,N*-DIETHYLCARBAMOYL)PIPERIDINO]ALKANES



Compd.	*n*	Acid Component	Human Plasma Cholinesterase Inhibition (I_{50} ± S.E.)[a]	Surface Pressure,[b] dynes/cm. at 0.01 *M*	Surface Pressure,[b] dynes/cm. at 0.005 *M*	Surface Tension,[c] dynes/cm. ± S.E.[d] at 0.01 *M*	Surface Tension,[c] dynes/cm. ± S.E.[d] at 0.005 *M*
X	1	HBr	$(17.5 \pm 1.0) \times 10^{-5}$ *M*	3.34	1.67	67.39 ± 0.02	69.06 ± 0.02
XI	2	HCl	$(99.3 \pm 1.8) \times 10^{-5}$ *M*	3.38	2.22	66.99 ± 0.03	68.11 ± 0.04
XII	3	HBr	$(42.0 \pm 1.7) \times 10^{-5}$ *M*	2.00	0.94	68.87 ± 0.04	69.93 ± 0.04
XIII	4	HCl	$(27.1 \pm 1.3) \times 10^{-5}$ *M*	5.15	4.72	65.18 ± 0.05	65.54 ± 0.05
XIV	5	HBr	$(15.0 \pm 0.3) \times 10^{-5}$ *M*	3.58	1.90	66.95 ± 0.04	68.63 ± 0.05
XV	9	HBr	$(2.59 \pm 0.06) \times 10^{-5}$ *M*	4.78	4.98	65.63 ± 0.04	65.43 ± 0.03

[a] Summarized from a paper by Lasslo and co-workers (17). [b] The surface pressure is the corrected surface tension of a redistilled water control minus the corrected surface tension of the solution. [c] The static surface tension is reported. [d] Standard error (18).

pressure at these concentrations and the inhibitory characteristics, previously reported by Lasslo and co-workers (17), are given in Tables I, II, and III.

The graphic interpretation of surface pressure values (at 0.01 *M* concentration) is illustrated along with the corresponding plot of the values determined by Lasslo and co-workers (17). Whereas in Fig. 1 the correlation between surface pressure and inhibitory effect is striking, no such relationship is apparent in Fig. 2. (In Fig. 2 the straight line representing surface pressure was computed by the method of least squares.)

It is indeed interesting to note the parallel between the cholinesterase inhibition and surface pressure of the mono[3-(*N,N*-diethylcarbamoyl)-piperidino]alkanes (Fig. 1, Table I) and the lack of it in the corresponding bis[3-(*N,N*-diethylcarbamoyl)piperidino]alkanes (Fig. 2, Table III).

In addition, it is instructive to compare the parallel between the surface activity and cholinesterase inhibition elicited by certain specific members of our groups of compounds. Considering the carbamoylpiperidinodecanes, for example, it can be seen that the *monosubstituted* alkane is the more potent one, both in terms of enzyme inhibition as well as in its ability to lower surface tension. In the case of the corresponding ethanes, the *bis-substituted* derivative more strongly inhibits the

enzyme system and is, at the same time, more powerful in lowering surface tension.

The determined surface tension values seem to be consistent with Ciusa's (19) results, reported for some corresponding pyridinium derivatives, and with Gadebusch and Cavallito's (20) figures for their α,ω-bis(2,2′-dipyridylamino)alkane bisquaternary salts.

In the course of determining the static surface tension, it was observed that, for a few compounds, values of dynamic surface tension varied considerably from the static value (*e.g.*, III, 5.6 dynes/cm.; IV, 5.3 dynes/cm.; XI, 9.4 dynes/cm.; XIII, 4.6 dynes/cm.). In most cases, however, variation from the static value was small, *i.e.*, 1 to 2 dynes/cm.

The pH of all solutions of our mono[3-(*N,N*-diethylcarbamoyl)piperidino]alkanes lay between 5.40 and 6.05 and between 5.65 and 6.50 for the corresponding pyridinium compounds. In the series of bis[3-(*N,N*-diethylcarbamoyl)piperidino]-alkanes, the pH of all solutions fell between 5.10 and 6.15, except for compounds X and XI (Table III). The values for 0.01 and 0.005 *M* solutions of X were 3.65 and 3.80, respectively; those for XI were 4.55 and 4.70, respectively.

SUMMARY

The static surface tension of 0.01 and 0.005 *M* aqueous solutions of a series of mono- and bis[3-(*N,N*-diethylcarbamoyl)piperidino]alkanes and some pyridinium analogs has been measured utilizing the ring method.

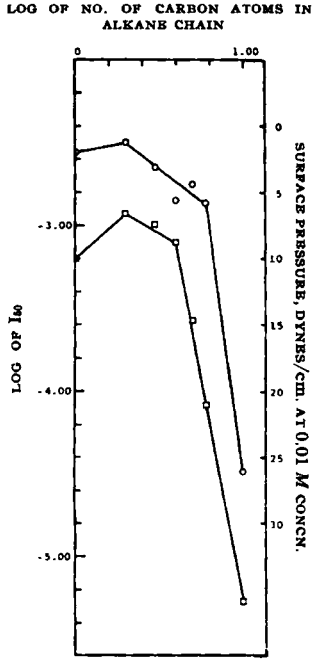


Fig. 1.—Graphic comparison of inhibitory (□—□) and surface-active (○—○) characteristics of mono[3-(*N,N*-diethylcarbamoyl) piperidino] alkanes.

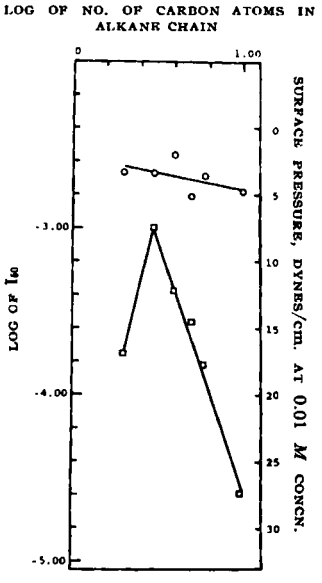


Fig. 2.—Graphic comparison of inhibitory (□—□) and surface-active (○—○) characteristics of bis[3-(*N,N*-diethylcarbamoyl) piperidino] alkanes.

A parallel was observed between the ability of mono[3-(*N,N*-diethylcarbamoyl)piperidino]alkanes to lower surface tension and their inhibition of human plasma pseudo-cholinesterase. No such relationship was noted in the case of the corresponding bis-substituted alkanes.

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Correlation of Amount of Metabolite Excreted and Its Excretion Rate with Available Surface Area of Tolbutamide in Dosage Form

By EINO NELSON, STUART LONG, and JOHN G. WAGNER

Tolbutamide was administered to normal human test subjects in the form of thin cylindrical disks having an initial surface area of 6.60 cm.² per 0.5 Gm. dose and in the form of spherical granules prepared by coating spherical inert core granules with this drug. The initial surface areas of the granules used were 36.9, 63.1, and 98.6 cm.² per 0.5 Gm. dose of tolbutamide. Excretion rate of carboxytolbutamide, the metabolite of tolbutamide, was determined following dose ingestion. Perfect rank-order correlation was shown between per cent of a dose as metabolite excreted in the urine in 48 hours and surface area of tolbutamide in the dosage form. Likewise, there was perfect rank-order correlation between mean maximum excretion rate of carboxytolbutamide and surface area of tolbutamide in the dosage form.

WHILE THERE is now good recognition of the qualitative aspects of the effect of surface area of drug on absorbability, little work has been done on the quantitative aspects of this factor, although the effect of surface area on the absorption of tetracycline and several of its salts has been previously studied in cases of solution rate-limited absorption and nonsolution rate-limited absorption (1, 2). This communication reports the results of studies conducted using dosage forms of the antidiabetic drug tolbutamide,[1] with known surface areas in which a direct relationship was shown between either mean maximum excretion rate of carboxytolbutamide,[2] the metabolite of tolbutamide, or percentage of this metabolite recovered in the urine in 48 hours and average surface area of tolbutamide when drug was taken as thin cylindrical disks or spherical granules.

EXPERIMENTAL

Dosage Forms.—Thin cylindrical disks, 0.168 cm. in thickness and 1.28 cm. in diameter, were prepared by compressing tolbutamide with 1% calcium stearate in a single punch tablet compressing machine. The apparent density of the disks was 1.16 Gm./cm.³. Each disk contained 250 mg. of tolbutamide; hence, two disks contained a 0.5-Gm. dose of tolbutamide.

Three lots of spherical granules of tolbutamide were prepared in a manner similar to that previously described (3), except that no external coating was applied. By varying the size of the core granules and the thickness of the coated tolbutamide layer, the surface areas per 0.5 Gm. dose of tolbutamide were altered from one lot of granules to the next.

Particle size variation was estimated by measuring the diameters of 100 individual granules of each lot with a micrometer. The average and median diameters, the standard deviations, and the coefficients of variation are shown in Table I. Percentage of granules by number less than diameter against diameter is plotted on normal probability graph paper in Fig. 1. Approximately 95% of the granules of each lot gave linear plots which indicated this fraction approximates the normal Gaussian distribution. There was some skewness to the left, *i.e.*, toward smaller diameters. The medians, standard deviations, and coefficients of variation were estimated from the linear portions of the plots shown in Fig. 1, and these are reported in Table I also. In a similar manner, particle size variation of the core granules was estimated. The variation in particle size of the core granules was of the same order of magnitude as the particle size variation of the coated granules. Wachtel and LaMer (4) reported an average coefficient of variation of 12.6% for so-called monodisperse emulsions and an average coefficient of variation of 10% for aerosols. Hence, the smaller coefficients of variation of the core and coated granules, approximately 4–6%, indicate very narrow particle size ranges.

More accurate estimates of average diameters of the core and coated granules were obtained by measuring about 1,000 granules of each lot. This was done by lining up the granules side by side in a trough in groups of 110 to 130 and measuring the distance from the first to the terminal granule. The average diameters determined by this method are shown in Table II and are within 1 to 3% of the average diameters obtained with the 100 individually measured granules of each lot. From the average diameters and weights shown in Table II, the apparent densities of the coated tolbutamide layers on the granules were calculated to be 0.997, 0.930, and 1.02 Gm./cm.³ for granules A, B, and C, respectively.

Calculation of Surface Areas and Doses.—Each lot of granules was considered sufficiently homogeneous and of particle size range narrow enough to estimate the surface area from the average diameters of the core granule and the coated granules. To illustrate the method of calculation, the data for granules C (Table II) are used. The surface area of an average coated granule was π (0.1195)² or 0.0449

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The authors acknowledge the assistance of Mr. K. W. Riebe, who prepared the cylindrical disks.

[1] Tolbutamide is 1-butyl-3-*p*-tolylsulfonylurea.

[2] Carboxytolbutamide is *p*-(butylcarbamoyl) sulfamoylbenzoic acid.

TABLE I.—PARTICLE SIZE VARIATION OF THE COATED GRANULES

	Granules A	Granules B	Granules C
Based on 100 Granules of Each Lot:			
Av. (cm.)	0.129	0.135	0.117
S.D. (cm.)	0.0072	0.0076	0.0051
Coefficient of variation (%)	5.6	5.6	4.4
Median (cm.)	0.129	0.136	0.117
Based on Linear Portion of Normal Probability Plot:			
Median (cm.)	0.129	0.137	0.117
S.D. (cm.)	0.0066	0.0064	0.0046
Coefficient of variation (%)	5.1	4.7	3.9

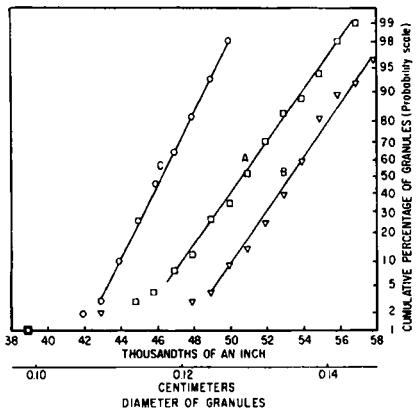


Fig. 1.—Particle size distributions of the granules. Key: O, granules C; □, granules A; ▽, granules B.

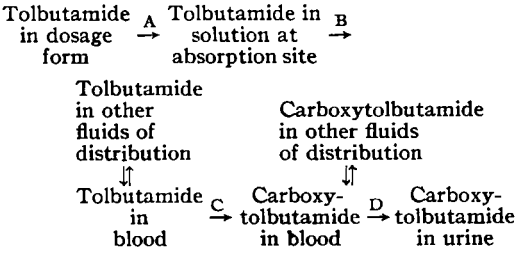
cm.²; hence, the surface area of 2195 granules was 98.6 cm.². The surface area of an average core granule was π (0.1075)² or 0.0363 cm.²; hence, the surface area of 2195 granules was 79.7 cm.². The average surface area during dissolution of the coated layer was then (98.6 + 79.7)/2 or 89.1 cm.² per 0.5-Gm. dose of tolbutamide. The weight of coated granules required was obtained by dividing the dose (in milligrams) by the potency or 500/157 or 3.185 Gm. The coated granules weighed 14.5103 Gm. per 10,000 or an average of 1.45103 mg./granule. The number of granules administered was therefore 3185/1.45103 or 2195 granules. The corresponding calculated quantites for granules lots A and B are shown in Table II.

The surface area of the disks calculated from their dimensions was 3.30 cm.²/disk or 6.60 cm.² per 0.5-Gm. dose of tolbutamide. Since their final surface area after dissolution would be theoretically zero, their average surface area during dissolution would be 3.30 cm.² per 0.5 Gm. of tolbutamide.

Conduction of Tests.—Drug in 0.5-Gm. doses was ingested orally by *normal* human test subjects after overnight fast, and no food was taken until 1 hour after ingestion of the dose. The doses of the drug as spherical granules were contained in an appropriate number of hard gelatin capsules. Urine was collected at 2-hour intervals for the first 12 hours and then in pooled 12-hour collections for 48 or 60 hours. The designations (by initial) of sex, age, and weight in kilograms of the test subjects were, respectively, E-M-41-77, C-F-30-57, and P-M-31-72. The various preparations were taken serially in the order disks and granules A, B, and C, respectively. At least 1 month elapsed between tests.

Analytical Method.—Suitable diluted urine aliquots were assayed for carboxytolbutamide by the method of Nelson *et al.* (5). Suitable blank and binding corrections were made on the initial analytical values as prescribed in the assay.

THEORY



It is known from previous work (6, 7) that (*a*) B, C, and D are or could be expected to be first-order processes within experimental error; (*b*) process D is about 12 times more rapid than process C; (*c*) process B is at least 10 times more rapid than process C; and (*d*) tolbutamide is nearly quantitatively oxidized to carboxytolbutamide (*i.e.*, $f \cong 1$).

Hence, assuming that tolbutamide entering the blood during the absorption process rapidly equilibrates with the blood and other fluids of distribution, one would expect that the rate of excretion of the metabolite, carboxytolbutamide, would be greatly influenced by the rate(s) of process A. Since rate of dissolution is theoretically directly proportional to surface area, one would expect the rate of excretion of the metabolite to be related to the surface area of tolbutamide in the administered dosage form. Also, since one would expect the dosage form to be at the absorption sites in the gastrointestinal

TABLE II.—AVERAGE DIAMETERS, AVERAGE WEIGHTS, POTENCIES, DOSES, AND SURFACE AREAS OF GRANULES

	Granules A	Granules B	Granules C
Av. diam. of coated granules (cm.)	0.1326	0.1368	0.1195
Av. diam. of core granules (cm.)	0.0937	0.1151	0.1075
Av. wt. of coated granules (Gm. per 10,000 granules)	15.9875	19.8227	14.5103
Av. wt. of core granules (Gm. per 10,000 granules)	8.5312	14.7800	12.0109
Potency (mg. tolbutamide per Gm. of granules)	467	235	157
Wt. of granules equiv. to 0.5 Gm. tolbutamide (Gm.)	1.071	2.131	3.185
No. of granules equiv. to 0.5 Gm. tolbutamide	669	1075	2195
Total surface areas for No. of granules equiv. to 0.5 Gm. of tolbutamide			
Coated granules	36.9	63.1	98.6
Core granules	18.5	44.7	79.7
Av. during dissolution of coated layer	27.7	53.9	89.1

TABLE III.—AMOUNTS OF CARBOXYTOLBUTAMIDE (mg.) EXCRETED BY TEST SUBJECTS AFTER INGESTION OF 500-mg. DOSES OF TOLBUTAMIDE IN SEVERAL DOSAGE FORMS[a]

Subject	Time, Hr. 2	4	6	8	10	12	24	36	48	60
Thin Cylindrical Disks D, 6.6 cm.²/0.5 Gm. Dose Tolbutamide										
E[b]	10	30	47	61	71	84	124	...	219	
C[b]	2	9	21	33	46	54	93	...	124	
P	1	12	24	34	43	49	89	...	140	
Mean	4	17	31	43	53	62	102	...	161	
Granules A, 27.7 cm.²/0.5 Gm. Dose Tolbutamide										
E	10	19	38	73	116	126	211	246	269	...
C	4	21	45	78	99	120	187	230	252	...
P	0	17	37	62	86	104	171	209	229	...
Mean	5	19	40	71	100	117	189	228	250	...
Granules B, 53.9 cm.²/0.5 Gm. Dose Tolbutamide										
E	6	23	52	86	115	146	191	260	287	319
C	8	87	163	222	276	312	449	486	500	516
P	6	41	79	120	156	184	322	402	432	465
Mean	7	50	98	143	182	214	321	382	406	433
Granules C, 107.6 cm.²/0.5 Gm. Dose Tolbutamide										
E	43	121	203	272	333	379	510	556	580	599
C	32	137	222	294	348	387	534	573	590	590
P	22	72	119	158	199	227	366	448	480	504
Mean	32	110	181	241	293	331	470	526	550	564

[a] Theoretical maximum recovery, 555 mg. [b] Data for these subjects are also reported in another communication (6).

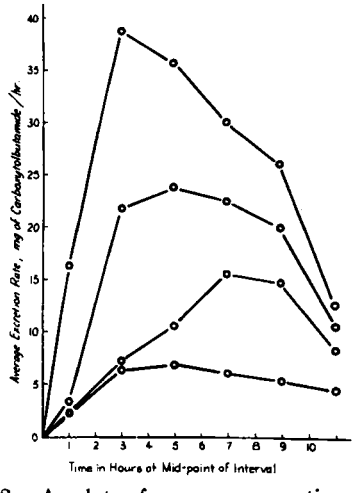


Fig. 2.—A plot of average excretion rate of metabolite against time. The curves from top to bottom are for granules C, granules B, granules A, and the disks, respectively.

tract for some finite time or range of time, the amount of metabolite excreted in a given time should be related to the surface area of tolbutamide in the administered dose. Due to the complexity of the biological situation, as illustrated by the model above, it is not possible to estimate quantitatively the rate of process A as a function of time from the rate of appearance of the metabolite in the urine. The amount of metabolite in the urine at a given time will be a function of the volumes of many of the "compartments" involved and many of the rate and/or permeability constants involved. We tested the feasibility of making rank-order correlations between extent and rate of excretion of carboxytolbutamide and available surface area of tolbutamide in the administered dosage form.

RESULTS

Amounts of carboxytolbutamide excreted in various times are given in Table III. Figure 2 is a plot of average excretion rate of carboxytolbutamide (milligrams per hour) against time at the midpoint

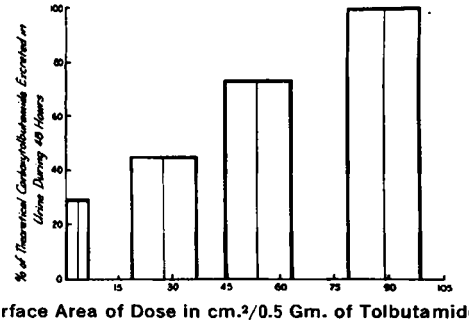


Fig. 3.—A perfect rank-order correlation of per cent of theoretical metabolite excreted in the urine in 48 hours with surface area of tolbutamide in the dosage form. The center line in each bar gives the average surface area during dissoluton.

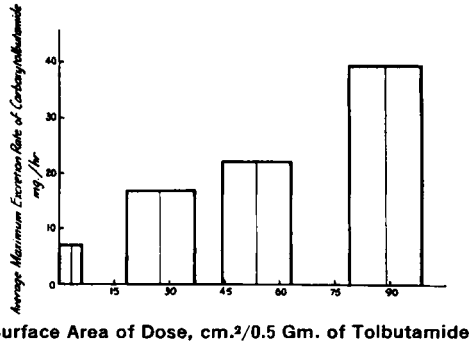


Fig. 4.—A perfect rank-order correlation of average maximum excretion rate of metabolite in the urine with surface area of tolbutamide in the dosage form. The center line in each bar gives the average surface area during dissolution.

of the collection intervals over which the rates were calculated. Such excretion rate-time plots are much more sensitive and show curvature not nearly as evident on a cumulative amount excreted *versus* time plot.

Figure 3 shows perfect rank-order correlation of per cent of theoretical carboxytolbutamide excreted in the urine in 48 hours with surface area of tolbutamide in the dosage form.

Figure 4 shows a perfect rank-order correlation of average maximum excretion rate of carboxytolbutamide in the urine with surface area of tolbutamide in the dosage form.

These results show that in normal human subjects available surface area of tolbutamide in the dosage form can have a pronounced effect on extent and rate of excretion of the metabolite, carboxytolbutamide, in the urine. Indirectly, they show that available surface area of tolbutamide in the dosage form can influence the rate and extent of absorption of tolbutamide if the surface area is restricted in the range studied.

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don) XXIV

Vinaspine, Vincathicine, Rovidine, Desacetyl VLB, and Vinaphamine

By GORDON H. SVOBODA and ALBERT J. BARNES, JR.

The continued phytochemical investigation of this pantropical plant has resulted in obtaining vinaspine and vinaphamine as free bases and vincathicine, rovidine, and desacetyl VLB as the sulfates. The total number of alkaloids obtained from this plant, utilizing selective extraction, column chromatography, and gradient pH techniques, is now 49.

This investigation was pursued in an effort to elucidate the alkaloid composition of this pantropical plant as completely as possible (1). Occasionally, there is a spillover of leurocristine[1] (and leurosidine) into the B fraction. This investigation centers on the chloroform eluate of so-called post-leurocristine B fractions.

Rechromatography of the benzene-soluble material from the above fractions on deactivated alumina yielded only the known alkaloid perivine directly. Application of the gradient pH technique to each individual fraction yielded the new alkaloid vinaspine only as the base; while vincathicine, rovidine, and desacetyl VLB were obtained only as sulfates. A new alkaloid, vinaphamine, was obtained from crude amorphous leurosidine-containing material from the B fraction.

These new alkaloids are listed in Table I,

Table I.—New Alkaloids from *Vinca rosea* Linn.

Name	M. p., °C.	pK'a in 33% DMF	U.V. $\lambda_{max.}^{EtOH}$ mμ
Vinaspine	235–238	7.85	225, 281, 289
Vincathicine (sulfate)	>320 dec.	5.10, 7.05	231, 264, 300
Rovidine (sulfate)	>320 dec.	4.82, 6.95	214, 265, 286
Desacetyl VLB (sulfate)	>320 dec.	5.40, 6.90	214, 266, 294
Vinaphamine	229–235	5.15, 7.0	214, 262, 292

along with certain pertinent physical data. Their infrared spectra are reproduced separately (Figs. 1–4) as additional aids to their identification.

EXPERIMENTAL AND DISCUSSION[2,3]

Rechromatography of 3.659 Kg. of the chloroform eluate of post-leurocristine material from the B fraction from 90% leaf in benzene on 120 Kg. of deactivated alumina yielded 29.580 Gm. of perivine

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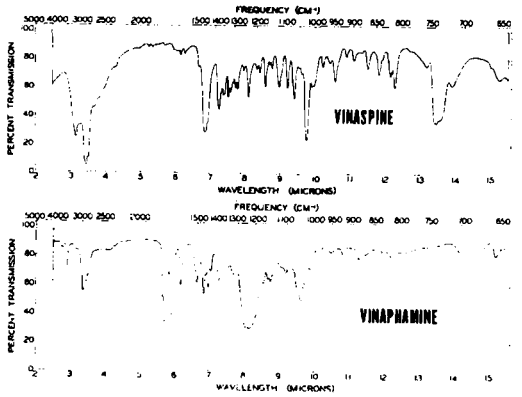


Fig. 1.—Infrared spectra of vinaspine and vinaphamine.

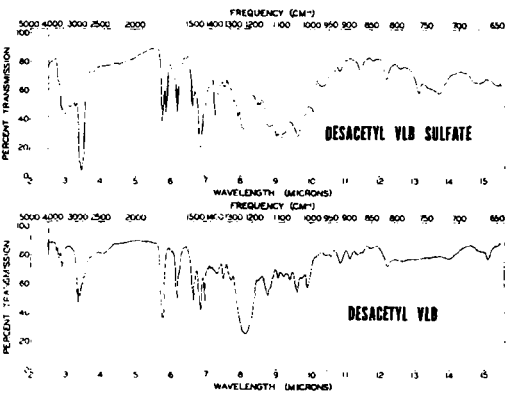


Fig. 3.—Infrared spectra of desacetyl VLB sulfate and desacetyl VLB.

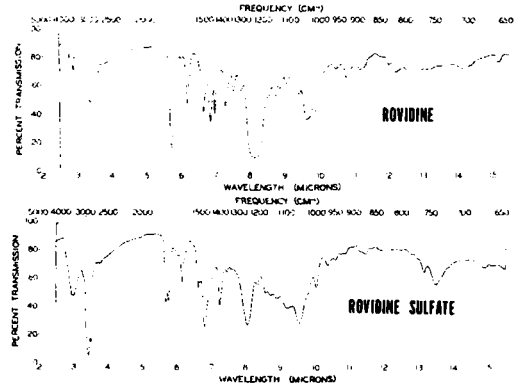


Fig. 2.—Infrared spectra of rovidine and rovidine sulfate.

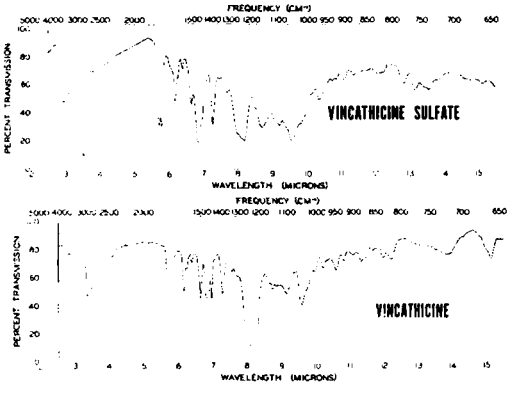


Fig. 4.—Infrared spectra of vincathicine sulfate and vincathicine.

TABLE II.—CHROMATOGRAPHY OF POST-LEUROCRISTINE FRACTIONS

Fraction[a]	Eluting Solvent	Wt., Gm.	Compd.	Wt., Gm.	Crystallizing Solvent
1	Benzene	67.9	...	...	...
2–5	Benzene–chloroform (3:1)	112.5	...	...	...
6	Benzene–chloroform (1:1)	38.8	Perivine	14.505	Methanol, acetone
7	Benzene–chloroform (1:1)	45.4	Perivine	15.075	Methanol, acetone
8–13	Benzene–chloroform (1:1)	370.3	...	...	...
14–21	Benzene–chloroform (1:3)	851.4	...	...	...
22–27	Chloroform	640.0	...	...	...
28–30	Chloroform–methanol (19:1)	387.4	...	...	...

[a] Fraction 1 was 1440 L. in volume; all others were 360 L.

(4). Attempts to crystallize directly other alkaloids from the fractions were unsuccessful. Data are given in Table II.

Utilization of the gradient pH technique with 20-Gm. aliquots of the fractions listed in Table II yielded 0.085 Gm. of vinaspine along with 1.3265 Gm. of leurosine (4), 0.0265 Gm. of mitraphylline (5), 4.267 Gm. of perivine (4), and 1.132 Gm. of lochnerine (4). Residues from the pH levels were subjected to sulfate formation and yielded 7.08 Gm. of vincathicine sulfate in addition to indicating the presence of two new sulfates which were eventually determined as being rovidine and desacetyl VLB sulfates. The following known sulfates were also obtained: 0.373 Gm. of catharanthine (4), 0.420 Gm. of cathindine (6), 0.528 Gm. of perosine (6), 0.261 Gm. of perivine (4), 0.175 Gm. of sitsirikine[4] (4), 0.146 Gm. of VLB (4), and 1.012 Gm. of cavincidine (6). Complete gradient pH data are given in Table III.

Vinaspine.—The base crystallizes from methanol as blades which show parallel extinction under polarized light, m.p. 235–238°. pK'a 7.85, electrometric titration, 33% DMF. The ultraviolet spectrum is that of a simple 2,3-disubstituted indole; $\lambda_{max.}^{EtOH}$ 225 mμ (log $E_{1\ cm.}^{1\%}$ 3.07), 281 mμ (log $E_{1\ cm.}^{1\%}$ 2.37), 289 mμ (log $E_{1\ cm.}^{1\%}$ 2.29).

The infrared spectrum of a Nujol mull confirms the ultraviolet data and in addition shows no carbonyl absorption color.

While thin-layer chromatographic examination on silica in the system ethyl acetate–absolute ethanol

[4] Sitsirikine sulfate is usually obtained as a co-crystallizing mixture of sitsirikine and its dihydro derivative (7).

TABLE III.—WEIGHTS OF GRADIENT pH FRACTIONS AND YIELDS OF ALKALOIDS AND SULFATES

Fraction and Wt., Gm.	Acid Insol.	pH[b] 2.90	3.40	3.90	4.40	4.90	5.40	5.90	6.40	7.50	7.50 (CHCl3)	8.50 (CHCl3)
1 (20.0)	8.21	1.25	0.58	0.59 0.130 C·SO4	0.76	0.56	0.67	0.81	0.71	0.86	0.93	...
2 (20.0)	7.86	1.37	0.68	0.80	0.99 0.243 C·SO4	0.86	0.80	0.86	0.73	1.03	1.12	...
3 (20.0)	6.20	2.54	2.21	1.07	1.02	0.89	1.01	0.97	0.63	0.70	0.89	...
4 (17.98)	6.71	2.07	1.43	1.18	...	1.73	1.48	1.09	0.64	0.53	0.53	...
5 (15.3)	4.08	2.05	1.18	0.85	0.86	0.85	0.96	1.02 0.115P 0.026 PS·SO4	1.07 0.483P	1.71 1.121P	0.94 0.118P 0.055 CI·SO4	...
6 c.m.l.[a] (20.0)	4.69	2.46	0.82	0.95	1.48 0.118L	1.75 0.212L 0.031 VLB·SO4	1.55 0.047 VLB·SO4	1.47	1.28 0.099 P·SO4	2.27 0.830P	1.82 0.244P 0.021 PS·SO4	0.44
7 c.m.l.[a] (20.0)	3.47	2.09	0.66	0.86 0.0385L	1.19 0.235L	1.71 0.416L 0.019 VLB·SO4	1.84 0.084L	1.67 0.049 VLB·SO4	1.39 0.050 PS·SO4	2.48 0.689P 0.0175 S·SO4	2.52 0.431 PS·SO4	0.67 0.085 VA
8 (20.0)	0.83	1.16	0.48	0.47 0.020L	0.79 0.100L	1.09 0.063L	1.01	1.15	1.10	2.19 0.667P	2.36	0.54 0.104LN
9 (20.0)	1.20	1.35	0.45	0.49	0.85 0.040L	1.48 0.011M	1.47 0.0155M	1.98	2.05	3.32	4.28	0.57 0.267LN
10 (20.0)	0.94	1.12	0.45	0.46	0.83	1.34	1.36	2.06	2.52	3.04	4.73 0.761LN	0.65
11 (20.0)	0.83	1.01	0.43	0.60	1.08	...	2.63	2.27 0.162 P·SO4	2.56 0.283 CI·SO4	2.59	3.84	0.43
12 (20.0)	1.17	1.23	0.53	0.69	0.97	1.15	1.61	1.72	3.16	2.65 0.058 CI·SO4	4.47 0.024 CI·SO4	0.39
13 (15.01)	0.83	0.60	0.52	0.50	0.66	0.86	1.37	2.08	1.76	1.72 0.277 CCD·SO4	2.95 0.391 CCD·SO4	0.40 0.037 CCD·SO4
14 (20.0)	0.92	1.08	0.93	0.89	1.15	1.64	2.51	3.41	2.43	1.90 0.307 CCD·SO4	2.78	0.34
15 (20.0)	0.69	1.04	0.78	0.92	1.08	1.59	2.92	3.74	3.08	2.07	1.85	0.18
16 (20.0)	...	...	...	...	...	3.53	2.74	5.32 0.18 VC·SO4	4.66 0.34 VC·SO4	...	...	3.23

continued overleaf

TABLE III.—*Continued*

Fraction and Wt., Gm.	Acid Insol.	pH[b] 2.90	3.40	3.90	4.40	4.90	5.40	5.90	6.40	7.50	7.50 ($CHCl_3$)	8.50 ($CHCl_3$)
17 (20.0)	...	...	...	...	2.23	1.54 0.02 Mixt. of RV·SO_4, DA-VLB·SO_4, and LC·SO_4	3.34 0.05 Mixt. of RV·SO_4, DA-VLB·SO_4, and LC·SO_4 0.06 VC·SO_4	5.07 1.12 VC·SO_4	3.21 0.51 VC·SO_4	...	...	2.32
18 (20.0)	...	...	...	...	...	3.62	3.99 0.25 VC·SO_4	5.85 1.38 VC·SO_4	3.32 0.25 VC·SO_4	...	...	2.45
19 (20.0)	...	...	...	...	...	3.63	4.88 0.10 VC·SO_4	5.23 0.95 VC·SO_4	3.50 0.17 VC·SO_4	...	...	2.26
20 (20.0)	...	...	...	...	...	3.29	3.72	5.69 0.66 VC·SO_4	3.56	...	...	1.74
21 (20.0)	...	...	...	...	...	2.90	5.18	5.44 0.90 VC·SO_4	3.68	...	...	2.41
22 (20.0)	...	...	...	...	...	3.81	4.63	4.92 0.21 VC·SO_4	3.61	...	...	2.69
23 (20.0)	0.39	0.28	0.28	0.30	0.44	1.13	3.90	4.98	2.87	2.21	0.92	...
24 (20.0)	0.31	0.29	0.17	0.25	0.42	1.07	4.19	5.73	2.85	2.09	0.75	...
25 (20.0)	0.51	0.32	0.33	0.39	0.54	1.04	3.05	4.26	3.11	2.31	1.27	...
26 (20.0)	0.53	0.29	0.35	0.44	0.58	1.17	2.83	3.38	3.16	2.83	1.52	...
27 (20.0)	0.65	0.32	0.45	0.35	0.66	1.13	2.58	3.69	3.53	3.15	2.15	...
28 (20.0)	0.87	0.48	0.23	0.26	0.40	0.65	0.94	1.67	2.39	3.63	3.64	0.34
29 (20.0)	1.19	0.34	0.29	0.28	0.38	0.58	0.79	1.64	2.03	3.05	4.00	0.42
30 (20.0)	4.51	...	0.03	0.55	0.28	0.51	1.12	2.07	2.20	2.42	2.86	0.40

[a] c.m.l., crude mother liquor. [b] C·SO_4, catharanthine sulfate; P, perivine; PS·SO_4, perosine sulfate; CI·SO_4, cathindine sulfate; L, leurosine; VLB·SO_4, vincaleukoblastine sulfate; P·SO_4, perivine sulfate; S·SO_4, sitsirikine sulfate; VA, vinaspine; M, mitraphylline; LN, lochnerine; CCD·SO_4, cavincidine sulfate; VC·SO_4, vincathicine sulfate; RV·SO_4, rovidine sulfate; DA-VLB·SO_4, desacetyl VLB sulfate; LC·SO_4, leurocristine sulfate.

(1:1) showed vinaspine to have an R_f identical to that of lochnerine, spraying with ceric ammonium phosphate solution (8) produced a persistent gray-blue *versus* a pinkish-gray color.

No derivatives were prepared.

Vincathicine.—The sulfate crystallizes from ethanol as small blades, m.p. > 320° dec. The base could not be crystallized. pK'a 5.10, 7.05, electrometric titration, 33% DMF. The ultraviolet spectrum suggests similarities to the other dimeric alkaloids. $\lambda_{max.}^{EtOH}$ 213 mμ (log $E_{1\ cm.}^{1\%}$ 2.64), 264 mμ (log $E_{1\ cm.}^{1\%}$ 2.24), 300 mμ (log $E_{1\ cm.}^{1\%}$ 1.85).

The infrared spectrum of the base in chloroform shows it to be a dimeric alkaloid related to the other known dimerics.

The NMR spectrum shows it to be a dimeric alkaloid of the indole-vindoline type. The acetyl, methoxyl, and (*N*) methyl functions are still intact, while the indole is shown to have been oxidized to an oxindole.

Anal.—Found: C, 61.19; H, 6.68: N, 5.94; O, 22.52; S, 3.74.

The base, dissolved in isopropanol, readily forms a crystalline methiodide with excess methyl iodide. Recrystallization from isopropanol yielded a sample of small spheres, m.p. > 320° dec.; its hydrochloride crystallizes readily from ethanol-ether as irregular, submicroscopic crystals, m.p. > 320° dec.

Rovidine.—While the presence of this alkaloid was indicated in certain crude crystalline sulfates (shown in Table III), workable amounts were not obtainable therefrom. This entity subsequently was found to occasionally accompany crude crystalline leurosidine and leurocristine sulfate at pH levels 5.40 and 5.90. Rechromatography and sulfate formation in the usual manner yielded the pure compound. The base could not be crystallized.

The sulfate crystallizes from ethanol as very thin blades, m.p. > 320°. pK'a 4.82, 6.95, electrometric titration, 33% DMF. The ultraviolet spectrum is that of a dimeric alkaloid of the VLB sulfate type; $\lambda_{max.}^{EtOH}$ 214 mμ (log $E_{1\ cm.}^{1\%}$ 2.71), 265 mμ (log $E_{1\ cm.}^{1\%}$ 2.18), 296 mμ (log $E_{1\ cm.}^{1\%}$ 2.05); inflections at 286, 310 mμ.

The infrared spectrum of a chloroform solution of the base also shows it to be closely related to VLB, appearing to contain all of the same functional groups.

The NMR data confirm the I.R. observations.

No derivatives were made.

Anal.—Found: C, 60.76; H, 6.74; N, 5.89; O, 22.06; S, 3.53.

Desacetyl VLB.—As with rovidine, the presence of this compound was indicated in Table III. Rechromatography of leurocristine sulfate crude mother liquors eventually yielded workable amounts of the pure compound.

The sulfate crystallizes from ethanol as blades, m.p. > 320° dec. The free base crystallizes from aqueous ethanol as blades with parallel extinction, m.p. 205–210°. pK'a 5.40, 6.90, electrometric titration, 33% DMF. The ultraviolet spectrum is that of a dimeric indole-indoline alkaloid and is similar to that of VLB sulfate; $\lambda_{max.}^{EtOH}$ 214 mμ (log $E_{1\ cm.}^{1\%}$ 2.76), 266 mμ (log $E_{1\ cm.}^{1\%}$ 2.27), 294 mμ (log $E_{1\ cm.}^{1\%}$ 2.10); inflections at 286, 310 mμ.

The infrared spectrum of a chloroform solution of the base shows it to be closely related to VLB, displaying a less intense carbonyl function. Both the I.R. and NMR spectra were identical to authentic desacetyl VLB (9).

Anal.—Found: C, 59.26; H, 6.68; N, 8.57; O, 21.63; S, 3.71.

Vinaphamine.—While this alkaloid was not encountered during the investigation of the fractions as originally defined, it was associated with crude amorphous leurosidine obtained occasionally from the B fraction at pH levels 5.40, 5.90, and 6.40. Prudent selection of fractions which contained vinaphamine as a major component and leurosidine as a minor component (based on thin-layer monitoring) and crystallization from methanol yielded pure vinaphamine. The base crystallizes from methanol as thin blades with inclined extinction, m.p. 229–235°. pK'a 5.15, 7.0, electrometric titration, 33% DMF. The ultraviolet spectrum suggests a dimeric indole-indoline chromophore; $\lambda_{max.}^{EtOH}$ 214 mμ (log $E_{1\ cm.}^{1\%}$ 2.78), 262 mμ (log $E_{1\ cm.}^{1\%}$ 2.24), 292 mμ (log $E_{1\ cm.}^{1\%}$ 2.08); shoulders at 224, 284, 300 mμ.

The infrared spectrum of a chloroform solution resembles that of a VLB type of dimer, exhibiting an extra carbonyl function at 5.95 μ.

The NMR spectrum resembles that of the other dimeric alkaloids.

No derivatives were made.

Anal.—Found: C, 67.54; H, 6.77; N, 8.70; O, 17.22.

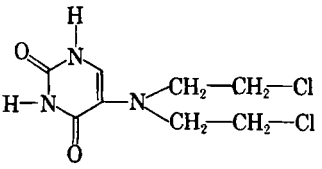
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Qualitative and Quantitative Tests for Uracil Mustard

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

5-[BIS(2-CHLOROETHYL)AMINO] URACIL; $C_8H_{11}Cl_2N_3O_2$; mol. wt. 252.10. The structural formula of uracil mustard may be represented as



Physical Properties.—Uracil mustard occurs as a cream-white, odorless, crystalline powder, and melts at about 200° dec., U.S.P. XVI Class Ia. It is slightly soluble in acetone and in alcohol, very slightly soluble in water, and practically insoluble in benzene and in chloroform.

Identity Tests.—Mix 5 mg. of uracil mustard and 50 mg. of benzoin (benzoylphenylcarbinol) in a 10 × 75 mm. test tube and cover the tube opening with a moistened strip of Congo red test paper. Immerse the tube into an oil bath at 170°: the test paper begins to turn blue within 1 minute.

A 1 in 40,000 solution of uracil mustard in alcohol exhibits an ultraviolet absorbance maximum at about 256 mμ [absorptivity (1%, 1 cm.) about 226], a shoulder at about 295 mμ, and a minimum at about 233 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of uracil mustard in postassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests.—Dry about 1 Gm. of uracil mustard, accurately weighed, *in vacuo* over silica gel for 18 hours: it loses not more than 0.5% of its weight.

Char about 0.1 Gm. of uracil mustard, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.5%.

Dissolve about 20 mg. of uracil mustard in 10 ml. of alcohol, add 2 drops of nitric acid and 1 ml. of silver nitrate T.S.: no opalescence is produced (absence of chloride ion).

Place about 500 mg. of finely powdered potassium nitrate in the cup of a Parr peroxide bomb, add about 250 mg. of finely powdered sucrose, and mix with a glass rod. Transfer to the cup about 250 mg. of finely powdered uracil mustard, accurately weighed, and again mix. Place a 5-Gm. portion of sodium peroxide in the cup, mix, and add a 10-Gm. portion of the peroxide, mixing the entire contents of

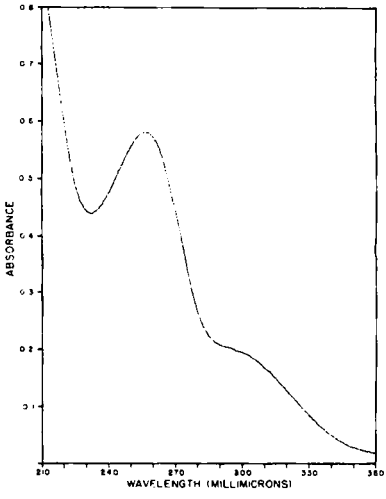


Fig. 1.—Ultraviolet absorption spectrum of uracil mustard in alcohol (25 mcg. per ml.). Beckman model DK-2A spectrophotometer.

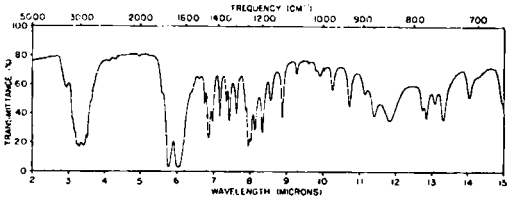


Fig. 2.—Infrared spectrum of uracil mustard in potassium bromide disk (0.5%). Perkin-Elmer model 21 spectrophotometer; sodium chloride prism.

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the cup thoroughly with the glass rod. Brush any particles clinging to the glass rod into the cup with a camel hair brush. Cover the cup and shake for 1 to 2 minutes, tapping the cup sharply several times to loosen any material clinging to the sides or bottom of the cup. Remove the cover, brush into the cup any particles adhering to the cover or to the rim of the cup, place the electric ignition head on the cup, and tap the base sharply on a hard surface to compact the charge. Assemble the bomb and ignite, observing the precautions given under *Sulfur and Halogen Determination*, U.S.P. XVI, p. 908, and the instructions in the bomb manufacturer's manual. After the bomb has cooled, rinse the exterior of the cup with water and dismantle the bomb. Immerse the ignition head into a 1-L. beaker containing about 100 ml. of hot water to dissolve any of the fusion mixture which may be adhering to its underside and rinse the head with hot water, catching the rinsings in the beaker. With a pair of tongs lay the fusion cup on its side in the same beaker of hot water, covering it immediately with a watch glass. After the fusion mixture has dissolved, remove the cup and rinse it into the beaker with hot water. Allow the solution to cool, add 50 ml. of dilute nitric acid (1 in 1), slowly with stirring, and filter, washing the filter with water. To the filtrate add 500 mg. of hydrazine sulfate and, very slowly with stirring, add 25 ml. of 0.1 *N* silver nitrate, avoiding exposure to strong light. Filter the mixture through a tared sintered-glass crucible of medium porosity, wash the precipitate with 300 ml. of water in divided portions, dry at 140° for 1 hour, cool, and weigh. The weight of the dried precipitate, multiplied by 0.2474, represents the weight of chlorine in the sample. The chlorine content is not less than 27.6% and not more than 28.7%, calculated on the dried basis.

Assay

Transfer about 200 mg. of uracil mustard, previously dried *in vacuo* over silica gel for 18 hours and accurately weighed, to a 100-ml. volumetric flask, dilute to volume with alcohol, and mix. Pipet 20 ml. of this solution into a 50-ml. volumetric flask containing 20 ml. of water, mix, add 1.0 ml. of 5% acetic acid solution, and mix. Transfer 6.0 ml. of 8-hydroxyquinoline T.S. to the flask, mix, add 3.0 ml. of sodium carbonate solution (1 in 10), dilute to volume with water, mix, and allow to stand 150 minutes. Determine the absorbance of this solution and of a similar solution prepared with uracil mustard reference standard in 1-cm. cells with a suitable spectrophotometer at about 466 mμ, using a reagent blank to set the instrument. Record the absorbance of the sample solution as A_u and that of the standard solution as A_s and calculate the amount of $C_8H_{11}Cl_2N_3O_2$, in milligrams, in the amount of sample taken by the formula $W(A_u/A_s)$, where W is the exact weight of uracil mustard reference standard taken. The amount of uracil mustard found is not less than 97.0% and not more than 103.0% of the weight of the sample taken.

DOSAGE FORMS OF URACIL MUSTARD

Uracil Mustard Capsules

Identity Test.—Transfer the contents of two uracil mustard capsules to a 100-ml. volumetric flask, add alcohol to volume, and mix. Centrifuge a portion of this mixture until clear. The ultraviolet absorption spectrum of the solution exhibits a maximum at 256 ± 3 mμ, a shoulder at 295 ± 3 mμ, and a minimum at 233 ± 3 mμ, comparable to that of a similar solution of uracil mustard reference standard.

Assay.—*Standard Preparation.*—Transfer about 50 mg. of uracil mustard reference standard, previously dried *in vacuo* over silica gel for 18 hours and accurately weighed, to a 50-ml. volumetric flask, dissolve in alcohol, dilute to volume with alcohol, and mix.

Assay Preparation.—Transfer as completely as possible the contents of not less than 20 uracil mustard capsules to a tared weighing bottle and weigh accurately. Transfer to a glass-stoppered centrifuge tube an amount of the mixed powder, equivalent to about 10 mg. of uracil mustard and accurately weighed, add 10.0 ml. of alcohol, mix thoroughly by inversion, and centrifuge.

Procedure.—Pipet 4 ml. of water into each of three glass-stoppered test tubes. Pipet 4 ml. of the *Assay Preparation* and of the *Standard Preparation*, respectively, into separate tubes; prepare a blank by adding 4.0 ml. of alcohol to the remaining tube and mix. To each tube add 1.0 ml. of 5% acetic acid and 2.0 ml. of 8-hydroxyquinoline T.S., mixing after each addition. Add 3.0 ml. of sodium carbonate (1 in 10) to each tube, mix well, and allow to stand for 150 minutes. Filter the assay sample through dry filter paper and determine the absorbances of the solutions in 1-cm. cells using a suitable spectrophotometer at the wavelength of maximum absorbance at about 466 mμ using the blank to set the instrument at zero absorbance. Record the absorbance of the solution from the *Standard Preparation* as A_s and that from the *Assay Preparation* as A_u and calulate the weight, in milligrams, of $C_8H_{11}Cl_2N_3O_2$ in the portion of uracil mustard capsules taken by the formula $10C(A_u/A_s)$, where C is the concentration, in milligrams per milliliter, of uracil mustard reference standard in the *Standard Preparation*. The amount of uracil mustard found is not less than 90% and not more than 110% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Uracil mustard,[1] synthesized by Lyttle and Petering (1), is an orally active alkylating agent of the nitrogen mustard class which is used in the palliative treatment of certain neoplasms affecting the reticuloendothelial system.

Identity Tests.—Identification of uracil mustard as an aliphatic halogen compound is based on its reaction with molten benzoin (2). The reaction yields a halogen hydride which can be detected readily in the gas phase by the action on Congo paper. The ultraviolet absorption spectrum for uracil mustard cannot be used alone for identification since an intermediate in its synthesis, 5-[bis-(2-hydroxyethyl)amino]uracil, has a similar spectrum with a nearly identical molar absorptivity. However, these identity tests together with the infrared spectrum provide a satisfactory identification of uracil mustard.

[1] Marketed only under the generic name.

Quantitative Methods.—The chloride content of uracil mustard determined by a sodium peroxide fusion and gravimetric analysis gave an average value of 28.0 ± 0.3%.[2] The colorimetric analysis of uracil mustard using 8-hydroxyquinoline reagent in alkaline solution serves to differentiate uracil mustard and the 5-[bis(2-hydroxyethyl)amino] uracil intermediate. Conformity to Beer's law was observed from 10–40 mg. of uracil mustard per 50 ml. of reaction solution. Analysis of commercial uracil mustard capsules by this method gave an average value of 96.2 ± 4.3%.[2] The colorimetric method of Petering and Van Giessen (3) provides an alternate quantitative procedure and an additional identity test for uracil mustard and related alkylating agents. This method employs the reaction of alkylating agents with 4-(*p*-nitrobenzyl)pyridine (NBP) producing a chromophore with a maximum at about 600 mμ and may be adaptable to the capsule formulation, although limited investigation in this laboratory did not yield quantitative results.

[2] Maximum deviation from the mean value.

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Technical Articles

Experiences with Unit-to-Unit Variations in Tablets

By LEON LACHMAN and HANNA D. SYLWESTROWICZ

The literature reports on the subject of interunit dosage and weight variations of tablets indicate that past studies were mainly concerned with analyzing the finished product to detect gross departures from the desired quality. These studies, however, were cognizant of the several stages of manufacture before the compression step could contribute to the end tablet variability. In this investigation, the variability in several stages of tablet manufacturing (dry mixing, granulating, lubricating, and tableting) was evaluated for a particular tablet formulation. The importance of employing correct sampling procedures to obtain random samples to be used for the accurate estimation of the sources of variability and product uniformity is illustrated. The relationship that exists between tablet weights and drug concentration was determined. Since systematic sampling is commonly employed in the in-process control of tableting operations, the information that can be gained from this type of sampling procedure and random sampling is presented and discussed.

THE TERM "unit-to-unit variation of potency" of solid dosage forms has developed considerable significance in recent years. Several reports have appeared in the literature on this subject (1–5). It has been demonstrated (1, 2) that substantial variations can exist in the potency between individual tablets which would not be detected by test methods devised to determine average drug content.

Realizing the importance of this situation, the Quality Control Section of the Pharmaceutical Manufacturers Association initiated an extensive study of this problem. As a result of this study, recommendations have been submitted to the United States Pharmacopeia and National Formulary revision committees that these compendia include a two-limit attribute statistical test in their specifications for tablet composition-uniformity to measure intertablet dosage uniformity (6).

Tablets have received the most attention and study with regard to interunit dosage variation (1–5) because they are the most acceptable dosage form on the U. S. market for the administration of orally effective therapeutic agents and account for a major part of the pharmaceutical sales.

Although these studies were cognizant of the several stages of manufacture before the compression step could contribute to the over-all end variability of the drug concentration in the tablets, none of these investigators studied in detail the variability of each of these steps; instead, they sampled the finished product to detect gross departures from the desired quality. However, to insure acceptable quality of the finished product, the successive phases of the manufacturing process must be evaluated to

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The authors express their appreciation to Professor A. P. Dempster, Harvard University, for his valuable suggestions; to Dr. H. Bornhold for his critical review of the text; to Mrs. D. Chen for her assistance with the statistical computations; to Dr. C. Rehm for analytical assistance; and to Mr. Ronald Kilgore for his technical assistance.

Quantitative Methods.—The chloride content of uracil mustard determined by a sodium peroxide fusion and gravimetric analysis gave an average value of 28.0 ± 0.3%.[2] The colorimetric analysis of uracil mustard using 8-hydroxyquinoline reagent in alkaline solution serves to differentiate uracil mustard and the 5-[bis(2-hydroxyethyl)amino] uracil intermediate. Conformity to Beer's law was observed from 10–40 mg. of uracil mustard per 50 ml. of reaction solution. Analysis of commercial uracil mustard capsules by this method gave an average value of 96.2 ± 4.3%.[2] The colorimetric method of Petering and Van Giessen (3) provides an alternate quantitative procedure and an additional identity test for uracil mustard and related alkylating agents. This method employs the reaction of alkylating agents with 4-(*p*-nitrobenzyl)pyridine (NBP) producing a chromophore with a maximum at about 600 mμ and may be adaptable to the capsule formulation, although limited investigation in this laboratory did not yield quantitative results.

[2] Maximum deviation from the mean value.

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Technical Articles

Experiences with Unit-to-Unit Variations in Tablets

By LEON LACHMAN and HANNA D. SYLWESTROWICZ

The literature reports on the subject of interunit dosage and weight variations of tablets indicate that past studies were mainly concerned with analyzing the finished product to detect gross departures from the desired quality. These studies, however, were cognizant of the several stages of manufacture before the compression step could contribute to the end tablet variability. In this investigation, the variability in several stages of tablet manufacturing (dry mixing, granulating, lubricating, and tableting) was evaluated for a particular tablet formulation. The importance of employing correct sampling procedures to obtain random samples to be used for the accurate estimation of the sources of variability and product uniformity is illustrated. The relationship that exists between tablet weights and drug concentration was determined. Since systematic sampling is commonly employed in the in-process control of tableting operations, the information that can be gained from this type of sampling procedure and random sampling is presented and discussed.

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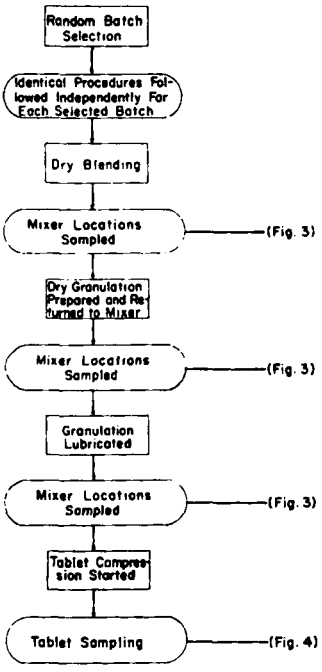


Fig. 1.—Sequence of production sampling.

pinpoint the sources of variability. Only in this way can they be minimized, with resultant optimal quality levels for each stage of the manufacturing process.

The causes for variability of drug content in the final product can be broken down into two major factors: (*a*) variations in tablet weight and (*b*) heterogeneous drug distribution in the tablets. If a homogeneous drug distribution is found, the problem lies at the compression step; where a heterogeneous drug distribution is found, the problem most likely is at the stages of granulation manufacture.

In practice, several situations could arise which involve variations in tablet weight or heterogenous drug distribution in the tablets or both. A brief description follows.

(*a*) For a tablet preparation which exhibits a large variability in tablet weight and homogeneity of drug distribution in the tablets, a high correlation coefficient between tablet weight and drug content which approaches unity results. The large tablet weight variability in turn causes a large variability in drug content. To improve such a process, the factors that can contribute to this large tablet weight variability must be evaluated. Factors such as hopper load, machine settings and adjustments, size distribution of the granulation, segregation of the granulation, and flow properties of the granulation can influence the weight variability of the tablets. Since there is homogeneity in drug distribution, it should be possible to predict the drug content in the tablets from the weight data.

(*b*) In the case where the tablets exhibit small weight variability and homogeneous drug distribution, again the correlation coefficient is high approaching unity. However, such a situation indicates that the production operation is satisfactory, and no further study is required. Here, as for (*a*) it should be possible to predict the drug content in the tablets from the weight data.

(*c*) For a tablet formulation which exhibits large variations in tablet weight and a heterogeneous drug distribution in the tablets, the correlation coefficient would be small and not significant. To improve such an operation, it is necessary to investigate the factors responsible for heterogeneous drug distribution—completeness of the mixing at the several stages of granulation manufacture, whether separation or segregation of the granulation occurs during compression—and the factors responsible for large variations in tablet weight presented earlier.

(*d*) In the situation where a tablet preparation exhibits small variability in tablet weight and heterogeneous drug content in the tablets, a correlation coefficient approaching zero is obtained. Here it is necessary to study the factors that can contribute to this heterogeneity of drug content.

In the remainder of this report, information will be presented that was obtained from an evaluation of several of the critical steps concerned with the manufacture of a tablet formulation which exhibited low tablet weight variability but high drug content variability in the tablets. The methods developed to estimate the variability at the end of the mixing, granulating, lubricating, and tableting procedures will be described, and the importance of the sampling plan will be illustrated. It will be shown that by pinpointing the stage of manufacture responsible for intertablet dosage variation, studies then can be undertaken readily to determine the cause of this variation, and corrective measures can be instituted.

EXPERIMENTAL

The tablet formula chosen for investigation contained about 5% drug and weighed approximately 1.0 Gm. The tablets were prepared in the production department with production personnel and in accordance with the established manufacturing procedure. The granulations for these tablets were prepared by the customary wet granulation tech-

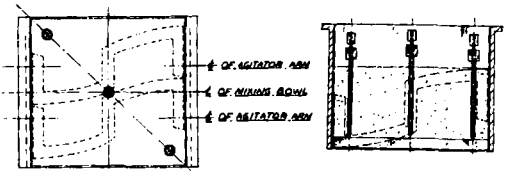


Fig. 2.—Right, plan view; left, section view of Readco mixing bowl with theft sampler positions.

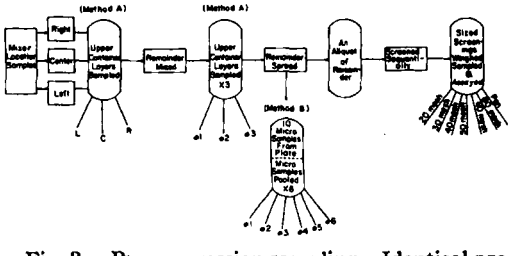


Fig. 3.—Precompression sampling. Identical procedures were followed independently for the dry mix, dry granulation, and lubricated granulation.

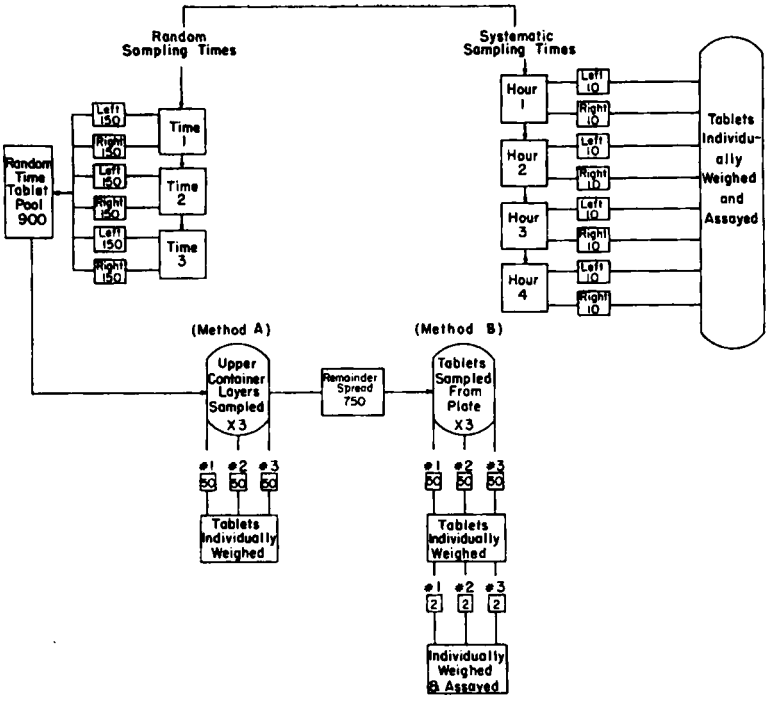


Fig. 4.—Tablet sampling.

niques. The entire batch of lubricated granulation was placed in a master hopper which fed two hoppers on a Stokes model 540 double rotary tablet press, and tablets were produced at a rate of 1200 per minute. Normal machine adjustments were made by the tablet operator to keep the weights of the tablets under control.

Sampling Plans

Since the production order for the tablet formulation under study consisted of eight individual batches of 240,000 tablets per batch, three batches were selected at random for study which will be known subsequently as batches K_4, K_6, and K_8. Each batch was processed individually and controlled accordingly. A schematic illustration of the sampling performed during the several stages in the manufacture of the tablet preparation is presented in Fig. 1. In the subsequent sections of this report, each stage will be treated in detail.

Method A.—*Sampling of Dry Mix, Dry Granulation, and Lubricated Granulation.*—In studying the variability at the end of the mixing, granulating, and lubricating stages of the manufacturing process for each of the three batches, three location samples of 300 Gm. were taken from the mixer with a sample thief at (*a*) the end of the dry blending period, (*b*) when the dried screened granulation was returned to the mixer for lubrication, and (*c*) after lubrication. The samples were taken along the diagonal of the mixer at three locations illustrated by Fig. 2. The location points were designated left (L), center (C), and right (R).

From each location sample one analysis sample equivalent to the weight of one tablet was taken at random by *Method A*, weighed on a semimicro analytical balance, and assayed. The remaining material in the three location samples was blended. Three random samples, each equivalent to the weight of one tablet, were taken by *Method A;* six random samples, each equivalent to the weight of one tablet, were taken by *Method B* and assayed. Figure 3 summarizes these sampling plans.

Method A consisted of removing samples for analysis equivalent to the weight of one tablet from the top portion of the samples in glass bottles. *Method B* consisted of spreading the sample from the glass bottle on a rectangular surface, 8 × 10 in., then taking approximately 10 small quantities from different locations on the spread material with a microspatula which made up an analysis sample equal to the weight of one tablet.

In addition, 100-Gm. aliquots were taken by *Method B* from the blended samples and screened sequentially. The distribution of the 100 Gm. of material on the particular size screens was determined and will be presented as per cent of the initial aliquot. The material on each size screen was sampled and analyzed for drug concentration.

Method B.—*Random and Systematic Sampling of Tablets.*—The same three random batches used to study the several stages of granulation manufacture were also used to study the variability of the compression operation. As illustrated in Fig. 4, the random sampling plan employed was to take three samples of 300 tablets in sequence (150 from the left side and 150 from the right side of the press) during the compression of each batch of tablets on the Stokes model 540 tablet press at three random times.

The three samples of 300 tablets were mixed together for each batch to form a composite sample of 900 tablets. Then three random laboratory samples of 50 tablets each were taken by two methods. *Method A* consisted of taking the three samples of 50 tablets each from a bottle holding the 900 composite sample of tablets. *Method B* consisted of placing the remaining tablets from the 900 in the bottle onto a circular disk of such diameter that the tablets formed a single layer on the disk.

TABLE I.—VARIATIONS IN DRUG CONCENTRATION FOR SAMPLES TAKEN AT THE VARIOUS STAGES OF GRANULATION PREPARATION[a]

	Dry Mix			Dry Granulation			Lubricated Granulation		
	K_4	K_6	K_8	K_4	K_6	K_8	K_4	K_6	K_8
Location samples, *Method A*									
L	37.3	47.4	54.1	32.5	40.4	35.9	36.9	37.7	40.7
C	54.1	52.2	52.3	37.3	38.4	35.0	40.4	40.7	38.5
R	53.2	52.0	39.9	52.2	38.5	32.1	39.9	38.0	33.3
Random samples, *Method A*									
1	55.2	51.9	56.1	41.9	35.0	53.9	34.1	39.5	39.1
2	55.6	53.4	56.7	37.6	37.5	47.6	36.1	37.4	37.4
3	54.8	53.5	57.3	39.0	36.2	50.4	32.2	36.6	35.7
Random samples, *Method B*									
1	49.1	51.6	49.0	48.3	46.3	52.1	43.7	45.4	46.4
2	48.2	53.2	47.1	53.3	47.0	50.8	45.6	45.9	53.6
3	47.3	51.4	47.7	52.3	44.2	47.6	44.9	43.3	46.1
4	47.5	52.5	47.4	47.7	45.9	54.2	44.3	44.1	49.9
5	49.6	55.1	46.6	48.1	43.4	58.3	45.6	45.1	47.0
6	47.6	51.1	46.3	44.8	47.7	52.3	46.7	46.4	47.4
Means	48.2	52.5	47.4	49.1	45.8	52.6	45.1	45.0	48.4

[a] Milligrams per tablet weight.

Subsequently, 50 tablets were taken three times at random from this disk. Each of the 50 tablets in the random laboratory samples was weighed on a semimicro analytical balance individually. From the second set of three samples of 50 tablets, two analysis samples composed of two individual tablets were taken from each of the three samples of 50 tablets by a random method, weighed, and assayed. Subsequently, it will be illustrated that the sampling method is important in insuring that the samples selected are, in fact, random.

Method C.—*Systematic Sampling.*—This is commonly employed in the in-process control of tableting operations. This sampling plan is also outlined in Fig. 4. It consisted of taking 10 tablets from the left and right compression sides of the Stokes model 540 tablet press at 1, 2, 3, and 4 hours for the same batches of tablets previously described. Individual weights and assays were performed on all tablets. The 4-hour period was chosen since this is approximately the time required for the compression of a batch of tablets. During this 4-hour period, normal machine adjustments were made by production personnel in their weight control of the tableting operation. This could involve a different number of adjustments to the right and left compression sides of the press and can be made at different times during the compression of a batch of tablets.

RESULTS AND DISCUSSION

Dry Mix, Dry Granulation, and Lubricated Granulation

The drug content of location and random samples taken by *Methods A* and *B* for the three production batches are presented in Table I.

It is evident from the data in Table I that the method of taking samples is extremely important. For the dry mix, the random samples taken by *Method A* are generally substantially higher in drug content than those taken by *Method B*. However, the reverse is true for the dry granulation and lubricated granulation. If one were to use the results obtained by *Method A*, a completely different interpretation would have been given to the situation which prevailed in the production of this granulation.

Consequently, unless correct sampling techniques are employed, the data obtained can be very misleading concerning the prevailing conditions of drug concentration and uniformity at the several stages in the production of the tablet granulation.

The reason for this phenomenon becomes clear after observing the size distribution of this granulation and the corresponding drug content for the different sized granules as shown in Table II.

It would appear that the drug seems to be more concentrated at the larger granules for this particular tablet preparation. The reason for this occurrence possibly can be explained as follows.

Since the drug is poorly water soluble, it would be expected to be found at the granule, if it was adhered to the granule surface by the gum used in the granulating liquid or if it was encapsulated in the granule. Because the large granules exhibit a smaller surface area than the small granules, there is less chance for the drug to dislodge with further processing if it were adhered to the surface of the granule. In addition, the large granules can more readily encapsulate the drug particles fully, thus further insuring its presence in these size granules.

It is clear that unless this granulation is well mixed, so that the various size granules are randomly distributed in the blend, problems can arise when samples are taken. Furthermore, due to the substantial difference in drug concentration for the

TABLE II.—DRUG CONCENTRATION IN DIFFERENT SIZED GRANULES FROM SIZE DISTRIBUTION OF LUBRICATED GRANULATION

Screen Size	% on Screen	Drug Concn.,[a] mg./0.5 Gm.
20	1	56.4
30	13	46.0
40	24	36.5
50	25	20.0
60	13	11.2
80	15	6.7
Pan	10	4.8

[a] Theoretical concentration = 23.3 mg. per 0.5 Gm

various size granules, if unmixing were to take place when transferring the granulation from the sample thief into the glass bottle, the results presented in Table I under *Method A* could occur.

Although it is realized that the location samples which were taken by *Method A* are most likely incorrect indications of the drug distribution in the granulation, the data will be used on a hypothetical basis to illustrate the information that can be gained through an analysis of the various stages of the tablet manufacture. However, the three random samples taken by *Method A* were not used again in this study, but only the six taken by *Method B*, for which it is fairly certain that sampling was done correctly.

The variability of the drug concentration in the mixer load after the several stages of granulation manufacture was determined from an analysis of the random and location samples. Since the per cent active ingredient present in the formulation is small, statistical analysis was performed on the assay values which were transformed into logarithms as suggested in the statistical literature (7).

The analysis of variance for the location samples is summarized in Table III. There were significant interactions between batches and locations for all three stages of production. This would indicate that the mixes in the mixer were not uniform for the three batches. For the dry mix and dry granulation it was not possible to state which location of the mixer would always be low or high, since the results differed from one batch to another. However, in the analysis of the lubricated granulation, differences between locations were significant when the interaction between batches and locations was used as an error term. These location differences indicated that the samples taken from the center of the mixer had a tendency to be consistently higher in drug content when compared with the samples taken from the right side of the mixer. This held true for the three batches as shown in Table I and could be extended further to hold for the eight batches of the production order.

The results can possibly be explained in two ways: (*a*) the mixes were heterogeneous or (*b*) the samples were not taken properly, making it appear as if the drug distribution were heterogeneous. As illustrated earlier, improper selection of location samples is definitely a factor, but it is also felt that heterogeneity of the mixes is a contributing factor. To show that this is the case, analysis of variance was computed for the data at each stage of the operation on the six random samples from each of three batches. These results are summarized in Table IV.

From this analysis, it was proved that there existed significant differences between the means of drug content of each batch for the three stages of production. However, since the procedures of manufacture and the formulas for the three batches were the same, there was no reason to believe that the mean drug content per batch should differ. It is anticipated that if the random samples were taken for analysis from many locations (*e.g.*, 15 or 20) the mean drug content from batch to batch for each stage of the granulation manufacture would not differ significantly from one another.

Table V presents the standard deviations for the six random samples of each stage in the production of each batch.

Table III.—Analysis of Variance on Random Samples and Location Samples of Three Stages of Granulation Manufacture

Source of Variation	D.F.[e]	Dry Mix			Dry Granulation			Lubricated Granulation		
		Sum of Squares	Mean Square	F Test	Sum of Squares	Mean Square	F Test	Sum of Squares	Mean Square	F Test
Total	26	0.03765707	...	...	0.12979881	...	...	0.04908239	...	...
B (Batches)	2	0.00711237	0.00355618	0.9428	0.00286934	0.00143467	0.2222	0.00107681	0.00053840	0.4751
L (Location differences)/	3	0.00630858	0.00210286	0.5575	0.07876028	0.02625342	4.0655	0.03687527	0.01229175	10.8472[b]
B × L	6	0.02262974	0.00377162 = S_2^2	35.2192[b]	0.03874539	0.00645756 = S_2^2	10.2786[b]	0.00679904	0.00113317 = S_2^2	3.9245[a]
B × Random error	15	0.00160638	0.00010709 = S_1^2	...	0.00942380	0.00062825 = S_1^2	...	0.00433118	0.00028874 = S_1^2	...
Random	...	...	...	...	...	...	...	...	...	...

[a] Significant at 5% level. [b] Significant at 0.1% level. [c] S_1^2 was used to test B × L interaction. [d] S_2^2 was used to test B and L. [e] D.F. = degrees of freedom. [f] +, includes contrasts between L, C, R, and random.

Table IV.—Analysis of Variance on Six Random Samples per Batch of Three Stages of Granulation Manufacture

Source of Variation	D.F.[d]	Dry Mix			Dry Granulation			Lubricated Granulation		
		Sum of Squares	Mean Square	F Test	Sum of Squares	Mean Square	F Test	Sum of Squares	Mean Square	F Test
Total	17	0.00814692	...	...	0.02007812	...	...	0.00799471	...	...
Between batches	2	0.00654054	0.00327027	30.5375[c]	0.01065432	0.00532716	8.4793[b]	0.00366344	0.00183172	6.3436[a]
Within batches	15	0.00160638	0.00010709 = S^2	...	0.00942380	0.00062825 = S^2	...	0.00433127	0.00028875 = S^2	...

[a] Significant at 5% level. [b] Significant at 1% level. [c] Significant at <0.1% level. [d] D.F. = Degrees of freedom.

TABLE V.—STANDARD DEVIATIONS OF DRUG CONTENT FROM RANDOM SAMPLES TAKEN AT THE VARIOUS STAGES OF GRANULATION MANUFACTURE

Batch	Dry Mix	Dry Granulation	Lubricated Granulation
K_4	0.93	3.16	1.07
K_6	1.50	1.65	1.15
K_8	0.98	3.57	2.88

The nine standard deviations shown in Table V do not differ significantly. However, if a greater number of samples were taken from the mixer at more than three locations for each batch, significant differences between batches could possibly be obtained. This would suggest that the degree of mixing was insufficient and also different for the three batches.

It would be expected that the standard deviation for consecutive stages in the granulation manufacture should decrease since the mix is being exposed to further mixing. However, as the data in this table illustrate, the standard deviation *increases* as we go from dry mix to dry granulation. This indicates that there are certain factors acting which interfere with the improvement of the drug uniformity. On the other hand, as we proceed from dry granulation to lubricated granulation, the standard deviation *decreases* as is expected.

Tablets

Before performing analysis of variance on the data, studies were undertaken to investigate the homogeneity of variances and the types of distribution exhibited by the tablet weights and tablet drug content. This was done on the original data and on the logarithms transformation. It is necessary to determine the above two factors, since for a valid application of analysis of variance, homogeneity of variances and normality of distributions are the necessary prerequisites. The logarithms transformation of data was investigated since it was anticipated that it would facilitate bivariate analysis of variance (simultaneous analysis of tablet weight, tablet drug content, and per cent drug concentration in the tablets).

The weight variances of the tablet samples from the random and systematic sampling plans were investigated, and no departures from homogeneity were observed. Tests of skewness (assymetry) and kurtosis (steepness of peak) were performed (7) on the data from each batch and on a composite of all batches for each sampling plan; no gross departures from normality were observed. There was, however, a tendency toward leptokurtosis for the pooled data. This is not a surprising result when a mixture of distributions is analyzed (8). The same tests performed on the data transformed to logarithms showed no significant departure from normality, but a tendency toward negative skewness.

Tests for normality performed on the distributions of tablet drug content and on their logarithms for each batch and for a pool of the three batches showed that both distributions exhibited no departures from normality.

Random Time Samples.—*Weights.*—In this analysis the three sets of 50 tablets taken by *Method A* and the three sets of 50 tablets taken by *Method B* were compared to determine whether the sampling

TABLE VI.—ANALYSIS OF VARIANCE OF TABLET WEIGHTS OF RANDOM TIME SAMPLES TAKEN BY METHODS *A* AND *B*

Source of Variation	*Method A and Method B*			*Method A*			*Method B*		
	D.F.	Sum of Squares	Mean Square	*F* Test	D.F.	Sum of Squares	Mean Square	*F* Test	Sum of Squares	Mean Square	*F* Test
Total	899	0.12155779	...	...	449	0.01893859	...	...	0.10190115	...	...
Batches (B)	2	0.00440295	0.00220147	41.0416[c]	2	0.00247227	0.00123648	15.0186[b]	0.00194845	0.00097422	24.6139[c]
Methods (M)	1	0.00071705	0.00071705	13.3678[c]	...	...	...	...	...	...	...
B × M	2	0.00001948	0.00000974	...	...	...	...	...	...	...	...
Samples within batches (pool)	12	0.00073149	0.00006095	...	6	0.00049400	0.00008233 = S_2^2[a]	2.2731[a]	0.00023749	0.00003958 = S_2^2	0.1750
Within samples of 50	882	0.11568682	0.00013116	...	441	0.01597162	0.00003622 = S_1^2[d]	...	0.09971521	0.00022611 = S_1^2	...
Pool (B × M samples within batch)	14	0.00075097	0.00005364 = S^2	...	...	...	...	...	...	...	...

[a] Significant at 5% level. [b] Significant at 1% level. [c] Significant at <0.1% level. [d] S_1^2 was used to test samples within batches. [e] S_2^2 was used to test batches.

method has a bearing on the randomness of the sample.

By analysis of variance, it was shown that there was a significant difference between sampling *Methods A* and *B*. These data are summarized in Table VI. The samples taken by *Method A* resulted in a significant difference between samples within batches, indicating that the samples were *not random*. This would suggest that the composite sample of 900 tablets was not adequately mixed to give random samples by *Method A*. However, the samples taken by *Method B* were *proved random*. Here again, as illustrated earlier for the sampling of the various stages in granulation manufacture, the sampling technique is extremely important in the collection of data for evaluation.

The analysis of variance has also shown highly significant differences between the tablet weight means of batches. There appear to be no valid reasons to believe that these differences really exist, but are probably due to time-to-time or adjustment-to-adjustment variability during the whole production of an order. It is believed that if a greater number of random tablet samples (which could be smaller in size than 50) would be taken per batch, these differences that were found, probably would not exist.

Random Time Samples.—*Weights, Assays, and Per Cent Drug Concentration.*—To determine whether a relationship existed between the weights and drug content of the individual tablets, analysis of variance was performed on the logarithms of the weight and drug content data, and analysis of variance was performed on the logarithm of the per cent drug concentration data.

The analysis was performed on 18 tablets, six from each of the three batches. There were no significant differences between batches for tablet weights, drug content, and per cent drug concentration. In addition, the samples within the batches were not significantly different from one another, indicating proper random sampling.

The correlation coefficient between tablet weight and drug content was not significant. This means that from the data available, it was not possible to trace dependence of drug content on tablet weight.

Systematic Samples.—*Weights, Assays, and Per Cent Drug Concentration.*—The analysis was performed on 240 tablets (three batches, two sides of the tablet press, four intervals, 10 tablets per sample) and analyzed by analysis of variance for the *Random Time Samples*.

When the data were analyzed as a nested classification, the differences between samples within batches were highly significant for tablet weight, assay, and per cent drug concentration.

Since the eight samples of 10 tablets each per batch were not selected at random but at four time intervals from each compression side of the tablet press, analysis of variance was performed on the basis of a factorial experiment to determine the sources of between-samples–within-batches variability.

The results summarized in Table VII indicate significant interaction between batches and hours for the tablet weights. When each batch was analyzed separately, no significant differences were found between hours.

The data in Table VIII summarize the mean

TABLE VII.—ANALYSIS OF VARIANCE OF SYSTEMATIC SAMPLING DATA[c]

Source of Variation	D.F.	y = Log Tablet Wt.		x = Log Drum Amt.		z = Log % concn.	
		SS_y	MS_y	F Test	SS_x	MS_x	F Test	SS_z	MS_z	F Test
Total	239	0.0011599	0.0000048	...	0.0393486	0.0001646	...	0.0436741	0.0001827	...
Batch (B)	2	0.0000429	0.0000214	1.6461	0.0005922	0.0002961	1.0020	0.0008667	0.0004333	0.8943
Hr (H)	3	0.0000245	0.0000122	0.9384	0.0028231	0.0009410	3.1855	0.0030132	0.0010044	2.0730
Side (S)	1	0.0000247	0.0000247	1.9000	0.0005682	0.0005682	1.9234	0.0003557	0.0003557	0.7341
B × H	6	0.0003359	0.0000559	4.3000[a]	0.0037760	0.0006293	2.1303	0.0060295	0.0010049	2.0740
B × S	2	0.0000203	0.0000102	0.7846	0.0119128	0.0059564	20.1638[b]	0.0125549	0.0062774	12.9564[b]
H × S	3	0.0000255	0.0000085	0.6538	0.0007453	0.0002484	0.8408	0.0011264	0.0003754	0.7748
B × H × S	6	0.0000783	0.0000130 = S^2	...	0.0017727	0.0002954 = S^2	...	0.0029072	0.0004845 = S^2	...
Within samples of 10	216	0.0006078	0.0000028	...	0.0171583	0.0000794	...	0.0168205	0.0000779	...

[a] Significant difference at 5% level. [b] Significant difference at 1% level. [c] Correlation coefficient between y and x = $r_{y,x}$ = 0.1464 D.F. 216.

tablet weights for the four time intervals for the three batches of tablets. It is evident from the data in this table that there exists no consistency in batches concerning the time of low or high weight tablets.

TABLE VIII.—INFLUENCE OF TIME ON MEAN TABLET WEIGHT

Batch	Time, Hr. 1	2	3	4
K_4	1.0698	1.0630	1.0461	1.0620
K_6	1.0678	1.0621	1.0700	1.0661
K_8	1.0653	1.0699	1.0590	1.0655

As indicated by the data in Table VII, analysis of the drug content results showed highly significant interaction between batches and compression sides of the tablet machine. This significant interaction indicates that from batch to batch the difference between sample means from each compression side of the tablet press is not consistent for the three batches. When each batch was analyzed separately, the results obtained showed that batch K_4 gave tablets on the left side of the machine which were significantly higher in concentration compared to those obtained from the right side of the machine. The data in Table IX summarize the mean drug content values for the tablets from the left and right sides of the double rotary press.

TABLE IX.—MEAN DRUG CONTENT RESULTS SHOWING BATCH-BY-SIDE INTERACTION OF TABLETS

Batches	Tablet Press, Left Side	Tablet Press, Right Side
K_4	49.5	47.38
K_6	48.13	49.08
K_8	48.70	49.29

The results of the analysis of variance on per cent drug concentration are shown in Table VII; they indicate that the conclusions here are the same as from the analysis of variance of drug content. The highly significant interaction between batches and compression sides indicates that factors taking place during the compression operation are influencing the per cent drug concentration differently on each side of the tablet press.

The correlation coefficients between tablet weights and drug content were computed from within sample variances and covariance and were not significant. The values are given in Table VII. This is not surprising when we compare the "within samples variances" for weights and drug content in Table VII. It is obvious that the variability of the drug content data is comparatively large compared to the weight data. This is clearly illustrated by the results in Table X, which show that the coefficient of variation for drug content is about five times that of the coefficient of variation for tablet weights.

From the bivariate analysis of variance data, we find that about 50% of the variability for weights, drug content, and per cent drug concentration can be attributed to such factors as compression sides, hours, batches, and their interactions. This tends to point to the fact that the tableting operation can be improved to some extent, but will not alter the fact that the major problem lies in the heterogeneity of drug distribution in tablets.

Precision of Assays

For this study the assay precision was estimated by analysis of variance on 80 assays performed in replicate and had a standard deviation of 0.061 mg., which is about 0.1%.

Tablet Quality

According to the intertablet potency test recommended to the U.S.P. and N.F. by the Quality Control Section of the Pharmaceutical Manufacturers Association (6), the assays of 10 individual tablets are to fall within 85–115% of the mean value of the official purity rubric to meet the standards for tablet potency variability. From Table X, it is seen that batch K_4 exhibits the greatest potency variability. By taking three standard deviations around the mean drug concentration, 99% of the population should be covered. Three standard deviations around the mean drug concentration of the tablets for batch K_4 give a range of 43.6–53.7 mg. The ±15% around the theoretical drug concentration of 50 mg. for these tablets gives a permissible range for intertablet dosage variation of 42.5–57.5 mg. Consequently, the results obtained for the three batches of tablets studied indicate that they meet the tolerances of intertablet dose variations as recommended by the Quality Control Section of the Pharmaceutical Manufacturers Association. However, the range obtained for batch K_4 is rather broad, indicating that it would be desirable to correct the sources of variability that exist in the stages of granulation manufacture to insure minimum intertablet dosage variations.

SUMMARY AND CONCLUSIONS

This report has been concerned mainly with a description of certain methodology and sampling plans appropriate for obtaining the components of variation in the manufacture of tablet dosage forms. To illustrate clearly the effectiveness or ineffectiveness of the methodology and sampling plans, the production manufacture of a tablet formulation was followed from the dry blending operation to the final compression of the granulation into tablets.

The precompression stages studied were the dry blending, granulating, and lubricating. Each stage

TABLE X.—COMPARISON OF MEANS, STANDARD DEVIATIONS, AND COEFFICIENT OF VARIATION OF THE WEIGHTS AND DRUG CONTENT FOR SYSTEMATIC SAMPLED TABLETS

Batches	Wt., Gm. Mean ± S.D.	Coeff. of Variation, %	Drug Content, mg. Mean ± S.D.	Coeff. of Variation, %
K_4	1.0647 ± 0.0066	0.62	48.67 ± 1.68	3.46
K_6	1.0664 ± 0.0063	0.59	48.60 ± 1.33	2.74
K_8	1.0649 ± 0.0068	0.64	49.00 ± 1.24	2.53

was analyzed to estimate its variability. The following findings are briefly summarized.

1. By performing a size analysis on the granulation and then determining the corresponding drug content of each sized fraction, it was possible to ascertain whether the drug is uniformly distributed throughout the different granules.
2. If the drug is not uniformly distributed, the technique of sampling is extremely important in obtaining a true representation of the drug distribution in the mix. Misleading estimates of variance were produced by the samples taken by *Method A*.
3. To determine the uniformity of drug content at different locations in the mixer, location samples were compared with random samples. Through the computation of standard deviations, it is possible to estimate the homogeneity of drug content in the mixer and to compare the same stage of manufacture for different batches.
4. In determining the standard deviation of the drug content at the different stages of granulation manufacture, it is possible to pinpoint the stage which is interfering with good mixing.

To evaluate weight and drug content variability of the tablets, random and systematic sampling plans were employed. The systematic sampling plan gave more information about the tablet compression operation than the random sampling plan. The following information was gained from this study.

1. The sampling technique is important for obtaining random samples.
2. The influence of compression time and compression sides of a double rotary press on tablet weight and drug content can be determined by analysis of variance.
3. The relationship between tablet weight and drug content was determined by computing the correlation coefficient and was not significant. This was because for each of the three batches of tablets studied, the coefficient of variation for drug content was about fivefold that of the tablet weight.

Although this report described certain methodology and sampling plans, it should be realized that similar and possibly more extensive information could be obtained through the use of other sampling plans. However, before any sampling plan is decided upon, careful consideration must be given to the factors that are to be evaluated. Only then is it possible to design the correct methodology and sampling plans which would result in the accumulation of sufficient random samples to evaluate adequately each factor and eliminate the possibility of taking samples from materials which may have undergone unmixing.

Additional studies presently are underway on different dosage forms using modified sampling plans to obtain maximum information regarding the relationship that may exist between drug heterogeneity in the several stages of granulation manufacture and the final tablets.

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Notes

Titrimetric Assay of Sulfonamides by Diazotization Using Ferrocyphen as Indicator

By W. M. BANICK, Jr., and J. R. VALENTINE

A study has been made concerning the use of ferrocyphen [dicyano-bis-(1,10-phenanthroline)-iron(II) complex] as an internal, reversible indicator for the diazotization titration of sulfonamides. Many of the sulfonamides of pharmaceutical interest can be determined using the ferrocyphen visual end point. The titration procedure is simple and rapid.

Most of the sulfonamides of pharmaceutical interest have the general formula $H_2NC_6H_4SO_2NHR$, in which the amino group and the sulfonamide group are in a *para* position to each other. Many different methods have been described (9) for the analysis of these sulfonamides. Diazotization of the primary amino group appears to be the preferred method, probably because it is applicable to nearly all sulfonamides and employs a titrant which is stable, readily available, and easily standardized.

The end point in the diazotization titration may

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Most of the sulfonamides of pharmaceutical interest have the general formula $H_2NC_6H_4SO_2NHR$, in which the amino group and the sulfonamide group are in a *para* position to each other. Many different methods have been described (9) for the analysis of these sulfonamides. Diazotization of the primary amino group appears to be the preferred method, probably because it is applicable to nearly all sulfonamides and employs a titrant which is stable, readily available, and easily standardized.

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TABLE I.—TITRIMETRIC ASSAY OF SULFONAMIDES USING FERROCYPHEN AS INDICATOR

Common Name	Structure H_2N—SO_2NHR, R=	% Purity[a,b]
Sulfanilamide	H	100.2
Sulfaguanidine	—C(=NH)NH_2	99.7
Sulfadiazine	2-Pyrimidinyl	99.9 (99.5)
Sulfamethazine	4,6-Dimethyl-2-pyrimidinyl	100.2 (100.4)
Sulfacetamide	Acetyl	100.0 (99.6)
Sulfachloropyridazine	6-Chloro-3-pyridazinyl	99.4 (99.9
Sulfachloropyrazine	6-Chloro-3-pyrazinyl	100.4 (100.2)
Sulfamethoxypyrimidine	6-Methoxy-4-pyrimidinyl	[c]
Sulfamethoxypyridazine	6-Methoxy-3-pyridazinyl	[d]
Sulfamerazine	4-Methyl-2-pyrimidinyl	[d]
Sulfapyridine	2-Pyridinyl	[d]
Sulfathiazole	2-Thiazolyl	[d]

[a] Average of at least four determinations. [b] Values in parentheses were obtained by a diazotization procedure employing a starch-iodide end point (2). [c] End point not stable. [d] Visual end point detection was impossible since titration gives colored products.

be determined potentiometrically (2, 7), amperometrically (6), with starch-iodide external indicator (9), with diphenylbenzidine disulfonic acid (1), or with orange IV (8).

Schilt (5) recently described the use of a metal complex as an internal, reversible indicator for the diazotization titrations of some primary aromatic amines (aniline, *p*-bromoaniline, *o*-chloroaniline, and 2,4-dichloroaniline). He proposed the trivial name "ferrocyphen" for the dicyano-bis-(1,10-phenanthroline)-iron (II) complex.

We have studied the use of this indicator in the diazotization titration of 12 sulfonamides of pharmaceutical interest. Seven of the sulfonamides can be titrated using ferrocyphen as indicator. The titration procedure using ferrocyphen as indicator is characterized by speed and simplicity.

EXPERIMENTAL

Reagents

Ferrocyphen [Dicyano-bis-(1,10-phenanthroline)-Iron (II)].—The reagent was prepared as the dihydrate using the procedure described by Schilt (4).

Ferrocyphen Solution, 1.0%.—Dissolve 0.50 Gm. of the reagent in 50 ml. of concentrated sulfuric acid.

Sodium Nitrite, 0.1 M.—Dissolve 14.2 Gm. of reagent grade sodium nitrite in distilled water to give 2000 ml. of solution. Standardize against purified sulfanilic acid as follows. Dissolve an accurately weighed sample of about 0.7 Gm. in 25 ml. of distilled water and 5 ml. of 1.0 *M* sodium hydroxide. Add 200 ml. of 6 *M* hydrochloric acid, 1.0 ml. of the ferrocyphen solution, and titrate with the 0.1 *M* sodium nitrite. At the end point, the color will change from yellow to violet. The end point is stable at least 3 minutes. Run a blank titration on the reagents.

Sulfonamides.—All the sulfonamides used in the investigation were equivalent to U.S.P. grade.

Procedure

Accurately weigh about 0.5 Gm. of the sulfonamide and dissolve in 100 ml. of 6 *M* hydrochloric acid. Add 1.0 ml. of the 1.0% ferrocyphen solution and titrate with 0.1 *M* sodium nitrite. Introduce the titrant under the surface of the solution with a buret having an elongated tip. In the vicinity of the end point, withdraw the tip and complete the titration dropwise to a pale violet or rose end point which is stable at least 3 minutes. Run a blank titration.

$$\% \text{ Purity} = \frac{A \times M \times F.W.}{G \times 10}$$

where A is the net ml. of M molar sodium nitrite, $F.W.$ is the formula weight of the sulfonamide, and G is the Gm. of sample.

RESULTS AND DISCUSSION

The results obtained for 12 sulfonamides analyzed by the diazotization procedure are summarized in Table I. Since previous investigators (2, 6) have shown that titration at 5–10° is not a necessary requirement for the diazotization titration, all the titrations using ferrocyphen as indicator were performed at room temperature (25°). The purity values obtained using ferrocyphen as indicator are in good agreement with the values obtained by a procedure employing a starch-iodide end point (3).

In all the titrations for which quantitative data were obtained, the ferrocyphen responded reversibly in the same way as described by Schilt (5). The diazotization of all the sulfonamides and the indicator response were extremely rapid in the 6 *M* hydrochloric acid.

Visual end point detection was impossible for the last four compounds in Table I because titration gives colored products (yellow or yellow-orange) which obscure the end point.

Precision.—The standard deviation calculated using 29 degrees of freedom is ±0.24%.

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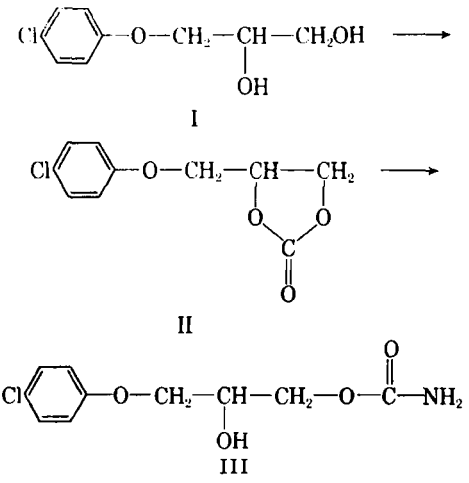
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Determination of Residual Chlorphenesin in Chlorphenesin-1-carbamate

By ARLINGTON A. FORIST and RAY W. JUDY

A rapid, sensitive, and specific method for the determination of residual chlorphenesin in chlorphenesin-1-carbamate has been developed. The procedure involves oxidation with periodic acid in 50 per cent ethanol followed by spectrophotometric determination of the formaldehyde produced. Neither chlorphenesin-1-carbamate nor the possible trace by-products interfere. Over the range 2 to 5 per cent chlorphenesin, a mean recovery ± standard deviation of 99.6 ± 2.1 per cent has been found.

CHLORPHENESIN-1-CARBAMATE[1] (III), a new muscle relaxant (1), is prepared from chlorphenesin (I) *via* the cyclic carbonate (II). The isomeric chlorphenesin-2-carbamate is also formed in the



I

II

III

reaction. Likely by-products in the bulk drug are therefore this isomer, the cyclic carbonate, and chlorphenesin. The isomer content may be determined by nuclear magnetic resonance spectroscopy (2) and residual carbonate is readily determined by a quantitative infrared assay based on the carbonyl absorption at 1800 cm^{-1}. The present report describes a simple spectrophotometric procedure for the determination of residual chlorphenesin.

EXPERIMENTAL

Reagents.—The following reagents were used: periodic acid, 0.1 *M;* sodium arsenite, 1 *M*— a mixture of 25 Gm. of arsenious oxide and 11.3 Gm. of sodium hydroxide dissolved in water and diluted to 250 ml.; sulfuric acid, 2 *N;* sulfuric acid, 2:1 (v/v), prepared by mixing 2 vol. of concentrated sulfuric acid, slowly with cooling, with 1 vol. of water; chromotropic acid reagent—a 0.5-Gm. sample of recrystallized chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) dissolved in 50 ml. of water and slowly diluted, with mixing and cooling, to 250 ml. with the 2:1 sulfuric acid.

Apparatus.—Glass-stoppered tubes prepared by sealing the ends of 24/40 outside ground joints, a boiling water bath, and a Beckman model B spectrophotometer equipped with 1-cm. cells were employed.

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[1] Trademarked as Maolate by the Upjohn Co.

Procedure.—Ten to twelve milligrams of sample, containing no more than 10% residual chlolphenesin, are accurately weighed into a 10-ml. volumetric flask and dissolved in 1 ml. of 95% ethanol. One milliliter of 0.1 *M* periodic acid is added, the solution is mixed, and oxidation is allowed to proceed for 15 minutes. One milliliter of 1 *M* sodium arsenite is then added, followed by 1 ml. of 2 *N* sulfuric acid. After at least 10 minutes, the resulting solution is diluted to 10 ml. with water and mixed thoroughly. One milliliter of this solution is then transferred to a glass-stoppered test tube, followed by 10 ml. of the chromotropic acid reagent. The solution is mixed thoroughly, and the stoppered tube is immersed in a boiling water bath for 30 minutes. The sample is then removed, cooled, and its absorbance determined at 570 mμ *versus* a reagent blank similarly treated. Chlorphenesin content is calculated from a linear standard curve.

RESULTS AND DISCUSSION

The present procedure is based on the production of formaldehyde from chlorphenesin by oxidation with periodic acid. The method is a modification of the procedure of Lambert and Neish (3) with the oxidation conducted in 50% ethanol to achieve complete solution of the samples involved. Results in Table I show that formaldehyde production from chlorphenesin is complete within 15 minutes at room temperature, and the resulting formaldehyde is stable for at least 2 hours in the reaction mixture.

Success of the present approach requires that chlorphenesin-1-carbamate, its isomer, and the cyclic carbonate be stable to hydrolysis and oxidation under the conditions employed. Results in Table I show that the two carbamates and the cyclic carbonate are completely stable for at least 45 minutes in the oxidation medium. Similar stability of carbamates in periodic acid was observed in earlier studies with the antibiotic novobiocin (4). Therefore, the present method is specific for chlorphenesin in the system under study.

TABLE I.—EFFECT OF OXIDATION TIME ON FORMALDEHYDE PRODUCTION

Oxidation Time, Min.	A_{570}			
	I[a]	II[b]	III[c]	IV[d]
15	0.341	0	0	0
30	0.340	0	0	0
45	0.341	0	0	0
60	0.347	...	...	...
90	0.346	...	...	...
120	0.346	...	...	...

[a] Chlorphenesin, 0.5-mg. sample. [b] Cyclic carbonate, 3.0-mg. sample. [c] Chlorphenesin-1-carbamate, 10-mg. sample. [d] Chlorphenesin-2-carbamate, 1.56-mg. sample.

TABLE II.—ABSORBANCE AS A FUNCTION OF CHLORPHENESIN SAMPLE SIZE

Sample, mg.	A_{570}	A_{570}/mg.[a]
0.10	0.062	0.620
0.20	0.139	0.695
0.30	0.194	0.647
0.40	0.260	0.650
0.50	0.323	0.646

[a] Mean ± standard deviation = 0.652 ± 0.027.

Absorbance at 570 mμ follows Beer's law over the range 0.10 to 0.50 mg. of chlorphenesin per sample (Table II). A mean absorbance per milligram ± standard deviation of 0.652 ± 0.027 has been observed, indicating adequate precision.

Application of the method to typical synthetic mixtures of chlorphenesin and chlorphenesin-1-carbamate is shown in Table III. Over the range 2 to 5% chlorphenesin, a mean recovery ± standard deviation of 99.6 ± 2.1% has been found.

The method described is rapid, sensitive, and specific for chlorphenesin in the system studied. The range 1 to 10% chlorphenesin is covered by the method as outlined; different ranges may be accommodated by suitable changes in sample size.

TABLE III.—DETERMINATION OF CHLORPHENESIN IN SYNTHETIC MIXTURES WITH CHLORPHENESIN-1-CARBAMATE

Chlorphenesin, %		
Taken	Found	Recovery, %[a]
1.96	1.95	99.5
2.91	2.98	102.4
3.85	3.81	99.0
4.76	4.63	97.3

[a] Mean ± standard deviation = 99.6 ± 2.1.

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Interaction of Low Molecular Weight Polyethylene Glycols with Sorbitol Solution

By JOSEPH ROBINSON, MILO GIBALDI, NORMAN D. WEINER, and JOSEPH L. KANIG

A single-phase solid material is produced when liquid polyethylene glycols and sorbitol solution U. S. P. are mixed. Various factors which influence this interaction have been explored. Temperature, components, component ratio, and soluble salts affect the rate of the interaction but do not affect the product itself. A nonspecific mechanism of dehydration of the sorbitol solution is apparently involved.

OBSERVATIONS MADE in our laboratories had shown that a waxlike solid material was produced when two widely used pharmaceutical liquids, polyethylene glycol 400 U.S.P. and sorbitol solution U.S.P., were intimately mixed. This solid substance was found to be soluble in water in all proportions and to melt in a range of 35 to 40°.

A survey of the literature indicated that this phenomenon had never been reported. This fact, in addition to the potential pharmaceutical applications of a material with such solubility and fusion characteristics, prompted further investigation.

Both sorbitol and polyethylene glycol (PEG) are ordinarily chemically inert. The oxyethylene moiety of PEG, however, is known to associate with compounds that possess an "active" hydrogen atom. This type of hydrogen bonding occurs between PEG and water, alcohol, and phenols (1). The strong tendency of PEG to form hydrogen bonds has been shown to be of pharmaceutical importance in such instances as the inactivation of salicylates (1) and a number of phenolic preservatives (2). An illustration of the extent of this association is the strong solvation of the polymer in water, which results in its complete solubilization.

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Accepted for publication March 19, 1964.

A study was initiated to investigate certain variables which would provide an insight into the possible mechanism of this liquid-liquid interaction.

EXPERIMENTAL RESULTS

Procedure.—Sorbitol solution U.S.P. [containing 70% (w/w) D-sorbitol in water] and the various low molecular weight polyethylene glycols used in this investigation were maintained at 25° for at least 1 hour prior to mixing. Mixing of the components was accomplished in a 600-ml. beaker immersed in a water bath at 25 ± 0.2°. The total weight of the mixture used in all experiments was 100 Gm.

The addition of the liquid PEG to sorbitol solution results in two distinct liquid phases. Gentle stirring immediately produces a hazy cloud within the mixture. Upon continued stirring, the viscosity of the mixture gradually increases until a solid material, with no apparent liquid phase, is produced. It was noted that an increase in the rate of stirring greatly decreased the time required to solidify the mixture. Intermittent manual stirring required about 2 hours to form the solid, whereas high-speed agitation with a model F Lightnin mixer produced equivalent results in considerably shorter periods of time. The transition from the fluid to the solid state was clearly defined and gave rise to an end

TABLE II.—ABSORBANCE AS A FUNCTION OF CHLORPHENESIN SAMPLE SIZE

Sample, mg.	A_{570}	A_{570}/mg.[a]
0.10	0.062	0.620
0.20	0.139	0.695
0.30	0.194	0.647
0.40	0.260	0.650
0.50	0.323	0.646

[a] Mean ± standard deviation = 0.652 ± 0.027.

Absorbance at 570 mμ follows Beer's law over the range 0.10 to 0.50 mg. of chlorphenesin per sample (Table II). A mean absorbance per milligram ± standard deviation of 0.652 ± 0.027 has been observed, indicating adequate precision.

Application of the method to typical synthetic mixtures of chlorphenesin and chlorphenesin-1-carbamate is shown in Table III. Over the range 2 to 5% chlorphenesin, a mean recovery ± standard deviation of 99.6 ± 2.1% has been found.

The method described is rapid, sensitive, and specific for chlorphenesin in the system studied. The range 1 to 10% chlorphenesin is covered by the method as outlined; different ranges may be accommodated by suitable changes in sample size.

TABLE III.—DETERMINATION OF CHLORPHENESIN IN SYNTHETIC MIXTURES WITH CHLORPHENESIN-1-CARBAMATE

Chlorphenesin, %		
Taken	Found	Recovery, %[a]
1.96	1.95	99.5
2.91	2.98	102.4
3.85	3.81	99.0
4.76	4.63	97.3

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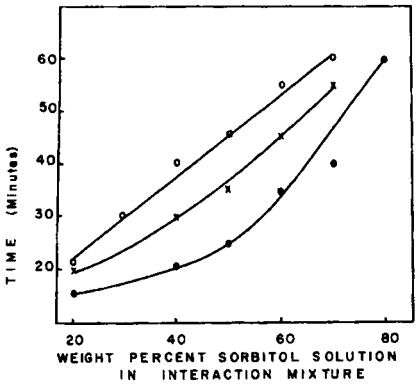


Fig. 1.—The effect of PEG molecular weight and sorbitol solution concentration on solidification time. *Key:* O = PEG 200; × = PEG 300; ● = PEG 400.

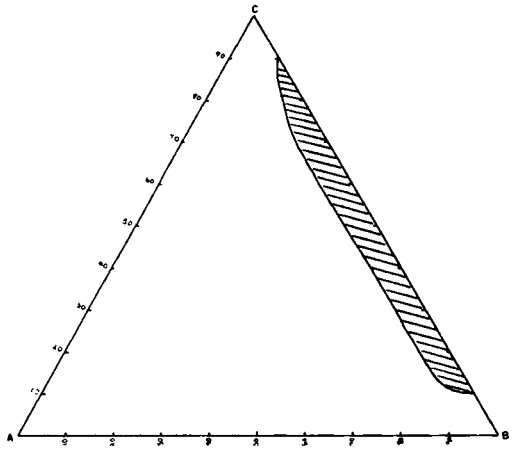


Fig. 2.—Ternary plot indicating critical component ratios of A, excess water; B, PEG 400; and C, sorbitol solution. (Shaded area = solid state; unshaded area = liquid state.)

point which was reproducible to within 1 minute at a given speed of mixing. Since the rate of mixing was critical in determining the end point, the Lightnin mixer was locked at a constant speed of about 1500 r.p.m. measured by a tachometer.

Effect of Composition.—Varying amounts of PEG 200, 300, or 400 were added to a sufficient quantity of sorbitol solution to prepare 100 Gm. The mixtures were treated as outlined above, and the time required to achieve a solid end point for each mixture was recorded. Figure 1 demonstrates that this time is increased with an increase in sorbitol solution concentration in the mixture. In addition, this plot indicates that the interaction rate is dependent on the ethylene oxide content of the PEG used. A decrease in the ethylene oxide content results in an increase in the time required for solidification. Mixtures containing more than 90% of either liquid component failed to solidify within 2 hours of mixing.

Solubility determinations revealed that crystalline D-sorbitol exhibited less than 1% solubility in PEG 400 with no apparent viscosity change. Excess amounts of D-sorbitol produced suspensions of this material in the PEG with no solidification occurring even when a 50% (w/w) suspension was prepared and stirred for several hours. No changes occurred when these suspensions were stored under ambient conditions for 3 months in sealed containers. It would thus appear that water is required for the interaction to occur.

To determine the extent of the role of water in the interaction, various mixtures of water, sorbitol solution, and PEG 400 were prepared. Figure 2 illustrates the phase analysis derived from these experiments. This ternary diagram indicates that excess water above 7% results in a clear, single-phase solution, regardless of the sorbitol solution–PEG ratio present. An excess water content of between 0 and 7% resulted in the formation of a solid, provided a proper ratio of sorbitol solution–PEG existed.

There were no intermediate stages of solidification. A given mixture either remained fluid or else became a solid mass. The solid never varied in its apparent consistency. The "melting point" of the solid was between 35 and 40° in all cases.

Effect of Temperature.—Raising the temperature of the interaction mixture resulted in a significant increase in the time required for solidification. The results of one series of such experiments are depicted in Fig. 3.

Effect of Addition of Soluble Salts.—Two grams of either powdered sodium chloride or powdered calcium chloride were added to an interaction mixture consisting of 50% (w/w) of sorbitol solution and PEG 400. The mixtures were stirred under controlled conditions and the solidification point determined. In each case, the effect of the salts was observed merely as a kinetic phenomenon. Both salts significantly increased the time for solidification to occur. This is indicated in Table I. Salts, however, did not affect the solidification products, which were indistinguishable in consistency from the control samples.

Effect of Dehydration of Sorbitol Solution.—Since it appeared that the water content of the interaction mixture was critical to the formation of the solid, a series of experiments was conducted to evaluate the effect of physical and chemical dehydration on sorbitol solution. The addition of excess amounts of soluble salts to sorbitol solution resulted in the formation of a clear, rigid gel after about 1 week. Salts such as sodium chloride and sodium citrate were equally efficient in their ability to produce the gels.

Anhydrous ethyl alcohol was carefully layered on top of sorbitol solution. This resulted in the gradual solidification of the lower sorbitol layer. A clear gel resulted upon standing for 2 weeks.

Continuous heating of sorbitol solution in an open vessel at 80° produced a very hard, clear gel after 3 days.

TABLE I.—EFFECT OF SALT ON INTERACTION RATE

Salt	Time Required to Reach End Point, Min.
Sodium chloride	32
Calcium chloride	35
Control (no salt)	15

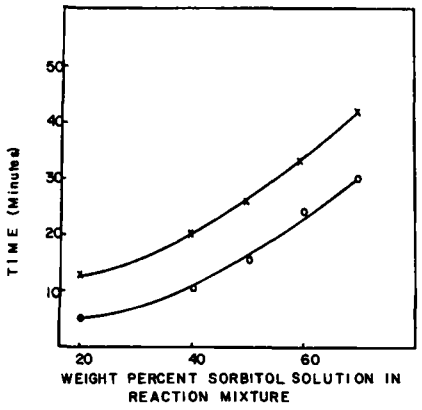


Fig. 3.—The effect of temperature on solidification time of PEG 400 and sorbitol solution mixtures. Key: O = 25°; × = 30°.

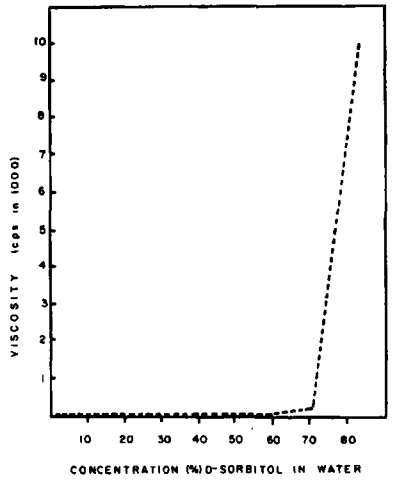


Fig. 4.—Viscosity of sorbitol solutions *versus* D-sorbitol concentration.

DISCUSSION

A study of the data indicated that a nonspecific mechanism seemed to be involved in the formation of the solidified mixture. This conclusion was based on the apparent similarity in properties of the solid obtained under the wide range of experimental conditions described. A possible explanation is that low molecular weight polyethylene glycols, mixed with sorbitol solution, produce a dehydration effect on the solution which results in a polymeric material consisting of associated molecules of sorbitol. The formation of this polymer and its dispersion throughout the system could conceivably result in an extremely rigid state possessing a high internal fluidity.

The transition from the liquid to the solid state occurred over a fairly narrow concentration range of water in the system. This is in consonance with the fact that the viscosity of sorbitol solution is nearly independent of sorbitol concentration up to 70% (w/w). Immediately beyond this concentration a fiftyfold increase in viscosity occurs over a 13% concentration range (from 70 to 83%) (3). This unusual rheological behavior is depicted in Fig. 4.

Density data available on solutions of different concentrations of D-sorbitol in water also indicate that beyond the 70% concentration marked deviation from linearity occurs. This is indicative of a reduced molar volume and association of molecules in the system. In view of these considerations, a slight dehydration of 70% sorbitol solution would result in a disproportionate increase in the viscosity. This contention is borne out by the results obtained with those experiments where dehydration of the sorbitol solution was accomplished by physical and chemical means, *viz.*, salts, ethyl alcohol, and evaporation at elevated temperatures.

The proposed mechanism is supported by the data obtained with the PEG–sorbitol solution experiments. As indicated in Fig. 1, the reduction of sorbitol solution content in a system resulted in a decrease in the time required for the solid to form. The decrease in time is directly related to the decrease in the water content of the mixtures with lower sorbitol solution content. A relatively smaller amount of water, therefore, needs to be removed by the correspondingly larger amounts of PEG to induce solidification.

Figure 1 also shows that the time required for solidification is dependent on the molecular weight of the PEG used. The affinity of polyethylene glycol for water is related to its ethylene oxide content. By the same token, the rate at which it is capable of dehydrating a material is equally dependent on the ethylene oxide content. This is in agreement with the order of interaction time evidenced in Fig. 1.

The effect of temperature (Fig. 3) is likewise related to the dehydration effect. The association of water with the oxyethylene moiety of PEG is critically temperature dependent (1). The bonding of water to this polymer will be considerably weakened with increasing temperature, and the affinity of this moiety for water will be correspondingly decreased. The expected decrease in interaction rate with increasing temperature is demonstrated in Fig. 3.

The prolonged reaction time observed with the addition of salts to the PEG–sorbitol solution mixture (Table I) may possibly be attributed to two factors acting in concert. (*a*) The addition of salts to sorbitol solution results in an increased viscosity. This increased viscosity may be responsible for a slower rate of mixing with PEG and therefore a slower rate of interaction. (*b*) The rate of dehydration of the sorbitol solution by the PEG will be dependent upon the thermodynamic activity of the water in the sorbitol solution. The addition of salts will decrease this activity and therefore prolong this interaction time.

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Elevated Temperature Studies on Stability of Ascorbic Acid in Certain Fruit Juice and Aqueous Vehicles

By M. C. UPRETY and B. REVIS

The stability of ascorbic acid was investigated in certain fruit juice and aqueous vehicles between pH 2.5–6.5 at 37 and 55° in the presence of 0.2 per cent citric acid, 0.01 per cent EDTA, 0.01 per cent propyl gallate, 0.2 per cent ferrous gluconate, 0.2 per cent cysteine hydrochloride, and 0.2 per cent potassium *m*-bisulfite. Lime juice–sorbitol–glycerol (2:2:1) retained 70 per cent ascorbic acid at pH 6.5 after 120 days. Citric acid also protected ascorbic acid. EDTA gave little protection to ascorbic acid. The loss of ascorbic acid in aqueous solution at some pH values was counteracted by 0.9 per cent sodium chloride. Dihydrosteptomycin sulfate in the concentration of 0.33 mg./ml. gave protection to ascorbic acid at all pH levels, except at pH 6.5 (37°) and at pH 5.5 (55°), where a destructive effect was noticed. Stability of ascorbic acid was a function of pH, temperature, vehicle, and head space in the container.

SINCE THE pharmaceutical industry is concerned more with the shelf life of a liquid vitamin mixture, the stability of ascorbic acid was studied at 37 and 55° employing certain fruit juice bases, some of which have been used earlier (1) together with some other formulations. The study was conducted at higher temperatures because any liquid vitamin mixture stable at a higher temperature keeps its quality for a longer time at a correspondingly lower temperature (2). This observation of Garrett has been confirmed by McLeod *et al.* (3), Uprety *et al.* (4), recently by Agrawal *et al.* (5). These higher-temperature tests would also be useful in predicting the stability at room temperature, thus shortening the time normally taken by such storage studies at room temperature.

A separate experiment was run with an aqueous solution of ascorbic acid at room temperature, 37°, and 55° to study the rate of destruction of ascorbic acid and to discover whether a formula could be evolved to correlate the stability of ascorbic acid with temperature and pH. Studies were continued for 3 months or until the retention of the vitamin dropped below 30%.

EXPERIMENTAL

Vitamin (Ascorbic Acid).—This was obtained from F. Hoffmann-La Roche and Company, Ltd., Basel, Switzerland.

Vehicle.—Fruit juices, sorbitol (E. Merck), and glycerol (BDH) were used. The formulations employed are given in Table I. A 5.0 mg./ml. quantity of ascorbic acid was added to the vehicle containing 0.2% (w/v) methyl parahydroxybenzoate (Rhodia, France). After adjusting to the required pH, the preparations were stored in 2-oz. amber-colored, glass-stoppered bottles in incubators at 37 and 55°.

Ascorbic acid was estimated by the 2,6-dichlorophenol-indophenol method.

Antioxidants.—The antioxidants examined for their effects on the stability of ascorbic acid in an aqueous solution were propyl gallate (Ward Blenkinsop, and Company, Ltd., London, England), ethylenediaminetetraacetate and potassium metabisulfite (E. Merck, here referred to as EDTA and KMS, respectively) cysteine hydrochloride (E. Merck), ferrous gluconate (Nila Products Ltd., Bombay, India), and citric acid (BDH). The concentrations of these antioxidants and other formulations are given in the Table I.

After adjusting the pH, a 0.5% solution of ascorbic acid was dispensed into Pyrex test tubes ($6 \times {}^{3}/_{4}$ in.) and kept at room temperature, 37° and 55°. The test tubes were closed with rubber stoppers. Samples were drawn periodically for recording the changes in the retention of ascorbic acid. A similar experiment was conducted using physiological saline. The effect of 0.33 mg./ml. of dihydrostreptomycin sulfate (Pfizer Dumex, Bombay, India) was also examined in an aqueous solution at 37 and 55° between the pH range 2.5 to 6.5.

RESULTS

Ascorbic acid in glass distilled water was lost completely at the end of 60 days both at 37 and 55° in the control and in the presence of 0.02% EDTA, 0.01% propyl gallate, 0.02% ferrous gluconate, 0.02% cysteine hydrochloride, and 0.2% citric acid at all pH values between 2.5 to 6.5, but 0.2% KMS protected ascorbic acid at pH 3.5 (37°) to the extent of 65%, followed by 0.02% EDTA which gave a slight protection up to 30% at pH 2.5. After a storage period of 120 days, KMS in the concentration used had no protective effect on ascorbic acid. (A tabulation of data is not given.)

Table I shows that at 55° and a storage period of 60 days all ascorbic acid added in the different vehicles was oxidized, except for lime juice – sorbitol – glycerol (2:2:1), in which there was a 30% retention at pH 2.5, 3.5, and 5.5 and 40% at pH 6.5. Samples kept at this temperature changed color turning toward deep brown. Inclusion of antioxidants (concentration shown in Table I) failed to improve the stability of ascorbic acid. In apple juice at the end of 60 days, practically all the ascorbic acid was degraded at 37° between the pH range 2.5 to 5.5, but a 35% retention was observed at pH 6.5. EDTA (0.01%) stabilized ascorbic acid at pH 3.5 and 4.5 to the extent of 66 and 48%, respectively. At other pH values, no such protection was noticed. Propyl gallate (0.1%) also protected ascorbic acid at pH 3.5, 4.5, and 6.5. Generally, citric acid protected vitamin C in apple juice at 37° in the concentration used. Ferrous gluconate did not seem to have an influence on the retention of ascorbic acid in apple juice at 37° within the experimental pH range.

Apple juice – glycerol (1:1) was a better vehicle than apple juice up to pH 5.5. The addition of

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TABLE I.—PER CENT RETENTION OF ASCORBIC ACID

No.	Vehicle	Antioxidants or Metal Binder	Days	pH Values 2.5	3.5	4.5	5.5	6.5
		Temperature, 37° C.						
1	Pineapple juice	Control	60	60.00	28.9	2.9	49.3	52.5
2	Apple juice	Control	60	1.43	1.40	1.43	1.45	35.0
3	Lime juice	Control	30	38.3	24.0	8.3	3.0	17.2
4	Lime juice–sorbitol–glycerol (2:2:1)	Control	72	29.4	61.4	75.4	75.4	89.6
			120	20.3	44.2	47.4	45.2	70.0
5	Apple juice 75% (w/v) glycerol (1:1)	Control	48	44.8	59.2	53.5	43.6	21.8
			79	37.0	32.0	20.0	15.4	2.6
6	Apple juice	0.01% EDTA	72	Nil	66.6	48.1	Nil	Nil
7	Apple juice	0.01% Propyl gallate	72	Nil	68.74	79.3	Nil	88.4
			120	Nil	Nil	Nil	Nil	53.5
8	Apple juice	0.2% Citric acid	64	34.2	41.04	34.2	26.2	95.2
			105	Nil	20.6	17.5	17.5	36.1
9	Apple juice	0.2% Ferrous gluconate	60	2.3	2.3	2.3	7.0	22.4
10	Apple juice 75% (w/v) glycerol (1:1)	0.02% EDTA	49	67.7	58.2	42.0	42.8	61.1
			79	53.7	33.3	17.0	29.4	44.9
11	Apple juice 75% (w/v) glycerol (1:1)	0.02% Propyl gallate	46	58.3	56.8	33.5	36.1	28.1
			63	43.2	43.3	20.4	25.4	10.2
		Temperature, 55° C.						
1	Pineapple juice	Control	60	2.5	4.8	3.2	3.8	5.0
2	Apple juice	Control	60	3.0	3.0	3.0	3.0	1.5
3	Lime juice	Control	30	10.6	6.7	2.7	3.0	2.8
4	Lime juice–sorbitol–glycerol (2:2:1)	Control	72	27.3	36.8	Nil	30.1	40.2
			120	6.4	5.7	Nil	5.1	Nil
5	Apple juice 75% (w/v) glycerol	Control	48	9.4	16.4	2.3	2.3	12.2
6	Apple juice	0.01% EDTA	72	Nil	10.6	Nil	Nil	34.2
7	Apple juice	0.01% Propyl gallate	72	Nil	Nil	Nil	41.6	Nil
8	Apple juice	0.2% Citric acid	64	2.7	2.7	2.7	2.7	5.5
9	Apple juice	0.2% Ferrous gluconate	60	2.3	2.3	4.7	4.7	4.8
10	Apple juice 75% (w/v) glycerol	0.02% EDTA	49	36.0	13.6	22.6	2.3	2.3
11	Apple juice 75% (w/v) glycerol	0.2% Propyl gallate	46	5.16	5.16	2.58	2.58	2.58

EDTA to the above vehicle showed better stability at pH 2.5, 5.5, and 6.5. Propyl gallate (0.02%) had no effect on the stability of ascorbic acid in vehicle *5*.

Pineapple juice was better than apple or lime juice as a carrier of ascorbic acid. At the end of 120 days at pH 6.5, lime juice – sorbitol – glycerol (2:2:1) showed 70% retention of ascorbic acid at 37°, whereas at pH 3.5, 4.5, and 5.5 the retention was about 45%. A pH value of 2.5 showed a poor retention in this vehicle.

Room Temperature.—Examination of the data in Table II shows that the best retention of ascorbic acid was at pH 3.5, and the maximum degradation was at pH 5.5 at the end of 50 days. At other pH levels, about 60% destruction occurred. The decomposition of ascorbic acid in physiological saline followed the same pattern as that in distilled water at all pH levels except 5.5, where a protective action was noticed.

At 37°.—About 75% of ascorbic acid was lost after 40 days in distilled water at all pH levels except 3.5. The maximum destruction was at pH 2.5 and the minimum at 3.5. The maximum destruction was at pH 5.5 and the minimum at pH 3.5 in physiological saline. At pH 2.5, NaCl (0.9%) promoted the stability. At other pH values, physiological saline had no influence on the stability within the range of experimental error. The addition of 0.33 mg./ml. of dihydrostreptomycin sulfate to the aqueous solution of ascorbic acid had a stabilizing tendency up to pH 5.5; but at pH 6.5 this concentration of dihydrostreptomycin sulfate exhibited a deleterious effect.

At 55°.—About 70% ascorbic acid was lost at the end of 30 days in distilled water. The destruction was more with the rise in pH up to 4.5; however, at pH 5.5, which appears to be a critical value, the retention was fairly good to the extent of about 52%. At pH 6.5, a complete loss of ascorbic acid occurred at the end of 20 days. In saline, under similar conditions, a greater stability of ascorbic acid was observed at pH 6.5. There seemed to be no influence at other pH values. Dihydrostreptomycin sulfate, in the concentration used, had a stabilizing effect on ascorbic acid at all pH values except 5.5, where a destructive effect was noticed after a period of 40 days in distilled water.

At the end of the experimental period no change in pH was noticed, although a buffer was not used. All the samples containing ascorbic acid developed color. A greater degree of coloration was shown by the samples containing citric acid, the intensity of color was proportional to the degradation of ascorbic acid.

TABLE II.—PER CENT RETENTION OF ASCORBIC ACID IN AQUEOUS SOLUTION

Vehicle	pH	Days 4	10	20	30	40	50
		Room Temperature					
Distilled water	2.5	97.0	93.4	80.0	67.4	57.8	48.5
	3.5	99.79	96.5	87.9	75.1	65.9	54.8
	4.5	94.93	91.2	71.2	64.1	50.0	44.5
	5.5	94.9	86.4	71.2	61.7	41.2	23.6
	6.5	92.11	88.5	75.5	65.7	51.9	36.3
Physiological saline	2.5	49.79	94.6	78.0	73.0	58.6	51.2
	3.5	94.93	91.2	83.2	73.2	65.2	54.8
	4.5	92.30	96.3	78.5	69.0	45.9	44.5
	5.5	100.0	100.0	85.2	75.1	58.9	51.2
	6.5	97.51	96.05	73.0	68.2	47.0	40.8
		Temperature, 37° C.					
Distilled water	2.5	97.6	80.4	57.4	37.4	14.4	...
	3.5	99.8	88.5	65.7	50.2	35.2	...
	4.5	92.3	78.9	61.7	42.7	24.2	...
	5.5	89.87	81.3	61.7	45.1	25.3	...
	6.5	94.79	80.9	68.2	53.9	25.9	...
Physiological saline	2.5	97.51	91.07	73.0	53.5	37.5	...
	3.5	89.87	88.8	76.1	61.7	43.3	...
	4.5	87.45	81.3	71.7	69.0	25.0	...
	5.5	92.1	85.8	65.7	48.7	19.9	...
	6.5	94.6	88.5	68.2	53.5	33.3	...
Distilled water dihydrostreptomycin (0.33 mg./ml.)	2.5	...	82.2	62.4	45.2	22.8	...
	3.5	...	91.2	73.2	55.8	39.3	...
	4.5	...	79.8	63.7	48.2	31.7	...
	5.5	...	99.7	77.0	62.5	37.0	...
	6.5	...	80.4	51.6	36.6	12.4	...
		Temperature, 55° C.					
Distilled water	2.5	85.1	54.2	30.0	4.9	Nil	...
	3.5	86.0	58.1	17.0	2.3	Nil	...
	4.5	80.9	44.3	9.5	1.1	Nil	...
	5.5	93.6	78.9	52.2	30.7	Nil	...
	6.5	77.8	32.7	Nil	Nil	Nil	...
Physiological saline	2.5	85.5	65.7	36.5	17.0	...	...
	3.5	86.0	62.1	23.6	4.6	...	...
	4.5	83.9	62.1	23.6	2.3	...	..
	5.5	83.0	68.2	39.0	24.2	...	
	6.5	90.7	78.4	51.2	29.2	...	...
Distilled water dihydrostreptomycin (0.33 mg./ml.)	2.5	...	71.4	30.7	17.2	...	...
	3.5	...	73.6	47.2	25.8	...	...
	4.5	...	61.5	7.8	2.1	...	...
	5.5	...	79.9	36.0	11.1	...	...
	6.5	...	87.1	68.9	38.6	...	...

DISCUSSION

The stability of ascorbic acid, evidenced from this study, is a function of pH and temperature. At pH 2.5, the stability is markedly decreased. This finding is in support of Ganguly (6), who showed that at low pH air and copper ions decreased stability of an aqueous solution of ascorbic acid.

It is interesting that KMS stabilizes ascorbic acid to a fair extent. This finding confirms our earlier finding (1) and also lends support to the view expressed by Schroeter (7). It is essential that a proper concentration of this antioxidant be added because the mechanism of the protection of ascorbic acid depends upon the availability of the S ions.

EDTA in a concentration of 0.2% also gave some protection to ascorbic acid in an aqueous solution. This finding is in agreement with that of Bartilucci and Foss (8) and also of Ch'en (9). Probably the concentration used in the present investigation was rather low, a factor which accounts for the reduced stability.

Other antioxidants used did not offer protection to ascorbic acid in distilled water, either because the concentrations were insufficient or because the samples were analyzed after storing them for a longer period.

At 55°, most of the ascorbic acid was easily destroyed. This discovery clearly shows that at such a high temperature the oxidation is rapid. A preparation which can be made stable at 55° for 1 month would theoretically be stable for about 5–6 months at 25°. This may shorten the time usually taken in the analytical study at room temperature by one-fifth if attempts are made to stabilize liquid vitamin mixtures at 55°.

Lime juice – sorbitol – glycerol (2:2:1) at pH 6.5 showed a retention of about 40% at 55°. Therefore, this vehicle affords maximum protection among all the vehicles tried, even without the aid of an antioxidant. Our earlier finding (1) that lime juice – sorbitol (1:1) and lime juice – glycerol (1:1) are good vehicles for externally added ascorbic acid has been confirmed and modified with the inclusion of glycerol in this study. The retention of

TABLE III.—PER CENT RATE OF DESTRUCTION OF ASCORBIC ACID IN AN AQUEOUS SOLUTION FOR 24 HOURS

pH	Room Temp.	37° C.	55° C.
2.5	1.05	2.14	3.5
3.5	1.096	1.62	4.15
4.5	1.11	1.894	4.525
5.5	1.53	1.87	2.39
6.5	1.274	1.85	6.73

ascorbic acid in this vehicle at 55° would roughly be equivalent to about 1 year at room temperature.

In apple juice the ascorbic acid degraded completely up to pH 5.5 at 37°. This may be explained with the presumption that apple juice may contain a high concentration of polyphenol-oxidase to catalyze the oxidation of ascorbic acid. The addition of 0.01% EDTA protected ascorbic acid at pH 3.5 and 4.5. This protection can be explained on the basis of the Cu ions being arrested by this metal binder. Propyl gallate also exhibited a protective action at pH 3.5, 4.5, and 6.5, in conformity with the finding of Ch'en (9).

Citric acid in the concentration used protected ascorbic acid in apple juice at all the pH values. Presumably there may exist a sort of competitive inhibition, which may explain the mechanism by which citric acid could stabilize ascorbic acid.

The substitute of 50% of the apple juice by glycerol improved the stability of ascorbic acid at 37° up to pH 5.5. This finding is in agreement with that of Bandelin and Tuschhoff (10), who showed that the addition of polyhydric alcohols tends to produce a stabilizing effect on ascorbic acid.

Pineapple juice showed a good stability at 37° compared to lime juice and apple juice, probably because pineapple juice may contain less ascorbic acid oxidase or other metallic ions injurious to ascorbic acid.

Ascorbic acid showed a 70% retention in vehicle 4 after a period of 4 months at pH 6.5. This is in conformity with the finding of Bartilucci and Foss (8), who showed that optimum pH for the stability of ascorbic acid in solution lies between 6.0 and 7.0.

An aqueous solution of ascorbic acid at room temperature showed the best retention at pH 3.5 and maximum degradation at pH 5.5. This deteriorating action of pH was counteracted by the addition of 0.9% NaCl to the medium. A similar pattern was followed at 37°. The destructive action at pH 3.5 and 37° was further counteracted by physiological saline. This shows that 0.9% NaCl is effective in counteracting the deleterious effect of pH and temperature in an aqueous solution —which may have something to do with the ionic equilibrium in an aqueous solution.

At 55°, the destruction in an aqueous solution was more with the rise in pH. This is in agreement with Bendelin and Tuschhoff (10), who showed that the rate of oxidative decomposition of an ascorbic acid solution increases with the rise in pH values. But a 30.7% retention of ascorbic acid at 55° after a period of 30 days could not offer an explanation. Again, at pH 6.5 NaCl in a concentration of 0.9% was effective in counteracting the oxidation of ascorbic acid.

Dihydrostreptomycin sulfate, in the concentration of 0.33 mg./ml. had a tendency to stabilize ascorbic acid at 37 and 55° at all pH values, except at pH 5.5 and 55°.

Citric acid, in the concentration used, turned the vehicle containing ascorbic acid brown. This has also been shown by Lamden and Harris (11), who stated that the water of crystallization of citric acid promoted browning at 49°. In fact, all the samples turned brown, but the intensity differed and the maximum was observed in those containing citric acid.

The oxidation of ascorbic acid is reported to follow two paths: (*a*) polymerization to furfural and (*b*) oxidation to diketogulonic acid and finally to oxalic acid. The color development is probably lacking in (*b*). Various workers (1, 4) have shown that sulfur, in the form of cysteine, methionine, and KMS, exercised a stabilizing action on ascorbic acid. The stability is likely to be due to sulfur. The mechanism involved is probably formed through a loose bond between S and the hydrogen ions of ascorbic acid. However, this loose bond appears susceptible to changes in temperature and pH due to molecular agitation.

Table III shows the rate of destruction of ascorbic acid in an aqueous solution at three temperatures. The rate of destruction at pH 6.5 is six times more at 55° than at room temperature; at other pH values the rate of destruction varies from two to four times, depending upon the pH. At 37°, the rate of destruction is roughly one and one-half times more than at room temperature. Thus, an idea about the prediction of stability at lower temperature by conducting studies at higher temperatures is given.

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Solubility of Several Solutes as a Function of the Dielectric Constant of Sugar Solutions

By ANTHONY N. PARUTA*

The solubility of several pharmaceutical solutes has been determined in sucrose solutions of varying concentration. These syrup vehicles possess dielectric constant values lower than pure water, their respective dielectric constants decreasing with increasing concentration. The effect of both the concentration and the dielectric constant of sucrose in syrup vehicles upon the solubility of several solutes has been determined. The solubility of the solutes studied are seen to increase directly with increasing sucrose concentration and inversely with dielectric constant for the range and systems studied.

THE COMMON USE of syrup vehicles in liquid pharmaceutical preparations indicates a need for determining certain properties of these dissolution media. It has often been assumed that a given solute will be about equally soluble in water and in syrup. To test this assumption, the solubility of various solutes was determined in sucrose solutions of varying concentration.

The useful concept of the "polarity" of dissolution media may be advantageously applicable to sugar solutions *via* a parameter which in some fashion quantitates "polarity." The parameter chosen was the dielectric constant as a function of sucrose concentration.

The dielectric constant of sucrose solutions has been determined by Maryott and Malmberg (1) and Akerlof (2). These workers found that the dielectric constant of these solutions decreased with increasing concentrations of sugar. Simple syrup has a dielectric constant of about 60, placing it about halfway between water, $\epsilon = 78$, and glycerin, $\epsilon = 42$, on the dielectric constant scale.

As the polarity of the dissolution media decreases, *i.e.*, hydroalcoholic mixtures, the solubility of semipolar solutes increases, due to a medium of more favorable polarity. Increased solubility of semipolar solutes in mixtures of pharmaceutical solvents of decreasing polarity has been reported previously (3-5).

This study was undertaken to determine the solvency characteristics of syrup vehicles and to illustrate the polarity-dielectric constant relationship of dissolution media (6, 7).

EXPERIMENTAL

Materials.—The solutes used in this study were recrystallized from water-ethanol mixtures and dried to constant weight at 40° in a convection oven. The solutes studied were quinine alkaloid, phenobarbital, PABA, and sulfanilamide. Sugar solutions were prepared from commercial sucrose in distilled water. Solvents used to establish a calibration dielectric constant curve were from fresh unopened bottles assuming labeled water contents were true values. Calibration values were subsequently corrected for water content.

Equipment.—Sargent chemical oscillometer model V, Sargent thermomonitor water bath equipment, and Beckman DK-2 ratio recording spectrophotometer were used.

Dielectric Constant Determinations.—The dielectric constant of sucrose solutions, from 10 to 80% w/v in 10% steps was determined by the use of a Sargent chemical oscillometer. A calibration curve was prepared by measuring the apparent capacitances of anhydrous liquids of known dielectric constants (8). The prepared sucrose solutions were measured in a 10-ml. cell in the order of increasing viscosity. Each solution was determined at least three times with about 1 minute between each reading. Each successive solution was measured in the above fashion after rinsing the cell twice with the solution to be measured. Since day-to-day variations were found in the calibration curve, water and other solvents were also tested on a given day for adjustment of the calibration curve. It is interesting to note that these variations led to a family of calibration curves which were continuously parallel over the dielectric constant range studied. The dielectric constant of the sugar solutions was determined from the prepared calibration curve. The relative error associated with these determinations and graphical interpretation is felt to be about ±0.5% or about ±0.4 dielectric constant units.

Solubility Determination.—The solubility of the solutes studied was determined in 15-ml. vials attached to a submerged rotating plastic disk. The plastic disk unit was attached to a dispersator motor fitted with an aluminum shaft and submerged with attached samples in an 8-gal. water bath. The temperature of the bath was maintained at 24.6 ± 0.2° by a Sargent thermomonitor unit. Solubility equilibration was about 72 hours; this rather long period was needed because of the relatively high viscosity of the syrup vehicles.

Solubility Analysis.—After equilibration, samples of appropriate volume were withdrawn through a fine glass-wool plug fitted to a pipet with a short rubber tube. The pipet was overdrawn, the plug removed, and the pipet wiped clean with a towel. The sample was placed in a volumetric flask and diluted to the appropriate volume for subsequent analysis. The concentration of solute in solution was determined by a Beckman DK-2 spectrophotometer from sample absorbance and previously established Beer-Lambert law plots for each solute. The absorption maxima for the solutes were as follows: PABA, 270 mμ; sulfanilamide, 259 mμ; quinine alkaloid, 327 mμ; and phenobarbital, 269 mμ (sh). These absorption maxima were from 2 to 4 mμ greater than the values reported in the literature.

RESULTS AND DISCUSSION

The experimentally determined dielectric constants of sucrose solutions of varying concentration

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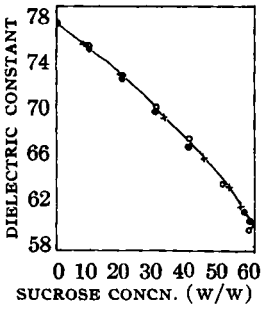


Fig. 1.—A plot of the dielectric constant variation of sucrose solutions reported by Maryott (●), Akerlof (○), and the author (×).

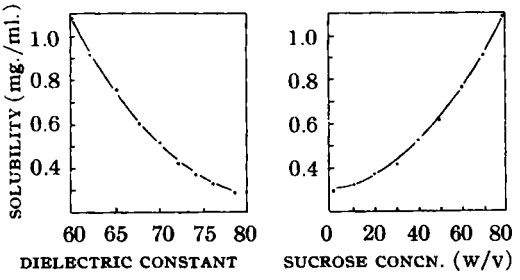


Fig. 2.—A plot of the solubility of quinine alkaloid in mg./ml. at 25°C. as a function of dielectric constant and sucrose concentration.

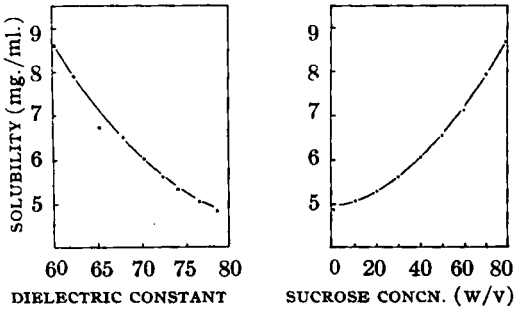


Fig. 3.—A plot of the solubility of *p*-aminobenzoic acid in mg./ml. at 25°C. as a function of dielectric constant and sucrose concentration.

are shown in Fig. 1. The values obtained have been converted to the w/w notation from density measurements. This was done to show the comparison to data previously reported (1, 2). The values compare favorably with other workers, although the data reported by Akerlof are slightly lower at higher concentrations.

The solubility of the solutes used in this study are shown in Figs. 2–5. In each case, the solubility has been plotted as a function of the dielectric constant and sucrose concentration. Each point is the average solubility of three runs, the sucrose concentration varying from 10 to 80% in 10% increments. The solubility of each of these solutes increases with increasing sucrose concentration or decreasing dielectric constant. The use of hydroalcoholic mixtures in increasing or maintaining solubility of semipolar solutes in solution is well known. In this study, the solubility is increased by a series of successive dissolution media of more favorable polarity. Thus, the observed increase may be explained on the basis of decreasing polarity of syrup vehicles as reflected by their respective dielectric constants.

Quinine alkaloid and *p*-aminobenzoic acid gave smooth nonlinear curves when solubility was plotted *versus* either the dielectric constant or concentration of these syrup vehicles. These curves did not give a first-order or linear relationship when the log solubility was plotted *versus* the dielectric constant. The solubility of the solutes in distilled water compared favorably with the values reported in the official compendia.

The solubility of phenobarbital and sulfanilamide showed an approximately linear relationship with dielectric constants and sucrose concentration. The results are shown in Figs. 4 and 5. The rates of change of solubility per dielectric constant unit for phenobarbital and sulfanilamide were 0.06 and 0.2 mg., respectively. The solubility of the above solutes also compared favorably with values reported in the literature.

The ratio of the solubility of these solutes in 80% w/v syrup vehicle relative to distilled water is presented in Table I. The ratios vary from a low value 1.5 for sulfanilamide to a striking 3.7 for quinine alkaloid.

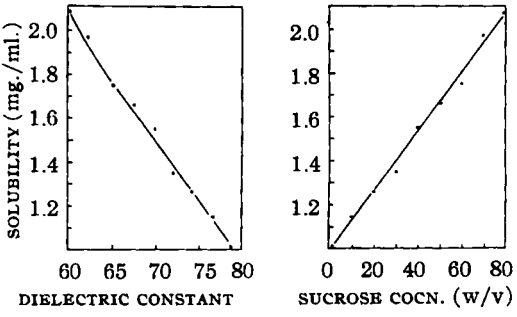


Fig. 4.—A plot of the solubility of phenobarbital in mg./ml. at 25°C. as a function of dielectric constant and sucrose concentration.

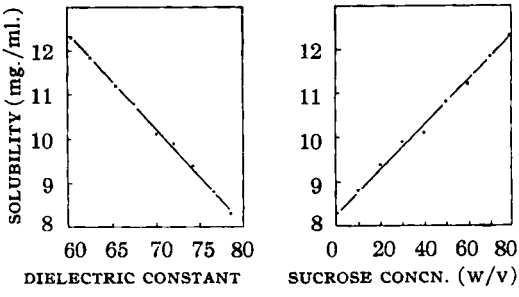


Fig. 5.—A plot of the solubility of sulfanilamide in mg./ml. at 25°C. as a function of dielectric constant and sucrose concentration.

TABLE I.—SUMMARY OF THE SOLUBILITY RATIO FOR THE SOLUTES STUDIED

Solute	Solubility, mg./ml. A, Water	Solubility, mg./ml. B, 80% w/v Sucrose	Ratio B/A
Quinine alkaloid	0.3	1.1	3.7
Phenobarbital	1.0	2.1	2.1
PABA	4.8	8.6	1.8
Sulfanilamide	8.3	12.3	1.5

This simple study leads to manifold consequences which may be advantageously utilized by the formulator and researcher. Control of solubility as a function of dielectric constant variation might have many applications such as increasing the solubility of drugs by using additives such as sucrose or other sugars. On the other hand, decreased solubility may also be useful in a kinetic sense. Since a drug, such as aspirin suspension, will only degrade depending upon the amount of drug in solution, decreased solubility could lead to increased stability to some degree. This approach could be quite useful for aspirin, sulfonamides, antibiotics, and other common pharmaceutical suspensions.

Preliminary work indicates that methocel and other viscosity-inducing agents in solution have dielectric constants higher than that of pure water. These vehicles also possess the proper consistency for physical suspension, stability, and palatability. Increased chemical stability *via* decreased solubility or increased polarity (dielectric constant) is a definite possibility. Studies along these lines are being conducted in these laboratories and results will be reported in future communications.

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Effects of Various Growth Regulators on *Datura meteloides*

By JAMES H. BENNETT and LEO A. SCIUCHETTI

Datura meteloides **D.C. was treated during a 4-week period with Amo-1618, CCC, AMAB, Phosphon, B995, and CO-11. Growth was not significantly affected by the treatments. The concentration of alkaloids was increased 26 per cent in the roots of the Amo-1618 group and 17 per cent in the roots of the Phosphon group. The total alkaloid content was increased 25 per cent in the leaf-tops of the CO-11 group, 32 per cent in the roots of the Amo-1618 group, and 19 per cent in the roots of the B995 group. Increases of about 18 per cent in chlorophyll concentration were found in the Phosphon, B995, and CO-11 groups. The results of a selective solvent extraction of the leaf-tops are reported.**

NUMEROUS CHEMICAL COMPOUNDS have been recently reported to cause a marked decrease in stem and petiole elongation (1–9). These compounds can be conveniently classified into three chemical groups, *viz.*, the quaternary ammonium compounds, the quaternary phosphonium compounds, and a few selected organic acids. Members of the first group include 4-hydroxy-5-isopropyl-2-methylphenyl trimethylammonium chloride, 1-piperidine carboxylate (Amo-1618), (2-chloroethyl) trimethylammonium chloride (CCC), and allyltrimethylammonium bromide (AMAB). Reduction of internode length resulting in shorter plants has been reported in various plants by treatment with Amo-1618 (1–6), CCC (4, 6, 7), and AMAB (6, 7). CCC and AMAB have been shown to increase the alkaloid content of *Nicotiana tabacum* L. and decrease that of *N. rustica* L. (6).

A phosphonium compound which has been reported to inhibit plant growth is 2,4-dichlorobenzyltributylphosphonium chloride (Phosphon) (2, 3, 6, 8, 9). This compound and the three aforementioned ones are considered to be antigibberellins (1, 3, 6, 9–11). Lockhart (12) concludes that Phosphon and CCC retard stem elongation by partially blocking the system which provides gibberellin to the growth mechanism. Among the organic acids reported to retard plant growth are *N*-dimethylaminomaleamic acid (CO-11) and *N*-dimethylaminosuccinamic acid (B995) (13). These two compounds are closely related to maleic hydrazide (MH), which is considered by some to be an inhibitor of auxin (14, 15).

Since these compounds with reported growth-retarding activity had not been tested on *Datura* spp., it was decided to determine their effects on the growth and synthesis of certain chemical constituents of *Datura meteloides*. In addition, since some of these compounds appear to be antigibberellins and since gibberellin treatment generally reduces the concentration of alkaloids in many medicinal plants (16, 17), it was of interest to note whether a "growth retardant" would cause an increase in the concentration of alkaloids of the plants.

EXPERIMENTAL

Procedure.—Seventy uniform plants were grown under greenhouse conditions in a soil mixture composed of 1 part sand, 2 parts sandy loam, and 10 Gm. of organic fertilizer[1] per gallon can. On July 12, 1962, *Daturas* which were about 34 days old were labeled according to the following plan for treatment: Amo-1618-treated plants, CCC-treated plants, AMAB-treated plants, CO-11-treated plants, B995-treated plants, Phosphon-treated plants, and control (untreated) plants. Each of the above groups consisted of 10 plants. On that date the plants were randomly arranged on a greenhouse bench, and treatment was instituted. Treatments

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Research paper No. 464, School of Pharmacy, Department of Pharmacognosy, Oregon State University.

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. *Anal.* —5% total nitrogen, 3% available phosphate, 2% available potash.

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Since these compounds with reported growth-retarding activity had not been tested on *Datura* spp., it was decided to determine their effects on the growth and synthesis of certain chemical constituents of *Datura meteloides*. In addition, since some of these compounds appear to be antigibberellins and since gibberellin treatment generally reduces the concentration of alkaloids in many medicinal plants (16, 17), it was of interest to note whether a "growth retardant" would cause an increase in the concentration of alkaloids of the plants.

EXPERIMENTAL

Procedure.—Seventy uniform plants were grown under greenhouse conditions in a soil mixture composed of 1 part sand, 2 parts sandy loam, and 10 Gm. of organic fertilizer[1] per gallon can. On July 12, 1962, *Daturas* which were about 34 days old were labeled according to the following plan for treatment: Amo-1618-treated plants, CCC-treated plants, AMAB-treated plants, CO-11-treated plants, B995-treated plants, Phosphon-treated plants, and control (untreated) plants. Each of the above groups consisted of 10 plants. On that date the plants were randomly arranged on a greenhouse bench, and treatment was instituted. Treatments

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Research paper No. 464, School of Pharmacy, Department of Pharmacognosy, Oregon State University.

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. *Anal.* —5% total nitrogen, 3% available phosphate, 2% available potash.

TABLE I.—CONCENTRATION OF ALKALOIDS[a] IN *Datura* PLANT PARTS

Treatment	Leaf-Tops mg./Gm.	Leaf-Tops Control, %	Stems mg./Gm.	Stems Control, %	Roots mg./Gm.	Roots Control, %
Control	4.18	...	3.33	...	5.95	...
Amo-1618	3.78	90.3	3.43	103.0	7.48	125.6
AMAB	4.00	95.7	3.20	96.2	6.42	107.9
Phosphon	4.55	108.9	3.80	114.3	6.98	117.2
CCC	4.55	108.9	3.32	99.9	6.13	102.9
B-995	3.73	89.1	3.48	104.5	6.58	110.5
CO-11	4.60	110.1	3.32	99.9	6.45	108.4

[a] Alkaloids were calculated as scopolamine.

TABLE II.—TOTAL ALKALOID CONTENT[a] OF *Datura*

Treatment	Leaf-Top mg.	Leaf-Top Control, %	Stems mg.	Stems Control, %	Roots mg.	Roots Control, %	Per Plant mg.	Per Plant Control, %
Control	18.68	...	13.39	...	15.65	...	47.72	...
Amo-1618	17.50	93.7	14.89	111.1	20.64	131.9	53.03	111.1
AMAB	19.08	102.1	13.86	103.5	17.78	113.6	50.72	106.3
Phosphon	21.43	114.2	14.78	110.4	16.12	103.0	52.33	109.7
CCC	21.79	116.6	14.11	105.5	16.31	104.2	52.21	109.4
B-995	17.68	94.6	13.57	101.3	18.56	118.6	49.81	104.4
CO-11	23.32	124.82	13.78	102.9	17.22	110.0	54.32	113.8

[a] Calculated from dry weight and alkaloid analyses data: per plant = leaf-tops + stems + roots.

TABLE III.—CHLOROPHYLL CONTENT[a] IN LEAF-TOPS OF *Datura* Plants

Treatment	Chlorophyll *a* mg./Gm.	Chlorophyll *a* Control, %	Chlorophyll *b* mg./Gm.	Chlorophyll *b* Control, %
Control	2.54	...	2.27	...
Amo-1618	2.17	81.9	2.07	91.2
AMAB	2.26	89.0	2.09	92.1
Phosphon	3.00	118.1	2.74	120.8
CCC	2.64	103.9	2.28	100.4
B995	2.92	115.0	2.52	111.0
CO-11	2.73	107.4	2.23	98.2

[a] Based upon the analysis of 1-Gm. samples from a pool of 10 plants per group.

with Amo-1618, CCC, AMAB, CO-11, and B995 consisted of spraying the plants to run-off with aqueous solutions containing 1000 p.p.m. of the chemicals.[2] Paper shields were employed to prevent the spray from contacting the soil. Due to its phytotoxicity when applied to the aerial parts of plants (3), the Phosphon[3] was applied in the form of a soil drench. Each plant of this group received 100 mg. of the chemical. Subsequent applications of the chemicals were made at weekly intervals for a period of 4 weeks. The habit of the plants was observed daily during this time. Height measurements were taken at 2-day intervals. On the day prior to harvesting (August 11) the plants were examined for the number of flowers, the number and size of capsules, the number of internodes, and the stem diameter. The division of the plants into their morphological parts at harvest time, fresh and dry weight determinations, pulverization, and storage of the powdered material were conducted in a manner previously described (18).

Growth Effects.—Statistical analysis of height and dry weight data indicated no significant changes from the treatments. However, the B995 and CO-11 groups were about 90 and 94%, respectively, as tall as controls. This would indicate slight retardation of stem elongation. No significant changes were noted at harvest time in the number of flowers and capsules or in the stem diameters of the variously treated groups.

Analysis for Alkaloids.—The alkaloid analyses, performed on pooled samples by the Brummett-Sciuchetti method (17), indicated variable trends (Table I). Two extractions were made per group, and each group was analyzed in duplicate. When duplicate determinations did not agree, two further extractions were performed on each group. The concentration of alkaloids in the roots of the Amo-1618 group increased about 26%. Increased concentrations, varying from 9 to 17%, were found in the organs of the Phosphon group (Table I). Likewise, increases of approximately 10% were noted in the roots of the B995 group and in the leaf-tops of the CO-11 group.

Total Plant Alkaloids.—The total alkaloids per plant and per plant organ were obtained by multiplying the dry weight of the plant part by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table II). The following increases were noted in total alkaloid content: 32% in the roots of the Amo-1618 group, 25% in the leaf-tops of the CO-11 group,

[2] The authors are indebted to Dr. Paul C. Marth, Crops Protection Research Branch, U.S.D.A., Beltsville, Md., for the supply of Amo-1618, CCC, AMAB, and CO-11 employed in this study and to Dr. J. A. Riddell, Naugatuck Chemical Co., Bethany, Conn., for the supply of B995.

[3] The technical grade of Phosphon was furnished through the courtesy of Dr. Charles R. Downing, Agr. Chem. Group, Virginia-Carolina Chemical Corp., Richmond, Va.

19% in the roots of the B995 group, and 17% in the leaf-tops of the CCC group. It is of interest to note that all organs of the AMAB, Phosphon, CCC, and CO-11 groups indicated higher total alkaloid content than the controls.

Chlorophyll Content.—The analysis for chlorophyll content was conducted by a modification (17) of the method described by Gjerstad (19). A minimum of two duplicate determinations was carried out on each sample. Although no apparent macroscopic differences were observed in the green color of the leaves of the treated groups, an effect was found when the leaf-tops were analyzed for the concentrations of chlorophyll *a* and *b* (Table III). Plants treated with Amo-1618 exhibited about a 19% reduction in chlorophyll *a* and a 9% decrease in chlorophyll *b*. Decreases of a smaller magnitude were also found in the AMAB group. Significant increases of about 20% were noted for both pigments in the Phosphon group. The B995 group demonstrated a similar trend.

Selective Solvent Extraction.—To determine the effects of the growth regulators on various other plant constituents, an abbreviated selective solvent extraction was performed by following the general sequence of solvents proposed by Dragendorff (20). The method employed 2-Gm. samples of dried leaf-tops material. This material was completely extracted in a Soxhlet continuous extraction apparatus with the following sequence of solvents: petroleum ether U.S.P., anhydrous ether C.P. grade, alcohol U.S.P. (92.3% w/w), and distilled water. The weights of the dry extracts were obtained by evaporation of the solvent on a water bath followed by oven drying at 48.5° for 24 hours. No significant differences were found in the petroleum ether or ether extractives of the treated groups. However, the alcohol-soluble extractives of the Phosphon and CO-11 groups were increased over controls 37 and 25%, respectively. The general trend was a higher content of alcohol-soluble extractive in all treated groups. Increases of 18, 25, and 44% were noted in the water-soluble extractives of the CCC, Amo-1618, and CO-11 groups, respectively. A 17% decrease was noted in the Phosphon group.

DISCUSSION AND CONCLUSIONS

The principal response of sensitive plants to "growth retardants" is a reduction in internode length, resulting in shorter plants. Plants from all treated groups were shorter than controls. There appeared to be an apparent retardation of growth, indicated by the reduced height of the treated groups. However, when height and dry weight data were subjected to statistical analysis, the differences were insignificant.

An interesting aspect of the experiment was an increased concentration of alkaloids in many of the organs of the treated groups. The 26% increase in the roots of the Amo-1618 group was considered significant. All treated groups synthesized more total alkaloids per plant. The total alkaloids in the leaf-tops were increased about 15, 17, and 25% in the Phosphon, CCC, and CO-11 groups, respectively. The total alkaloids of the roots were increased by all treatments. The increases of 32% in the Amo-1618 group and 19% in the B995 group were considered significant. Previous investigations demonstrated that gibberellin treatment usually induces a reduction in the concentrations of alkaloids in the aerial parts of *Daturas* (16–18). Several workers (1, 3, 6, 9–11) consider Amo-1618, CCC, AMAB, and Phosphon to be antigibberellins. Thus, it appears that there is a relationship between gibberellin action, growth, and alkaloid synthesis. In this experiment some of the growth retardants increased alkaloid synthesis. This suggests that the effects of some of the chemicals employed were antigibberellin in nature regarding alkaloid synthesis. This tends to confirm the reports of other investigators that some of these compounds are antigibberellin in nature (1, 3, 6, 9–11).

Of the chemicals employed, Amo-1618 and Phosphon induced the greatest changes in plant composition. The former caused a 26% increase in root alkaloid concentration, a 32% increase in total root alkaloid content, a 25% increase in the water-soluble extractive, and a 19% decrease in the concentration of chlorophyll *a*. Phosphon induced increases in the concentration of alkaloids in all the plant organs and caused an increased total alkaloid content in the morphological parts of the plant. Furthermore, about a 20% increase was found in the chlorophyll *a* of this group and a 37% increase in the alcohol-soluble extractive. Appreciable differences from controls were also noted in the CO-11, CCC, and B995 groups. The following increases were found in the CO-11 group: 25% in the total leaf alkaloids, 25% in the alcohol-soluble extractive, and 44% in the water-soluble extractive. All of the organs of the CCC group indicated increased total alkaloid content. Increases of 17% in total leaf alkaloid content and 18% in the water-soluble extractive were noted. A higher concentration of chlorophyll *a* and a 19% gain in total root alkaloids were found in the B995 group.

It is the authors' opinion that more pronounced effects, especially on internode elongation, might have been induced if a five to tenfold increase in the concentration of the chemicals had been employed or if the plants had been treated and harvested at an earlier stage of plant development.

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Determination of Hyoscyamine Hydrobromide in a Multicomponent Tablet

By ELLIOT B. BECK, MELVIN H. PENNER, and JOSEPH M. TALMAGE

The quantitative assay of hyoscyamine hydrobromide in a multicomponent tablet formulation is described. The method consists of tablet treatment with sulfuric acid, an ether extraction from an alkaline aqueous solution, followed by quantitative column chromatography to separate the alkaloid from the nonalkaloidal tablet components.

THE DETERMINATION of the parasympatholytic alkaloid, hyoscyamine hydrobromide, in a urinary analgesic, antispasmodic, sedative tablet combination[1] proved to be a formidable problem. The problem was amplified by the minimal dosage per tablet of 0.3 mg. of the alkaloid to 150 mg. of phenazopyridine hydrochloride.[2] No problems were encountered due to the presence of 15 mg. of butabarbital.

Phenazopyridine hydrochloride is measured at its ultraviolet absorption peak at 240 mμ in aqueous acid without prior separation. Butabarbital can be assayed by triturating the tablets with methanol, extracting an acid solution with chloroform, re-extracting the chloroform with sodium hydroxide, and finally determining the ultraviolet absorption at 240 mμ. The assay procedures for butabarbital and phenazopyridine hydrochloride were precise and accurate.

Separation of hyoscyamine hydrobromide and phenazopyridine hydrochloride by liquid-liquid extraction proved difficult because the phenazopyridine hydrochloride distributed itself between both phases. Phenazopyridine hydrochloride also interfered with the usual methods of alkaloid quantitation. Thus, simple acid-base titration methods (1, 2), titration with picric acid (3), extraction and colorimetry of the picrate salt (4), precipitation with ammonium reineckate (5), and estimation by ultraviolet absorption spectroscopy yielded invalid results because of interference due to phenazopyridine hydrochloride. Attempts at reducing the azo linkage in phenazopyridine hydrochloride with $TiCl_3$ (6) or $Na_2S_2O_4$ similarly proved unsatisfactory.

It became apparent that a complete separation of the two compounds was required to assure an accurate assay for the solanaceous alkaloid. Chromatographic methods of analysis were investigated and limited success was achieved using a unidimensional multisolvent paper technique. This method consisted of chromatographing the mixture in an ethyl acetate–acetic acid–water solvent system in which hyoscyamine hydrobromide has an R_f value of 0.00 and most of the phenazopyridine hydrochloride migrates down the paper. The papergram is then placed in *n*-butanol–acetic acid–water where the alkaloid has an R_f value of 0.65. This method gave incomplete recoveries because of the difficulty in preparing a sufficiently concentrated extract of the tablet to allow for proper spotting on the paper chromatogram.

A liquid-liquid partition chromatographic column method was evolved based upon experience gained with paper chromatographic methods. A description of this method along with results obtained follows.

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[1] Pyridium (150 mg.), 0.3 mg. of hyoscyamine hydrobromide, and 15 mg. of butabarbital. Marketed as Dolonil by the Warner-Lambert Pharmaceutical Co., Morris Plains, N. J.

[2] Marketed as Pyridium by Warner-Chilcott Laboratories, Division of Warner-Lambert Pharmaceutical Co.

PROCEDURE

Reagents.—*Buffer pH 7.*—Dissolve 10 Gm. of KH_2PO_4 in 500 ml. of distilled water, adjust to pH 7.0 with 1 *N* NaOH, and dilute to 1 L. with distilled water.

Buffer pH 11.2.—Dissolve 1 Gm. of Na_3PO_4 in 1 L. of distilled water and adjust to pH 11.2.

Ethyl Acetate/Acetic Acid/Water Chromatographic Solvent.—Equilibrate 990 ml. of ethyl acetate, 10 ml. of glacial acetic acid, and 50 ml. of water in a separator and allow the phases to separate. Save both phases; the upper layer is used as the mobile phase and the lower layer as the stationary phase.

Sodium Picrate Reagent.—Dissolve 1 Gm. of picric acid in 50 ml. of distilled water, adjust pH to 7.0 with 1 *N* NaOH, and dilute to 100 ml. with water.

Sample Preparation.—Determine the average weight of not less than 15 tablets and reduce them to a fine powder. Weigh accurately a portion of the powder equivalent to about 3 mg. of hyoscyamine hydrobromide and transfer it to a 100-ml. centrifuge tube. Add 60 ml. of 1 *N* H_2SO_4 and heat the sample on the steam bath for 1 hour with occasional agitation. Centrifuge the suspension and filter the supernatant liquid through a medium porosity sintered-glass funnel into a 100-ml. volumetric flask. Wash the centrifuge tube with an additional 20 ml. of 1 *N* H_2SO_4, resuspend the solids, and repeat the centrifugation. Filter the supernatant liquid through the same funnel into the volumetric flask and dilute to volume with 1 *N* H_2SO_4. Pipet a 25-ml. aliquot into a separator, make alkaline (about pH 9) with 10% NH_4OH, and extract with 1 × 75 and 2 × 50 ml. of ether. Evaporate the combined ether extracts to about 20 ml. on the steam bath with a current of air directed above the solution.

Standard Preparation.—Dissolve 60 mg. of hyoscyamine hydrobromide N.F. in 200 ml. of 1 *N* H_2SO_4 and dilute a 10-ml. aliquot to 100 ml. with 1 *N* H_2SO_4. Pipet a 25-ml. aliquot into a separator, make alkaline (about pH 9) with 10% NH_4OH, and proceed as directed for the sample.

Column Preparation.—Mechanically stir 10 Gm. of Solka-Floc BW-40 (Gurntly Brown Co.) with 200 ml. of the mobile phase for about 15 minutes; then add 5 ml. of stationary phase and stir for an additional 30 minutes. Place a pledget of glass wool into a chromatographic column (150 × 25 mm.) and pack the column with the adsorbant to a height of 50 mm. using positive pressure. Place a pledget of glass wool on top of the column bed and wash the column with 20–30 ml. of mobile phase.

Chromatographic Development.—Quantitatively transfer the prepared sample and standard to

TABLE I.—RESULTS OF ASSAY OF SYNTHETIC MIXTURES

Analysis No.	% Recovery	Analysis No.	% Recovery
1	98.3	9	102.8
2	100.3	10	104.3
3	99.1	11	103.5
4	99.5	12	101.4
5	100.3	13	96.5
6	100.7	14	103.3
7	99.0	15	93.9
8	104.3		
$\bar{X}$ =	100.4%	$(X - \bar{X})^2$ =	121.6
σ =	3.0%	3σ =	9.0%

TABLE II.—RESULTS OF TRIPLICATE ASSAY[a] FOR HYOSCYAMINE HYDROBROMIDE

Analysis No.	Assay, mg./Tablet	Recovery, % of Theoretical
	Tablet *A*	
1	0.300	100.0
2	0.290	96.7
3	0.306	102.0
	Tablet *B*	
1	0.293	97.6
2	0.295	98.4
3	0.300	100.0
	Tablet *C*	
1	0.296	98.8
2	0.304	101.5
3	0.294	98.0

[a] The average assay is 99.2% ± 1.8% of the theoretical value.

separate columns, washing the vessel with small increments of mobile phase and collect the eluate at a flow rate of approximately 3 ml./minute. When the vessel has been washed free of phenazopyridine hydrochloride (absence of yellow color), wash the column with an additional 100 ml. of mobile phase. Discard these washings. Elute the column with 0.1 *N* HCl and collect in a 100-ml. volumetric flask until just up to volume, remove the organic phase with a syringe, and dilute to volume with an additional portion of 0.1 *N* HCl which has passed through the column.

Color Development.—Adjust a 25-ml. aliquot of each sample and standard to pH 7 with 1% NH_4OH and transfer each solution quantitatively to separators using 25 ml. of pH 7 buffer. Add 3 ml. of sodium picrate solution to each separator and extract with 3 × 25 ml. of $CHCl_3$, filtering each $CHCl_3$ layer carefully through glass wool. Extract the combined $CHCl_3$ layers with 2 × 35 ml. of pH 11.2 buffer collecting the buffer solution in 100-ml. volumetric flasks. Dilute to volume with pH 11.2 buffer.

Determine the absorbance of the sample and standard solutions *versus* pH 11.2 buffer at lambda maximum about 355 mμ in a suitable spectrophotometer using matched 10-cm. cells. These values are inserted into

$$\frac{A}{A_s} \times \frac{\text{Av. tablet wt.}}{\text{sample wt.}} \times \frac{\text{std. wt.}}{20} = \text{mg. hyoscyamine HBr/tablet} \quad \text{(Eq. 1)}$$

where A and A_s are the absorbances at 355 mμ of the sample and standard, respectively.

DISCUSSION

To evaluate the precision and accuracy of the method, synthetic mixtures of hyoscyamine hydrobromide and tablet formulation mixtures were prepared and assayed. The results of this study are shown in Table I.

Three individual lots of tablets were assayed in triplicate for hyoscyamine hydrobromide. The results of this study are shown in Table II.

RESULTS

A precise and accurate method has been developed for the determination of hyoscyamine hydrobromide in a tablet formulation. The method is generally applicable to other combinations of alkaloids and amines. The method circumvents the interference due to azo substances which exhibit atypical liquid-liquid partition characteristics.

The method can be easily manipulated and lends itself quite readily to routine control measurements.

SUMMARY

A method has been presented for the separation and assay of small amounts of hyoscyamine hydrobromide in complex tablet mixtures by partition column chromatography. The method has accuracy and precision well within the limits required for good quality control and stability testing.

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Comparison of Triphenyltetrazolium and Blue Tetrazolium for Determination of Prednisolone

By FRIEDA M. KUNZE and JOEL S. DAVIS

Blue tetrazolium (3,3'-dianisole bis[4,4'-(3,5-diphenyl)tetrazolium chloride]) and 2,3,5-triphenyltetrazolium chloride are compared as colorimetric reagents for the assay of prednisolone. The effect of oxygen, light, temperature, and base concentration on the rate of color development and the stability of the blue tetrazolium reaction were investigated and found to be negligible. The procedure is applicable to other adrenocortical steroids.

A NUMBER of investigators have reported that the Mader and Buck (1) triphenyltetrazolium procedure for the determination of adrenocortical steroids described by the U.S.P. XVI gave poor reproducibility even though precise timing of the reaction was observed and meticulous care was taken in the cleaning of glassware. Johnson *et al.* (2) demonstrated the effect of oxygen, light, and base concentration on both the intensity of color and the rate of its formation with pure hydrocortisone, hydrocortisone acetate, prednisolone, and fludrocortisone; they recommended a procedure to control variance due to these factors. Callahan *et al.* (3) extended this study and applied the procedure to tablet formulations. They concluded, "The conditions found necessary to obtain satisfactory reproducibility are use of low actinic glassware, complete evaporation of the chloroform extracts under nitrogen, use of smaller tetramethylammonium hydroxide concentration and its addition last, flushing the empty part of the flasks containing the reagent solution with nitrogen, development of the color in a 30° water bath, and the use of ethanol with controlled water content, such as U.S.P. 95% ethanol." While we found that Callahan's procedure would give reproducible results, it was hoped that a simpler method of equally acceptable accuracy could be developed for the routine analyses of pharmaceuticals.

The use of blue tetrazolium as a colorimetric reagent for steroid determinations has been reported in the literature with varying degrees of success. A rapid and sensitive blue tetrazolium procedure was published by Rechnagel and Litteria (4). Reproducible results were obtained when these precautions were observed: recrystallization of the blue tetrazolium from ether, the use of twice redistilled ethanol, the substitution of ethanolic sodium hydroxide solution for tetramethylammonium hydroxide solution, and restriction of the water content of the reaction mixture using anhydrous ethanol. In their study of those factors which influence the triphenyltetrazolium reagent, Johnson *et al.* (2) also compared that reagent with blue tetrazolium. They concluded that the advantages of greater sensitivity and freedom from interference by oxygen of the blue tetrazolium reagent are more than outweighed by the disadvantages of high and continuously varying blank readings. Smith and Halwer (5) used blue tetrazolium as one means of characterizing triamcinolone and the related 16 alpha hydroxy steroids. Because of the instability of the blue tetrazolium solution, they recommended that the reagent be prepared fresh daily.

Johnson and Francois (6) reported the successful use of Dajac B.T. brand blue tetrazolium without further purification for steroid studies. We investigated this particular reagent for its suitability in routine analyses. The reagent proved to be both sensitive and stable; a solution stored for 3 months in the dark showed no change in color development. The absorption of colors was proportional to concentration of the steroid in the 50–450 mcg. range examined. The maximum deviation was 0.7% from the mean in 126 analyses of U.S.P. prednisolone reference standard made over a 5-week period.

EXPERIMENTAL

Reagents

We used the following reagents: alcohol U.S.P., 95%; blue tetrazolium solution–dissolve 50 mg. of blue tetrazolium[1] in 10 ml. of alcohol by warming in a hot water bath and swirling; cool, filter, and protect solution from light. Dilute tetramethylammonium hydroxide—dilute 1 ml. of U.S.P. test solution to 20 ml. with alcohol.

Assay

Standard Preparation.—Using an accurately weighed amount of U.S.P. reference standard corresponding to the steroid under study, prepare an alcohol solution containing about 10 mcg./ml.

Sample Preparation.—For bulk steroids, prepare as directed under *Standard Preparation*. Transfer 20.0 ml. of the solution to a glass-stoppered 50-ml. conical flask for color development. For tablet preparations, weigh and finely powder not less than 10 tablets. Weigh accurately a portion of powder equivalent to about 5 mg. of the active ingredient and transfer it to a 125-ml. separator with the aid of 15 ml. of water. Extract the steroid using four 25-ml. portions of chloroform, filtering each portion through chloroform-washed glass wool into a 250-ml. volumetric flask. Add chloroform to volume and mix. Pipet 10 ml. of this solution into a glass-stoppered 50-ml. conical flask, evaporate the solution to dryness on a steam bath with a gentle current of air, cool, and dissolve the residue in 20.0 ml. of alcohol.

Color Development.—Into separate glass-stoppered 50-ml. conical flasks, pipet 20.0 ml. of the *Standard Preparation* and 20.0 ml. of alcohol to serve as a blank. To these flasks and to the flask containing the *Sample Preparation*, add 2.0 ml. of blue tetrazolium solution and mix. Add 2.0 ml. of dilute tetramethylammonium hydroxide and mix. Allow the solutions to stand in the dark for

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[1] Dajac, B. T., the Borden Co., Philadelphia, Pa., has been found satisfactory and was used for these studies.

90 minutes and, with a suitable spectrophotometer, determine the absorbances of the sample and standard solutions at the point of maximum absorbance at about 525 mμ relative to the reagent blank.

DISCUSSION

Melting point specifications and infrared and ultraviolet spectra do not distinguish between lots of blue tetrazolium suitable for steroid assay and those yielding a high blank and low color development. A simple suitability test is required prior to use of a new lot of this reagent. The absorbance of the colors developed must be proportional to the concentration of steroid; when assayed as directed under *Color Development*, the absorbance of the 200-mcg. level of hydrocortisone, corrected for the reagent blanks, should be no less than 0.500. Examination of nine lots of blue tetrazolium obtained from four different distributors indicates that such a test excludes unsuitable blue tetrazolium and yet allows considerable latitude for lot to lot variation. A high blank *per se* (above 0.100) does not necessarily preclude use of a particular lot of reagent. Of nine lots examined, two had absorbance blank values of 0.111 and 0.127, four were between 0.030 and 0.060, and three were unsuitable.

Those factors which have been reported to affect the intensity and stability of colors obtained with triphenyltetrazolium salts were studied and applied to the blue tetrazolium procedure. A series of tests using prednisolone showed that flushing the flasks with nitrogen prior to color development increased the color intensity by about 2%. Since reproducible absorption values were obtained without such nitrogen treatment, this precautionary step was eliminated. The use of a 1:10 dilution of tetramethylammonium hydroxide instead of the 1:20 dilution specified in the method gave no significant change in the absorbance values. Within the time interval examined, the order of the addition of the base and the color reagent has no effect on these values. Variations in room temperature between 22° and 30° did not affect reproducibility of the results. The use of actinic glassware showed no advantage over the use of clear glass when the solutions in the latter were protected from sunlight and incandescent light. Exposure to sunlight greatly increased the values obtained for the blanks when clear glass was used. In view of these observations, we recommend that direct sunlight be excluded from the laboratory when the reagents are added to the solutions and when the spectrophotometric measurements are being made. While the color developed with prednisolone and blue tetrazolium continued to increase in intensity over 18 hours, the rate of color development had decreased after 90 minutes to a point where it had negligible effect on the results.

RESULTS

Comparison of the U.S.P. XVI method modifications A and B represents work completed in this laboratory as a portion of a collaborative study designed and conducted by The Upjohn Co. Modification A reverses the order of the addition of triphenyltetrazolium and tetramethylammonium hydroxide. Modification B incorporates the precautions recommended by Callahan. The blue tetrazolium procedure was added to the study by the authors so that all three techniques could be studied under similar conditions. In each analysis 250 mcg. of prednisolone was used. The concentrations used were too high for optimum absorbance values for the blue tetrazolium reaction; however, it was felt that comparison would be simplified and additional variables avoided if equal aliquots of the same solutions were used throughout. The three methods were compared on three different days by two analysts. Each analyst prepared separate standards and weighed and extracted two samples of ground tablet formulation on each test day. Colors were developed in duplicate.

TABLE I.—RECOVERY OF PREDNISOLONE FROM TABLETS IN PER CENT OF LABEL CLAIM

	U.S.P. XVI Method Modification A	U.S.P. XVI Method Modification B	Blue Tetrazolium Procedure
Analyst A			
Day 1	100.5	96.7	99.6
	99.7	96.0	99.5
Day 2	100.3	99.6	100.4
	100.3	99.6	100.5
Day 3	101.1	99.6	101.1
	100.4	98.7	100.9
Analyst B			
Day 1	98.7	100.9	100.5
	96.6	102.4	100.1
Day 2	99.5	101.0	101.2
	98.7	99.8	101.2
Day 3	100.3	102.9	101.3
	100.0	102.2	101.1
Max. range	96.6 to 101.1	96.0 to 102.9	99.5 to 101.3

The results of this study are presented in Table I. While the results by all three procedures showed fair reproducibility, those obtained by the blue tetrazolium method were by far the most consistent. This fact and the ease with which this procedure can be applied would make it appear to be the method of choice.

The blue tetrazolium reaction is applicable to other adrenocortical steroids. It was used to quantitate those fractions isolated by paper chromatography in the procedure "Determination of the Major Component of Some U.S.P. Adrenocortical Steroids" which is presented in a separate paper (7).

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N,N'-Thiocarbonylbis(Cyclic Nitrogen Heteroparaffins)

By RONALD E. ORTH

A series of thioureas, in which the two amido nitrogens are incorporated into separate rings, are prepared by the action of thiophosgene on hetercyclic amines. The infrared spectra are studied and the data correlated with that of prior investigations. The results of preliminary pharmacological testing are reported.

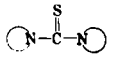
THIOUREA and its many derivatives have proven their therapeutic effectiveness in a variety of well-known pharmaceuticals (1). Incorporation of thiourea into cyclic structures tends to lower the toxicity of the parent compound (2). Though many of these cyclized thioureas are known, few attempts have been made to synthesize both of the amido-nitrogens of thiourea into separate rings (3). *N,N'*-Thiocarbonyldipiperidine was first synthesized from dipiperidyl thiuram disulfide (4); in 1924, a method for preparing symmetrically tetra-substituted thioureas was introduced by Dyson *et al.* (5). Rieds' recent synthesis of *N,N'*-thiocarbonylbis(3,5-dimethylpyrazole) and *N,N'*-thiocarbonyldiimidazole is the first preparation of *N,N'*-di-*N*-heteroaromatic thiocarbonyl compounds (6).

This investigation deals with the synthesis of a number of symmetrical *N,N'*-thiocarbonylbis-(cyclic *N*-heteroparaffins) such as Fromm prepared (4) by a modified version of the Dyson method (5).

Infrared Spectral Information.[1]—All the compounds show strong absorption at or near 1480 cm.$^{-1}$, and this band is assigned to the —N—C=S moiety. Weak absorption in the 660 to 667 cm.$^{-1}$ is also assigned to this moiety. A band in the region 1360 to 1370 cm.$^{-1}$ is believed due to the C=S group. Absorption in the 1280 to 1300 cm.$^{-1}$ region appears to be caused by the N—C group (7). Absorption for the Ried *N,N'*-thiocarbonylbis *N*-heteroaromatics is in the same regions for the major bands as the compounds used in this research (6).

Preliminary Pharmacology.—The investigation of *in vitro* anthelmintic, C.N.S., schistosomicidal, and trichomonacidal activity, as well as a broad antibacterial screening has led to negative expectations.

TABLE I.—*N,N'*-THIOCARBONYLBIS (CYCLIC NITROGEN HETEROPARAFFINS)



N,N'-Substituted Thiocarbonyls	% C[a] Calcd.	% C[a] Obs.	% H[a] Calcd.	% H[a] Obs.	% N[a] Calcd.	% N[a] Obs.	% S[a] Calcd.	% S[a] Obs.	M.p., ° C.
Dipyrrolidyl-	58.7	58.7	8.70	8.82	15.2	15.2	17.4	17.1	127–28
Dipiperidyl-	62.2	62.2	9.45	9.44	13.2	13.4	15.1	14.9	
Bis(2-ethylpiperidyl)-	67.2	67.2	10.5	10.6	...	...	...	...	150–160/2 mm.[b]
Bis(2-methylpiperidyl)-	64.9	65.2	10.0	10.1	11.7	11.1	...	...	146–154/2 mm.[b]
Bis(3-methylpiperidyl)-	65.0	65.0	10.0	9.97	11.7	11.7	13.3	13.2	148–158/4 mm.[b]
Bis(4-methylpiperazyl)-	54.5	54.9	9.10	9.49	23.1	22.3	13.2	13.1	82–4
Dimorpholyl-	50.0	50.3	7.40	7.89	13.0	12.9	14.8	14.6	89–90
Bis(1,2,3,4-tetrahydroisoquinolyl)-	74.0	73.4	6.49	6.49	9.09	8.84	10.4	10.3	173–75
Bis(1,2,3,4-tetrahydroquinolyl)-	74.0	73.7	6.49	6.49	9.09	8.93	10.4	10.0	168

[a] Duplicate C, H, N, and S by Dr. G. Weiler and Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, England. [b] Yellow to brown oil.

EXPERIMENTAL

General Synthesis, Isolation, and Purification of the N,N' - Thiocarbonylbis(cyclic N - heteroparaffins).—Two-tenths mole of the corresponding cyclic amine is dissolved in 100 ml. of ether (or acetone), and placed in a three-necked reaction flask fitted with a separator, reflux condenser, and stirrer. The flask is placed in an ice bath; 0.05 mole of thiophosgene is dissolved in 50 ml. of ether or acetone and added dropwise from the separator to the reaction flask, stirring constantly. Reflux 1 hour past the addition of the last drop of thiophosgene solution. Heating will be necessary during this last hour of reflux. The reaction is violent at the start, so the thiophosgene addition must be made very slowly. The amine hydrochloride separates as an insoluble solid and is removed from the ethereal or acetone solution by filtration after the reaction is completed. The filtrate is decolorized with charcoal, dried over anhydrous sodium sulfate, and evaporated to either a thick viscous yellow-brown oil or a low-melting white or yellow solid, depending on the specific case. The product is vacuum distilled at low pressures (near 1 or 2 mm.). The oils are washed with ligroin repeatedly, and the solid products recrystallized from ethanol. Yields of about 50% are the rule. (See Table I.)

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[1] Beckman IR-8 spectrophotometer; 0.1 mm. path; chloroform solvent.

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Sensitive Biological Tests for Medical Grade Plastics
I. Toxicity of Organo-Tin Stabilizers

By MARCEL NIMNI

Tissue implantation and the measurement of inflammation by the dye extravasation technique are sensitive and rapid methods for screening plastics for toxicity. The subcutaneous and the intramuscular implantation of polyvinyl chloride plastics containing organo-tin stabilizers produces severe local tissue reaction. The degree of extravasation of trypan blue at the site of the injection of a suitable plastic extract is also an indication of compatibility with animal tissues. Mineral oil was found to be a suitable, nonirritating agent for extracting the plastic. The same techniques are suitable for screening raw materials before incorporation into the plastic formulation. Tin stabilizers, such as dibutyltin dilaurate, caused irritation in dilutions as great as 1:10,000.

PLASTIC MATERIALS, because of their versatility and low cost, find increasing usage in the pharmaceutical and medical fields (1). To keep pace with varying needs in diversified applications, the plastic industry has developed many different formulations (2, 3). It has been of primary importance to investigate carefully among other properties, the biological inertness of the plastics which contact drugs or tissues. Sensitive bioassay procedures are used to detect any toxic effects of the plastic resins themselves, as well as of the ingredients which may be added to the basic polymers for purposes of stability, flexibility, or coloring. The effectiveness of the subcutaneous or intramuscular implantation in animals, as a means of detecting acute and chronic toxicity of plastics, has been reported previously (4–8).

Herewith are described our results obtained using the rat and the rabbit as test animals: (*a*) tissue reactions caused by the subcutaneous injection of small amounts of plastic additives or implantation of plastic materials (polyvinyl chloride) containing such additives, and (*b*) a sensitive technique for rapidly detecting an inflammatory response by measuring the increased capillary permeability of intravenously injected dye at the site of the intradermal injection of a plastic extract.

EXPERIMENTAL

Tissue Implantation.—Wistar rats and New Zealand white rabbits were used as test animals. Rats were implanted subcutaneously with test samples under light ether anesthesia. After shaving the dorsal area, a transverse incision through the skin was made in the lumbar region. Using the blunt end of a forceps, the skin was separated from the underlying connective tissue, and the plastic sample was positioned using another forceps in the subcutaneous tissue, not closer than 30 mm. from the wound. The incision was sutured with 11-mm. nickel-silver wound clips.

Rabbits were anesthetized with thiopental sodium (20 mg./Kg.) and ether. For routine testing, intramuscular implants were made with an automatic pellet implanter[1] at the level of the dorsal paravertebral musculature. In some instances, larger sections were implanted surgically. In all instances, the procedures were performed in an aseptic manner.

At the end of the experimental periods, either 4 days, 1 week, 2 weeks, or 2 months, the animals were sacrificed and the areas observed for macroscopic changes. The plastic specimen and its surrounding tissue were fixed in neutral formalin solution for histologic examination.

A 1% solution of trypan blue in normal saline (0.9% sodium chloride in distilled water) was used to measure local inflammatory response by the degree of dye extravasation. Extracts of plastics were prepared by autoclaving (121° for 60 minutes) 10 Gm. of plastic, cut into small particles, in 10 ml. of mineral oil. Normal saline extracts were prepared in a similar way. The dorsa of the rabbits were carefully clipped to remove hair and 0.1 ml. of the extract was injected intradermally with a 27-gauge needle. A 20% ethanol solution was used as a control irritant. Fifteen minutes after the last intradermal injection, the 1% dye solution was ininjected intravenously at a level of 1 ml./Kg. Response was graded on the basis of amount of dye accumulation at the injection site and classified as none, mild, moderate, and marked (9). The response with 20% ethanol was considered as marked inflammation.

Commercially available dibutyltin dilaurate and other organo-tin stabilizers from various sources were tested. Dilutions of these compounds were made prior to injection, using mineral oil, and were sterilized before use.

RESULTS

The tissue reaction, from a subcutaneous implant of polyvinyl chloride containing a tin stabilizer, is shown in Fig. 1. This specimen had been maintained *in situ* for 1 week. At that time, the animal exhibited swelling of the area as well as all the other signs which accompany an inflammatory reaction. On exposing the subcutaneous tissue, a large capsule was visible surrounding the implant; there was evidence of increased vascularization and fluid accumulation. Intramuscular implantation of similar plastic caused tissue reactions evidenced by a whitish zone of necrotic tissue surrounding the sample. These reactions have been described and illustrated by previous investigators (7, 8). Figure 2 shows a transverse section of a tin stabilized plastic sample surrounded by necrotic epidermis and fat. There is an abscess formation walled off by a thick layer of proliferating fibrous tissue. The white area is caused by retraction of the plastic during the process of fixation.

Figure 3 shows the subcutaneous tissue of a rabbit at a site of injection of 0.1 ml. of dibutyltin dilaurate.

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[1] Manufactured by Sterol Derivatives, Inc., Los Angeles, Calif.

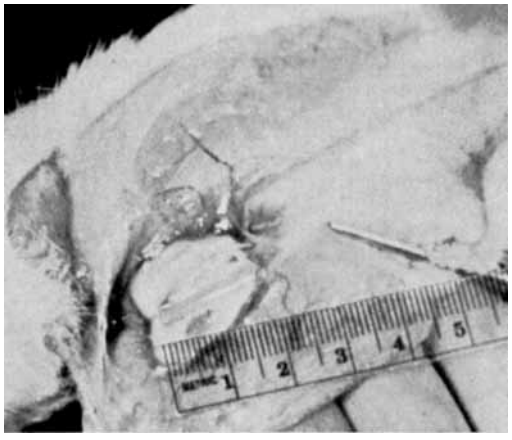


Fig. 1.—Fibrous connective tissue capsule walling off a tin-containing plastic material which has caused a significant inflammatory reaction, 1 week following its subcutaneous implantation.



Fig. 2.—Photomicrograph showing a plastic formulation stabilized with an organo-tin compound, surrounded by proliferating granulation tissue, abscess formation, necrotic epidermis, and fat.

After 24 hours, the area showed signs of necrosis, edema, hemorrhage, multiple cystic spaces, and fibrinous exudate. Eosinophils with occasional polymorphonucleocytes surrounded the area. The whole picture is suggestive of an acute allergic reaction.

As a part of this investigation, the changes in capillary permeability caused by the intradermal injection of different extracts of the plastics and stabilizers were also determined. Vital dyes, when injected intravenously, become bound to plasma proteins, and their accumulation in the treated skin sites reflects the degree of inflammation. Table I summarizes the observations made in this connection.

It can be seen that the irritating plastic containing a tin stabilizer gave a marked positive reaction soon after the injection of the dye when the extract was made using mineral oil as a solvent. Using normal saline, the reaction was scarcely visible and would be termed nonirritating. Plastics without tin gave no signs of tissue reaction irrespective of oil or saline extraction.

Dibutyltin dilaurate caused a marked inflammatory response even when considerably diluted in mineral oil. Dilutions of 1:10,000 still gave evidence of inflammation. It should be pointed out that this mineral oil solution is insoluble in body fluids; presumably, therefore, the tissue reaction occurs with those molecules of solute which are on the periphery of the infiltrating fluid. This makes these concentrations in a mineral oil vehicle comparable to that in a plastic formulation which is maintained in contact with tissues.

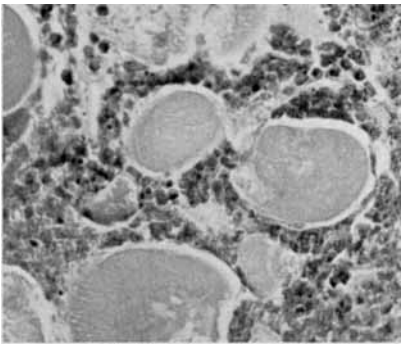


Fig. 3.—Subcutaneous and muscular tissues of a rabbit following the injection of 0.1 ml. of dibutyltin dilaurate. The response is indicative of an acute allergic reaction.

TABLE I.—INFLAMMATORY RESPONSE[a]

Sample	Dilution	Degree of Inflammatory Response[b] Recorded at Different Time Intervals				
		10 Min.	20 Min.	30 Min.	60 Min.	120 Min.
Mineral oil (MO)	...	0	0	0	0	0
Normal saline (NS)	...	0	0	0	0	0
Tin-containing plastic in MO	...	++	+++	+++	++++	++++
Tin-containing plastic in NS	...	0	0	+	+	+
Tin-free plastic in MO	...	0	0	0	0	0
Tin-free plastic in NS	...	0	0	0	0	0
Dibutyltin dilaurate	1:50	++	+++	++++	++++	++++
	1:100	++	+++	+++	+++	+++
	1:1000	++	++	+++	+++	+++
	1:5000	++	++	++	++	+++
	1:10000	0	0	+	++	++
Tin-containing stabilizer A	1:1000	++	++	+++	++++	++++
Tin-containing stabilizer B	1:1000	0	+++	+++	++++	++++
Tin-containing stabilizer D	1:1000	0	++	+++	++++	++++
Tin-containing stabilizer E	1:1000	++++	++++	++++	++++	++++
Tin-containing stabilizer F	1:1000	++	++	++	++	++
20% ethanol	...	++++	++++	++++	++++	++++

[a] Measured by the degree of dye extravasation caused by the subcutaneous injection of plastic extractables and of different concentrations of organo-tin stabilizers. [b] Degree of inflammatory response: 0, none; +, slight; ++, moderate; +++, marked; ++++, severe.

DISCUSSION

The presence of organo-tin compounds in a plastic formulation, added for stabilization purposes, renders the plastic irritating to animal tissues. Its subcutaneous as well as intramuscular implantation in rats or rabbits causes a severe tissue reaction, reflected by edema, cellular necrosis, proliferating granulation tissue, as well as encapsulation by fibrous tissue.

These results confirm the observations of previous investigators which indicate that the tissue implantation technique is one sensitive means of determining the suitability of plastic formulations for medical applications. Our findings indicate that the rat is a satisfactory animal for routine testing of plastics as well as the raw materials proposed for their formulation.

Implantation reveals tissue reaction, if any, within 2 to 4 days, whereas inertness is reflected by excellent tolorance during many weeks of implantation.

The subcutaneous implantation of solid materials and the injection of liquid constituents are valuable in screening the ingredients which are compounded for the different formulas. Macroscopic as well as microscopic examination of the areas of contact are of immediate importance in determining the nature of the response.

The degree of dye extravasation, at the site of intradermally injected extracts to the rabbit, was a rapid and sensitive method for detecting toxicity. Extracts, prepared by autoclaving the plastics in test with mineral oil, increased the sensitivity of the method. Using this method, it was determined that dilutions of 1:10,000 of organo-tin stabilizers still were able to cause an inflammatory response. In this connection it should be mentioned that dibutyltin dilaurate has recently shown to be teratogenic when injected into the yolk sac of fertile eggs prior to incubation (10). Because of this reactivity in tissue, plastics with organo-tin compounds should not be used in applications involving intimate and/or prolonged contact with tissue, in view of other effective stabilizers that do not cause reaction.

In addition to the test described, it is also recommended that all plastic materials which shall be in contact with tissue or body fluids be screened for the presence of pyrogens, vasomotor activity, antigenicity, ocular irritation, hemolytic effects, chronic toxicity in various species, and *in vitro* with isolated muscle systems.

Additional work continues in this area. The author would like to stress the importance of biologically screening the individual constituents that are used in making up all formulations as well as testing the completed plastic formulation.

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Thimerosal as a Preservative in Biological Preparations I. Application of Polarography to the Determination of Thimerosal in Aqueous Solutions and Vaccines

By J. BIRNER and J. ROS. GARNET

Thimerosal, in the low concentration commonly used as an antiseptic and bacteriostatic agent in biological preparations such as vaccines, may be determined polarographically and rapidly and with a degree of accuracy comparable to that obtained by the commonly used agar-cup technique.

It has been observed that the concentration of thimerosal[1] added to vaccines and other biological preparations diminishes with storage (1). The loss has been attributed to absorption of the substance by the rubber with which the containers are often sealed or to physical or chemical interaction with one or another of the components of the rubber. Although there is general agreement that reduction of its concentration does occur, methods for the precise measurement of the extent of the reduction have been largely dependent upon biological techniques.

In some earlier studies in these laboratories, the bacteriostatic activity of antiseptics was measured by the well-known agar-cup assay method. With this technique, the growth of selected microorganisms (*Staphylococcus aureus*, strain B313, and *Bacillus subtilis*, strain 8236, of the National Type Culture Collection) on nutrient agar is inhibited within a zone, the diameter of which is approximately proportional to the concentration of antiseptic placed in the cup.

A comparison of the clear zone formed by a chosen volume of the sample and equal volumes of standard concentrations of the thimerosal affords a measure of the concentration of the antiseptic in the sample—hence, a measure of its diminution.

However, the present authors have used for some time a polarographic method (2) which they consider preferable for speed and accuracy to the rather cumbersome biological method. It is being applied successfully with routine samples having a thimerosal content of not less than 20 p.p.m.

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[1] Thimerosal, sodium ethylmercurithiosalicylate, is official in the "British Pharmacopoeia" as Thiomersal. It is trademarked as Merthiolate.

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Thimerosal, in the low concentration commonly used as an antiseptic and bacteriostatic agent in biological preparations such as vaccines, may be determined polarographically and rapidly and with a degree of accuracy comparable to that obtained by the commonly used agar-cup technique.

It has been observed that the concentration of thimerosal[1] added to vaccines and other biological preparations diminishes with storage (1). The loss has been attributed to absorption of the substance by the rubber with which the containers are often sealed or to physical or chemical interaction with one or another of the components of the rubber. Although there is general agreement that reduction of its concentration does occur, methods for the precise measurement of the extent of the reduction have been largely dependent upon biological techniques.

In some earlier studies in these laboratories, the bacteriostatic activity of antiseptics was measured by the well-known agar-cup assay method. With this technique, the growth of selected microorganisms (*Staphylococcus aureus*, strain B313, and *Bacillus subtilis*, strain 8236, of the National Type Culture Collection) on nutrient agar is inhibited within a zone, the diameter of which is approximately proportional to the concentration of antiseptic placed in the cup.

A comparison of the clear zone formed by a chosen volume of the sample and equal volumes of standard concentrations of the thimerosal affords a measure of the concentration of the antiseptic in the sample—hence, a measure of its diminution.

However, the present authors have used for some time a polarographic method (2) which they consider preferable for speed and accuracy to the rather cumbersome biological method. It is being applied successfully with routine samples having a thimerosal content of not less than 20 p.p.m.

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[1] Thimerosal, sodium ethylmercurithiosalicylate, is official in the "British Pharmacopoeia" as Thiomersal. It is trademarked as Merthiolate.

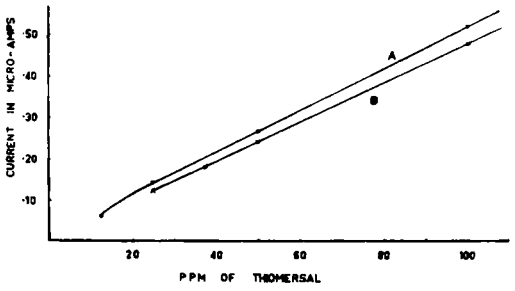


Fig. 1.—Standard curve for thimerosal. Key: A, in water; B, in triple antigen vaccine.

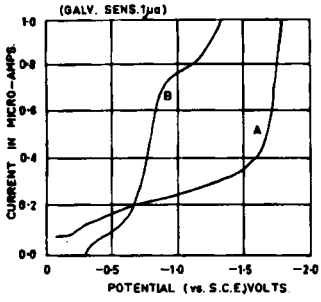


Fig. 2.—Key: A, 10 ml. of water + 2 ml. of 0.1 *N* KNO_3 + 1 ml. of 0.1% gelatin (blank); B, 10 ml. of 100 mcg./ml. of thimerosal in water + 2 ml. of 0.1 *N* KNO_3 + 1 ml. of 0.1% gelatin.

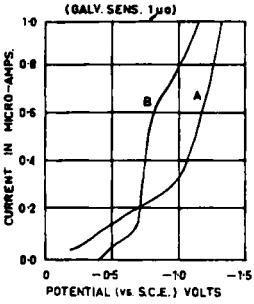


Fig. 3.—Key: A, 10 ml. of triple antigen vaccine + 2 ml. of 0.1 *N* KNO_3 + 1 ml. of 0.1% gelatin (blank); B, 10 ml. of 100 mcg./ml. of thimerosal in triple antigen vaccine + 2 ml. of 0.1 *N* KNO_3 + 1 ml. of 0.1% gelatin.

EXPERIMENTAL

Equipment.—A Tinsley self-recording polarograph was used at damping 3 with an H-type cell against a saturated calomel electrode and a saturated potassium chloride/agar bridge maintained at 25 ± 0.5°, in a thermostatically controlled water bath.

The drop time in open circuit in distilled water was 4.4 seconds per drop. The *M* value of mercury was 2.10 mg. per second.

Procedure.—Estimations were carried out in duplicate in 10-ml. quantities of the sample, to each of which was added 2 ml. of 0.1 *N* potassium nitrate in glass-distilled deionized water to serve as supporting electrolyte and 1 ml. of 0.1% gelatin as maximum suppressor.

Two waves characteristic of thimerosal were registered at −0.75 v. and −1.35 v., respectively, but for our purposes, only the first wave (shown in Fig. 2) was used for measurement. Figure 3 shows the comparable polarogram for triple antigen.[2]

[2] Triple antigen is an alternative name for diphtheria, tetanus, and pertussis vaccine B.P.

Standard curves for thimerosal in aqueous solution and in triple antigen and pertussis vaccines were established in the following manner.

Standard Curve for Thimerosal in Aqueous Solution.—Three reference solutions containing respectively 50, 25, and 12.5 parts of thimerosal per million of deionized water were prepared from a 0.01% solution which in turn was derived from a 1% stock solution.

Ten milliliters of reference solution were pipeted into a dry test tube, and 2 ml. of 0.1 *N* potassium nitrate and 1 ml. of 0.1% gelatin were added. The contents of the tubes were mixed and, after transfer to a dry polarographic cell, freed from oxygen by bubbling in oxygen-free nitrogen.

Polarograms of each of the four standard concentrations were recorded and the standard curve was plotted from the values obtained. (See Fig. 1, curve A.)

Standard Curve for Triple Antigen Vaccine.—A standard curve for a series of comparable concentrations of thimerosal in triple antigen was constructed in the same manner. (See Fig. 1, curve B.)

RESULTS AND DISCUSSION

In constructing the standard curve for thimerosal in triple antigen, it was noted that the values registered for a concentration of 12.5 p.p.m. were less than those to be expected by extrapolation of the curve for higher values. Hence, values for the assay of thimerosal in solutions containing this quantity or less represent only a qualitative indication of the presence of the antiseptic. Values registered in this region of the curve indicate that the "limit" of assay is about 20 p.p.m., and anything less than 12.5 p.p.m. can only be regarded as indicating the presence of a trace.

The standard curve for pertussis vaccine was essentially identical with that derived from triple antigen.

Figure 2 shows two polarograms, curve B representing that of an aqueous solution containing 100 parts of thimerosal per million and curve A the "blank"—a solution of the same composition, except that it lacked thimerosal. In curve B only the first thimerosal wave, appearing at −0.75 v. half-wave potential, is shown.

Figure 3 represents comparable polarograms recorded for solutions in which triple antigen vaccine is substituted for water as the diluent. Curve B represents a solution of thimerosal in the vaccine and curve A the thimerosal-free blank. Replacement of triple antigen by pertussis vaccine furnishes solutions giving an analogous picture.

SUMMARY

The polarographic method of assay makes it practicable to determine quickly low concentrations of thimerosal in aqueous solution or in vaccines, provided the antiseptic is present in an amount not less than 20 p.p.m. Using standard curves, thimerosal concentrations in unknown strength above this limit are capable of being determined to within 5%.

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Thimerosal as a Preservative in Biological Preparations II. Formation of a Thimerosal-Zinc Complex

By J. BIRNER and J. ROS. GARNET

Thimerosal reacts with zinc ions to form a zinc-thimerosal complex which possesses antiseptic properties comparable to those of thimerosal itself. The kinetics of the reaction can be demonstrated using polarography.

THIS PAPER discusses a previously undescribed reaction in which a thimerosal[1]–zinc complex is formed. The subject matter of the paper stemmed from an investigation of the effect of storage on the thimerosal content of certain biological preparations held in containers sealed with various types of rubber closure.

In the course of the investigation it was observed that generally, after an initial small and rapid fall, the concentration of thimerosal in dilute solution in rubber-capped glass containers continued to decrease at a rate which appeared to be related to the type of closure used.

The estimations were carried out by the polarographic technique mentioned in a previous paper (1).

After only a brief period of storage, in some instances the characteristic polarographic wave at −0.75 v. half-wave potential for thimerosal could not be detected. Instead, there appeared another at −1.0 v. half-wave potential which subsequently was found to be due to the zinc ion.

Reznek (2) has observed that zinc ions are leached from rubber closures when the rubber is allowed to remain in contact with acid substances. Davisson *et al.* (3) reported that traces of copper ion present in the water used in the preparation of poliomyelitis vaccine inactivate thimerosal. The addition of ethylenediamine tetracetic acid (EDTA) to chelate the copper was recommended.

EXPERIMENTAL

Extraction of Zinc from Rubber.—Three types of rubber combination cap and stopper of the pattern used as closures for vaccine multidose containers were used in the experiments: (*a*) a red natural rubber with the inner surface lacquered with a film of epoxy resin polymer, (*b*) a black carbon-filled natural rubber and, (*c*) a grey natural rubber.

Five of each type were boiled in 15 ml. of glass-distilled, deionized water. A portion of the extract was used for the Rush and Yoe (4) colorimetric test for zinc, and the rest was polarographed by the procedure described in a previous paper (1).

Formation of Thimerosal-Zinc Complex.—To investigate the possibility of a reaction between thimerosal and zinc ions, polarograms were recorded for a series of solutions consisting of approximately 10 ml. of test solution together with supporting electrolyte and suppressor in the proportions previously indicated (1).

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[1] Thimerosal, sodium ethylmercurithiosalicylate, is official in the "British Pharmacopoeia" as Thiomersal. It is trademarked as Merthiolate.

A, nothing added ..... See Fig. 1
B, 100 mcg. of zinc ion in 10 ml. of aqueous solution (without thimerosal) See Fig. 2
C, 100 mcg. of zinc ion in 0.1 ml. of aqueous solution ..... See Fig. 3
D, 200 mcg. of zinc ion in 0.2 ml. of aqueous solution .....
E, 300 mcg. of zinc ion in 0.3 ml. of aqueous solution .....
F, 400 mcg. of zinc ion in 0.4 ml. of aqueous solution ..... See Fig. 4
G, 10 mg. of solid EDTA disodium salt See Fig. 5
H, 10 mg. of solid EDTA + 400 mcg. of zinc ion in 0.4 ml. of aqueous solution ..... See Fig. 6

All but solution *B* in the series above contained 1000 mcg. of thimerosal furnished by 10 ml. of a 0.01% aqueous solution of the antiseptic with additions as indicated. Solution *B* was 10 ml. of an aqueous solution of zinc sulphate.

Antiseptic Quality of the Thimerosal-Zinc Complex.—To test a supposition that the thimerosal and zinc might form a complex which is itself capable of functioning as an antiseptic in the manner of thimerosal, an additional series of experiments was undertaken.

Samples of 0.01% solutions of thimerosal in water and triple antigen[2] were stored for 10 months at 25°. They were separated into five groups: (*a*) unaltered solutions to serve as controls, (*b*) solutions to which 40 mcg./ml. of zinc ion had been added, (*c*) solutions to which solid zinc oxide been added, (*d*) to which fragments of white rubber caps had been added, and (*e*) solutions to which fragments of black rubber caps had been added.

At the conclusion of storage, the thimerosal content of each of the solutions was determined by both the polarographic and the biological methods. For the biological estimation, two different test organisms were used according to the recognized standard procedure mentioned in a previous paper (1). The results of both tests are recorded in Table I.

RESULTS AND DISCUSSION

Zinc was present in all three extracts obtained by boiling the rubber closures in distilled water, an indication that these ions are readily extracted from the rubber.

An inspection of the polarograms of solutions *C*, *D*, *E*, and *F* reveals that a reaction between thimerosal and zinc occurred.

The zinc wave at −0.1 v. is neither apparent in the polarogram of solution *C* (Fig. 3) nor in those of solutions *D* and *E*. Its appearance in solution *F* (Fig. 4) indicates the presence of an excess of zinc and a consequent alteration in the pattern of the thimerosal wave.

The polarogram of solution *H* (Fig. 6) shows the chelating action of EDTA on zinc ions. Comparison

[2] Triple antigen is an alternative name for diphtheria, tetanus, and pertussis vaccine B.P.

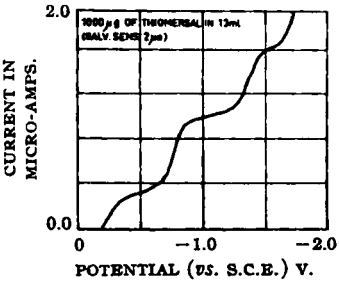


Fig. 1

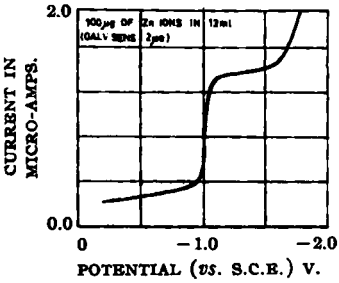


Fig. 2

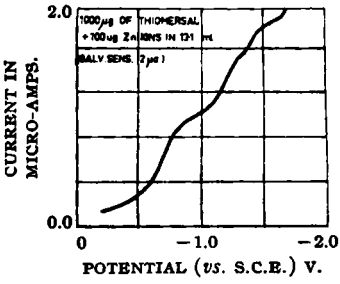


Fig. 3

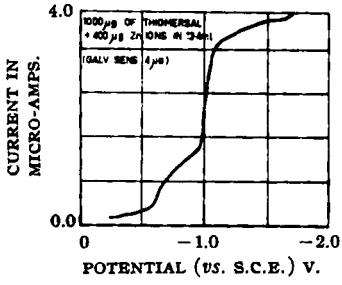


Fig. 4

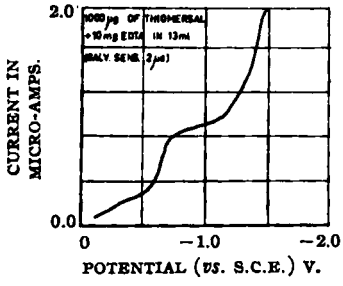


Fig. 5

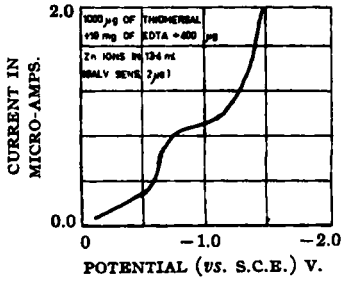


Fig. 6

TABLE I.—ACTIVITY OF 100 mcg./ml. of THIMEROSAL

Added to Soln.	After 10 Mo. Storage at 25°C.[a] Water Soln. — Biological Assay Against *B. subtilis* and *S. aureus*	Water Soln. — Polarographic Assay	Triple Antigen — Biological Assay Against *B. subtilis* and *S. aureus*	Triple Antigen — Polarographic Assay
Nil	+	+	+	+
$ZnSO_4$	+	+	+	+
ZnO (solid)	+	+	+	+
White rubber cap	−	−	−	−
Black rubber cap	−	−	−	−

[a] +, Thimerosal present; −, thimerosal absent.

of Figs. 5 and 6 shows that the shape and height of the first thimerosal wave are identical, irrespective of the presence or absence of zinc ions.

The appearance of a second thimerosal wave is obscured by the presence of EDTA which, in solutions *G* and *H*, was used in a concentration of 0.075%. Both of the solutions produced polarograms in which the first thimerosal wave appeared at a lower half-wave potential than was apparent in the other polarograms. This is consistent with the observations of Benesch and Benesch (5) concerning the effect of pH on the solution.

The supposition that the thimerosal-zinc complex behaves as an antiseptic appeared to be confirmed by the results of the comparative assays given in Table I. Loss of antiseptic activity is apparent only in those solutions of thimerosal which were stored in contact with rubber; hence, the inference is warranted that the loss of activity which occurs is not due to the presence of zinc released from the rubber.

SUMMARY

The addition of zinc ions to dilute solutions of thimerosal results in the formation of a thimerosal-zinc complex which behaves as an antiseptic in much the same manner as uncomplexed thimerosal.

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Simple Lecithin-Protein Model

By PHILIP S. VASSAR and JOHN S. MILLAR

Latex particles may be coated by various lecithins and protein to study their electrophoretic mobilities using the technique of cell electrophoresis. It was demonstrated that the degree of purity of L-α-lecithin profoundly affected the particle mobility; exposure of lecithin-coated latex to gamma globulin also resulted in significant differences in mobility related to the purity of the lecithin.

STUDIES OF protein monolayers at the oil/water interface are considerably less numerous than those at the air/water interface because of the technical difficulties encountered, although the sensitive technique of cell electrophoresis has been somewhat neglected in this regard (1). Recently, we have utilized a simple model that appears to provide a reproducible technique for studying the electrophoretic mobilities of lecithin-protein complexes. Briefly, the method depends upon coating polystyrene latex particles[1] (6–14 μ diameter) with lecithin, followed by protein, and measuring the particle mobility with cell electrophoresis.

The cell electrophoretic procedure has been described in detail previously (2); the electrophoretic solution consists of a phosphate-sucrose buffer (pH 7.0, ionic strength 0.083). The particle-coating technique consists of suspending the latex in pure absolute ethanol containing lecithin, at room temperature, until a surface film of lecithin begins to form. The latex particles are then washed repeatedly in buffer solution and electrophoretic mobilities determined at 25 ± 0.1°.

Commercial egg lecithin and synthetic L-α-lecithin were used in addition to purified L-α-lecithin[2] to coat the latex. Protein coating experiments were performed by suspending latex or latex-lecithin particles in phosphate-sucrose buffer containing 0.5% bovine gamma globulin at room temperature for 1 hour, followed by repeated washings in buffer solution and cell electrophoresis determinations.

The results, summarized in Table I, reveal the following findings: (*a*) egg lecithin increases latex electrophoretic mobility, synthetic lecithin slightly decreases mobility, and pure lecithin markedly decreases mobility; (*b*) gamma globulin decreases latex-lecithin mobilities, and, with pure lecithin, decreases the mobility below that found with latex-protein alone. Analysis of the commercial synthetic L-α-lecithin revealed the presence of phosphorus-containing impurities,[3] thus accounting for the rather high mobilities obtained with this compound.

TABLE I.—ELECTROPHORETIC MOBILITIES[a] OF LATEX PARTICLES

Specimen	Electrophoretic Mobilities, u./sec./V./cm.
Uncoated latex	1.85
Latex/γ globulin	0.40
Latex/egg lecithin	2.45
Latex/egg lecithin/γ globulin	1.05
Latex/synthetic lecithin	1.65
Latex/syn. lecithin/γ globulin	0.70
Latex/pure lecithin	0.80
Latex/pure lecithin/γ globulin	0.25

[a] Mean negative mobilities, corrected to viscosity of water at 25°C., have S.D.'s <0.03 based on 20 timings per specimen.

These findings are consistent with the fact that the polar head of lecithin is a zwitterion with nearly zero net charge at a neutral pH (3) and has low electrophoretic mobility in isotonic saline (4). It is not possible, on the basis of the present experiments, to determine whether variations in degree of lecithin and/or protein coating account for differences in mobilities, whether underlying phosphate charges are capable of "showing through" the protein, or whether the various lecithins change the molecular configuration of the protein. Recent work has discussed the binding of proteins to lipids (3, 5, 6), the effects of various soap and salt concentrations on latex particle electrophoresis (7, 8), and the problems of interpreting surface electrokinetic potentials (9).

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[1] Polystyrene latex particles were donated by Dr. J. W. Vanderhoff, Dow Chemical Co., Midland, Mich.

[2] Pure L-α-lecithin was supplied by Dr. Erich Baer, Toronto, Ontario, Canada.

[3] We are grateful to Dr. Donald Hanahan, Seattle, Wash., for analysis of the commercial L-α-lecithin.

Rodent Repellency of Some Substituted Phthalimides

By JAMES E. FEARN* and JAMES B. DEWITT

Twenty-eight substituted phthalimides were either synthesized or procured, tested for rat repellency, and their indices of repellency computed. The data obtained were analyzed with the objective of correlating rat repellency with chemical structure and configuration. Although the results do not afford the postulation of a precise correlation, they do suggest that such a correlation exists.

THE FISH AND WILDLIFE SERVICE has long shown an interest in chemical substances which will deter or inhibit rodent attack upon packaged commodities. Tests upon more than 6500 compounds have shown that repellent activity may be correlated with composition and molecular configuration. Hydrocarbons, carboxy acids, ethers, alcohols, and esters have little repellency; but activity may be increased by introduction of nitrogen as a ring constituent or in substituent amido, amino, or nitro groups. Among the series of compounds showing promise in this respect, the *N*-substituted phthalimides are particularly interesting. Bellack and DeWitt (1) have reported the preparation and testing of 14 of these compounds along with several other groups of cyclic imides. The activity of some members of this series indicated that a more complete study of these compounds was advisable; therefore, a number of phthalimides containing substituents both on the imino nitrogen and at various positions in the benzene nucleus were synthesized or procured from cooperating agencies and industries. These compounds were screened according to the procedure of DeWitt *et al.* (2) and the repellency indices computed and tabulated.

Since investigations at this agency often have indicated that the addition of a nitro group to an aromatic nucleus tends to accentuate repellent activity, the decision was reached to prepare 3-nitro analogs of eight of the phthalimides previously studied.

PREPARATION OF COMPOUNDS

The compounds in Table I and half of those in Table II were prepared in laboratories of the Fish and Wildlife Service, Laurel, Md. *N*-(2-Bromoethyl)-phthalimide and its 3-nitro and 4-nitro analogs were prepared by Gabriel's synthesis as described by Weygand (3). The other imides were prepared by heating the amine with phthalic anhydride or phthalic acid to form the amine salt. This reaction product was then heated under reduced pressure to approximately 150° for varying periods of time in the manner reported by Bogert and Boroschek (4). *N*-(3-Cyanopropyl)-phthalimide was prepared in this fashion and converted to *N*-(3-carboxypropyl)-phthalimide through alkaline hydrolysis by the method described by Vogel (5). All synthesized compounds were recrystallized, usually from absolute alcohol or from alcohol containing 1–10% by weight of water, to a constant melting point. These melting points compared favorably with those given in the literature. These compounds were soluble in acetone, a little less soluble in alcohol, and very slightly soluble in water. Melting points for synthesized compounds are indicated. Compounds submitted by cooperating agencies were tested as received without purification.

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* Present address: National Bureau of Standards, Washington, D. C.

REPELLENCY TESTS

Individually caged laboratory rats weighing between 150 and 250 Gm. were given a choice of two food containers, one containing 20 Gm. of ground laboratory food and the other a 2% mixture of the test compound in the same food. Daily food consumption was determined through the 4-day test period. By substituting the data in the Bellack-DeWitt formula

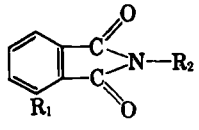
$$K = 100 - 1/100W \times (8T_1 + 4T_2 + 2T_3 + T_4) \times (U_1 + U_2 + 2U_3 + 4U_4 + 8X)$$

where W represents the body weight of the test animal in kilograms, $T_1 - T_4$ are grams of treated food consumed on the respective days, $U_1 - U_4$ are grams of untreated food consumed the respective days, and X represents grams of untreated food remaining at the end of the test period, the repellency indices (K) were computed. K values of 85 or higher indicated sufficient repellency to warrant more exhaustive investigation, such as barrier tests or simulated warehouse studies. Compounds showing indices of less than 85, but approaching it, often suggested other compounds of possible merit as repellents.

RESULTS AND DISCUSSION

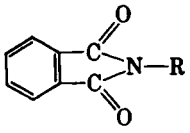
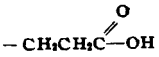
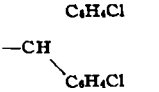
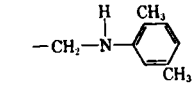
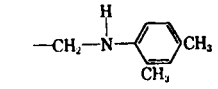
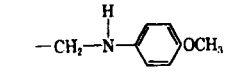
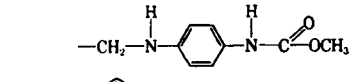
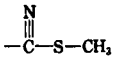
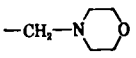
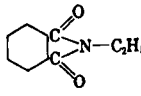
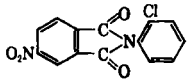
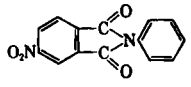
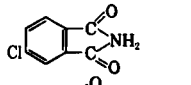
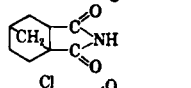
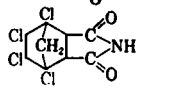
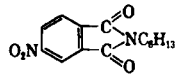
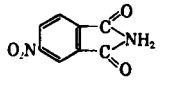
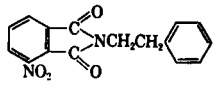
Results from the preparation and testing of 3-nitro analogs of previously studied phthalimides are given in Table I. The findings indicate that when the carbon chain of the *N*-substituent exceeded three carbon atoms, the compound containing the 3-nitro substituent was less repellent than the corresponding compound with an unsubstituted benzene nucleus. When the carbon chain was three carbon atoms or less in length, the converse was true. *N*-Bromoethyl-phthalimide gave a K value of 84, which was intermediate between that of the

TABLE I.—RELATIVE REPELLENCIES OF *N*-SUBSTITUTED PHTHALIMIDES AND 3-NITRO-*N*-SUBSTITUTED PHTHALIMIDES



R_2	Repellency Index, K $R_1 = H_1$	Repellency Index, K $R_1 = NO_2$	M.p., °C. $R_1 = NO_2$
CH_3	62.5	72	111
C_2H_5	53.8	87	104
C_3H_7	71.4	93	84
i—C_3H_7	77.2	81	92
i—C_4H_9	97.3	55	76
C_5H_{11}	95.4	93	74
C_6H_{13}	90.4	49	68
C_8H_{17}	64.6	59	71

TABLE II.—RAT REPELLENCY INDICES AND MELTING POINTS OF SUBSTITUTED PHTHALIMIDES

R	*K* Value	M.p., °C.
Part One		
		
	83	82
	80	78
	65	
	70	
	88	
	71	
	74	
	77	169
	91	138
	91	
	69	
Part Two		
	89	
	99	61
	87	69
	89	
	96	
	61	
	58	
	87	
	66	83

3-nitro analog [96] and the 4-nitro analog [82]. *N*-Butyl phythalimide and *N*-butyl-3-nitrophthalimide exhibited approximately the same repellency, 100 and 99, respectively.

Consideration of the compounds will indicate effects of changes in structure on the index of repellency. For example, *N*-(*o*-chlorobenzyl)-4-nitrophthalimide is decidedly more repellent than the corresponding *p*-chloro isomer. It is also evident that *N*-[2-(3-methyl-pyridyl)]-phthalimide is more repellent than *N*-[2-(6-methyl-pyridyl)]-phthalimide. It appears that molecular configuration and especially electronic and spatial phenomena connected with certain highly significant atoms in the molecule give rise to physical properties which cause rats to avoid contact with these substances, even though they have a low order of toxicity. The exact nature of these phenomena is not understood to the degree that precise predictions of repellency from structure may be attempted; but enough general information now is available to enable the investigator to reject, without testing, compounds with little likelihood of repellency. The result has been that the percentage of compounds tested that display appreciable repellency has increased decisively.

Data obtained from these studies suggest that a definite and precise relationship between rat repellency and chemical structure will eventually be determined, but that this relationship is complex and involves many factors which are not readily discernible. These data serve to support the contentions of Bellack and DeWitt that certain substituents tend to reduce the repellent activity of other substituents in given basic molecular structure. These results further suggest that a substituent which will enhance repellency when attached to one molecule may inhibit repellency when attached to another; *N*-(2-bromoethyl)-phthalimide is more repellent than *N*-(2-bromoethyl)-4-nitrophthalimide, while phthalimide ($K=18$) is much less repellent than 4-nitrophthalimide ($K=87$). Findings also support the idea that the lengthening of an aliphatic hydrocarbon chain of an *N*-substituent beyond four carbon atoms causes a diminution rather than an increase in repellent effect.

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Altered Response of Stressed Rats to Hypercholesterolemic Diets

By FAWZI DABORA* and MICHAEL M. CLAY

Rats were stressed for 2 weeks by restriction of movement, then fed a diet containing cholesterol, cholic acid, and calciferol for 2 weeks. The prestressed rats developed higher levels of plasma cholesterol and atherosclerosis than did unstressed controls. Plasma cholesterol increased in prestressed rats when the diet was supplemented with 250, 500, and 2000 U.S.P. units of calciferol. The degree of atherosclerosis was not directly related to the degree of hypercholesterolemia in all cases.

ENVIRONMENTAL "STRESS" is one of the factors being studied in the elucidation of the mechanisms of atherosclerosis. Increases in serum cholesterol levels have been reported in students in response to the "stress" posed by examinations (1–4). Page *et al.* (5), however, failed to find a causal relationship between changes in plasma levels of cholesterol, lipoprotein, or triglycerides and short-term "stresses" (tournament bridge playing and performance of surgery).

The contradictory results obtained in human studies may be due to nonuniform definitions of the state referred to as "stress" as well as to the varying periods of exposure to an unpleasant environment. Also, human studies are limited by lack of knowledge of prestudy experience and inability to study blood vessels for evidence of atherosclerosis.

After 14 days of partial restriction of movement of rats, Gabel and Clay (6) reported that systolic blood pressures were elevated. Further studies in this laboratory have demonstrated that peripheral plasma corticosterone levels were sharply increased by restriction of movement in rats. Page and Brown (7) reported that rats fed a diet containing 4% cholesterol, 2% cholic acid, and radioactive iodine for 6 months failed to develop atherosclerosis although plasma cholesterol levels increased.

The present study represents an attempt to induce the deposition of fat in the aorta of the rat by combining several of the treatments used by the above authors.

MATERIALS AND METHODS

Male Wistar strain rats were used. The rats were sacrificed by decapitation and blood was collected upon completion of each treatment. Plasma total cholesterol was assayed with Bloor's modification of the Liebermann-Burchard reaction, and plasma cholesterol esters were assayed by the same method after precipitation of free cholesterol with digitonin. Liver total lipids were determined gravimetrically after extraction with alcohol-ether. The thoracic aortas were removed, stripped free of adventitia, and fixed in 10% formalin. After longitudinal splitting, the flattened vessels were stained with Sudan IV and counterstained with hematoxylin.

Effects of Stress and/or High Cholesterol Diet.—The combined effect of three factors, stress, excessive cholesterol ingestion, and added dietary calciferol, were studied. Five groups of eight rats (140–215 Gm.) were subjected to five test conditions.

Group *1*.—Normal diet,[1] "stress free."
Group *2*.—Normal diet; restriction of movement for 14 days.
Group *3*.—Normal diet; "stress free" for 14 days, followed by a special diet,[2] "stress free" for 14 days.
Group *4*.—Normal diet; restriction of movement for 14 days, followed by the special diet, "stress free" for 14 days.
Group *5*.—Normal diet; complex stress: restriction of movement, heat (31 ±2°), and bright light for 14 days, followed by the special diet, "stress free" for 14 days.

Restriction of movement was imposed by housing a single rat in a clear plastic cage measuring 5.5 × 2.5 × 2.5 inches. The dimensions permitted the animal limited movement. Food and water were supplied at all times *ad libitum*.

Effects of Graded Calciferol Dosage.—Thirty-two rats (190–250 Gm.) were maintained on the normal diet with restriction of movement for 14 days. They were then divided into four equal groups and maintained for 14 days under "stress free" conditions on Turtox vitamin D deficient diet (General Biological Supply House Inc.), supplemented with 4% cholesterol and 2% cholic acid. Group *1* was fed the above diet only. Groups *2*, *3*, and *4* were fed the above diet supplemented with 250, 500, and 2000 U.S.P. units calciferol per 15 Gm. of food, respectively.

RESULTS

The results of the study on the effects of stress and/or high cholesterol diet are summarized in Table I. Group *2* (stressed) differed from Group *1* (unstressed) only in the reduction of food intake by about 50% and the presence of minute sudanophilic aortic areas in the former group. Feeding the special diet to Groups *3*, *4*, and *5* resulted in significantly elevated plasma total cholesterol, plasma cholesterol esters, and per cent liver lipids when compared with the values of Group *2* ($p = <0.01$ in each instance). Plasma total cholesterol, plasma cholesterol esters, and per cent liver lipids of Group *4* (restriction stress) and Group *5* (complex stress) were higher after the 14 days on the special diet than those of Group *3* (unstressed). The differences were significant for Group *4* only for plasma total cholesterol ($p = <0.02$) and per cent liver lipids ($p = <0.001$). Only the difference in per cent liver lipids of Group *5* ($p = <0.01$) was significant.

Aortic sudanophilia was present in seven of eight rats of Group *3*, seven of eight of Group *4*,

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[1] The normal diet consisted of Wayne Lab-Blox pellets (Allied Mills).
[2] The special diet consisted of powdered Wayne Lab-Blox pellets to which was added 4% cholesterol, 2% cholic acid, and 250 U.S.P. units of calciferol per 15 Gm. of diet. The calciferol content of Wayne Lab-Blox is 444.5 U.S.P. units per 100 Gm.

TABLE I.—EFFECTS OF STRESS AND CHOLESTEROL DIET

Treatment (Group)	Plasma Total Cholesterol, mg./100 ml.	Plasma Cholesterol Esters, mg./100 ml.	Total Liver Lipids, % wet wt.
1. Stress free, 14 days	84 ± 9[a]	55 ± 6	6.10 ± 0.46
2. Restriction, 14 days	88 ± 9	57 ± 10	5.65 ± 0.74
3. Stress free, 14 days; cholesterol, 14 days	279 ± 111	200 ± 102	10.25 ± 1.83
4. Restriction, 14 days; cholesterol, 14 days	382 ± 78	250 ± 72	13.34 ± 2.07
5. Triple stress, 14 days; cholesterol, 14 days	304 ± 80	238 ± 69	12.40 ± 0.91

[a] Standard deviation.

TABLE II.—EFFECT OF GRADED DOSES OF CALCIFEROL

U.S.P. units[a] Added Calciferol (Group)	Plasma Total Cholesterol, mg./100 ml.	Plasma Cholesterol Esters, mg./100 ml.	Total Liver Lipids, % wet wt.
1. 0	240 ± 103[b]	170 ± 64	15.36 ± 7 60
2. 250	293 ± 46	204 ± 24	13.56 ± 0.74
3. 500	348 ± 84	235 ± 75	11.73 ± 0.66
4. 2000	440 ± 115	257 ± 75	12.86 ± 1.76

[a] Seven of eight surviving rats in each group. [b] Standard deviation.

and six of seven surviving rats of Group 5. In Group 3, numerous small zones occurred in five rats, while generalized distribution occurred in two rats. In Group 4, numerous small zones occurred in one rat, while generalized distribution occurred in six rats. In Group 5, generalized distribution occurred in six rats.

The results of the study on the effects of graded calciferol dosage are summarized in Table II. Plasma total cholesterol and plasma cholesterol esters increased with each increment in calciferol dosage. Increases from group to group were significant only in the comparison between per cent total liver lipids of Groups 2 and 3 ($p = <0.001$) and plasma total cholesterol and per cent total liver lipids of Groups 3 and 4 ($p = <0.05$ and <0.02, respectively).

The aortas of five of the rats of the four experimental groups failed to show evidence of atherosclerosis. Although the aortas of the remaining rats showed varying degrees of atherosclerosis, there was no consistent pattern of atherosclerosis from group to group. Furthermore, there was no relationship between degree of hypercholesterolemia and severity of atherosclerosis between individual rats of each group.

DISCUSSION

Changes in fat metabolism, similar to those described here, have been reported elsewhere in rats only after thyroidectomy or thyroid depression (8). The temperature in the restriction cages was about 30°, although room temperature was maintained at 21–22°. The effect of elevated environmental temperature and restriction of movement upon thyroid gland activity is not known.

The differences reported among the rats maintained on four levels of calciferol cannot be attributed to different levels of food or cholesterol intake. Mean daily food intake ranged from 12.5 to 13.7 Gm. among the four groups. Mean daily food intake among the three cholesterol fed groups of the stress study were also relatively uniform, ranging from 18.1 to 18.8 Gm.

Page *et al.* (7) suggested that resistance of rats with normal or depressed thyroids to an atherogenic diet containing 4% cholesterol and 2% cholic acid was due to lack of vascular tissue response. The data presented here suggest that stress can overcome aortic resistance. Hauss *et al.* (9) have reported increased connective tissue metabolic activity in various organs of the rat, including the aorta after imposition of stress. They reported a similar finding in excised human atherosclerotic aortas. Similar stress induced changes in the aortic wall might predispose the tissue to the deposition of fat when a diet rich in cholesterol is subsequently fed.

Rats subjected to a triple stress showed more severe atherosclerosis but lower levels of hypercholesterolemia than did rats subjected to a single stress. When comparing individual rats within each group, there was no relationship between degree of hypercholesterolemia and atherosclerosis. Similar findings were made in the rats treated with graded doses of calciferol.

The mechanism whereby calciferol increased plasma free cholesterol and cholesterol esters is obscure. It has been suggested (9) that arteriosclerosis with calcium deposition in the vessel wall might be a precursor to fat deposition.

SUMMARY AND CONCLUSIONS

Prestressed rats showed higher plasma total cholesterol, plasma cholesterol esters, and per cent total liver lipids as well as intensity of aortic sudanophilia than did unstressed rats after 2 weeks on a cholesterol rich diet. The intensity of the plasma changes were accentuated by providing increased calciferol intake. Stress-induced alterations in aortic connective tissues and its mucopolysaccharides may play a role in fat deposition in the aorta when a diet rich in cholesterol is subsequently administered. The degree of atherosclerosis was not directly related to the degree of hypercholesterolemia in all cases.

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Alkalinized Glutaraldehyde, a New Antimicrobial Agent

By P. M. BORICK, F. H. DONDERSHINE, and V. L. CHANDLER†

A high degree of microbiological activity was demonstrated when aqueous glutaraldehyde solutions were alkalinized with the appropriate buffer. The potent bactericidal, tuberculocidal, fungicidal, sporicidal, and virucidal activity of alkalinized glutaraldehyde was not affected by the presence of serum and retained activity for a minimum of 2 weeks. Acidic glutaraldehyde solutions, stored at room temperature, are highly stable. Alkalinized glutaraldehyde solutions show a significant change in pH and loss of glutaraldehyde after 2 weeks. They have a mild odor and exhibit a relatively low order of toxicity as they are only slightly irritating to the skin when not removed. No damage to lensed instruments was observed with 2 per cent solutions.

THE IMPORTANT ROLE played by the environment in the spread of disease has emphasized the need for effective chemical disinfectants. The use of delicate instruments and equipment which cannot be heat-sterilized makes an extensive microbiological testing program essential in searching for potent antimicrobial agents (1). Adequate disinfection may not be achieved by agents destroying only bacterial forms. Antimicrobials intended for universal application should possess practical bactericidal, tuberculocidal, fungicidal, sporicidal, and virucidal activity as well. They should not be harmful to individuals or cause damage to instruments (6).

This is a report on alkalinized glutaraldehyde solutions[1] which meet most of these requirements.

EXPERIMENTAL

Test Agent.—The dialdehyde, glutaraldehyde (Union Carbide Chemical Co., N. Y.), undergoes typical aldehydic reactions to form acetals, cyanohydrin, oximes, and hydrazones. Oxidation results in glutaric acid, while reduction produces 1,5-pentanediol. Aqueous glutaraldehyde solutions are stable for at least 2 years while still acidic with little change in pH. Alkalinized glutaraldehyde solutions show a significant change in pH after 2 weeks.

Glutaraldehyde—$OCH(CH_2)_3CHO$—Antimicrobial action was demonstrated upon the addition of 0.3% sodium bicarbonate to 2% aqueous glutaraldehyde solutions; this alkalinization was necessary to elicit activity (5). When alkalinized, 2% aqueous glutaraldehyde solutions continued to show microbicidal activity for a minimum of 2 weeks at room temperature. These solutions were nonflammable and of low volatility. Glutaraldehyde concentrations were confirmed by the bisulfite iodometric technique of Siggia (7).

Test Organisms.—The following test organisms were used:

(*a*) Nonsporeforming bacteria: *Staphylococcus aureus*, American Type Culture Collection (ATCC) 6538; *S. aureus*, penicillin-resistant strain (Hunterdon Medical Center, Flemington, N. J.); *Streptococcus pyogenes*, ATCC 12384; *Diplococcus pneumoniae*, Type III (W. J. Nungester, University of Michigan); *Escherichia coli*, ATCC 6880; *Pseudomonas aeruginosa*, ATCC 10145; *Proteus vulgaris*, ATCC 6380; *Klebsiella pneumoniae*, ATCC 132; *Mycobacterium tuberculosis*, ATCC 7690.

(*b*) Sporeforming bacteria: *Bacillus globigii*, *Bacillus subtilis*, *Clostridium tetani*, *Clostridium perfringens* (L. S. Ortenzio, U. S. Department of Agriculture).

(*c*) Fungal strain: *Trichophyton interdigitale*, ATCC 640.

(*d*) Viruses: Poliomyelitis virus, Types I and II; Coxsackie virus, B-1; Echo 6; Herpes simplex; Vaccinia; Influenza A-2, Asian; Adeno virus, type 2; mouse hepatitis virus, MHV-3 (M. Klein, Temple University).

The bactericidal action of alkalinized 2% aqueous glutaraldehyde solutions was determined by the modified use-dilution method of Ortenzio and Stuart (4). This technique employs polished stainless steel cylinders (penicylinders) as carriers of the inoculum. After determining that each test organism had a uniform resistance to phenol, the inoculum was dried onto the penicylinders. The contaminated penicylinders were immersed in 10 ml. of the test agent and, after predetermined exposures, transferred to 40 ml. of eugonbroth (BBL) for incubation at 37° for 48 hours. All negative broth tubes were challenged for bacteriostasis by reinoculation.

Testing of alkalinized glutaraldehyde solutions on a human strain of *M. tuberculosis* was accomplished by exposing approximately 10,000 tubercle bacilli/ml. of saline to the test solution for 10 minutes at 30° (8). Samples of this mixture were used to inoculate Lowenstein-Jensen slants and inject the inguinal glands of guinea pigs. The slants were incubated for 4 weeks at 37°. The animals were sacrificed after 6 weeks, and all involved organs were examined for tubercle lesions.

Sporicidal tests were performed according to the method of the Association of Official Agricultural Chemists (5). This method contains features not found in earlier procedures. These are the inclusion of 2.5 *N* HCl as a standard reference solution to insure that the spores meet a prescribed resistance, porcelain penicylinders (Fisher Scientific Co., N. Y.) as inoculum carriers, and maintenance of 20° as the testing temperature. Each porous penicylinder holds approximately 1.3×10^6 spores. The test method and the stasis challenge were conducted as previously described. Incubation was in fluid thioglycollate broth (BBL) for 7 days at 37°.

Fungicidal action of aqueous, alkalinized glutaraldehyde solutions was assessed by exposing conidial suspensions of *T. interdigitale* in accordance with Association of Official Agricultural Chemists pro-

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[1] Marketed as Cidex Solution by Arbrook Division, Ethicon, Inc., U. S. pat. 3,016,328.

TABLE I.—BACTERICIDAL ACTIVITY OF AQUEOUS, ALKALINIZED 2% GLUTARALDEHYDE SOLUTIONS AT 20° C.

	Immediate Challenge	Challenged 2 Wks. After Activation	Challenged 4 Wks. After Activation
No. of samples tested	11	11	5
Mean pH	8.3	7.9	7.7
Mean % glutaraldehyde	2.02	1.71	1.50
	—Killing Time, Min.—		
S. aureus	<1	<1	<1
S. aureus, penicillin resistant	<1	<1	<1
Str. pyogenes	<1	<1	<1
D. pneumoniae	<1	<1	<1
E. coli	<1	<1	<1
Ps. aeruginosa	<1	<1	<1
P. vulgaris	<1	<1	<1
K. pneumoniae	<1	<1	<1

TABLE II.—TUBERCULOCIDAL ACTIVITY OF AQUEOUS, ALKALINIZED 2% GLUTARALDEHYDE SOLUTIONS AT 30° C.

	M. tuberculosis growth	
	Lowenstein-Jensen	Guinea Pig
2% Aqueous glutaraldehyde	−[a]	−
Aqueous control	+[b]	+
Saline control	+	+

[a] −, No growth and absence of lesions. [b] +, Growth and presence of lesions.

cedures (3). Subcultures were incubated at 25° for 10 days.

Antiviral activity of alkalinized glutaraldehyde solutions was determined according to the method reported by Klein (2).

RESULTS

Table I presents the bactericidal testing results of aqueous, alkalinized 2% glutaraldehyde solutions against Gram-positive and Gram-negative, nonsporeforming microorganisms. According to use-dilution techniques, the originally observed level of bactericidal activity appears to be maintained for 4 weeks after alkalinization and at pH 7.7 to 8.3 and at glutaraldehyde concentrations of 1.50 to 2.02%.

Table II describes the tuberculocidal activity of aqueous, alkalinized glutaraldehyde solutions against *M. tuberculosis* after 10 minutes exposure at 30°.

Table III hows the results of the same lots of aqueous, alkalinized 2% glutaraldehyde solutions when tested against the spores of aerobic and anaerobic bacteria. According to AOAC-20° testing, the originally observed level of sporicidal activity appears to be maintained for 8 weeks after activation at pH 8.3 to 7.7 and glutaraldehyde concentrations of 2.02 to 1.39%. It is interesting to note the change in pH and the loss of glutaraldehyde during these tests.

When preparations of alkalinized glutaraldehyde were tested against conidial suspensions (5×10^6/ml.) of *T. interdigitale* (Table IV), an exposure of 30 seconds did not yield viable spores.

Aqueous, alkalinized glutaraldehyde solutions prevented the multiplication of all viruses tested in less than 10-minute exposure at the 2% level (Table V). The susceptibility of the mouse hepatitis virus to glutaraldehyde does not necessarily infer a similar susceptibility to human hepatitis virus. However, since this is one of the few experimental hepatitis viruses which could be tested, the results are significant. Viability controls were positive for all strains tested.

The ability of antimicrobial agents to maintain their activity in the presence of organic material is extremely important, and alkalinized glutaraldehyde was tested with increasing concentrations of sterile bovine serum (Table VI). It is noteworthy that the serum was neither precipitated nor coagulated by the activated microbicide, and that all of the species tested appeared to be sensitive to alkalinized glutaraldehyde at the original levels previously noted regardless of the presence of organic matter.

The oral LD_{50} in mice was determined in our laboratories to be approximately 352 mg./Kg. of glutaraldehyde. Toxicological studies indicate that contact, as well as vapors, were nonirritating to the skin of laboratory animals or human subjects (9).

DISCUSSION

The addition of slight amounts of sodium bicarbonate to glutaraldehyde with its concomitant rise of pH resulted in broad antimicrobial activity accompanied by no detectable deleterious effects on the tissues of test animals. Both the aqueous and alcoholic preparations showed considerable antimicrobial activity (5) when this was done. It was felt that the aqueous preparation deserved greater attention since it possessed predictably a lower irritant and inflammability index than the alcoholic preparation. In addition, it is characterized by a lower volatility and will not exert solvent damage to adhesives in lenses, instruments, plastics, rubber, or other material.

The findings presented here strongly support the view that alkalinized glutaraldehyde is a highly effective germicidal agent. It exerts its activities

TABLE III.—SPORICIDAL ACTION OF AQUEOUS, ALKALINIZED 2% GLUTARALDEHYDE SOLUTIONS AT 20° C.

	Immediate	Post Activation Challenge 2 wks.	4 wks.	6 wks.	8 wks.
No. of samples tested	11	11	5	4	2
Mean pH	8.3	7.9	7.7	7.7	7.7
Mean % glutaraldehyde	2.02	1.79	1.50	1.40	1.39
			Killing Time, Hr.		
B. globigii	<3	<3	<3	<3	<3
B. subtilis	<3	<3	<3	<3	<3
Cl. tetani	<3	<3	<3	<3	<3
Cl. perfringens	<3	<3	<3	<3	<3

TABLE IV.—FUNGICIDAL ACTION OF AQUEOUS ALKALINIZED 2% GLUTARALDEHYDE SOLUTIONS AT ROOM TEMPERATURE

No. of samples tested	5
Mean pH	7.8
Mean % glutaraldehyde	1.41
	Killing Time, Min.
T. interdigitale	<0.5
Phenol 1:60, control	3–5

TABLE V.—VIRUCIDAL ACTIVITY OF AQUEOUS, ALKALINIZED 2% GLUTARALDEHYDE SOLUTIONS

Virus	Activity
Poliomyelitis, types I & II Echo type 6 Coxsackie B-1 Herpes simplex Vaccinia Influenza A-2, Asian Adeno type 2 Mouse hepatitis (MHV3)	complete inhibition in <10 min.

TABLE VI.—INFLUENCE OF SERUM ON THE MICROBICIDAL ACTION OF AQUEOUS, ALKALINIZED 2% GLUTARALDEHYDE

	Bovine Serum Concn., %		
	5	10	20
Mean pH			
Mean % glutaraldehyde			
Vegetative Bacteria	Killing Time, Min.		
S. aureus	<1	<1	<1
S. aureus, penicillin-resistant	<1	<1	<1
Str. pyogenes	<1	<1	<1
D. pneumoniae	<1	<1	<1
E. coli	<1	<1	<1
Ps. aeruginosa	<1	<1	<1
P. vulgaris	<1	<1	<1
K. pneumoniae	<1	<1	<1
Spores	Killing Time, Hr.		
B. globigii	<3	<3	<3
B. subtilis	<3	<3	<3
Cl. tetani	<3	<3	<3
Cl. perfringens	<3	<3	<3
Fungi	Killing Time, Min.		
T. interdigitale	<0.5	<0.5	<0.5

against Gram-positive and Gram-negative bacteria; it is very efficient in preventing germination of aerobic and anaerobic bacterial spore suspensions. Despite the contention that aqueous solutions of germicidal agents usually do not exert a tuberculocidal effect *in vitro*, the antimycobacterial activity demonstrated would exclude alkalinized glutaraldehyde from this category. While antifungal activity was tested with only one filamentous species, the speed with which conidia were eradicated suggests an efficient fungicidal agent. Since a high degree of microbicidal activity was demonstrable with alkalinized glutaraldehyde, one cannot deny the very broad antimicrobial capabilities possessed by this molecule. Its use in medical and commercial preparations for disinfection would seem appropriate.

SUMMARY AND CONCLUSIONS

The findings presented support the conclusion that aqueous, alkalinized 2% glutaraldehyde solution is a highly effective microbicidal agent. This broad spectrum antimicrobial killed Gram-positive and Gram-negative bacteria as well as tubercle bacilli within the minimum exposure time of the test. Alkalinized glutaraldehyde solutions killed aerobic and anaerobic spores in addition to the conidia of a common dermatophyte. Aqueous alkalinized 2% glutaraldehyde solutions were shown to be potent virucidal agents as well. The *in vitro* activity of this microbicide was not affected by the presence of bovine serum in the concentrations used.

Acidic glutaraldehyde solutions were stable for at least 2 years, while alkalinized 2% solutions retained full antimicrobial activity for 2 weeks. However, a diminution of glutaraldehyde concentration with changes in pH occurred with time when alkalinized glutaraldehyde solutions were tested during this period.

Aqueous glutaraldehyde solutions have a mild odor, low volatility, are nonflammable, and will not damage lensed instruments or equipment.

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Steroid Sapogenins VII. Identification and Origin of 25D-Spirosta-3,5-diene Among the Fenugreek Sapogenins

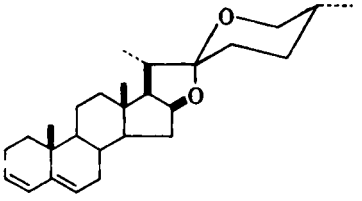
By M. S. BEDOUR, D. EL-MUNAJJED, M. B. E. FAYEZ, and A. N. GIRGIS

A reinvestigation of a certain fraction of the fenugreek sapogenin mixture led to the isolation of diosgenin, tigogenin, and gitogenin along with a fourth product identified with 25D-spirosta-3,5-diene. No evidence was found for trigonellagenin, which has been reported previously to occur in the same fraction. Evidence is presented which suggests that the diene is actually an artifact derived from diosgenin and produced early during the acid hydrolysis of the natural saponins.

THE STEROID sapogenins of fenugreek, *Trigonella foenum graecum*, seeds have had a few sporadic investigations. In 1943, Soliman and Mustafa (1) first alluded to the presence of a steroid sapogenin in the alcoholic extract hydrolysate of the seeds, shortly afterward Marker *et al.* (2) identified it with diosgenin. In a later communication (3), Marker *et al.* reported that the sapogenin mixture was made up mostly of diosgenin along with a small quantity of gitogenin and a trace of tigogenin. Soliman and Mustafa (4) then reported once again on the steroid sapogenins of fenugreek seeds and confirmed Marker's findings with respect to the presence of diosgenin and gitogenin, they did not mention tigogenin. Moreover, Soliman described another steroid sapogenin which he isolated in appreciable amounts from the mixture and, assuming it to be new, named it "trigonellagenin."

In the present search for steroid sapogenins in various sources from the local flora, fenugreek seeds were considered on account of their reputed (3) attractive diosgenin content. The processing of the seeds was conducted essentially according to the description of Wall *et al.* (5), where saponin-rich concentrates were prepared using *n*-butanol, and hydrolysis was effected by boiling in 4 *N* hydrochloric acid solution for 3 hours. The sapogenin mixture was next solvent-fractionated in the manner described by Soliman *et al.* (4) (*cf. Experimental*); the present report is concerned with that fraction from which the latter authors isolated trigonellagenin. A careful resolution of this fraction (of acetylated material) on alumina gave us tigogenin, diosgenin, and gitogenin along with a fourth adjunct having low polarity. The latter was purified with difficulty because of much contaminant matter and evidently possessed properties, m.p. 162–163°, $[\alpha]_D$ − 179°, different from those reported by Soliman *et al.* for trigonellagenin, m.p. 188–190°, $[\alpha]_D$ − 72.9°, acetate, m.p. 156–158° (4). No other products were isolated in appreciable amounts from this fraction.

The new product was soon realized to be nonhydroxylic, for it was recovered unchanged after treatment with acetic anhydride under normal acetylation conditions as well as after treatment with alkali. It also exhibited no apparent hydroxyl absorption in the infrared spectrum. However, its color reaction with the tetranitromethane reagent, taken together with the characteristic absorption (triplet max. at 229, 234, and 245 mμ) exhibited in the ultraviolet region of the spectrum, indicated the presence of a heteroannular conjugated diene system of the type present in cholesta-3,5-diene. That the compound possessed a steroid sapogenin nucleus with an intact spiroketal side chain was evident from the infrared spectrum which contained pronounced absorptions at 985, 920, 900, and 868 cm.$^{-1}$, with the 900 cm.$^{-1}$ peak stronger than that at 920 cm.$^{-1}$, an indication of 25D configuration. The substance was finally established to be 25D-spirosta-3,5-diene (I) by direct comparison with the synthetic product.



I

With regard to the origin of 25D-spirosta-3,5-diene, it is safe to say that it could not have possibly existed as a glycoside, engaging the hydroxyl of the open ring F in the corresponding furostane, in the natural fenugreek saponins—not only because of the experimental evidence reported here but also because of the established (6) fact that saponins, like their aglycones, possess intact spiroketal side chains. The isomerizing effect of mineral acids on the configuration at C-25 has long been recognized and is now well established (7). The susceptibility of steroid sapogenins to the dehydrating action of mineral acid does not, however, seem to have been sufficiently appreciated. We are in possession of evidence which strongly suggests that the 25D-spirosta-3,5-diene was an artifact produced from diosgenin, the principal sapogenin of fenugreek, during the acid hydrolytic processing of the saponins. This was sought by the direct treatment of chromatographically pure diosgenin with hydrochloric acid. The treatment (1 hour reflux, 4 *N* HCl) was milder than that normally employed for the hydrolysis of the saponins (3 hours reflux, 4 *N* HCl). Chromatographic resolution of the reaction mixture gave a crop constituting 18%, which was shown to be 25D-spirosta-3,5-diene and which was identical in all aspects with the fourth product isolated from the sapogenin mixture of fenugreek seeds. This reaction might also have led to some isomerization at C-25 as well as other unpredictable complications. This was evident from the continued analysis of the reaction mixture on thin layers of silica gel, although the period of reaction (1 hour) was far short of the time known (7) to be necessary for the attainment of equilibration. In a study of the hydrochloric acid-catalyzed equilibration of 25D and 25L spirostanes, Wall *et al.* (7) described an impure product, m.p. 130–140°, which was the sole crystalline substance

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resulting in low yield from refluxing yamogenin, 25L-spirost-5-en-3β-ol, with concentrated hydrochloric acid in ethanol for 96 hours. The product, which they rightly designated 25D-spirosta-3,5-diene, must evidently have resulted from the isomerization of the side chain to the more stable 25D configuration preceded by dehydration in ring A. The circumstances of its formation and its survival under such conditions are evidence to the durability of its structure, an observation which we endorse and which finds support in the results in this report.

We have also prepared the diene (I) by treatment of diosgenin methanesulfonate with pyridine under reflux, and the product was identical with the diene preparations already discussed.

In the literature (8) 25D-spirosta-3,5-diene had been previously identified among the sapogenin mixtures isolated from *Heloniopsis orientalis*, Thunb. and *Mianthemum dilatatum*, Wood. In both sources diosgenin was a prominent constituent of the mixture; therefore, it is likely that the diene was not a genuine natural product. Wall and Serota (9) have also described its synthesis by treatment of diosgenin tosylate with sodium iodide in acetone under pressure.

EXPERIMENTAL

The melting points were determined on a Kofler block and are uncorrected; the optical rotations were measured in chloroform in a 1-dm. tube at room temperature. The infrared spectra were determined with a Perkin-Elmer Infracord 137 spectrophotometer in sodium chloride cells in carbon disulfide solution.

Processing of Plant Material.—The powdered seeds of fenugreek were defatted by extraction with light petroleum, and a quantity (10 Kg.) was exhausted with hot ethanol. The extract was then concentrated and diluted with water (2 L.). The solution was repeatedly washed with light petroleum and again concentrated to 4 L. The saponins [1] were taken up in *n*-butanol saturated with water. This was done after the aqueous solution had first been treated with sodium chloride (5% w/v) and adjusted to pH 4 with hydrochloric acid. Concentration of the *n*-butanol extracts to a small volume was followed by treatment with hydrochloric acid and adjustment of the solution to 4 *N*. After a reflux period of 3 hours, water was added and the mixture worked up through chloroform. The dark resin so obtained was freed from much phenolic matter by refluxing in benzene (1 L.) with an alcoholic solution (300 ml.) of sodium hydroxide (50 Gm.) for 20 minutes. Working up the product gave 39 Gm. of crude sapogenin matter.

Isolation of 25D-Spirosta-3,5-diene.—The crude material mentioned above was fractionated using solvents according to the prescription of Soliman and Mustafa (4). Extraction of the mixture with light petroleum (b.p. 70–80°) in a Soxhlet apparatus removed 30 Gm. of brownish semisolid (*A*); then extraction with ether removed 3.9 Gm. of partly crystalline material (*B*). Fraction *A* was acetylated (acetic anhydride-sodium acetate, 2 hours of heating at 100°), and the product (32 Gm.) then was treated with 150 ml. of cold methanol. The insoluble part was again treated with boiling methanol (400 ml.) for 30 minutes and filtered while hot from insoluble matter. The hot filtrate was allowed to cool and again freed from deposited matter. The filtrate thus obtained was evaporated to dryness to give 8.58 Gm. of the fraction from which Soliman and Mustafa (4) isolated trigonellagenin acetate by repeated crystallization. Chromatographic resolution of this fraction on an alumina (210 Gm.) column gave the following characterized products.[2]

(*a*) A fraction (0.72 Gm.) eluted with light petroleum which after repeated crystallization from ethanol gave 25D-spirosta-3,5-diene, m.p. 162–163° (undepressed), $[\alpha]_D$ − 179°. [Reported m.p. 164°, $[\alpha]_D$ − 175° (9)]. The infrared absorption spectrum was identical with that determined for an authentic sample prepared from diosgenin (*vide infra*). The ultraviolet absorption spectrum (ethanol) exhibited triplet maximal absorption with peaks at 229, 234, and 245 (sh) mμ (ε 28000, 28600, and 18800, respectively). The substance gave a strong orange color reaction with tetranitromethane in chloroform.

(*b*) A fraction (1.04 Gm.) eluted with benzene/light petroleum (1:1) which afforded tigogenin acetate, m.p. 204–208°, undepressed by authentic sample, $[\alpha]_D$ − 71.63°. [Reported m.p. 204°, $[\alpha]_D$ − 73° (5)]. The infrared absorption spectrum was superimposible on that of authentic material.

(*c*) A fraction (0.35 Gm.) eluted with the same solvent mixture which after repeated crystallization gave diosgenin acetate, m.p. 190–194° (undepressed), $[\alpha]_D$ − 107°. The identity was further confirmed by comparison of the infrared absorption spectra. [Reported m.p. 202°, $[\alpha]_D$ − 115° (10)].

(*d*) A fraction (0.77 Gm.) removed with the same eluent which afforded gitogenin diacetate, melting point and mixed m.p. 233–236°. The infrared absorption spectrum was identical with that of authentic material.

(*e*) A fraction (0.75 Gm.) eluted with 3% chloroform in benzene which afforded tigogenin, melting point and mixed m.p. 201–204°, $[\alpha]_D$ − 78°. [Reported m.p. 206°, $[\alpha]_D$ − 67° (5)]. The identity was further confirmed by comparison of the infrared spectra and by acetylation to give a product identical in all respects with tigogenin acetate.

(*f*) A fraction (0.32 Gm.) removed with 3% ethanol in benzene which afforded gitogenin, m.p. 268–270° (undepressed), $[\alpha]_D$ − 80.6°. [Reported m.p. 268°, $[\alpha]_D$ − 63° (11)]. Acetylation of this material gave gitogenin diacetate, m.p. 233–237°, $[\alpha]_D$ − 91.7°. [Reported m.p. 237–242°, $[\alpha]_D$ − 98° (11)]. Infrared absorption spectra were identical with those of authentic samples.

Preparation of 25D-Spirosta-3,5-Diene.—A solution of chromatographically pure diosgenin (0.5 Gm.) in alcohol (150 ml.) was refluxed for 1 hour with enough concentrated hydrochloric acid to attain a 4 *N* mixture. After working up the reaction product, the residue (0.47 Gm.) was chromatographed on 35 Gm. of activated alumina. The course of fractionation was followed on thin layers of silica gel[3] using a mixture of acetone/benzene (1:5) for development and chlorosulfonic acid reagent for spraying the plates. Elution with dry benzene removed 0.086 Gm.

[1] The method of Wall *et al.* (5) for the enrichment of saponins was preferred to that described by Soliman and Mustafa (4) which involved the use of barium hydroxide and gave no satisfactory results in this investigation.

[2] The chromatography, having lasted several days, must have involved partial hydrolysis of the acetylated material.

[3] Actually it was adduced from various experiments that 1 hour reflux was optimal for the formation of the diene, R_f 0.95; for diosgenin, R_f 0.55 (same indicated solvent system).

of a crystalline product, m.p. 162–163°, which did not depress the melting point of the product isolated from the sapogenin mixture of fenugreek seeds. The infrared spectra were identical in all details. Elution with 2% methanol in benzene recovered most of the unchanged diosgenin.

Diosgenin Methanesulfonate.—This was prepared by treatment of a solution of diosgenin (0.34 Gm.) in pyridine (5 ml.) with methanesulfonyl chloride (2 ml.) at room temperature for 18 hours. Working up in the usual manner afforded 0.22 Gm. of the desired product which, after repeated crystallization from methanol, gave a pure sample, m.p. 162–163°, $[\alpha]_D$ − 108°.

Anal.—Calcd. for $C_{28}H_{44}O_5S$: C, 68.30; H, 8.94. Found: C, 68.27; H, 9.00.

A solution of this substance (0.1 Gm.) in pyridine (3 ml.) was refluxed for 10 hours. After dilution with water and isolation of the product, chromatography on a column of 4 Gm. of alumina gave a fraction (30 mg.) recovered with light petroleum. This was repeatedly crystallized from light petroleum-methanol to give a pure sample of 25D-spirosta-3,5-diene, melting point and mixed m.p. 160–162°, which was identical in all respects with the preparations described previously.

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Study of the Polyvinyl Alcohol-Borate-Iodine Complex III. Detection of Borates in Urine

By ANTHONY J. MONTE-BOVI, JOHN J. SCIARRA, and VINCENT DE PAUL LYNCH

A survey of the prevalence of boric acid toxicity indicates the difficulty encountered in detecting cases of poisoning by this commonly used substance. Because of this situation, an investigation was carried out utilizing a previously reported method for the detection of borates by the polyvinyl alcohol-borate-iodine reaction. The test was extended in this study to permit the detection of boric acid in the urine of poisoned animals. Rabbits were fed varying quantities of boric acid, and urine samples were collected. After concentration of the urine sample, the polyvinyl alcohol-iodine reagent was applied to the residue. Results of both *in vitro* and *in vivo* studies indicate that this method is capable of detecting as little as 0.3 mg. of boric acid (0.05 mg. B).

THE CHARACTERISTIC blue produced when solutions of polyvinyl alcohol (PVA) and iodine were brought into contact with boric acid has been reported in several diverse areas (1–5). Since most of these applications involved macroquantities of boric acid, there was need to determine the suitability of this method for the detection of smaller quantities and to extend the investigation further for the purpose of determining the possibility of utilizing this test for the detection of boric acid in animal fluids and tissues.

That such a test is desirable becomes apparent when one surveys the literature concerned with case histories of poisoning by boric acid. As early as 1904 (6) and as recently as March 1963 (7), attention was called to the toxic nature of boric acid. In a previous study, Sciarra (8) summarized the prevalence of poisonings due to boron compounds. Most cases reported in the literature involved children ranging in age from a few weeks to 12 years. In many of these instances it was noted that several days elapsed between the time of admission to the hospital and the time at which boric acid poisoning was first suspected. This problem is further complicated by the fact that the afflicted children usually were not given medical attention during the critical early stages of poisoning—apparently because the toxicity symptoms are not characteristic.

Many investigators have called attention to this problem. McNally and Rukstinat (9) commented on 58 cases, 28 of which proved fatal. Pfeiffer (10) revealed 86 cases, of which 42 were fatal. Goldbloom and Goldbloom (11) reported on 109 poisonings by boric acid. Valdes-Dapena and Arey (12) analyzed 175 cases, of which 86 resulted in death. It may be that there is some duplication in reporting these cases. Nevertheless, the records clearly indicate the prevalence of boric acid poisoning; and in spite of the many articles and warnings relative to the toxic nature of the boron compounds, poisoning continues. As late as 1962 (12) and 1963 (13, 18), accidental deaths due to the erroneous use of these compounds have been reported. It should also be noted that many of these investigators mention the probability that many such cases of poisoning go undetected, since the symptoms produced may be confused with those produced by other illnesses. This is especially true in cases of chronic toxicity; for acute poisoning usually demands rapid medical treatment which, while it may not be specific for boron compounds, will at least counteract the poison and prevent further damage to the victim. The problem is complicated further because, through the ingestion of certain foods boron can be found in the blood. A range of 0–0.72 mg./100 ml. of blood was reported (12). In addition, the expanding use of boron industrially

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THE CHARACTERISTIC blue produced when solutions of polyvinyl alcohol (PVA) and iodine were brought into contact with boric acid has been reported in several diverse areas (1–5). Since most of these applications involved macroquantities of boric acid, there was need to determine the suitability of this method for the detection of smaller quantities and to extend the investigation further for the purpose of determining the possibility of utilizing this test for the detection of boric acid in animal fluids and tissues.

That such a test is desirable becomes apparent when one surveys the literature concerned with case histories of poisoning by boric acid. As early as 1904 (6) and as recently as March 1963 (7), attention was called to the toxic nature of boric acid. In a previous study, Sciarra (8) summarized the prevalence of poisonings due to boron compounds. Most cases reported in the literature involved children ranging in age from a few weeks to 12 years. In many of these instances it was noted that several days elapsed between the time of admission to the hospital and the time at which boric acid poisoning was first suspected. This problem is further complicated by the fact that the afflicted children usually were not given medical attention during the critical early stages of poisoning—apparently because the toxicity symptoms are not characteristic.

Many investigators have called attention to this problem. McNally and Rukstinat (9) commented on 58 cases, 28 of which proved fatal. Pfeiffer (10) revealed 86 cases, of which 42 were fatal. Goldbloom and Goldbloom (11) reported on 109 poisonings by boric acid. Valdes-Dapena and Arey (12) analyzed 175 cases, of which 86 resulted in death. It may be that there is some duplication in reporting these cases. Nevertheless, the records clearly indicate the prevalence of boric acid poisoning; and in spite of the many articles and warnings relative to the toxic nature of the boron compounds, poisoning continues. As late as 1962 (12) and 1963 (13, 18), accidental deaths due to the erroneous use of these compounds have been reported. It should also be noted that many of these investigators mention the probability that many such cases of poisoning go undetected, since the symptoms produced may be confused with those produced by other illnesses. This is especially true in cases of chronic toxicity; for acute poisoning usually demands rapid medical treatment which, while it may not be specific for boron compounds, will at least counteract the poison and prevent further damage to the victim. The problem is complicated further because, through the ingestion of certain foods boron can be found in the blood. A range of 0–0.72 mg./100 ml. of blood was reported (12). In addition, the expanding use of boron industrially

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TABLE I.—DETECTION OF BORIC ACID ADDED TO AQUEOUS AND HUMAN URINE SOLUTIONS

Sample	H_3BO_3, mg.	B, mg.	Results[a] Aqueous Soln.	Results[a] Urine Soln.
1	0	0	−	−
2	50.0	8.75	++	++
3	25.0	4.40	++	++
4	12.5	2.20	++	++
5	6.3	1.10	++	++
6	3.2	0.56	++	++
7	2.5	0.44	++	++
8	1.3	0.23	+	+
9	0.6	0.11	+	+
10	0.3	0.05	+	+
11	0.15	0.03	*	*
12	0.03	0.005	−	−

[a] −, Negative test, as shown by the formation of rose-brown to yellow-brown; ++, strongly positive test, as shown by the formation of distinct blue to blue-black; +, weakly positive test, as shown by the formation of faint blue-red solutions leaving a distinctly blue ring around the edges of the dish as it stands for 10–15 minutes; *, inconclusive test, as shown by barely perceptible blue-red.

and in household products has increased the possibility of occupational poisoning by such substances (15).

Many tests have been developed for the purpose of detecting such boron compounds. The commonly known turmeric paper test has been unreliable (1) and unless carefully controlled will give false or confusing results. Smith *et al.* (15) report good results by using carminic acid to produce a color and measuring its intensity in a DU spectrophotometer. Draize and Kelley (16) also used a carminic acid reagent for a series of studies and reported good results.

Since the previously mentioned PVA-borate-iodine reaction gave reliable, accurate, and reproducible results on a macroscale, it was decided to investigate the possibility of adapting this test to the detection of lesser quantities of boric acid in both *in vitro* and *in vivo* conditions. The problems associated with the methods of detecting boron compounds in body fluids have already been reported (14–17).

EXPERIMENTAL

Detection of Boric Acid in Aqueous Solution.—A solution containing 50 mg. of boric acid U.S.P. in 10 ml. of water was prepared. Varying quantities of this solution were diluted as necessary, then placed into small porcelain casseroles in such volumes to yield a range of boric acid from 0.03 to 50 mg. as shown in Table I. A few drops of a 5% sodium hydroxide solution was added to render the solution slightly alkaline to litmus paper. This procedure decreases the volatility of boric acid by converting it to sodium borate. The resulting solution is then evaporated to dryness over a steam bath and re-acidified with 3–4 drops of hydrochloric acid to make the solution slightly acid to litmus; 3–4 drops of a 2% PVA solution[1] (51–05 or equivalent) and 1 drop of approximately 0.1 *N* iodine solution are added. A positive test is indicated by the appearance of a blue-black or blue color, the intensity of which depends upon the concentration of boric acid. A negative test produces a yellow, yellow-brown, or red-brown color. The results obtained are shown in Table I and represent the average of five determinations for each concentration of boric acid.

Detection of Boric Acid in Human Urine.—The above procedure was then adapted to detect the presence of boric acid in human urine and to determine whether the normal constituents found in human urine would interfere with the test and possibly lead to false results.

Samples of human urine were collected and tested for the presence of boric acid utilizing the procedure described under *Detection of Boric Acid in Aqueous Solution.* All samples gave negative results. Other samples of this same urine were then mixed with varying amounts of a solution containing 50 mg. of boric acid U.S.P. in 10 ml. of urine and diluted with urine if necessary to give the concentrations of boric acid as shown in Table I. The urine samples were then treated as for the aqueous solutions. The results are also shown in Table I. From this table, it can be seen that as little as 0.3 mg. of H_3BO_3 (0.05 mg. B) can be detected by this method.

Detection of Boric Acid in Animal Urine.—Since the addition of boric acid to voided human urine indicated the absence of naturally occurring substances which might interfere with the test, further investigation of its applicability to the detection of boron compounds in animal tissues and fluids was carried out.

Rabbits were chosen as the test animals for this study, mainly because of their ease of handling and because other investigators have used these animals for similar studies.

Initially, samples of urine were obtained by catheterization from normal animals maintained on similar routine diets. These samples were evaporated and treated as previously described under *Detection of Boric Acid in Human Urine.* All tests performed on these samples were negative and demonstrated that normal rabbit urine did not contain a substance which might interfere with this test.

Test animals were then given boric acid solutions by the oral and i. v. routes in concentrations as shown in Table II. Urine samples were collected by

TABLE II.—DETECTION OF BORIC ACID IN URINE OF RABBITS GIVEN BORIC ACID[a]

Dose of H_3BO_3 mg./Kg. of Body Wt.	Route of Administration	Time Period over which Urine was Collected, Hr.	Results[c]
50	I.V.	2	+
100	I.V.	1.5	++
50	oral	5	+
75	oral	5	+
110	oral	3	+
110	oral	4.5	+
150	oral	3	++
150	oral	4.5	+
215	oral	4	++
300	oral	4	++
300	oral	6	+
440	oral	4	++
570	oral	5	++
750	oral	5	++
1000[b]	oral	3	++

[a] Table II represents a minimum of three specimens at each dose level. [b] Lethal dose. [c] ++, Indicates a strongly positive test, as shown by the formation of distinct blue or blue-black; +, indicates a weakly positive test, as shown by the formation of faint blue-red solutions leaving a distinctly colored ring around the edges of the dish as it stands for 10–15 minutes; −, negative test would show as a rose-brown to yellow-brown.

[1] Marketed as Elvanol by E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.

catheterization. As shown in Table II, positive tests were obtained with the PVA-iodine reagent.

By a series of experiments which followed, rabbits were fed boric acid solutions of various concentrations (as shown in Table II) *via* a stomach tube. Samples of urine were collected by catheterization and tested for boron content. In all instances, control samples were collected from untreated animals. The results from these tests are shown in Table II.

DISCUSSION

As can be seen from Table I, as little as 0.3 mg. of H_3BO_3 (0.05 mg. B) can be detected in both aqueous and urine solutions with the PVA-borate-iodine reaction. The constituents normally found in urine did not interfere with the test, and quantities of boric acid above 0.3 mg. could easily be detected.

Starch, as an environmental contaminant, will give a positive test; therefore, its presence, if suspected, must be ruled out as suggested in a previous paper by Monte-Bovi and Sciarra (3).

The results of the PVA-iodine test when applied to samples of urine collected from animals treated or fed boric acid indicate the suitability of this test for these purposes. While we did not repeat these experiments on humans for obvious reasons, others (17, 19) have cited that boron compounds when absorbed by humans are found in body fluids and tissues.

While there are several other tests available for the detection of small quantities of boric acid as cited in the literature, the PVA-borate-iodine complex offers another accurate and sensitive method. This test can also be used as a confirmatory test when other methods give inconclusive results.

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Chromatographic Method for the Simultaneous Determination of Aspirin, Caffeine, and Acetaminophen

By K. THOMAS KOSHY

A procedure for the quantitative determination of aspirin, caffeine, and acetaminophen in a mixture is described using a modified chromatographic technique developed by Levine. In addition, this procedure can be used for the estimation of salicylic acid, if present in the mixture.

A REMARKABLE chromatographic technique for the analysis of mixtures of aspirin, caffeine, and phenacetin was developed by Levine (1) using a duplex column having aqueous solutions of sodium bicarbonate and sulfuric acid as immobile phases on a Celite support. The technique was extended by Heuermann and Levine (2) to the analysis of more complex mixtures. In principle, this technique is adaptable to the separation of acidic, basic, and neutral components in a mixture, provided suitable solvents are available. Since there are many formulations containing aspirin, caffeine, and acetaminophen (APAP), the purpose of this study was to determine these constituents in such a mixture. The study was further extended to the determination of *p*-aminophenol and salicylic acid in the presence of these components. The latter two are hydrolytic degradation products of APAP and aspirin, respectively.

EXPERIMENTAL

Preparation of Chromatographic Column.—Commercial acid-washed kieselguhr (Celite 545 Johns-Manville Corp.) was used in this study. It contained impurities which interfered in the analysis of *p*-aminophenol and was purified by boiling for 1 hour with concentrated hydrochloric acid, washing with water, and drying at 100°. The column was prepared as described by Heuermann and Levine (2). However, column arrangement was reversed. The column containing sulfuric acid as the immobile phase (column A) was mounted in such a manner that the effluent would flow into the column containing sodium bicarbonate as the immobile phase (column B). This was done to avoid contact of *p*-aminophenol with the sodium bicarbonate column since it was observed that this compound was not stable in the presence of bases.

Procedure.—Water-washed solvents were used throughout the chromatographic separation of the constituents. The sample size could be varied within wide limits depending on the composition of the material to be analyzed. Ethyl acetate was used as solvent for the sample. Chloroform could be used, provided the sample was first dissolved in a few milliliters of alcohol. However, excess alcohol should be avoided, as this would remove water from the column.

The columns mounted as described above were washed with 50 ml. of ethyl ether. The accurately

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Chromatographic Method for the Simultaneous Determination of Aspirin, Caffeine, and Acetaminophen

By K. THOMAS KOSHY

A procedure for the quantitative determination of aspirin, caffeine, and acetaminophen in a mixture is described using a modified chromatographic technique developed by Levine. In addition, this procedure can be used for the estimation of salicylic acid, if present in the mixture.

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EXPERIMENTAL

Preparation of Chromatographic Column.—Commercial acid-washed kieselguhr (Celite 545 Johns-Manville Corp.) was used in this study. It contained impurities which interfered in the analysis of *p*-aminophenol and was purified by boiling for 1 hour with concentrated hydrochloric acid, washing with water, and drying at 100°. The column was prepared as described by Heuermann and Levine (2). However, column arrangement was reversed. The column containing sulfuric acid as the immobile phase (column A) was mounted in such a manner that the effluent would flow into the column containing sodium bicarbonate as the immobile phase (column B). This was done to avoid contact of *p*-aminophenol with the sodium bicarbonate column since it was observed that this compound was not stable in the presence of bases.

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TABLE I.—ANALYSIS OF KNOWN MIXTURES OF APAP, ASPIRIN, CAFFEINE, AND *p*-AMINOPHENOL HYDROCHLORIDE

	Acetaminophen			Aspirin			Caffeine			*p*-Aminophenol HCl		
	Added, mg.	Found, mg.	Recovery, %	Added, mg.	Found, mg.	Recovery, %	Added, mg.	Found, mg.	Recovery, %	Added, mg.	Found, mg.	Recovery, %
1	491	491	100.0	977	960	98.3	103	103	100	...	...	...
2	330	325	98.5	647	627	96.9	64.3	65.9	102.5	...	...	...
3	650	657	101.1	1300	1303	100.2	137	136	99.3	...	...	...
4	500	512	102.4	507	499	98.4	104	103	99.0	...	...	...
5	511	500	97.9	999	981	98.2	162	159	98.2	...	...	...
6	503	506	100.6	1000	983	98.3	121	121	100.0	...	...	...
7	125	124	99.2	220.5	218	98.9	29.8	30.1	101.0	1.9	2.2	115.8
		125	100.0		215	97.5		30.1	101.0		2.0	105.3
		123	98.4		216	98.0		30.1	101.0		2.1	110.5
8	125	126	100.8	220.5	218	98.9	29.8	29.8	100.0	0.97	1.1	113.4
		127	101.6		218	98.9		30.1	101.0		1.2	123.7
		126	100.8		219	99.3		30.1	101.0		1.1	113.4
9	125	127	101.6	220.5	223	101.1	29.8	30.1	101.0	3.9	3.9	100.0
		127	101.6		222	100.7		30.1	101.0		3.9	100.0
		127	101.6		223	101.1		30.0	100.7		3.8	97.4

TABLE II.—ANALYSIS OF TWO COMMERCIAL TABLET FORMULATIONS OF APAP, ASPIRIN, AND CAFFEINE

	Acetaminophen			Aspirin			Caffeine		
	Labeled Amount, mg.	Found, mg.	Labeled Amount, %	Labeled Amount, mg.	Found, mg.	Labeled Amount, %	Labeled Amount, mg.	Found, mg.	Labeled Amount, %
1	125	120.4	96.3	230	229.8	99.9	30	29.0	96.7
		121.0	96.8		231.8	100.8		29.0	96.7
		119.3	95.4		230.5	100.2		29.0	96.7
2	162.5	165.0	101.5	325	314.0	96.6	32.5	32.5	100.0
		166.0	102.2		317.0	97.5		33.3	102.5
		162.0	99.7		317.0	97.5		33.5	103.1

weighed sample powder was dissolved in 100 ml. ethyl acetate in a volumetric flask. Quick dissolution was effected if the flask was heated gently on a steam bath for a few minutes. A suitable aliquot (2–5 ml.) was pipeted onto column A and allowed to run through column B into a 100-ml. volumetric flask. After all the solution had passed onto the adsorbent, the columns were washed with two 10-ml. portions of ether, allowing the first portion to run through the column before adding the second. More ethyl ether was passed through the columns until about 90 ml. was collected. The flask was removed and ether added to volume. This fraction contained APAP. A suitable aliquot was evaporated to dryness on a steam bath. The residue was dissolved in ethanol and diluted to a definite volume.

A second 100-ml. volumetric flask was placed as the receiver and the columns eluted with about 50–60 ml. of chloroform and brought to volume with more chloroform. This fraction contained caffeine. If necessary, a suitable dilution was prepared using chloroform as a diluent.

Column A was now removed but not discarded. Column B was eluted with 5 ml. of 10% glacial acetic acid in chloroform, followed by 90 ml. of 1% glacial acetic acid in chloroform. The effluent was collected in a 100-ml. volumetric flask and brought to volume with 1% glacial acetic acid in chloroform. This fraction contained aspirin and any salicylic acid that was present in the sample. Suitable dilutions, if necessary, were prepared with chloroform.

Column A containing *p*-aminophenol was eluted with approximately 40 ml. of ethyl ether to remove the chloroform from the previous operation. The ether washings were discarded. The Celite support was extruded from the column with air under pressure and collected in a 150-ml. beaker. The ether was evaporated by gentle heating on a steam bath. Exactly 20 ml. of 0.1 *N* HCl was added to the Celite, stirred to dissolve the *p*-aminophenol, and filtered through Whatman No. 44 filter paper. A 5-ml. aliquot of the filtate was used for the colorimetric determination of *p*-aminophenol using 1-naphthol as described by Greenberg and Lester (3).

The absorbance of each solution was determined on a Beckman model DU spectrophotometer against corresponding reagent blanks. The readings were taken at 245 mμ for APAP, 276 mμ for caffeine, 278 mμ for aspirin, 310 mμ for salicylic acid, and 580 mμ for *p*-aminophenol. The concentrations were calculated from the absorbance values of standard solutions of pure materials. The absorbance of aspirin is affected by acetic acid. Therefore, chloroform containing the same amount of acetic acid as the sample was used to prepare standard solutions of aspirin. This solution was freshly prepared for each analysis. In the determination of *p*-aminophenol, it was necessary to use a standard solution of *p*-aminophenol hydro-

TABLE III.—EFFECT OF COLUMN ON ABSORBANCE OF ASPIRIN SOLUTIONS

	A, 278 mμ		*A*, 310 mμ	
Aspirin, mcg./ml.	Original	Soln. Through Column	Original	Soln. Through Column
0.00	0.000	0.000		
24.17	0.171	0.170	0.000	0.020
48.34	0.360	0.355	0.000	0.028
72.51	0.550	0.540	0.000	0.036
96.68	0.730	0.720	0.007	0.049

TABLE IV.—ANALYSIS OF MIXTURES OF ASPIRIN AND SALICYLIC ACID (SA)

Added, mg.		Found, mg.		Recovery, %		
Aspirin	SA	Aspirin	SA	Aspirin	SA Exptl.	SA Corrected
24.16	00.00	23.80	...	98.5	...	...
24.16	3.02	24.16	3.62	100.0	119.9	100.0
24.16	6.03	24.21	6.51	100.2	108.0	99.2
24.16	9.05	23.94	9.67	99.1	106.9	101.0
24.16	12.07	24.76	12.50	102.5	103.6	99.3

chloride which was passed through the column and treated in the same manner as the sample. This standard was prepared by completely hydrolyzing APAP to *p*-aminophenol hydrochloride by refluxing for 30 minutes a solution of 0.2 Gm. of pure APAP in 45 ml. of 8 *N* HCl. After cooling, it was diluted to exactly 100 ml. with ethanol. A second dilution of 1 to 100 was made with ethyl acetate. Five milliliters of this solution was used as standard.

As a guide to the selection of sample size, the dilution of the effluent, and the preparation of the standard solutions, the following concentrations of materials gave the indicated absorbance reading: 50 mcg./ml. aspirin, 0.360; 10 mcg./ml. caffeine, 0.485; 5 mcg./ml. APAP, 0.430; 25 mcg./ml. salicylic acid, 0.740; and 2 mcg./ml. *p*-aminophenol, 0.370 (1-cm. cells).

RESULTS AND DISCUSSION

Results obtained on the analysis of synthetic mixtures of aspirin, caffeine, APAP, and *p*-aminophenol hydrochloride are given in Table I.

Quantitative recoveries were obtained for APAP, aspirin, and caffeine. There is good precision but variability in percentage recoveries for *p*-aminophenol. It may be mentioned that the quantities of *p*-aminophenol present in the mixtures are much higher than would normally be encountered in a tablet formulation of APAP. It is significant that *p*-aminophenol can be separated from the other components of the mixture, but the analysis of *p*-aminophenol itself is only of academic interest since the allowable limit for a tablet containing 125 mg. of APAP is only 0.06 mg.

Data on the analysis of two commercial tablet formulations of APAP, aspirin, and caffeine are shown in Table II. Most tablet excipients are either insoluble in ethyl acetate or would not absorb ultraviolet light in the concentration in the final dilutions used for spectrophotometric analysis. Therefore, this procedure should be applicable to other tablet formulations containing these active ingredients.

Attempts were made during this study to make simultaneous quantitative determinations of all components of a mixture of aspirin, APAP, caffeine, *p*-aminophenol, and salicylic acid. The results were always high for salicylic acid. Yet if there was no aspirin in the mixture, the results were very satisfactory for this compound. In these experiments, aspirin was allowed to remain on the column for the minimum time that was possible to prevent hydrolysis. Freshly prepared solutions of sodium bicarbonate were used to prepare the columns, as it was known that the pH of sodium bicarbonate increases on standing. The possibility of interference from impurities from Celite was ruled out because solvents alone passed through the column did not have absorbance at 310 mμ. A mixture of APAP, caffeine, and *p*-aminophenol hydrochloride passed through the columns under identical conditions did not cause interference. Levine (1) had reported results on the analysis of two mixtures of aspirin, phenacetin, caffeine, and salicylic acid. Although results for salicylic acid were high in both cases, no comments were made by the author. A solution of aspirin in chloroform containing 1.5% acetic acid has practically no absorption at 310 mμ, the wavelength of maximum absorption for salicylic acid. However, a solution of aspirin, which has been passed through the column, shows a slight absorption at this wavelength. This is illustrated in Table III.

It appears that the interference may be the result of slight hydrolysis of aspirin or some interaction of aspirin with the column material. This phenomenon was observed in all the experiments. The absorbance at 310 mμ for the aspirin solution passed through the column would cause an appreciable percentage error in the salicylic acid analysis because of the relatively small concentration of the latter in a realistic mixture of the two substances. This seemingly explains the results obtained from mixtures of aspirin and salicylic acid as shown in Table IV. The per cent recovery of aspirin was good, that of salicylic acid was high, but decreased as the salicylic content was increased.

Table III reveals that for solutions of aspirin passed through the columns, which gave absorbance readings of 0.360 and 0.550 at 278 mμ, the corresponding readings at 310 mμ were 0.028 and 0.036. Since it is possible to adjust the concentration of aspirin in a mixture containing salicylic acid to obtain a reading of about 0.4 at 278 mμ, it would seem possible to obtain a good approximation of salicylic acid content by subtracting 0.03 from the observed absorbance at 310 mμ. This correction factor was applied, and the results obtained are shown in the last column of Table IV. As can be seen, the corrected results are within 1% of theoretical, apparently validating this correction factor. However, this value might vary slightly under different operating conditions and should be determined experimentally.

SUMMARY

Mixtures of acetaminophen, aspirin, and caffeine were separated by a chromatographic procedure and determined quantitatively by ultraviolet spectrophotometry. This method is applicable to stability samples containing hydrolytic degradation products of acetaminophen and aspirin—namely, *p*-aminophenol and salicylic acid, respectively. In addition, salicylic acid can also be determined to obtain a reasonable estimation of aspirin stability.

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Ultraviolet Absorption of *trans*-3-Halo-2-decalones

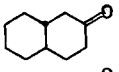
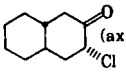
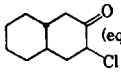
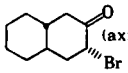
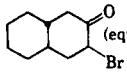
By O. KRISTIANSEN, E. E. SMISSMAN, and E. M. KOSOWER*

THE AVAILABILITY of some *trans*-3-halo-2-decalones from other research (1) allowed an examination of the solvent effect on the $n \rightarrow \pi^*$-transition. The results of the investigation of the ultraviolet spectra of these ketones in a series of solvents chosen for their range of Z-values (2) are reported in Table I.

The pattern of results for the 3-halo-2-decalones resembles that previously summarized for the conformationally fixed cyclohexanones (3). The change in $n \rightarrow \pi^*$-transition energy on substitution is fairly similar in both series as shown by the Δ-values listed in Table II.

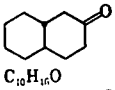
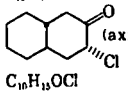
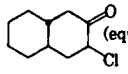
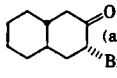
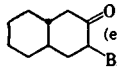
As noted before (3), the solvent sensitivities of the $n \rightarrow \pi^*$ transitions of the halo ketones is considerably lower than that for the unsubstituted ketones. It is imperative, then, that derived quantities (like Δ) for which some attempt at theoretical interpretation is made be recorded in the same nonpolar solvent.

TABLE II.—Δ-VALUES

	Δ (Kcal./mole)[a]	Cyclohexanones[a, b]
		
 (axial)	−5.42	−4.7
 (equat.)	+0.58	+1.89
 (axial)	−7.02	−7.4
 (equat.)	+0.59	+0.2

[a] Represents the difference in the $n \rightarrow \pi^*$ transition energy of the derivative and the parent ketone in a nonpolar solvent. [b] *Reference 3.*

TABLE I.—ULTRAVIOLET SPECTRA OF KETONES[a]

	Solvent			
	Isooctane (60.1)[b] $\lambda_{max.}$, Å.(ε$_m$)	Acetonitrile (71.3)[b] $\lambda_{max.}$, Å.(ε$_m$)	Methanol (83.6)[b] $\lambda_{max.}$, Å.(ε$_m$)	Water (94.6)[b] $\lambda_{max.}$, Å.(ε$_m$)
 $C_{10}H_{16}O$	2885 (18) 99.10	2845 (19) 100.49	2825 (22) 101.20	2783 102.73
 (axial) $C_{10}H_{15}OCl$	3052 (41) 93.68	3038 (44) 94.11	3026 (37) 94.48	...
 (equat.)	2868 (20) 99.68	2878 (23) 99.34	2846 (24) 100.46	...
 (axial)	3105 (104) 92.08	3103 (102) 92.14	3038 (102) 92.73	...
 (equat.)	2868 (28) 99.69	2855 (30) 100.14	2838 (31) 100.74	... ...

[a] Transition energies are given in Kcal./mole immediately below the figure for $\lambda_{max.}$. [b] Z-value (*Reference 2*).

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Synthesis and Activity of Phthalylglutamic Acid Analogs as Potential Folic Acid Antagonists in Bacteria

By W. A. ZYGMUNT and W. T. COMER

Four phthalylglutamic acid analogs were found to have no activity as inhibitors of bacterial growth in chemically defined media. More specifically, the compounds were not active as folic acid antagonists in bacteria. Cell permeability studies on these substances have not been conducted yet; consequently, this phenomenon is not ruled out as the mechanism whereby these compounds are inactive.

FAIGLE *et al.* (1) found that the principal metabolic products of *N*-(2,6-dioxo-3-piperidyl)phthalimide (thalidomide) in the dog were hydrolytic products and consisted chiefly of glutamic acid derivatives. *N*-Phthalyl-D,L-glutamic acid and *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid were shown to be two of the main hydrolytic products. Interestingly, thalidomide is the only sedative thus far described whose metabolites are glutamic acid derivatives. Kempner (2) reported that thalidomide antagonized the growth and sexual development of cockerels and suggested that thalidomide or certain of its metabolites may be folic acid antagonists. A clear illustration of the structural similarities between folic acid and some thalidomide metabolites also was shown by Faigle *et al.* (1). These workers suggested that the neurotoxic and embryotoxic effects of thalidomide or products of its metabolism may be due to an antagonism of glutamic acid or glutamine. This view was also held by Roath *et al.* (3), who showed that both isomers of thalidomide and certain degradation products of the drug inhibited development of human leucocytes. In addition, Frank *et al.* (4) found that thalidomide inhibited the growth of certain protozoa, and that this inhibition was reversed by nicotinic acid, adenine dinucleotide, and menadione.

On the basis that folic acid antagonists are known to be teratogenic (5) and that in many instances congenital malformations in animals are associated with vitamin deficiencies (primarily folic acid and riboflavin) and the suggestion of others that thalidomide metabolites may function as folic acid antagonists, it was of interest to test this hypothesis in a bacterial system. For this purpose, four phthalylglutamic acid analogs were synthesized and tested for possible antagonism of folic acid utilization and synthesis in a microorganism which requires preformed folic acid and in one which synthesizes all of its own vitamins and amino acids.

The test compounds, *N*-phthalyl-D,L-glutamic acid anhydride, *N*-phthalyl-D,L-glutamic acid, and *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid, were synthesized according to the methods of King and Kidd (6). The pure isomer, *N*-(*o*-carboxybenzoyl)-L-glutamic acid, was prepared according to the method of Nefkens *et al.* (7) for *N*-phthalyl-L-glutamic acid, followed by partial alkaline hydrolysis. This isomer had a corrected melting point of 159.5 to 160.5°, and $[\alpha]_D^{26} = -29°$ ($c = 1$, methanol). Identity of all compounds was confirmed by melting point determinations, elemental analyses for carbon, hydrogen, and nitrogen, infrared and NMR spectra, and optical rotations.

All compounds were tested for their ability to inhibit the growth of *Escherichia coli* B in the synthetic medium of Davis and Mingioli (8). In addition, they were tested for their ability to inhibit utilization of folic acid in Difco's folic acid assay medium, a modified medium of Capps *et al.* (9), using *Streptococcus faecalis* (ATCC 8043), a folic acid dependent bacterium. Neutralized solutions of test compounds, and of folic acid where applicable, were sterilized by Seitz filtration and portions added to previously autoclaved culture tubes containing graded levels of sterile distilled water and basal medium. Only freshly prepared solutions of test compounds were used. Final assay volumes were 6 ml. per culture tube (18 × 150 mm.). In the preparation of the inocula, precautions were taken to minimize the carry-over of preformed folic acid by appropriate washing of the cultures and by the use of small inocula. Incubation periods of 18–20 hours at 35° were used with both cultures. Growth was measured turbidimetrically as absorbance using a Coleman junior spectrophotometer at a wavelength of 620 mμ.

All of the glutamic acid analogs studied, at final concentrations of 1.0, 10, and 100 mcg./ml., fail to inhibit the growth of *S. faecalis* when cultured in the presence of 0.001 mcg./ml. of folic acid (a level required for about half maximal growth). At the concentrations tested, the ratios of inhibitor to metabolite screened were 1000:1, 10,000:1, and 100,000:1 (weight basis). In contrast, control tubes containing 4-amino-N^{10}-methylpteroylglutamic acid, a known folic acid antagonist, showed 64 and 95% inhibition of growth at inhibitor to metabolite ratios of only 1:1 and 8:1.

Growth of *E. coli* (a bacterium capable of synthesizing all of its own vitamins and amino acids) was not inhibited by concentrations up to 100 mcg./ml. of the glutamic acid analogs tested.

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Cell Surface Effects of a Phthalanilide Derivative

By P. S. VASSAR and W. L SANNE

Using the technique of cell electrophoresis to estimate surface charge density of mammalian tumor cells, a phthalanilide derivative (NSC 38280) caused a significant fall in cell electrophoretic mobility. These results suggest that the drug forms an irreversible complex with cell surface components, thereby lowering their zeta potential. The cell aggregation may account for the thromboembolism noted clinically.

A VARIETY of substituted phthalanilide derivatives possess antileukemic activity (1, 2). In view of the possibility that these compounds may form complexes with cell surface components, the present experiments were devised to study this phenomenon *in vitro*. The derivative used was the compound 2-chloro-4',4"-di(2-imidazolin-2-yl) terephthalanilide dihydrochloride (NSC 38280).[1]

The experimental system used was based on the sensitivity of cell electrophoresis to detect changes in charge density of cell surfaces following surface binding of drugs (3, 4). The cell electrophoresis equipment and technique have been described previously in detail (5). Briefly, the rectangular cell, maintained at 25°, was in the lateral position, and readings were taken at the first stationary level using a current of 4.0 ma. The phosphate-sucrose electrophoretic buffer had a relative viscosity of 1.14, pH 7.0, ionic strength 0.083, and osmolarity of 290 mosmoles. The tumor cells studied were Ehrlich ascites tumor cells, leukemic cells (buffy coat) from a patient with acute granulocytic leukemia, and cells from a patient with chronic lymphatic leukemia.

The washed cells were suspended in Hanks balanced salt solution containing NSC 38280 ($1.8 \times 10^{-4} M$, 100 mcg./ml.) for 30 minutes at room temperature; the cell concentration was 4×10^6/ml. The cells were then washed in phosphate-sucrose buffer and their electrophoretic mobilities determined. Control experiments, in the absence of NSC 38280, were run in parallel. Each experiment was repeated on three separate occasions. The results were highly reproducible, the figures in Table I represent the means of the experiments.

The results, summarized in Table I, show that exposure of tumor cells to NSC 38280 resulted in a highly significant ($P < .001$) drop in surface charge density. This finding suggests that the drug forms an irreversible complex with cell surface proteins and blocks surface anionic groups. In all instances, it is significant that tumor cells exposed to the drug underwent considerable aggregation presumably related to lowering of zeta potential. The mechanism of aggregation is probably complex, possibly initiated by London dispersion forces between small islands of adsorbed drug. These findings support the suggestion of York *et al.* (6) that phthalanilide-induced thromboembolism may be due to binding of the drug to cell surface components. The actual binding sites of the molecule include electrostatic forces of the imidazolin end-groups and hydrogen bonding at the amide bridges, although hydrophobic attractions have been suggested also (7).

During the course of these experiments an unexpected result emerged—namely, drug-treated red blood cells showed a slight increase in electrophoretic mobility: this effect was abolished if the red cells were pretreated with sialidase. At present the significance of these finding is obscure.

The possible relationship between NSC 38280 cytotoxicity and the cell surface effect is unknown. Phthalanilide derivatives have been shown to suppress protein synthesis (8), to complex with nucleic acids (7), and to be retained in leukemic ascitic fluid (9). In the present experiments a plausible explanation for the data may be some degree of denaturation of cell surface protein by the drug. It is of interest that another cytotoxic drug (chlorambucil) has been shown recently to affect cell surfaces (10).

TABLE I.—CELL SURFACE EFFECTS OF NSC 38280[a]

Tumor Specimen	Control u./sec./v./cm.	Control ESU/cm.$^{-2}$	NSC 38280 Treated u./sec./v./cm.	NSC 38280 Treated ESU/cm.$^{-2}$
Ehrlich's ascites tumor	1.33	2.66×10^3	1.05	2.10×10^3
Chronic lymphatic leukemia	1.45	2.90×10^3	1.18	2.36×10^3
Acute granulocytic leukemia	1.41	2.82×10^3	1.16	2.32×10^3

[a] Mean negative electrophoretic mobilities, corrected to viscosity of water at 25°, are expressed as u./sec./v./cm. Mobility values represent the average of 30 or more readings, with S.D.'s $\leq \pm 0.05$.

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[1] The authors are grateful to Dr. Joseph Burchenal for supplying this drug.

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Stability of Ascorbic Acid in the Presence of Ferrous and Ferric Ions

By M. C. UPRETY and V. K. MOHAN RAO*

It was reported earlier (1) that ferric (but not ferrous ions) have a deleterious effect on the stability of ascorbic acid in pharmaceutical preparations. Since sorbitol is a good vehicle for ascorbic acid, the effect of different concentrations of ferrous and ferric ions in 70 and 50% (w/v) sorbitol between the pH range 2.5 to 4.0 was examined. The results are presented in this communication.

The influence of ferrous sulfate (BDH) and ferric chloride (Riedel, De Haen; A. G. Seelze, Hanover) on the stability of 0.5% solution of ascorbic acid (E. Merck) was studied by storing the samples at the desired pH at 37° for 3 months in sigcol brown glass-stoppered bottles. Each sample contained 0.2% of *p*-hydroxy benzoate (Rhodia, France) as preservative. The vitamin was assayed by the 2, 4-dinitrophenyl hydrazine method, modified by Meyer *et al.* (2).

Results presented in Table I indicate that in 70% sorbitol ascorbic acid retention was best at pH 2.5. At the pH levels tried, the concentration of ferrous ions had no influence on the stability of this vitamin. Ferric ions, as reported earlier (1), had a deleterious effect, but varying concentrations of ions had no influence on the stability of ascorbic acid at pH 2.5 and 4.0.

Ferrous ion at the 0.175 and 0.150% level had no effect on the stability of ascorbic acid in 50% sorbitol at all the pH levels tried. Deleterious effect was noticed below 0.150% concentration of ferrous ions, unlike in 70% sorbitol; this effect was more at a higher pH level. This observation in 50% sorbitol might have something to do with the equimolar concentrations of iron and ascorbic acid. Generally, in the presence of ferric ions deleterious effect was observed at the three concentrations tried, but the change in pH had not affected materially the retention values of the ascorbic acid.

Table I.—Per Cent Retention of Ascorbic Acid[a]

Concn. of Iron in 100 ml.	% Retention		
	pH 2.5	pH 3.5	pH 4.5
	Vehicle 70% Sorbitol		
Nil (control)	73.6	58.2	54.3
Fe^{2+}			
0.175	66.0	66.5	62.5
0.150	72.3	68.8	62.1
0.125	72.3	59.8	62.2
0.100	72.2	...	
0.075	78.8	47.5	49.2
0.050	78.6	...	51.3
0.025	76.2	48.7	52.3
Fe^{3+}			
0.150	48.6	...	46.9
0.100	53.8	...	...
0.050	57.3	...	41.5
	Vehicle 50% Sorbitol		
Nil (control)	75.2	74.8	65.3
Fe^{2+}			
0.175	71.3	72.1	70.4
0.150	78.4	75.2	69.7
0.125	66.0	61.7	62.5
0.100	51.9	33.3	25.6
0.075	50.0	28.0	24.0
0.050	48.3	22.4	18.2
0.025	39.7	23.3	16.6
Fe^{3+}			
0.150	55.5	...	41.6
0.100	46.9	...	47.4
0.050	51.1		55.5

[a] Three-month storage period; temperature, 37°C.

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Bacteriostatic Property of *Aloe vera*

By LORNA J. LORENZETTI, RUPERT SALISBURY, JACK L. BEAL, and JACK N. BALDWIN*

Freeze-dried juice obtained from *Aloe vera* and heated for 15 minutes at 80° inhibited several test microorganisms.

SPECIES OF *Aloe* have had a long history as drug plants. This was pointed out by Morton (1) in a recent comprehensive review of *Aloe* from the standpoint of folk use and commercial exploitation. Fly and Kiem (2) recently carried out an invesigation to ascertain whether *Aloe vera* exhibits antimicrobial activity. They reported that macerates of the central gelatinous portion, of the green vascular portion, and of the complete leaf of *A. vera* did not exhibit, within the limits of their experiment, antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli*.

Since it has been established definitely in this laboratory that the fresh juice of *A. vera* L. contains a principle(s) which is inhibitory to certain microorganisms, the results are reported in this note.

EXPERIMENTAL

Leaves of *A. vera* L. were cut at the base and stood upright so that the juice could drain from the leaves into receptacles. If tested immediately, the fresh juice exhibited a marked zone of inhibition of *S. aureus* 209. However, the principle responsible for the inhibitory activity was found to be unstable. Preservatives as sodium bisulfite, sodium benzoate, and methyl paraben were ineffective; however, the principle could be temporarily preserved by refrigeration and preserved for an even longer period by heating the juice for 15 minutes at 80°. In all instances the juice would gradually turn dark. Once the juice became dark, the inhibitory property was lost. If the juice that had been heated for 15 minutes at 80° was freeze-dried, a buff-colored product resulted which was stable.

A solution of the freeze-dried juice (20 mg./ml. of distilled water) was tested by the agar diffusion technique for bacteriostatic activity against the following organisms: *S. auerus* 209, *E. coli*, *Streptococcus pyogenes*, *Corynebacterium xerose*, *Shigella paradysenteriae*, *Salmonella typhosa*, *Salmonella schottmuelleri*, and *Salmonella paratyphi*. After a period of incubation at 37° for 24 hours, significant inhibition of growth was evident on plates inoculated with *S. aureus* 209, *S. pyrogenes*, *C. xerose*, and *S. paratyphi*.

The whole leaf minus the juice, the leaf mesophyll, and the leaf epidermis were each separately freeze-dried and successively extracted with petroleum ether (b.p. 30–60°), ether, chloroform, ethanol, and distilled water. None of the extracts exhibited inhibitory activity against the test organisms.

Since the juice of *Aloe* is known to contain anthraquinone-type compounds, aloe-emodin, emodin, and chrysophanic acid were tested for inhibition of *S. aureus* 209. The results were negative.

CONCLUSIONS

While the freeze-dried whole leaf minus the juice, the leaf mesophyll, and the leaf epidermis of *A. vera* L. did not exhibit bacteriostatic properties, the freeze-dried juice previously heated for 15 minutes at 80° did inhibit *S. aureus* 209, *S. pyogenes*, *C. xerose*, and *S. paratyphi* using the agar diffusion test method.

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REFERENCES

(1) Morton, J. F., *Econ. Botony*, **15**, 311(1961).
(2) Fly, L. B., and Kiem, I., *ibid.*, **17**, 46(1963).

Communications

Determination of Isomeric Purity of Dextroamphetamine in Tablets and Capsules

Sir:

The tablet form of dextroamphetamine sulfate is widely used, has been official since the 15th revision of the "United States Pharmacopeia," and is produced by a large number of pharmaceutical companies. Enforcement work has brought to light several examples of adulterated products in which the *dextro* isomer has been replaced completely or in part by the less expensive racemate (1, 2). Nevertheless, the U.S.P. does not have a method to ascertain the isomeric purity of the drug present in the tablets. Because of the low specific rotation of dextroamphetamine sulfate (+23.5°) and the volatility of the base, certain derivatives are more suitable for determination of the optical activity. Acetylamphetamine has been particularly useful for this purpose. It can be prepared easily in good yield and its specific rotation (in chloroform) is about twice that of dextroamphetamine sulfate and of opposite sign (−44.0°). The main problem is the extraction and purification of the amine from the tablets prior to acetylation. Capsules of dextroamphetamine sulfate, especially those having sustained-release action, also present difficulties in the isolation of the base. In 1953, a procedure was de-

Bacteriostatic Property of *Aloe vera*

By LORNA J. LORENZETTI, RUPERT SALISBURY, JACK L. BEAL, and JACK N. BALDWIN*

Freeze-dried juice obtained from *Aloe vera* and heated for 15 minutes at 80° inhibited several test microorganisms.

SPECIES OF *Aloe* have had a long history as drug plants. This was pointed out by Morton (1) in a recent comprehensive review of *Aloe* from the standpoint of folk use and commercial exploitation. Fly and Kiem (2) recently carried out an invesigation to ascertain whether *Aloe vera* exhibits antimicrobial activity. They reported that macerates of the central gelatinous portion, of the green vascular portion, and of the complete leaf of *A. vera* did not exhibit, within the limits of their experiment, antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli*.

Since it has been established definitely in this laboratory that the fresh juice of *A. vera* L. contains a principle(s) which is inhibitory to certain microorganisms, the results are reported in this note.

EXPERIMENTAL

Leaves of *A. vera* L. were cut at the base and stood upright so that the juice could drain from the leaves into receptacles. If tested immediately, the fresh juice exhibited a marked zone of inhibition of *S. aureus* 209. However, the principle responsible for the inhibitory activity was found to be unstable. Preservatives as sodium bisulfite, sodium benzoate, and methyl paraben were ineffective; however, the principle could be temporarily preserved by refrigeration and preserved for an even longer period by heating the juice for 15 minutes at 80°. In all instances the juice would gradually turn dark. Once the juice became dark, the inhibitory property was lost. If the juice that had been heated for 15 minutes at 80° was freeze-dried, a buff-colored product resulted which was stable.

A solution of the freeze-dried juice (20 mg./ml. of distilled water) was tested by the agar diffusion technique for bacteriostatic activity against the following organisms: *S. auerus* 209, *E. coli*, *Streptococcus pyogenes*, *Corynebacterium xerose*, *Shigella paradysenteriae*, *Salmonella typhosa*, *Salmonella schottmuelleri*, and *Salmonella paratyphi*. After a period of incubation at 37° for 24 hours, significant inhibition of growth was evident on plates inoculated with *S. aureus* 209, *S. pyrogenes*, *C. xerose*, and *S. paratyphi*.

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TABLE I.—ISOMERIC PURITY OF COMMERCIAL DEXTROAMPHETAMINE SULFATE TABLETS AND CAPSULES

Mfr.	Product, mg.	Specific Rotation 1	Specific Rotation 2	Isomeric Purity, Av., %
A	Tablets, 5	−37.8°	−38.3°	93.2
B	Tablets, 5	−40.4°	−39.1°	95.2
C	Tablets, 5	−39.2°	−38.7°	94.3
D	Tablets, 10	−43.2°	−42.3°	98.6
E	Sustained-release capsules, 10	−38.7°	−39.5°	94.4

veloped by Welsh (1) for determination of the isomeric purity of dextroamphetamine in tablets based on the specific rotation of the acetyl derivative. This method, adopted by the Association of Official Agricultural Chemists (3), uses the titrated solution obtained after completion of the quantitative determination of dextroamphetamine sulfate as described in the official monograph. It has been recognized for some time that the assay of dextroamphetamine tablets in U.S.P. XVI is unsatisfactory and is, in fact, completely unsuitable for several of the preparations on the market (2). It will be replaced in U.S.P. XVII by a spectrophotometric method which utilizes partition chromatography for isolation and purification of the drug (4). However, this is a micromethod requiring only 5 mg. of dextroamphetamine sulfate, a quantity not enough for determination of the specific rotation. The difficulties of extracting a sufficient amount of dextroamphetamine from tablets and capsules have been eliminated by means of chromatography on aluminum oxide. The coloring agents and other excipients remain on the column, producing a clear colorless solution of dextroamphetamine which can be used for preparation of the acetyl derivative.

Procedure.—A pledget of fine glass wool is packed with a packing rod in the base of a chromatographic tube, 25 × 200 mm., and 5 Gm. of basic chromatographic alumina[1] is added to the tube and compressed firmly to a uniform mass. Dextroamphetamine sulfate tablets (or the contents of capsules), equivalent to about 130 mg. of dextroamphetamine sulfate, are reduced to a fine powder and mixed thoroughly in a mortar with 5 Gm. of basic chromatographic alumina.[1] After addition of 1.0 ml. of methanol and 0.5 ml. of stronger ammonia T. S., the mixture is triturated and, when uniform, transferred without delay to the chromatographic column and compressed with a packing rod. The mortar and pestle are wiped with a little glass wool which is inserted into the tube on top of the column. A 125-ml. separator containing 35 ml. of 0.1 *N* sulfuric acid is placed under the column as a receiver, and 60 ml. of chloroform is passed through the column. The separator is shaken vigorously for 1 to 2 minutes, the layers are allowed to separate, and the chloroform discarded. To the liquid in the separator is added 2.5 Gm. of sodium bicarbonate, and the mixture is swirled until most of the bicarbonate has dissolved. With a 1-ml. syringe, 1.0 ml. of acetic anhydride is injected rapidly into the contents of the separator. The separator is stoppered immediately and shaken vigorously until the evolution of carbon dioxide has ceased. The pressure is released as necessary through the stopcock. The solution is allowed to stand for 5 minutes, then extracted once with 50 ml. of chloroform. The chloroform extract is filtered through a pledget of cotton into a 100-ml. beaker. The cotton is rinsed with a little chloroform, and the solution evaporated to dryness on a steam bath in a current of air or nitrogen. The residue is heated and triturated with a glass rod until the odor of chloroform can be detected no longer. As the residue cools, it is induced to crystallize, powdered finely, and heated to 80° for 30 minutes.

After cooling in a desiccator, about 100 mg. of the crystalline acetylamphetamine, accurately weighed, is transferred to a 5-ml. volumetric flask and dissolved in chloroform. The optical rotation is determined by means of a 200-mm. semimicro polarimeter tube and the specific rotation calculated.

The procedure has been used for a number of commercial samples of dextroamphetamine sulfate tablets and capsules. The results in Table I indicate that the method has good reproducibility.

Optically pure dextroamphetamine sulfate has a specific rotation of +23.5° (1). On this basis, the official tolerance limits of +20 to +23.5° correspond to 92.6 to 100.0% of the *dextro* isomer. Dextroamphetamine sulfate of 92.6% isomeric purity would yield an acetyl derivative with a specific rotation of −37.5°.

(1) Welsh, L. H., *J. Assoc. Offic. Agr. Chemists*, **36**, 714 (1953).
(2) *Medical Letter on Drugs and Therapeutics*, **3**, No. 19, (Sept. 15, 1961).
(3) "Official Methods of Analysis of the Association of Official Agricultural Chemists," 9th ed., A.O.A.C., Washington, D. C., 1960, p. 528.
(4) Moody, J. E., THIS JOURNAL, **52**, 791(1963).

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[1] Aluminum oxide, Merck (Darmstadt), standardized according to Brockmann, was used in this work.

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Review Article

Toxicity, Untoward Reactions, and Related Considerations in the Medical Use of Plastics

By JOHN AUTIAN

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THE FIFTIES ushered into the medical and related professions a host of devices or items of one type or another composed in part or whole of a plastic material. Nearly a geometric increase in the use of these plastic items has occurred each year. The spectrum of uses today defies any ordinary listing, and it now appears possible that with the correct choice of plastic any desired property can be achieved. For the most part the medical and other ancillary professions have accepted these plastic items with a great deal of confidence—a confidence which perhaps is not entirely justified. Part of this acceptance of plastics for any and all uses stems from a lack of knowledge on the subject of plastics by clinicians, nurses, pharmacists, and hospital administrators. Other segments of the population are also finding that certain polymeric materials and their additives might cause harmful effects to the purchaser, as may be witnessed by recent findings in the United States that a plastic molding toy has caused over 1600 skin irritations.[1] It seems logical then that as more information is accumulated by medical and allied professions on plastics, a greater safety feature will be forthcoming, not only to patients but also to the general public. This review attempts to cover some of the problems encountered in the use of plastics in medicine and is intended to alert those interested in them that consequences—from very minor to extremely serious—may fall upon the user or patient if proper safeguards are not taken. Knowledge of this type can help all segments of the public health professions and their suppliers to produce and use only those items which insure absolute safety for intended use.

In this review the author has attempted to

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[1] From a U. S. Department of Health, Education and Welfare, Food and Drug Administration, News Release, May 17, 1963.

cover the general field (without claiming a complete coverage) of toxicity and untoward reactions which have or may have a direct or indirect effect upon the welfare of the patient.

TOXICITY AND UNTOWARD REACTIONS

General

Toxicity or tissue sensitivity reactions in animals can take place by two distinct routes: (*a*) by direct contact of the plastic material or product with tissue and (*b*) by indirect contact with tissue, such as injection or application of a solution (drug product, nutritional product, blood, etc.) which has had previous contact with a plastic. A number of reports have appeared in the past discussing the toxicity of various polymers and the other ingredients which are utilized to prepare a plastic product (1–8). For the most part these reports have dealt with industrial health problems in relation to the manufacturing of a plastic material. As is well known, many of the chemicals used in the synthesis of a polymer are highly toxic if proper care is not taken to safeguard the health of the worker. However, the discussion presented in this section will deal with the finished plastic material. Texts by Rolf (9) and Wesolowski (10) should be referred to for more detailed and specific information on the use of plastics in medicine and surgery.

Direct Effect on Tissue (Acute)

Pure Plastic (No Additives).—It is a natural biological consequence that any material introduced into tissue will show some degree of reactivity, even if this cannot be truly detected with present means of analysis. Results and conclusions must be viewed as relative and in comparison to a standard state. This "standard state," of course, poses a problem since different investigators may use different standards for comparison. Many of the conflicting reports, or at least differing opinions, dealing with plastics should be examined in the light of what has been said. One other additional factor is relevant, however, in regard to plastics, especially when the same generically named plastic has been used by more than one group with varying results. Since there are no standards for plastics to be used in medical practice, it is very probable that the "same" plastic might not actually be the same, even though the difference may not be apparent by visual observation.

A great body of evidence is readily obtainable to support the claim that a pure plastic material is inert when in contact with normal, healthy, unbroken, or untraumatized tissue such as the skin or mucous membrane (11–13). The use of synthetic yarns in various clothing apparel for a number of years by millions of persons with little incidence to tissue reactivity attests to this statement. Unfortunately, less information is available as to the effect of a pure plastic introduced into tissue of ill or diseased patients.

Early reports of tissue sensitivity, dermatoses, or other untoward reactions ascribed to a pure polymer may be—in the light of present knowledge—traced to the presence of monomers or other low molecular weight polymers rather than to the polymer itself. A good example of this may be seen by referring to the literature in both the dental and ophthalmic professions where acrylic resins were used (and, of course, are used) as prosthetic devices. For one reason or another in these early uses of the acrylic resins, the monomer was not completely polymerized and this caused the tissue response. Contact lenses have caused some difficulty to a small number of patients out of the nearly 5 million wearers. Recently, however, a report pointed out that 14 proved cases of blindness or near-blindness were attributed to contact lenses (14). One suspected cause of blindness is the possibility of a chemical impurity in the plastic migrating onto the surface of the eye.

Patty (15), LaVeen and Barberio (16), and Scales (17) have emphasized the importance of the removal of the monomers before actual medical use. There have been instances of tissue response to other polymeric materials, but most of these can also be traced to factors other than the polymeric material. Harris (12) states that the final polymerized chemical (plastic) is inert to tissue and cites the studies by Schwartz (18), Morris (11), Hine *et al.* (19), Zapp (20), and Calnan *et al.* (21), to support his contention. The reader should note, however, that these reports were based upon experiments on the skin.

Introduction of a pure plastic into the tissue (subcutaneous, intramuscular, intraperitoneal, etc.) has been found in numerous instances to be well tolerated by the host tissue (at least for short periods), but it would be incorrect to assume that tissue sensitivity will not appear. There is difficulty in assessing the results from this type of investigation since the reaction or lack of reaction will depend upon such factors as its particular site of implant, the size of implant, the degree of blood supply, the possible trauma effect of implantation, and the time of tissue contact. It is also possible that even here a reaction might have been caused by a contamination of one form or another rather than by the pure plastic.

Some authors have assumed that tissue re-

activity was due to the pure polymer after short contact with tissue. For example, Harrison *et al.* (22) indicated that Dacron, Ivalon, nylon, Orlon, and Teflon did produce some tissue sensitivity when subcutaneously implanted in dogs. In their study nylon was found to be the least acceptable, while Teflon was the most tolerated plastic in the series. Usher and Wallace (23) found that nylon, Orlon, Dacron, and Teflon were less tolerated in dogs (up to 1 week) than Marlex. Longer periods of contact with tissue (perhaps up to a year) might produce further tissue reactions, depending upon the particular plastic.

Little and Parkhouse (24) investigated a number of plastic materials as to their potential tissue reactivity in guinea pigs and noted that a correlation could be established between crystallite size in the plastic or the size of an added filler to the plastic and the incidence to fibroblast reactions. They employed X-ray diffraction methods to approximate the size of the crystallites or fillers. Results of their study demonstrated that the silicones and low-density and medium-density polyethylene produced little incidence to reactions whereas other types of plastics could elicit responses.

Compounded Plastics (Polymer plus Additives).—The propensity of toxic responses in animals and humans would seem to increase for those plastic materials which require the presence of other ingredients to impart a specific desired property. The evidence to support this previous statement is more difficult to find in the literature since little practical use is made of compounded plastics by surgeons as possible prosthetic devices. In recent years, however, more use is being made of compounded plastics such as polyvinyl chloride for tubings and protective coverings. For example, in-dwelling catheters and other types of catheters which are introduced into a natural body orifice or inserted through a surgical procedure will have contact with tissue which might be susceptible to a leached constituent from the plastic material. Actual clinical reports of this occurrence have been rare in the past, but results from several animal studies do indicate that toxicity of leached constituents is a real possibility. The work of Brewer and Bryant (25) may be cited as an example to demonstrate that even certain commercially available plastic devices used in medical practice can cause tissue sensitivity when implanted in various animal tissues for short periods of time.

Lawrence *et al.* (26) conducted a toxicity study on a number of commercially available plastic administration devices and found that out of 48 samples tested by intramuscular implantation in animals, 25 were found to cause a reaction after 1 week of contact. Most of these devices were tubings of the vinyl type and were packaged in sealed cartons ready for use. This particular investigation indicated that one or more ingredients in the tubings were being released to the tissue. Even though the actual offending agent was not ascertained, it was demonstrated that the plasticizers were not the causative agents in producing tissue reactions. Gas chromatographic techniques suggested that the offending agent was one of the additives incorporated in the plastic in minute quantities. Alcoholic extraction of the "toxic" tubings removed the offending agent from the tubing.

The above authors make a very poignant conclusion in their paper. They state the following:

> The question of course may now be raised that the tubings which are reported in this paper were never intended to be implanted either in animals or humans; consequently, for their intended use, they may be quite safe. Questions of this sort can best be answered by stating that good public health practice would seem to dictate that no plastic item should contain an ingredient which has a potential harmful ingredient that might leach into a solution to be administered to a patient.

Autian *et al.* (27) noted that one type of vinyl urinary bag caused tissue reaction in animals, while a similar type of bag from another manufacturer showed no ill effects.

On a number of occasions in-dwelling catheters have caused problems in patients. Bansmer *et al.* (28) documented a number of complications when catheters were placed into the inferior *vena cava*. Some of the complications were local thrombosis, thrombosis with embolism, suppurative thrombophlebitis with septicemia, and chemical necrosis. Derrick, in animal experiments, concluded that intra-aortic catheters cannot be left for a prolonged period of time without precipitating thrombosis and embolism to vital areas (29). Several reports have also appeared indicating that in-dwelling catheters have been severed and lost in the arm of patients (30). The break in the catheter may have been due to a bending action on the catheter with movements of the arm. Urinary catheters have increased the incidence to bacteriuria in patients (31). The exact reason or reasons for these catheters causing the infection is still unclear.

Direct Effect on Tissue (Chronic)—Cancer

Since no evidence has appeared in humans that a certain plastic material is the causative agent

in the production of tumors, there might be a natural tendency to discount these "inert" materials (plastics) as possible carcinogens. At the moment we must be content to view certain animal experimentations which have demonstrated that long implantations of plastic materials cause responses which would rightly be considered as carcinogenic.

Turner (32) appears to have been one of the first to detect the carcinogenic activity of plastics. In 1941 he reported his findings which showed that implanted Bakelite disks in rats gave rise to sarcomas at the site of implants. In the latter part of the forties, Oppenheimer *et al.* (33) observed that cellophane wrapped around rat kidneys for a period of 24 months gave rise to tumors. This was a chance discovery since these investigators were actually experimenting on hypertension. Further work by the Oppenheimer group reported in 1952 (34) that various types of plastic films caused a certain percentage of sarcomas in rodents tested. Druckrey and Schmahl (35) were able to produce sarcomas in rats at the site of implantation with regenerated cellulose film. These authors noted that the tumors were produced in a strain of rats which had shown no spontaneous disposition to sarcomas during a prior 12-year period.

In 1953, Oppenheimer *et al.* (36) reported the testing of a number of films by implantation in mice and rats. They found the production of malignant tumors when films of regenerated cellulose, pure and impure polyethylene, polyvinyl chloride, Silastic, Teflon, Dacron, polystyrene, and nylon were used. Druckrey and Schmahl (37), in a continuation of their earlier studies, reported that they had produced tumors in rats with a number of polymers, while the team of Laskin *et al.* (38) observed a 25% occurrence of fibrosarcomas in mice after implantation of methyl methacrylate film.

Further work by the Oppenheimer group (39–42), reports by Bering *et al.* (43), Nothdurft (44), Hueper (45–47), Russell *et al.* (48), and Bing (49) clearly show the incidence of sarcomas when various types of plastics are implanted in animals.

An analysis of the previous workers' data and conclusions offers two schools of thought concerning the causative mechanism of polymer cancer. A minority group, particularly Hueper (47) and Druckrey and Schmahl (35, 37), have advanced the hypothesis that a chemical or a physicochemical interaction between the polymer and the tissue is the chief causative factor in the production of tumors, while a much larger group of investigators, *i.e.*, Oppenheimer *et al.* (39), Nothdurft (44), and Russell *et al.* (48) support the physical or nonspecific theory of polymer carcinogenesis.

The chemical theory viewers postulate that such factors as end groups, free radicals, complex formation between the polymer and protein, degradation products of the polymer, and/or the presence of impurities in the plastic might initiate cancer production. On the other hand, those of the physical theory approach point out that many chemically unrelated substances cause cancer, but a specific substance in a number of physical forms, *i.e.*, film, perforated film, powder, size of implants, smoothness of implant, etc., may or may not cause cancer.

The physical theory school would seem to believe that induction of tumors by plastics is indirect and may be due, in a large part, to a general interference with normal cell growth at the interface between the plastic and the tissue. The critical factor, either stated or implied by this group, is the total uninterrupted surface contact. For example, films which have induced tumors will show less tendency to do so when they are perforated, and powders of the polymer show little or no tendency to cause tumors. Horning and Alexander (50) have observed a correlation between the size of the surface area of the implant and tumor production, while a recent report by Oppenheimer *et al.* (51) confirms that powdered plastic materials when implanted do not seem to act as carcinogenic agents.[2]

However, Hueper (47) disagrees with the physical theory group and reinterprets the results of other workers, as well as the results of his own study, to support the chemical or physicochemical thesis as the causative factor in polymer cancer. Therefore, it must be obvious that much more experimental work must be done to define clearly the causative factor which is responsible for the production of tumors when various types of plastics are implanted in animals.

In general, there appears to be a minimum induction period, depending upon the animal, before a tumor will develop from an implanted plastic. Oppenheimer *et al.* (41) noted that if implants are removed from their sheath (pocket or capsule which will form around an implant) within a 6-month period, no tumors will develop in rats. If the implants are kept in place for a longer period than 6 months and then removed, tumors will develop. However, tumor response,

[2] It should be noted that for the same unit weight of plastic, a powder will have more surface area than a film, but the powder will not have an uninterrupted contact with the tissue.

may be eliminated after the 6-month period, if both the implant and the sheath around the implant are removed. This would indicate that once the cells have reached a certain point of alteration, the reaction cannot be reversed even when the causative agent (plastic) is removed. Nothdurft (44) also paid attention to the sheath surrounding the implant as a factor to be considered in tumor production.

What has been noted in animals has, up to the present time, not been seen in man. In fact, Harris (52) points out that from his knowledge of 8000 cases of breast plasty in humans, not one case of cancer was noted due to the plastic implant. Several reports (53, 54) have indicated, however, that tumorlike manifestations in several humans could be traced to the use of aerosol hair products which contained polymeric material, but the evidence cannot be considered conclusive. The experiments performed in animals ranged for a period of several years which in man might mean an equivalent of 15 to 30 years. Since implanted prosthetic devices are of comparatively recent vintage, no sure prediction of either noncarcinogenic or carcinogenic activity can be postulated at this time. The surgeon must weigh the merits of implantation in saving or prolonging life with regard for the possible risk involved.

Indirect Effect on Tissue

Leaching of a Constituent into Solutions.—Another problem is the possibility of one or more ingredients from a plastic device leaching into blood, parenteral products, and other solutions during storage, collection, and administration. These will be referred to as "indirect effect on tissue" since the plastic itself will not have actual contact with tissue. Two questions might be raised here: (*a*) is an ingredient or ingredients being leached into a solution which will then be injected into animals or humans and (*b*) is that ingredient or ingredients toxic to the host?

If the answer to the first question is a definite "No," then there may be little need to seek an answer to the second. At first glance, the previous statement would seem reasonable, but a deeper penetration into the plastic problem will reveal a number of factors which will influence the migration rate of a constituent from the plastic into the solution. Of course, it is assumed here that the plastic is a compounded plastic or has been treated with one or more agents to improve the quality of the device. A plastic device in contact with saline solution may reveal no leaching, but in another solvent system or at another pH there may be significant quantities of the leached constituent in the solution. The problem becomes even more complicated when one considers the variety of parenteral products which could come in contact with a plastic device.

Several years ago, Autian and Brewer reported in a study on a disposable hypodermic needle having a plastic hub that a constituent was being released by certain needles from the plastic hub to a saline solution which on intravenous injection caused the death of mice (55). Further work by Brewer and Bryant (25) on various disposable devices demonstrated that compounded plastic materials may or may not cause a toxic effect when first exposed to several parenteral solvent systems and then injected by different routes into animals.

It is pertinent at this point of the review to mention that very little appears to be known concerning the toxicity of a number of agents which can be formulated into certain types of plastics. Certain countries, for example, the United States, have very stringent regulations on food packaging materials. Physical and biological testing must be performed on these packaging materials before they can be employed as food containers. The biological tests invariably involve long oral feeding tests in groups of animals to ascertain the safety of the substance. Unfortunately, this same information often appears to be used to indicate that the additives approved for food packages will also be safe when they are parts of plastic devices to be used in medicine. Of course, this assumption may be true, but on the other hand strict obedience to this doctrine could present possible harm. Documentation of this statement is not easy in regard to humans, but preliminary animal experiments by Meyers *et al.* (56) on a group of citric acid esters used as plasticizers revealed how false oral testing programs can be when they are extrapolated to parenteral administration. The aforementioned workers studied the behavior response to parenteral administration of these esters in rats, mice, frogs, and rabbits. They noted that all of the esters exhibited a marked effect on the central nervous system. A single dose (depending on the particular ester) killed these animals in a period of several hours. Death was not due to citrate intoxication as may have been anticipated, but due to another mechanism not yet elucidated. The question arises regarding what actions other ingredients used in plastic formulations may have on animals and upon sick persons by routes other than oral if they leach out into a solution.

The work of Autian and his group (57, 58) dramatizes the idea that the particular drug system will influence the rate of release of a constituent from a plastic material, and it should suffice to say that serious concern should be given to drug-plastic interactions as a potential hazard to patients.

Leaching of a Constituent and Other Consequences to Blood.—Clarity, disposability and nonbreakability as well as hemorepellent properties of certain plastics make these polymeric materials useful for devices to collect, store, and administer blood. Logistic advantages (59) also may be gained by the armed forces and civil defense organizations in the replacement of the conventional glass containers by plastic ones. However, problems may arise if the plastic material is not selected with care, properly formulated, and controlled in production and, finally, adequately tested.

It has been well known for a long time that the surface of a vessel can alter the viability of blood. As far back as the turn of the century, Bordet and Gengou (60) noted that clotting time was delayed when the surface of a vessel was coated with petrolatum or paraffin. Later, with the introduction of silicone coatings, the same results were obtained in a more acceptable manner (61). These coatings decreased the adhesive force between the blood and the glass (hemorepellent or nonwettable) and acted to smooth the surface of the vessel. Therefore, the use of plastics for blood appeared to be ideal, since a number of polymeric materials were also extremely hemorepellent.

Much research and development has gone into the present plastic containers used for the storage of blood. Walter (62) introduced the polyvinyl chloride container with the hope that this type of device would delay coagulation and cause less damage to blood. Since that time, various changes have been made in the formulation of the plastic material.

More convincing evidence is now available that the toxic effect imparted by certain containers is actually due to a release of a constituent from the container to the blood and not due to the surface of the container in contact with the blood. One must recognize, as mentioned before, that a plastic material might contain other ingredients to impart certain properties to the plastic. In the case of plastic containers for blood, plasticizers, stabilizers, and other additives are usually added to make possible the construction of the container as a flexible bag. Thus, there is the possibility of one of the additives leaching into the blood and causing the loss of viability. This, in fact, has been found to be the case. Strumia *et al.* (63) showed that certain types of containers (plastic, glass, and silicone-coated glass) might release a constituent which in turn would act as a toxic agent toward the blood cells, no matter if it were stored in plastic, glass, or silicone-coated glass,[3] and that the surface or nonwettability property of the container is not the important factor in blood survival.

Recent reports by Ballinger and Cohn (64) and Wiener (65) on the preservation of whole blood clearly indicate that there is no real proof that a plastic container will afford better protection to blood (erythrocytes) than glass. However, plastics seem to offer certain advantages over glass for prolonged platelet and leukocyte survival (66, 67). Storage of blood products, other than whole blood, in plastic bags have in some instances caused physical changes in the product. The same product stored in glass bottles revealed no changes.

Surface properties become important when blood is passed through a tube or tubing for one reason or another. It would seem logical to assume that less trauma would be imparted to blood when it is passing through a tubing having a smooth internal surface than one having a rough surface. Evidence to this effect has been reported by Stewart and Sturridge (68). Other important factors must also be considered as influencing the survival time of red blood cells, such as the flow rate, time of contact, particular material, and techniques used in circulating the blood through the tubing. All things being equal, however, the internal surface will play a role in helping to preserve or destroy the components of the blood which might be fragile to shock.

Today greater use is being made of extracorporeal devices for the circulation of blood in open heart surgery. The blood in these instances is oxygenated outside the body and then is made to pass into the body again, usually through one or more tubings. These tubings may be plastic and often are of the polyvinyl type which necessitates the addition of other ingredients to make a suitable tubing. That there is the possibility of one or more ingredients leaching into the blood, depending upon the polyvinyl formulation, which may produce a toxic effect, should be recognized.

Meyler *et al.* (69) showed that leaching from a

[3] Glass released small amounts of silica which acted as a toxic agent on blood. The silicone-coated glass also showed in certain instances toxic effects which might be explained on the basis that uninterrupted coatings of the glass probably had not been achieved.

plastic tube into blood can occur. In their study they noted that when they changed from glass tubing to a particular polyvinyl chloride tubing, premature cardiac arrest and ventricular fibrillation occurred on the isolated perfused rat's heart. The important point to be made here is that these authors indicated that some brands of polyvinyl chloride did show this adverse reaction.

Keith *et al.* (70) reported that one batch of plastic tubing enhanced the hemolytic effect on perfused blood. This observation suggested to Hirose and associates (71) that the significant increase in the incidence of renal complications during extracorporeal circulatory bypass in patients in their hospital might be due to the particular plastic and the ethylene oxide method used for sterilization. These investigators noted that ethylene oxide sterilization increased the tendency to hemolysis in blood samples stored in plastics but that the particular plastic would also influence the rate of hemolysis.[4]

There is, therefore, the very serious consequence in extracorporeal circulation in humans that the wrong brand of tubing might release a constituent into the blood and thereby produce one or more untoward reactions.

Another problem has arisen in the use of plastic tubing in heart lung machines. Usually the tubing or tubings are coated with silicone, and if the silicone actually has not been baked on the plastic, which in many cases might be the case, there is the possibility of the blood washing particles of the silicone from the plastic into the circulating blood. A report of this occurring in humans has been given by Lindberg *et al.* (72). In their study 10 patients had died after open heart surgery. On necropsy, clear, refractile emboli were found in the capillaries of the kidney, brain, and heart. The emboli were investigated and found identical to the silicone used in the coating of the tubings. Further work on animals substantiated the human results that silicone coatings can cause embolization and death. Similar results were noted by Helmsworth *et al.* (73), who found silicone emboli in the glomeruli of the kidney of patients and animals. The silicone was traced to the oxygenator pump which had been silicone treated for debubbling.

Here it is important to remember that as new materials are introduced into one or more of the components in various types of extracorporeal apparatus, it is imperative that the material be evaluated biologically to prevent any unnecessary hazard to the patient. Reuse of certain components by specific washing, rinsing, and sterilization should require further evaluation of the component since these treatments may affect the plastic material sufficiently which in turn may produce an untoward effect in the patient. Clearly, more research is needed in this area for better patient protection.

COMPLICATIONS ARISING FROM PLASTIC PROSTHESES

General

The use of synthetic materials as a prosthesis dates back to the year 1890 when Fraenkel repaired a bony defect in the skull by the use of celluloid (74). Since that time and especially within the last 15 years, an array of uses has been found for plastic materials within the body for both physiological and cosmetic needs (10). Many of these surgical procedures with subsequent implantations of the plastic prosthesis have permitted extension of human life or have given certain forms of comfort to the patient. Many problems still need to be studied in detail before an ideal synthetic device becomes possible. The following section attempts to bring some of these problems into focus with past experiences by various investigators.

Vascular Prostheses

The general success of surgical procedures in the replacement of segments of diseased or failing aortas and peripheral arteries with homografts soon made it evident that the supply of these homografts or even heterografts would not fulfill the anticipated needs. Thus, it was quite natural for the surgeon to turn to the various polymeric materials as synthetic vascular replacements.

One need not go too far back into the literature to note that most animal and clinical experiences with synthetic prostheses have occurred within the last 7 or 8 years, even though Deterling (75) refers to Vinylon-N yarn being used as a vessel substitute in dogs in 1951. Since 1957, the two most used plastic materials for synthetic grafts have been Dacron and Teflon; but Ivalon, nylon, Vinylon-N, Orlon, Fortisan, polyethylene, and polypropylene have had some initial success.

Much experience has now been accumulated on Ivalon. It might be of interest to review this material as a synthetic substitute for vessels from its original success to its final demise.

A number of excellent properties such as porosity, compressibility, ease in handling, and ease in molding made this material attractive

[4] It was found that if the ethylene oxide sterilized plastic was kept for 7 days prior to its contact with blood, the gas had no effect on the blood.

as a vessel substitute. Early reports by Shumway *et al.* (76), Ellis and Kirklin (77), Rob *et al.* (78), and Fitch and Denman (79) were extremely encouraging in regard to Ivalon as a vessel replacement; however, Deterling (75), as early as 1956, had some reservations in the use of Ivalon, for he noted that the material could not withstand the pressure in the thoracic aorta unless highly compressed. Creech *et al.* (80), in a report to the Society for Vascular Surgery in 1956, reported some unfavorable results with Ivalon in clinical practice. Rob (81) reversed his earlier impression of the value of Ivalon; in fact, he no longer advocates its use as an arterial replacement. This reversal of opinion also has been enunciated by Fitch *et al.* (82). They now state the following: "The use of Ivalon grafts as arterial conduits in the human patient is unjustified due to the hazards of thrombosis, aneurysmal formation, rupture, and anastomotic disruption." A recent report by Payne and Kirklin (83) on a number of patients having had reconstruction surgery performed on the right ventricular outflow tract with several plastic materials noted that Ivalon was an unsatisfactory material. Further support of the inadequacies of Ivalon is given by Adler and Darby (84), who noted marked changes in physical properties of Ivalon after tissue contact. In the light of present knowledge, it would appear that Ivalon no longer merits a position as a worthy substitute for vessel replacement either in man or other animals.

As early as 1956 an attempt was made to assess actual clinical experiences in the use of synthetic materials as vessel substitutes in humans. This assessment became a preliminary report which has been mentioned previously (80). The report relates the success of aortic replacements with one or more of the synthetic materials (*i.e.*, Ivalon, nylon, Fortisan, Orlon, Dacron, Vinyl-N, and Teflon). Less success was found for synthetic materials when these were used as replacements for peripheral arteries. Critical factors causing the failures of the peripheral vessels were the relatively small diameters of these vessels, the great length, and the crossing of flexion areas.

There appears to be general agreement today that vessels less than 5 mm. in diameter will fail. Most likely these failures will be due to the propensity of thrombi formation. Harrison (85, 86) found this to be the case with nylon, Dacron, Orlon, Ivalon, and Teflon in his studies. Further confirmation of this fact is given by Dale and his group (87, 88), as well as by Cate (89).

Some controversy exists as to the actual causative factor or factors inducing thrombi formation in synthetic vessels. Change in flow rate of blood and turbulence of flow due to the synthetic graft has been believed to be a factor in thrombi formation (90). Szilagyi (91) has viewed thrombi formation in synthetic grafts as possibly due to the nonreactivity of a particular plastic material. The implication here is that arteriogenesis will be delayed and that the intima will not adhere firmly to the synthetic vessel. A recent study of Phillips *et al.* (92) disputes both the turbulent flow and the nonreactivity theories as causative agents; in turn, it postulates that thrombosis formation is a direct consequence of a foreign body—especially if that body is relatively nonporous. They found in their study on Teflon that woven Teflon caused a greater degree of thrombi formation than the knitted material. The knitted Teflon, more porous than the woven form, permitted fibrous tissue to invade the interstices of the fiber, resulting in greater adherence of the fibrin lining in the vessel. Other investigators also have pointed out the importance of porosity in regard to synthetic materials used as vascular substitutes (90, 93–95).

Biogenic conditions will alter physical properties of synthetic materials, even though much still needs to be done to elucidate the actual mechanism underlying such physical changes. It is fairly well established now that polar polymeric compounds such as nylon, Orlon, etc., will show greater changes of properties than the very nonpolar compounds, exemplified by Teflon after long contact with tissue. Tensile strength measurements (for elastic properties) have been conducted for a number of synthetic polymers before and after implantation in tissues. For example, it has been reported that nylon will lose from 20 to 25% of its original tensile strength after 7 to 18 months of tissue contact (80). Other materials also will change significantly, but to varying degrees. This has prompted some to believe that elastic properties might be a critical factor to consider when a decision is made to use a specific synthetic material as a vessel substitute. Present evidence, however, gives little emphasis to tensile strength as a factor in success or failure of grafts (80). A revealing study by Newton *et al.* (96) apparently confirms this fact. In their studies (*in vivo*) they noted that various arterial substitutes showed differing elastic properties within a 6-month period; but after this time period, they all tended to stiffen and reach a constant

elastic value. They concluded that the constant value was due to the formation of scar tissue within the implanted arterial substitute.

As has been mentioned for synthetic materials, a degree of success has been made for grafting of vessels having larger diameters than 5 mm., while, conversely, little success with vessels smaller than 5 mm. A number of investigators recently have observed that homografts have proven more successful postoperatively over synthetic materials when surgical grafts were performed on smaller vessels falling below the inquinal ligament. Irvine *et al.* (97) noted this to be the case in a study of 95 patients who underwent a total of 106 femoropopliteal bypass treatments. Cockett and Maurice (98) in a 9-year observation of direct arterial surgery for claudication and ischaemia of legs, concluded that the synthetic materials were not functioning up to the level of homografts or other surgical techniques not involving synthetic materials.

It should be obvious that for vessels large or small other factors must enter the picture which can give rise to success or failure of synthetic grafts. In this respect it is interesting to note the suggestion of Wesolowski and associates (99), who have contributed a great deal to surgery in techniques and knowledge in regard to vessel and tissue substitutions. These investigators have concluded that the ideal synthetic vascular graft material should meet the following standards: (*a*) no toxicity or no allergenic potential, (*b*) no deterioration of the synthetic fiber upon biological implantation for prolonged periods of time, (*c*) desirable mechanical handling properties of being easily scrunched, crimped, and twisted, (*d*) very low implantation porosity, and (*e*) very high healing porosity or fibroblasting permeability. To achieve the last two requirements (*d* and *e*), Wesolowski *et al.* (99) fashioned compounded prosthetic vascular grafts (a core of resorbable material which has been wrapped with multifilament polymer). These compounded materials were found to be superior (in animals) to the conventional monoplastic material. Similar results with a gelatin-impregnated Dacron prosthesis by Jordan *et al.* (100) also have been reported.

Surgical skill has made it possible to replace faulty or defective cardiac valves in humans. Unfortunately, studies in canines and humans have revealed survival rates not as encouraging as had initially been anticipated. The most serious drawback to the synthetic valves has been the severity of thrombosis formed on the valve. A number of investigators—Frater and Ellis (101), Kolff *et al.* (102), and Muller *et al.* (103)—have observed this serious problem. Gott *et al.* (104) have noted the importance of electrical charge on the plastic (positive charge in comparison to the negative charge on the blood particles) and found that this charge could be reduced or eliminated by coating the valve with colloidal graphite. The coating resulted in producing an extremely smooth surface which apparently dissipated or reduced the positive charge, thereby decreasing the incidence to thrombosis. To date these authors have been able to coat polycarbonate, polyvinyl, and methyl methacrylate; their results appear encouraging. However, final success or failure must wait for long-term studies in both animals and humans.

Subcutaneous Prostheses

Reconstructive surgery on or near the surface of deformatives, either natural or induced by disease, accident, etc., has restored many patients to a normal manner of life. The synthetic materials have been used for this type of surgery for approximately 15 years with various degrees of success. As with other plastic implants, real problems have been encountered which have led to replacement of the original implant or to complete failure after varying periods of time.

Breast plasty has gained some popularity in the past to relieve psychic disturbance caused by actual or imagined hypoplastic breasts. Even though cosmetic acceptance can be achieved by the use of plastic implants behind the breast, the material gradually becomes firm and loses its initial resiliency (105, 106). The implant may also shrink in size or "fall" from its original position, thus creating further psychic disturbance. Hamit (106) lists a number of complications which might result from breast plasty, such as infection, drainage, increasing firmness of the plastic material, disruption of breast function or appearance, development of draining sinuses, and possible carcinogenic activities of the plastic material. Polyvinyl sponge has shown the greatest number of failures up to the present, and it is hoped that newer materials such as silicone rubbers and polyurethanes might prove more satisfactory. Harris (52) appears to be quite satisfied with a special type of polyurethane for breast plasty.

Various other reconstructive procedures on the face and other surface portions of the body have been performed with, in some instances, amazing success. Favorable initial results, however, must be weighed against long-term usage and the possible complications which will be ever

present with a foreign body. It will be interesting to see how plastics such as the dimethyl silicones and the various halogenated carbons will perform over long usage. Encouraging reports on these materials for subcutaneous prostheseis have been given by Brown *et al.* (107).

Synthetic Adhesives

One can see great advantages for adhesive materials which can replace the usual nail, clamp, cast, and other means of closing or holding segments of tissue or bone together. Fruitful progress has been made in this direction in the past 4 years in surgery with the advent of polymeric materials which, when actuated in some fashion, can become a tenacious adhesive for tissue.

In 1958, Manderino and Salvatore (108) were able to restore broken bones by the use of polyurethane material which acted as a very powerful adhesive agent, fixing the break in a very short period of time. Since then considerable use has been made of this material for the same purpose (109–111). To some it is considered as a form of "bone glue," but others are not quite so convinced of this fact (112). Up to the present no definite tissue reactions have been reported (which could be attributed to the polymer), but sufficient time must elapse before a complete evaluation can be made.

A great number of synthetic adhesive materials have been produced in the last decade, but for the most part these had little place in medical practice, since for one or more reasons they were quite noxious to tissue. Then in 1959, Coover and co-workers (113) demonstrated the unusual adhesive properties of alkyl 2-cyanoacrylates, the chief one being that the monomer (as a liquid) polymerizes when spread between two surfaces and pressed. The change from a liquid state to a solid (as the adhesive) takes place with little or no change in volume, enhancing the adhesive quality. Experimental surgical applications, in particular for blood vessel repairs, have been performed by a number of workers using the above-mentioned monomer (114–116). In general, few tissue responses have been noted by most workers, but a word of caution should be noted for these adhesive agents since there appear to be some differences in the compound when purchased from different sources.

Lewers *et al.* (117) have noted that certain of the synthetic adhesives have a distinct toxic effect. For example, in animals they found that both the monomer and the polymer caused death when injected into the liver and peritoneal cavity. Biological mechanism for death still has not been elucidated by these workers, and the possibility of carcinogenic activity is still to be decided. The above workers in their concluding remarks refer to a 1948 paper (118) in which the following sentence was quoted: "Clinical use of cellophane, polythene, or any other plastic carries with it the urgent requirement of knowledge of both the chemical and physical characteristics of the product being used." Lewers *et al.* (117) maintain this statement to be true with the synthetic adhesives.

PLASTICS AND DRUG ACTIVITY

In recent years pharmaceutical scientists have recognized the potential of polymeric materials as vehicles for prolonging the action of medicinal agents. Some success has been achieved in compounding the drug with an insoluble polymeric material which can then be taken by oral ingestion, the drug being released over a prolonged time period as the tablet or pellet travels along the alimentary tract. Similar dosage forms also have been suggested for long-term implantation of certain hormones. Advantages to both the patient and clinician of the above dosage forms are clear (one-time administration instead of multiple-drug administration) if certain unknown complications do not appear. Two hazards, even though they many appear only as remote possibilities, should be considered. The first of these is the release of a greater quantity of the drug than planned due to uncertain biological manifestations on the dosage form. The second is the possible irritating or sensitizing effect the particular plastic might have on the tissue when implantation therapy is employed. It remains to be seen whether these hazards will materialize in the future when more prolonged or sustained dosage forms are used.

Several investigators, in particular Garb (119), Scholtz (120), Sulzberger and Witten (121), and Hall-Smith (122), have noted that plastic films will aid the percutaneous absorption of a number of topical drugs. Such reports have stimulated other clinicians to consider the use of plastic films to decrease the usual concentration of the drug when applied topically. Lack of knowledge of the influence of these polymeric materials on the physical and chemical properties of drugs regarding their rate of penetration and diffusion into the skin may lead to unexpected toxic or untoward reactions. Vickers and Fritsch (123) have documented this fact with several cases where naphazoline was the test drug and Saran the plastic film. These aforementioned authors

give the following warning: "The knowledge of the vast increase in percutaneous absorption due to Saran occlusion might tempt clinicians to try to increase the effectiveness of other topical preparations with possible serious or even disastrous consequences."

DRUG-PLASTIC CONSIDERATIONS

Less recognized as a problem, probably due to the paucity of reports published, is the consequences which might result when a drug or drug product is kept in contact with a plastic material used as a container or administrative device (124–131). These problems may be collectively grouped into five general categories:

(*a*) *Permeation*—Oxygen or other gases permeating the plastic material and causing an incompatibility or, conversely, a volatile constituent in the drug product passing out of the container or device.

(*b*) *Leaching*—One or more ingredients migrating from the plastic into the drug solution.

(*c*) *Sorption* (including adsorption and absorption)—One or more constituents being removed from the drug solution into the plastic by a sorption process.

(*d*) *Chemical Reactivity*—One or more ingredients reacting by covalent bonding with the polymer or one of the additives in the plastic.

(*e*) *Alteration in the Physical Properties of Plastics*—Depending to an extent on one or more of the above or due to environmental effects, the container or device may undergo sufficient changes to no longer function as originally intended.

The above five considerations have been reviewed in some detail in previously published papers (132, 133) and thus will not be discussed any further in this review, except to emphasize that both the manufacturer and the user must share in the responsibility of safeguarding the health of the patient when plastic devices are to function as containers or devices for storing or administering drug products.

NEED FOR STANDARDS

An analysis of what has been reviewed in this paper should note that plastics are not so safe as originally thought. In no manner does this suggest that plastics should be banned or eliminated from medical practice. Such an expression would be ridiculous and certainly is not tenable to the many outstanding advantages to be gained by the use of the synthetic polymeric materials. Rather, a method should be sought which would insure that a particular plastic or plastic device will be safe for intended use. Standards must be created for "medical use" plastics as well as standards for the final plastic device. These standards obviously must include biological, physical, and chemical methods of testing. Finally, these standards must become part of the official compendia to insure legal status.

Autian (58) has published a guide to hospitals in the selection of plastic devices, while The University of Texas Medical Center has undertaken the task of developing its own standards for plastics [see report by Autian and Nicolaides (134)]. Brewer and Bryant were the first in this country to publish biological methods of testing of plastics to be used in medical practice (25). Workers in other countries recognizing the plastics problems have recommended also that stricter controls be maintained on plastics and have suggested a number of testing procedures (135–137). Guess and Autian (138) have presented a tentative protocol for the biological testing of plastic materials. In this country the Pharmaceutical Manufacturers' Association with support from the Society of Plastics Industry has developed a group of biological and physical chemical tests for plastics to be used with drug products.

Much work still remains to be done now and in the future before adequate standards are developed. Certainly, greater emphasis should be given to basic studies on plastics as they are related to medicine. In the past such support from industrial groups interested in plastics has been minimal, but it is hoped that a reversal of this trend will soon be forthcoming. There is also a need for better communication among groups working to manufacture and distribute plastic items to the medical profession. Often one group is not aware of what the other group has done to a plastic item. This point is clearly brought into focus by Bender (139), a consulting engineer, in his excellent article dealing with the trend toward plastics in surgery and medicine.

The plastics problem is such a vital health issue that it no longer can be kept as an "incidental" hazard with the ever-increasing use of plastics in all phases of medical practice. Good public health practice requires as much emphasis on measures to prevent a potential danger from becoming a serious reality as to combat the danger once it has occurred. Perhaps the best example of this in the past years is the pesticide issue brought into shocking public attention by Rachel Carson's "Silent Spring." Even though adverse criticism was given to the author for having overplayed the problem based upon

meager scientific evidence, it was interesting to note that the President's Science Advisory Committee has made a number of recommendations to prevent some of the possible consequences illuminated in the book "Silent Spring" (140). In much the same way, though on a much smaller scale, plastics for medical practice deserve equal attention.

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Research Articles

Determination of Drug Absorption Rates Without Chemical Assay

By GERHARD LEVY and KAREN E. MILLER*

This study illustrates the possibility of determining absorption rates of certain drugs without using chemical assays. The method is based on the determination of the time of onset of a suitable pharmacologic response under conditions where a constant drug concentration gradient across the absorbing membranes is maintained. While particularly suitable to studies with fish and other aquatic animals, the method may also be applicable to mammals for determining the absorption rate of certain volatile substances or aerosols administered by the pulmonary route and of certain dissolved drugs administered by intestinal perfusion.

ONE OF THE most important considerations in the pharmacologic and toxicologic evaluation of chemotherapeutic agents, pesticides, and other chemicals is their ability to pass across biologic membranes. Absorption studies ordinarily require chemical analysis of blood, urine, intestinal content, tissues, or of the solution from which the drug is being absorbed. At times, this requirement can represent an almost insurmountable barrier because of the lack of a sufficiently sensitive or specific analytical method. Recently, we have developed and tested a mathematical model which describes the relationship between drug absorption rate, drug concentration in the aqueous medium, and time of occurrence of a suitable pharmacologic effect in fish (1). This model is the basis for a novel method for the determination of drug absorption rates without chemical analysis and is described in this report.

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Levy and Gucinski have shown (1) that the time of death (T_L) of fish due to passive absorption of a drug is related to the concentration (C) of that drug in the aqueous medium in the following manner

$$\frac{1}{T_L} = \frac{DA}{L} C \qquad \text{(Eq. 1)}$$

where L is the lethal dose of the drug, D is the absorption rate constant, and A is the area of the absorbing membrane. This relationship is based on the following requirements: (*a*) absorption occurs by passive diffusion and therefore is not a saturable process; (*b*) the drug concentration gradient across the absorbing membranes remains essentially constant during the experiment; (*c*) the permeability characteristics of the membrane do not change with time or drug concentration over the time and concentration range of the experiment; (*d*) drug elimination is negligible during the time of the experiment; and (*e*) the pharmacologic end point (death) occurs without significant delay after a given amount of drug (the lethal dose) has been absorbed.

In essence, the requirement that absorption occur by passive diffusion is fulfilled by most nonphysiologic substances; an essentially constant concentration gradient can be maintained by using sufficiently high drug concentrations and relatively large

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Research Articles

Determination of Drug Absorption Rates Without Chemical Assay

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This study illustrates the possibility of determining absorption rates of certain drugs without using chemical assays. The method is based on the determination of the time of onset of a suitable pharmacologic response under conditions where a constant drug concentration gradient across the absorbing membranes is maintained. While particularly suitable to studies with fish and other aquatic animals, the method may also be applicable to mammals for determining the absorption rate of certain volatile substances or aerosols administered by the pulmonary route and of certain dissolved drugs administered by intestinal perfusion.

ONE OF THE most important considerations in the pharmacologic and toxicologic evaluation of chemotherapeutic agents, pesticides, and other chemicals is their ability to pass across biologic membranes. Absorption studies ordinarily require chemical analysis of blood, urine, intestinal content, tissues, or of the solution from which the drug is being absorbed. At times, this requirement can represent an almost insurmountable barrier because of the lack of a sufficiently sensitive or specific analytical method. Recently, we have developed and tested a mathematical model which describes the relationship between drug absorption rate, drug concentration in the aqueous medium, and time of occurrence of a suitable pharmacologic effect in fish (1). This model is the basis for a novel method for the determination of drug absorption rates without chemical analysis and is described in this report.

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where L is the lethal dose of the drug, D is the absorption rate constant, and A is the area of the absorbing membrane. This relationship is based on the following requirements: (*a*) absorption occurs by passive diffusion and therefore is not a saturable process; (*b*) the drug concentration gradient across the absorbing membranes remains essentially constant during the experiment; (*c*) the permeability characteristics of the membrane do not change with time or drug concentration over the time and concentration range of the experiment; (*d*) drug elimination is negligible during the time of the experiment; and (*e*) the pharmacologic end point (death) occurs without significant delay after a given amount of drug (the lethal dose) has been absorbed.

In essence, the requirement that absorption occur by passive diffusion is fulfilled by most nonphysiologic substances; an essentially constant concentration gradient can be maintained by using sufficiently high drug concentrations and relatively large

volumes of solution; drug elimination need not be considered in fish since apparently they do not metabolize drugs but eliminate them solely by passive diffusion through the gills (2); this effect would be negligible in magnitude under the experimental contions employed, in view of the high concentration gradient in the opposite direction. In species capable of metabolizing drugs, the requirement for negligible elimination during the time of the experiment can be satisfied in most instances by using drug concentrations high enough to cause death in a relatively short time. Fortunately, the experimental data will in themselves indicate if the specified requirements have been fulfilled, since a plot of reciprocal time of death *versus* drug concentration (which should be linear and intersect the origin) will not yield a straight line if the necessary conditions are not present. Compounds for which the presently described method is not suitable include (*a*) substances which affect the permeability of the membrane by denaturing or precipitating proteins or mucus, or which have an erosive or otherwise directly toxic effect (such substances would present similar difficulties when other methods are used to study their absorption); (*b*) substances which have a delayed, inductive, or other indirect pharmacologic effect.

As apparent from Eq. 1, the slope of a plot of reciprocal time of death *versus* drug concentration represents the value DA/L. If the lethal dose, L, is known, DA can be calculated. It is not possible in most instances to determine the absorption area, A, with any degree of accuracy. However, using animals of equal size (weight) in each experiment, it is possible to combine D and A to obtain a relative absorption rate constant K

$$K = DA \qquad \text{(Eq. 2)}$$

The purpose of the present study was (*a*) to determine if the lethal dose, L, of a drug absorbed by fish from an aqueous medium is essentially equal to the lethal dose when the drug is administered parenterally and—if this was so—(*b*) to test the proposed method by determining the relative rates of absorption of a series of four barbiturates known to differ in their absorption rates and partition coefficients (3).

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing about 7 Gm. (for the absorption study) and about 25 Gm. (for the determination of lethal dose as a function of route of administration), were used. In each of the studies all fish were from the same lot.

Secobarbital Assay.—The assay consisted of the extraction of secobarbital from homogenized goldfish tissue (adjusted to pH 6) into chloroform, followed by extraction of the chloroform phase with aqueous buffer of pH 10.2. Secobarbital in the aqueous buffer phase was determined by differential spectrophotometry, based on the difference in ultraviolet absorption spectra of barbiturates in strong alkali and in solutions of pH 10.2, respectively, as reported by Goldbaum (4).[1]

Individual goldfish, which died after immersion in secorbarbital solution, were homogenized in the semimicro container of a Waring Blendor after addition of 25 ml. of phosphate buffer (0.05 *M*, pH 6.0) and 3 Gm. of sodium chloride per 25 Gm. of fish tissue. The homogenate was extracted five times with 15-ml. portions of chloroform. A 30-ml. aliquot of the chloroform phase was reduced in volume to 5 ml. by evaporation, then extracted three times with 10-ml. portions of phosphate buffer (0.05 *M*, pH 10.2). Five milliliters of 3.75 *N* NaOH was added to one 10-ml. aliquot of the combined pH 10.2 phosphate buffer phase (solution *A*), while 5 ml. of phosphate buffer (pH 10.2) was added to another 10-ml. aliquot (solution *B*). The absorbance of solution *A* at 262 mμ was determined with a Beckman DU spectrophotometer, using solution *B* as a blank. A small correction had to be made for the apparent absorbance of sodium hydroxide, since each of the various lots of reagent grade sodium hydroxide available produced a small but reproducible and concentration-dependent absorbance.[2] Average recovery of secobarbital (in the range around 0.05 mg. per Gm. fish tissue) was 78%, and the data were corrected accordingly.

Determination of Lethal Dose by Injection.—Each goldfish was weighed in a tared beaker of water, and the appropriate amount of aqueous drug solution was injected intraperitoneally with a 0.25-ml. capacity tuberculin syringe with a 27-gauge, 1/4-in. regular point hypodermic needle. The concentration of the injected drug solution was such that 0.01 ml. of solution was injected per gram of body weight. In preliminary experiments, injections were also made intramuscularly at a point slightly lateral to the anterior point of the dorsal fin. After injection, the fish were placed in 1-gal. jars of aerated water, with separate jars used for each group of fish (on the basis of the administered dose). The jars were checked periodically, and dead fish were removed. A final count was made after 24 hours, although deaths occurred usually within the first 2–3 hours. Each drug was administered in from 6 to 10 logarithmically equally spaced dose levels, sufficient that at least the lowest dose caused no deaths, and the highest dose resulted in deaths of all fish in that group. Ten fish were used for each dose level (*i.e.*, 60–100 fish per drug). LD_{50} values were calculated by the Spearman-Karber method, as described by Irwin and Cheeseman (5) and amplified by Epstein and Churchman (6).

Determination of Time of Death.—Barbiturate solutions of various concentrations were prepared by dissolving the respective drugs in tris(hydroxymethyl)aminomethane (tris) buffer (0.05 *M*, pH 5.9),[3] then readjusting the pH to 5.9 where necessary. One-hundred-milliliter portions of the solutions were placed in 250-ml. capacity beakers. The temperature of the solutions was maintained at 22 ±1°. Single goldfish were placed in each beaker; the time of death, evidenced by cessation of gill and mouth movement, was recorded.

To eliminate bias in the time of death determina-

[1] Goldbaum actually used a buffer solution of pH 10.5. In our study, the use of such solutions for extraction yielded a turbid aqueous phase which made accurate spectrophotometric analysis impossible. Lowering the pH to 10.2 prevented the occurrence of this turbidity.

[2] *Caution:* Strong NaOH solutions will damage quartz cells on prolonged contact. Such solutions should remain in the cells for a period as short as possible, and the cells should be rinsed promptly with dilute acid.

[3] Tris was used despite its relatively poor buffer capacity at pH 5.9 because of the known innocuousness of this material to fish (1) and because it maintained the desired pH without difficulty under the experimental conditions.

TABLE I.—EFFECT OF ROUTE OF ADMINISTRATION ON LETHAL DOSE OF SECOBARBITAL

i.p. Injection, LD_{50}	1.49×10^{-4} mM/Gm.
	(S.D. $+ 0.09 \times 10^{-4}$, -0.07×10^{-4} mM/Gm.)
Av. drug content at death due to immersion in 25 mg. % secobarbital soln.	1.46×10^{-4} mM/Gm.
	(S.D. 0.09×10^{-4} mM/Gm.)

TABLE II.—EFFECT OF ROUTE OF INJECTION OF SECOBARBITAL ON LETHALITY OF GOLDFISH

Date	Dose, mM/Gm.	Lethality[a] i.p.	Lethality[a] i.m.
10–18–1963	1.54×10^{-4}	2/5	1/5
10–21–1963	1.92×10^{-4}	7/8	4/8
10–26–1963	1.92×10^{-4}	8/8	4/8
10–30–1963	1.92×10^{-4}	8/8	4/8

[a] Number of deaths/number of fish injected.

tions, each beaker of drug solution was marked solely with a code number by an individual not involved in the study. This code was broken only after all determinations had been completed.

Partition Coefficients.—Partition coefficients were determined using 0.1 *N* HCl and olive oil as the aqueous and organic phases, respectively. The phases were shaken at room temperature (about 22°) until equilibrium was attained (24 to 48 hours). The aqueous layer was removed, centrifuged, diluted with 0.5 *N* NaOH, and assayed spectrophotometrically.

RESULTS AND DISCUSSION

To determine the effect of route of administration on the lethal dose of a barbiturate, goldfish weighing about 25 Gm. were injected intraperitoneally with graded doses of secobarbital sodium. A number of other fish of the same lot was placed in an aqueous solution of 25 mg.% secobarbital, and each fish was removed immediately after death. The fish were then rinsed with water and assayed individually.[4] Results are recorded in Table I. The drug content of the fish at death due to immersion in secobarbital solution varied only slightly between individual fish, and a similar extremely narrow distribution was apparent in the mortality data after intraperitoneal injection of the drug. Strictly speaking, comparison of lethal doses observed upon drug administration by different routes requires that the per cent mortality *versus* injected dose data be expressed in terms of a weighted average lethal dose. However, in view of the steep rise of mortality with a small increase of administered dose, it was possible to use the LD_{50} values as a measure of the average lethal dose. The respective LD_{50} values were in no case more than 5% below the apparent weighted average lethal doses. Use of the former values was preferred because they could be calculated by objective and well-known procedures, while the latter required somewhat subjective manipulations. (This simplified approach cannot be used if lethal dose distribution is wide and if it differs significantly from normal.)

As evident from the data in Table I, the lethal dose of secobarbital upon intraperitoneal administration was the same as the lethal dose (body drug content) upon immersion of fish in drug solution. This is not surprising since the drug enters directly into the circulation by either route. In contrast, differences in lethal dose as a function of route of administration are quite common in higher animals for a number of reasons [reviewed by Levy (7)]. For example, lethal doses, upon oral administration of a drug, may be higher than parenteral lethal doses because of partial degradation of the drug in the gastrointestinal tract, incomplete absorption, partial biotransformation in the intestinal wall, different initial distribution, and sufficiently slow absorption (though rapid elimination), resulting in maximum drug levels considerably lower than those after parenteral administration. None of these factors apply to fish since absorption takes place predominantly across the gills, biotransformation of drugs does not occur [at least in the case of those drugs, including barbiturates, studied to date (2)], and elimination (which is by passive diffusion across the gills and requires a favorable concentration gradient) is negligible under the conditions of the experiment. Actually, elimination is favored in the experiments where the drug is administered parenterally since the fish are placed in pure water after they have been injected. However, death occurs rapidly, and elimination of barbiturates is hindered not only by protein binding but also by partial ionization of these drugs in the tissue fluids, so that this factor does not appear to be important. These results make possible the use of lethal dose data obtained by intraperitoneal injection of graded doses, together with slope values (DA/L) determined from time of death *versus* drug concentration data, to calculate relative absorption rate constants (DA or K).

In initial experiments, parenteral injections were made by the intramuscular route. Lethal dose values obtained in this manner were considerably higher than lethal dose values from immersion of fish in drug solution. The data in Table II demonstrate that, when equal doses of drug were administered by the intraperitoneal route, the lethality was consistently greater than when administered by the intramuscular route. It was found, in agreement with observations by Bergmann *et al.* (8) and Chavin (9), that some leakage of drug solution occurred after intramuscular injection and was responsible for the apparently higher lethal doses when drug was administered by this route. That physical injury caused the observed differences was ruled out since no deaths occurred when animals were injected by both routes with comparable volumes of water.

The relationship between reciprocal time of death and drug concentration in the aqueous medium is shown in Fig. 1 for four barbiturates. Since the absorption rate of weak acids by fish is affected by the degree of ionization of these drugs (1), the pH of the solutions was adjusted to 5.9, so that all the barbiturates were in essentially nonionized form. Five concentration levels (10 fish per concentration) were used in the cases of secobarbital and pentobarbital. Only two concentration levels (but 15 fish per concentration) were used for phenobarbital and barbital, respectively, because of limitations imposed by solubility and the long death times at low concentrations. Concentrations yielding times of death

[4] The use of relatively large (25 Gm.) fish was necessary to permit assay of individual fish. In all other parts of the study it was more convenient to use smaller (7 Gm.) fish.

greater than 100 minutes were not used because death occurred gradually, end points proved too difficult to determine, and results were accordingly variable.

Figure 2 is a plot of per cent mortality *versus* intraperitoneal dose (logarithmic scale) for the four barbiturates. Although each drug was administered in between six to 10 logarithmically equal spaced doses, only the highest of the LD_0 and the lowest LD_{100} values were plotted in addition to the intermediate values. As mentioned previously, the distribution of the mortality as a function of dose was extremely narrow. The LD_{50} for secobarbital was the same for 7 Gm. and 25 Gm. goldfish, although this could have been fortuitous.

Relative absorption rate constants for the four barbiturates were calculated from slope values (from data in Fig. 1) and LD_{50} values (from data in Fig. 2) and are shown in Fig. 3. Also shown in the figure are the relative absorption rates of these compounds from the rat colon, as reported by Schanker (3). Such data are comparable because, under the experimental conditions of the rat study (virtual colonic membrane pH 6.5), the drugs were predominantly nonionized (phenobarbital is 89% nonionized, while the other three barbiturates are 96% or more nonionized at pH 6.5). A perfect rank-order correlation is shown between the results obtained from fish and rats.

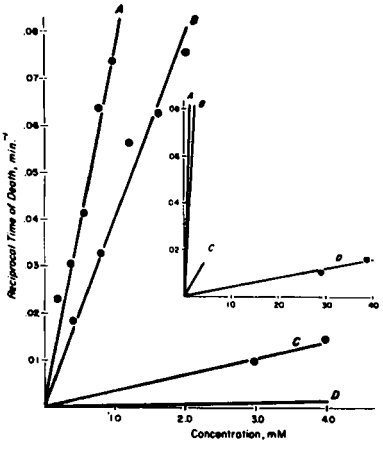


Fig. 1.—Reciprocal time of death of goldfish as a function of barbiturate concentration in pH 5.9 buffer at 22°. Key: *A*, secobarbital; *B*, pentobarbital; *C*, phenobarbital; *D*, barbital. Each data point represents an average of 10 fish (*A* and *B*) or 15 fish (*C* and *D*).

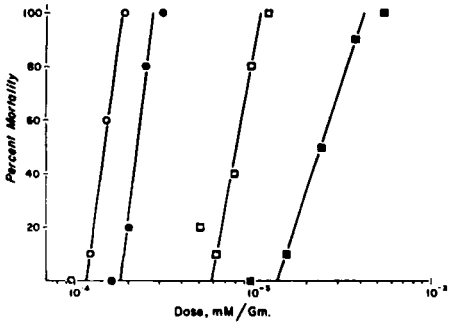


Fig. 2.—Mortality of goldfish as a function of dose of barbiturate injected intraperitoneally. Key: ○, secobarbital; ●, pentobarbital; □, phenobarbital; ■, barbital.

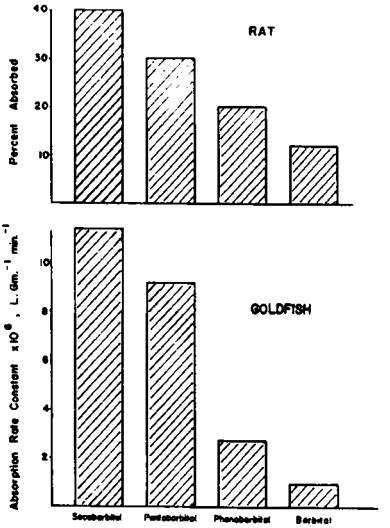


Fig. 3.—Relative absorption rates of four barbiturates in goldfish and from the rat colon. [Rat data from Schanker (3)]. The drugs were essentially nonionized under the conditions of both studies.

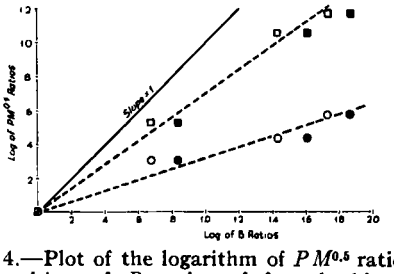


Fig. 4.—Plot of the logarithm of $PM^{0.5}$ ratios *vs.* the logarithm of *B* ratios of four barbiturates. (See *text* for explanation.) Squares represent goldfish data; circles represent rat data reported by Schanker (3). Solid symbols are based on chloroform: 0.1 *N* HCl partition coefficients; open symbols are based on olive oil: 0.1 *N* HCl partition coefficients.

From a theoretical study of the process of diffusion of substances across biologic membranes, Danielli (10) concluded that in the case of rapidly penetrating molecules

$$PM^{0.5} = B \cdot \text{Constant} \qquad \text{(Eq. 3)}$$

where *P* is the permeability, *M* is the molecular weight, and *B* is the lipoid-water partition coefficient of the penetrating substance. He pointed out that this relationship represents a first approximation, and that it is subject to appreciable deviation. Figure 4 is a plot of the logarithm of the $PM^{0.5}$ ratios (based on barbital $PM^{0.5} = 1$) *versus* the logarithm of the ratio of the respective partition coefficients (based on barbital $B = 1$) for the four barbiturates. Chloroform: 0.1 *N* HCl partition coefficients are those of Schanker (3), while olive oil:0.1 *N* HCl

TABLE III.—RATIO OF LD_{50} AND CT VALUES OF FOUR BARBITURATES IN GOLDFISH

Drug	LD_{50} mM/ Gm. $\times 10^{-4}$	Ratio of LD_{50} Values	CT, mM Min.	Ratio of CT Values
Secobarbital	1.46	1.0	12.8	1.0
Pentobarbital	2.26	1.5	24.7	1.9
Phenobarbital	7.85	5.4	290	22.6
Barbital	24.3	16.7	2614	204

partition coefficients were determined in the present study. Schanker's absorption data were corrected to account for the 11% ionization of phenobarbital and the lesser ionization of the other barbiturates under the conditions of his study. The goldfish absorption data are closer to a slope of unity (predicted by theory) than the rat absorption data obtained by Schanker. This may be due to the more severe and somewhat unphysiologic conditions of the rat perfusion method (involving surgery and prolonged perfusion) which may cause the intestinal membranes to become a somewhat less perfect lipoidal barrier. Alternatively, these results could reflect a true species difference or a number of other factors.

A frequently used index of relative toxicity for pesticides, compounds employed in chemical warfare, and other toxic agents is the CT value, which is the product of concentration of the poisonous agent and time of death due to exposure to that concentration of the poison. Levy and Gucinski (1) have shown, on the basis of theoretical considerations, that the CT value is a function not only of the inherent lethality but also of the rate of absorption of a compound. This fact is demonstrated rather convincingly by the results of the present study, as shown in Table III. For example, in a comparison of secobarbital to barbital, based on LD_{50} values, the former is 16.7 times more toxic than the latter. According to the CT values, secobarbital would be judged 204 times as toxic as barbital. The difference is due to the fact that, from Eq. 1

$$CT_L \approx \frac{L}{DA} \qquad \text{(Eq. 4)}$$

and, where animals of equal size are used and A cancels out,

$$\frac{C_1T_{L1}}{C_2T_{L2}} = \frac{L_1/D_1}{L_2/D_2} \qquad \text{(Eq. 5)}$$

where the subscripts refer to two different substances. Thus, the lower rate of absorption of barbital compared to secobarbital causes the ratio of CT values to be greater than the ratio of LD_{50} values. Obviously, the opposite would occur if the less toxic drug is absorbed more rapidly.

The results of this study demonstrate that the rate of absorption of certain drugs can be determined without chemical assay. The potential advantages of such a method are obvious: it may permit absorption rate studies with drugs which cannot be studied otherwise due to the lack of a sufficiently sensitive or specific analytical method. However, the theoretical considerations outlined in this and in previous report (1) should make it quite clear that the method is not suitable for all drugs. While it is particularly useful for fish and certain other aquatic animals, and although experimental verification has only been carried out with fish so far, the method is also applicable *in principle* to mammals, provided a constant drug concentration gradient across the absorbing membranes can be maintained. (This is often possible with aerosol application and with intestinal perfusion.) However, for reasons referred to previously, there may be practical limitations due to differences in lethal dose as a function of route of administration and/or due to rapid drug elimination. Further studies in this area are in progress.

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Effect of Electrophilic and Electrodotic Groups on the Titration of Amides and Other Weak Bases

By LESLIE G. CHATTEN and CONNIE K. ORBECK

The effect of electrophilic and electrodotic groups on the titration of amides in acetic anhydride and combinations of acetic anhydride with chloroform and acetone has been demonstrated. The presence of electrodotic groups enhanced the potentiometric end point, whereas electrophilic groups usually depressed the end point to such an extent that the compound was not titratable. Similar behavior was noted with acetanilide and its derivatives. Examination of the potentiometric first derivative showed an enhancement of the end point when electron donating groups were present.

PRIOR TO Wimer's work (1) on the potentiometric determination of amides in acetic anhydride, little had been reported on the titration of this weakly basic group of compounds. Acetic anhydride has been used alone as a titration medium or in combination with other solvents (2–10). A good summary has been presented by Wimer (1).

It has been demonstrated by Chatten and Harris (11) that the presence of electrophilic groups has a marked influence on the $E_{1/2}$ values of two groups of relatively strong organic bases when they are titrated in certain organic solvents.

It is the purpose of this investigation to demonstrate the influence of electrophilic and electrodotic groups on the titratability of acid amides and other weakly basic compounds. The solvent systems chosen were acetic anhydride alone, acetic anhydride combined with equal parts of chloroform, and acetic anhydride combined with equal parts of acetone.

EXPERIMENTAL

Apparatus

A Metrohm model E336 recording potentiometer, equipped with conventional glass and calomel electrodes, was used in all titrations. The calomel electrode was not modified.

Reagents and Solutions

Acetic anhydride, A.C.S.; chloroform, A.C.S.; acetone, A.C.S.; dioxane, A.C.S.; and perchloric acid, 70%, were employed.

A 0.1 *N* solution of perchloric acid in dioxane was prepared and standardized potentiometrically against primary standard potassium acid phthalate in glacial acetic acid.

All compounds titrated were Eastman Kodak white label grade and were analyzed without further purification.

Procedures

Acetic Anhydride.—A sample of the material to be titrated equivalent to 0.5–1.0 mmole was stirred in 50 ml. of acetic anhydride. Heat was used to aid solubilization if necessary. The sample was titrated with 0.1 *N* perchloric acid in dioxane and titration followed with a Metrohm model E336 recording potentiometer.

Chloroform–Acetic Anhydride System.—A sample of the material to be titrated equivalent to 0.5–1.0 mmole was stirred in 25 ml. of chloroform. Upon dissolving the compound, 25 ml. of acetic anhydride was added and the titration carried out as previously described. For those compounds which were difficult to dissolve in chloroform, heat was used, and the 25 ml. of acetic anhydride was added to assist in solution.

Acetone–Acetic Anhydride System.—The size of sample and quantity of acetone used was identical to *Chloroform–Acetic Anhydride*. Again, where the material did not dissolve in acetone by stirring, heat and the addition of 25 ml. of acetic anhydride were used.

RESULTS AND DISCUSSION

In his report, Wimer (1) showed that a wide variety of acid amides and a few acylated amines could be titrated in acetic anhydride. In addition, he showed that the presence of glacial acetic acid seriously depressed the magnitude of the inflection point if more than 10% of that solvent was incorporated in the titration medium.

In the present study, amides and acylated amines were chosen to contain various electrophilic or electrodotic groups. The influence of these groups on the titratability of the compound will be interpreted in the following discussion.

In addition, the effect of an aprotic solvent, chloroform, and a weakly protophilic solvent, acetone, upon the titration of the chosen substances was interesting.

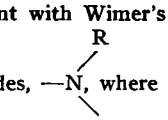
Table I reports the average of five determinations together with the standard deviation for titrations in acetic anhydride. For the other two solvent systems, the reported recoveries were the average of three determinations. In chloroform–acetic anhydride, the average range for the three determinations was 0.51%, with a low of 0.2 and a high of 0.9. The average range in acetone–acetic anhydride was virtually the same as that in the aforementioned solvent system as was the lowest and highest ranges. In each instance, the $\Delta E/\Delta V$ value was the average of all determinations for a compound in a particular solvent system.

An arbitrary limit of $\pm 5\%$ from theoretical has been selected as satisfactory for the quantitative recovery of the weak bases in this study. Upon examination of the data for the first three compounds, which were all derivatives of formamide, the first two, which have electrodotic groups, gave

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TABLE I.—TITRATION OF AMIDES IN ACETIC ANHYDRIDE ALONE AND IN COMBINATIONS

Compd.	Recoveries, %			$(\Delta E/\Delta V)_{Max.}$		
	Acetic Anhydride	Chloroform–Acetic Anhydride	Acetone–Acetic Anhydride	Acetic Anhydride	Chloroform–Acetic Anhydride	Acetone–Acetic Anhydride
N-Ethylformamide	100.5 ± 0.10	104.9	101.5	215	385	170
N,N-Dibutylformamide	104.5 ± 0.11	108.1	104.4	595	390	235
N,N-Diphenylformamide	No end point	No end point	No end point			
Acetamide	101.4 ± 0.77	No end point	No end point	260	...	...
2-Chloroacetamide	No end point	No end point	No end point			
2-Cyanoacetamide	No end point	No end point	No end point			
N,N-Dimethylacetamide	98.8 ± 0.20	106.5	98.3	185	290	505
N-Ethylacetamide	100.8 ± 0.38	105.1	100.3	150	275	325
N,N-Di-*n*-propylacetamide	102.0 ± 0.25	104.8	101.4	280	360	165
N-*n*-Butylacetamide	103.4 ± 0.32	106.8	103.0	255	325	190
2-Phenoxyacetamide	85.6 ± 0.35	No end point	No end point	110	...	...
1-Naphthaleneacetamide	98.5 ± 0.34	103.7	101.7	235	240	75
N-Methyl-*N*-1-naphthylacetamide	100.6 ± 0.30	102.7	No end point	200	100	...
Thioacetamide	98.7 ± 0.12	98.3	100.8	405	730	420
N,N-Diethylpropionamide	100.7 ± 0.28	102.9	102.8	270	450	210
N,N-Di-*n*-butylpropionamide	104.4 ± 0.35	105.9	102.9	330	315	175
Butyramide	101.6 ± 0.37	101.5	High result	385	315	...
Isobutyramide	100.2 ± 0.20	101.2	High result	285	320	...
Acrylamide	98.3 ± 0.29	98.0	No end point	215	150	...
2-Furamide	70.8 ± 0.93	72.7	No end point	220	150	...
Hexanamide	101.9 ± 0.22	104.9	No end point	280	305	...
N-Butyloctadecanamide	Insoluble	104.5	Insoluble	...	330	...
Benzamide	84.4 ± 0.78	99.7	No end point	245	180	...
o-Ethoxybenzamide	97.8 ± 0.21	99.5	98.3	335	265	165
p-Nitrobenzamide	No end point	No end point	Insoluble	...	...	...
Oxamide	Insoluble	Insoluble	Insoluble	...	...	...
Malonamide	100.4 ± 0.49	Insoluble	Insoluble	335	...	...
Adipamide	Insoluble	Insoluble	Insoluble	...	...	...

satisfactory recoveries in acetic anhydride and acetic anhydride combined with acetone. No explanation can be offered for their somewhat high recoveries in the chloroform–acetic anhydride combination, particularly when one considers the sharpness of the respective end points as illustrated by the $\Delta E/\Delta V$ values. As would be predicted, the third compound on which the nitrogen was substituted by the two electrophilic phenyl groups gave no detectable end point in the three solvent combinations. This finding is in agreement with Wimer's (1) observation that tertiary amides,  , where R is CH_3—C—, exhibited no measurable basic properties. He did not comment on secondary and tertiary amides where the R groups were electron donors. However, Table I shows that the first derivative values were much higher for *N,N*-dibutylformamide than they were for *N*-ethylformamide. This was to be expected. In addition, the $\Delta E/\Delta V$ values at the inflection point should have been greater for the *N*-alkylated acetamide derivatives than for acetamide itself. This was not found experimentally, and no logical explanation can be offered. However, it is worthwhile to mention that the *N*-alkylated derivatives were titratable in the other two solvent systems, which are less acidic and hence less potentrating than acetic anhydride alone, while acetamide was titratable only in acetic anhydride. This would indicate that the *N*-alkyl groups have enhanced the basicity of the amide moiety in these compounds. In addition, there was a further indication of this phenomenon when it was observed that the $\Delta E/\Delta V$ value for *N,N*-di-*n*-butylpropionamide was somewhat greater than that for *N,N*-diethylpropionamide. This was to be expected since the *n*-butyl groups should have a greater electron donating potential than the ethyl moieties.

It was expected that the chloro- and cyano-derivatives would not be titratable in the solvent systems because of the electrophilic influence of these groups. This was demonstrated experimentally. The phenoxy group is less electrophilic than the two aforementioned ones but still sufficiently so to cause depression of the end point which resulted in less than quantitative recovery.

The only compounds of the acetamide series which exhibited anomalous and unpredictable behavior were 1-naphthaleneacetamide and *N*-methyl-*N*-1-naphthylacetamide. One would have expected the naphthalene group to behave as a strong electrophile and thus result in compounds that would be untitratable. While they appeared to be slightly less basic than acetamide when acetic anhydride was the solvent medium, both of them behaved as stronger bases in the chloroform–acetic anhydride system. Naphthylene acetamide even gave a weak but detectable end point in the acetone–acetic anhydride mixture.

The behavior of thioacetamide was predictable since $\rangle C = S$ is less electrophylic than $\rangle C = O$; hence, the thio compound should be more strongly basic than acetamide. This has been demonstrated experimentally.

Wimer (1) has suggested that the low recoveries which he obtained with acrylamide and 2-furamide may have been due to reaction of these compounds with acetic anhydride. In the present work, the

TABLE II.—TITRATION OF ACYLATED AMINES IN ACETIC ANHYDRIDE ALONE AND IN COMBINATIONS

Compd.	Recoveries, %			$(\Delta E/\Delta V)_{Max.}$		
	Acetic Anhydride	Chloroform–Acetic Anhydride	Acetone–Acetic Anhydride	Acetic Anhydride	Chloroform–Acetic Anhydride	Acetone–Acetic Anhydride
Acetanilide	110.5 ± 0.95	[a]	[a]	145	...	...
o-Nitroacetanilide	[a]	[a]	[a]	...	...	...
m-Nitroacetanilide	[a]	[a]	[a]	...	...	...
p-Nitroacetanilide	[a]	[a]	[a]	...	...	...
N-Ethyl-4′-nitroacetanilide	[a]	[a]	[a]	...	...	...
2′-Chloroacetanilide	[a]	[a]	[a]	...	...	...
3′-Chloroacetanilide	[a]	[a]	[a]	...	...	...
4′-Chloroacetanilide	[a]	[a]	[a]	...	...	...
α-Cyanoacetanilide	[a]	[a]	[a]	...	...	...
Acetoacetanilide	[a]	[a]	[a]	...	...	...
o-Hydroxyacetanilide	[a]	[a]	[a]	...	...	...
p-Hydroxyacetanilide	[a]	[a]	[a]	...	...	...
N-*n*-Butylacetanilide	105.4 ± 0.32	109.0	102.8	160	200	120
n-Butyranilide	[a]	[a]	[a]	...	...	...
N-Ethylacetanilide	103.7 ± 0.32	103.8	[a]	290	155	...
O-Acetophenetidide	[a]	[a]	[a]	...	...	...
m-Acetotoluidide	106.2 ± 0.50	104.5	[a]	100	100	...
o-Acetotoluidide	107.9 ± 0.27	103.6	[a]	150	115	...
Benzanilide	[a]	[a]	[a]	...	...	...

[a] No end point.

findings for acrylamide would indicate that such a reaction did not occur since reasonably quantitative recoveries were obtained in two solvent systems. The $\Delta E/\Delta V$ values were smaller for both compounds in acetic anhydride than they were for acetamide, and this was probably due to the existence of a conjugated system of double bonds in both acrylamide and 2-furamide. Such a system would exert an electrophilic effect.

Benzamide would fall into the same category as acrylamide and 2-furamide, since the phenyl group would be conjugated with the carbonyl. It was interesting that quantitative recovery was not obtained for this material in acetic anhydride; but when chloroform–acetic anhydride was used, excellent results were obtained. This would indicate that the presence of an aprotic solvent may be beneficial in some instances. On the other hand, *o*-ethoxybenzamide gave satisfactory results in all three solvent systems; this was to be anticipated because of the electrodotic ethoxy group. As expected, *p*-nitrobenzamide was too weakly basic to be titrated in any solvent system. Salicylamide was investigated also, although it was not included in Table I. The compound was not titratable in the solvent systems used in this investigation, possibly because of hydrogen bonding between the hydroxyl hydrogen and the amide nitrogen.

The solvent system of acetone–acetic anhydride was probably less capable of enhancing the weakly basic properties of the compounds and could account for the failure to reach an end point in many instances. It was noteworthy, however, that when end points were detectable, the quantitative data generally agreed closely with those obtained in acetic anhydride alone.

Although several of the compounds gave recoveries which were above theoretical values, when chloroform–acetic anhydride was the solvent, others gave excellent results with readily detectable end points. While this manuscript was in preparation, Cowell and Selby (12) reported the titration of weak bases in acetic anhydride which contained up to 50% chloroform; they observed that this latter solvent had little effect on the shape of the titration curve. The findings of the present report confirmed this observation. Such a solvent mixture has the added advantage of widening the scope of these titrations, due to the solubilizing properties of chloroform. Such a system, then, should be considered as a desirable alternate to acetic anhydride in the titration of very weak bases.

There was little that could be said concerning the data in Table II, except to observe that acetanilide itself was so weakly basic that it was barely titratable even in acetic anhydride. This phenomenon can be attributed partially to the electrophilic properties of the acetyl group, but the existence of resonance in the molecule may also play an important part in decreasing its basicity. Obviously then, those derivatives which contained additional electrophilic groups such as nitro, chloro, and cyano were even less basic and hence not titratable. Only those derivatives possessing electrodotic groups showed improved titratability, as would be predicted. Data in acetic anhydride were based on five determinations, while the values in the other solvents were the average of three titrations.

CONCLUSION

From the experimental evidence presented in this report, it was concluded that, in most instances, an accurate prediction could be made regarding the titratability of amides and acylated amines by examination of the structure of the compound.

A solvent combination consisting of equal parts of chloroform and acetic anhydride offers a useful alternate medium for the titration of weak bases.

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Incorporation of Ornithine-2-C^{14} into Scopolamine

By FRED A. TURNER* and JAMES E. GEARIEN

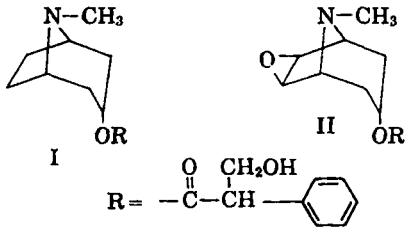
Radioactive hyoscyamine and scopolamine were isolated from 7-week-old *Datura stramonium* plants after the administration of ornithine-2-C^{14}. Degradation of the scopolamine showed that the radioactivity was located at the numbers 1 and 5 carbon atoms of the tropane ring.

THE ROLE OF ornithine as a precursor in the biosynthesis of hyoscyamine was demonstrated by Marion and co-workers (1), who isolated radioactive hyoscyamine (I) from 5-month-old *Datura stramonium* plants which had been fed ornithine-2-C^{14}. Degradation of the radioactive hyoscyamine showed that the radioactivity was located at carbons 1 and 5 of the tropane ring, and indication that at least most of the ornithine molecule was incorporated into hyoscyamine. Scopolamine (II) isolated from these plants proved to be inactive. These investigators suggested that either ornithine was not a direct precursor of scopolamine or that scopolamine was not being synthesized in 5-month-old plants.

Since further experiments by Marion (2) indicated that scopolamine synthesis had halted in mature plants, it seemed desirable to attempt to feed labeled ornithine to younger plants to establish its role in the biosynthesis of scopolamine. During the course of this investigation, a study by Leete (3) reported the isolation of radioactive scopolamine from 3-month-old *D. stramonium* plants which had been fed radioactive ornithine. Since no degradative studies of the alkaloid were reported, the study offers no evidence of whether ornithine is incorporated as a unit or is broken down into fragments before incorporation.

To determine the most favorable age for the administration of the radioactive ornithine, *D. stramonium* plants from the age of 2 to 12 weeks were analyzed for their alkaloidal content. The results of the analyses are listed in Table I.

The first alkaloid to be formed was scopolamine. Hyoscyamine was not formed in significant quantities until the fourth week, after which its concentration rapidly increased. After the seventh week, hyoscyamine was present in larger amounts than scopolamine.



From this study it appeared that a precursor must be fed to young plants if it is to be incorporated into scopolamine. Seven-week-old plants were chosen for the study since they were still synthesizing scopolamine and were of sufficient size to be handled conveniently.

When 7-week-old *D. stramonium* plants were injected with ornithine-2-C^{14}, both radioactive hyoscyamine and scopolamine were isolated. If ornithine was incorporated into scopolamine as a single unit through a biogenetic pathway similar to that suggested by Marion (1), the radioactive carbons of the scopolamine would be located at carbons 1 and/or 5 of the tropane ring. To establish if this was the biogenetic pathway, the isolated radioactive scopolamine was converted to hyoscyamine, which was hydrolyzed to tropine and degraded by a previously reported method (1).

Considerable difficulty was encountered in establishing a satisfactory method for the conversion of scopolamine to either hyoscyamine or tropine. Attempts to convert scopolamine to the corresponding episulfide, which could then be desulfurized to yield hyoscyamine, failed. Scopolamine was recovered unchanged when heated with aqueous solutions of thiourea or potassium thiocyanate at temperatures ranging from 25 to 115°.

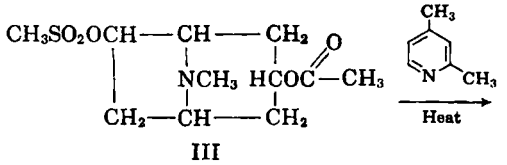
While the epoxide ring of scopolamine could be

TABLE I.—AGE-ALKALOID RELATIONSHIP OF YOUNG *D. stramonium*

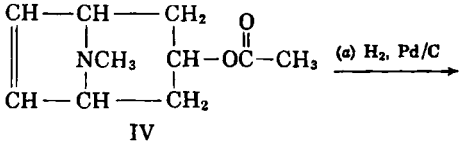
Age, Wk.	Wt. Hyoscyamine Base, mg.	Wt. Scopolamine Base, mg.	Scopolamine Hyoscyamine Ratio, Av.
2	0	6.58	...
	0	6.77	
3	0.76	10.00	17.7
	0.31	6.90	
4	5.26	12.55	2.48
	4.91	13.15	
5	6.38	13.08	2.12
	6.43	14.12	
6	13.20	17.05	1.26
	14.41	17.61	
7	15.05	19.39	1.18
	17.14	18.28	
8	22.82	17.69	0.82
	23.00	19.61	
10	34.01	24.22	0.72
	35.22	25.43	
12	35.81	25.20	0.69
	35.75	24.02	

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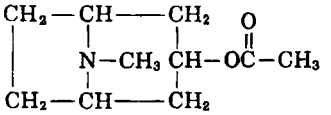
opened by hydrogenation to form 6-hydroxyhyoscyamine, it was not possible to remove the hydroxyl group by reduction of its tosyl derivative. 3-Acetoxytropane (V) was obtained when 3-acetoxy-6-methanesulfonyloxytropane (III) was desulfonated by heating with 2,4-lutidine in a sealed tube and the resulting 3-acetoxy-6,7-dehydrotropane (IV) hydrogenated. Unfortunately, the yield was too low to permit further degradation.



III



IV



V

Catalytic hydrogenation of scopolamine, followed by hydrolysis, provided the best synthetic route to tropine. This reaction has been reported by Fodor and co-workers (4), who suggested that the hydrogenolysis proceeds through a mechanism similar to that of nickel desulfurization. In view of this hypothesis, the alkaloid was hydrogenated with a large excess of W-28 Raney nickel. After alkaline hydrolysis, a 33% yield of tropine was obtained. Use of a more active catalyst resulted in a lower yield of tropine. When applied to the radioactive alkaloid, this procedure provided a sufficient quantity of tropine for the degradation.

The tropine was then degraded according to a modification of the procedure developed by Marion and co-workers (1). Mild oxidation of tropine (VI) by dilute acidic potassium dichromate (5) afforded tropinone (VII), which upon vigorous chromic acid oxidation yielded *N*-methylsuccinimide (VIII). Reaction of a large excess of phenylmagnesium bromide with the *N*-methylsuccinimide produced approximately equal amounts of 1-methyl-5-hydroxy-5-phenyl-2-pyrrolidone (IX) and 1-methyl-2,5-diphenylpyrrole (X) (6). The latter compound was oxidized to benzoic acid with acid potassium dichromate.

The specific activities of hyoscyamine and its hydrolysis products are shown in Table II, while the data for scopolamine and its degradation products are given in Table III.

The data in Table III indicate that all of the radioactive ornithine incorporated into scopolamine entered the tropane ring. The benzoic acid had essentially the same activity as that of tropine (one-half the specific activity since 2 moles of benzoic acid arose from 1 mole of tropine). This indicates that the No. 2 carbon atom of ornithine produces the bridgehead carbon atoms (No. 1 and No. 5) of the tropane ring. Ornithine-2-C^{14} is thus incorporated into both hyoscyamine and scopolamine.

TABLE II.—HYOSCYAMINE HYDROBROMIDE AND HYDROLYSIS PRODUCTS

Compd.	Specific Activity, c.p.m./mM × 10^{4a}
Hyoscyamine hydrobromide	5.10
Tropic acid	Traces
Atropic acid	Traces
Tropine picrate	5.11

[a] All specific activities have been corrected for efficiency and self-absorption.

TABLE III.—SCOPOLAMINE HYDROBROMIDE AND DEGRADATION PRODUCTS

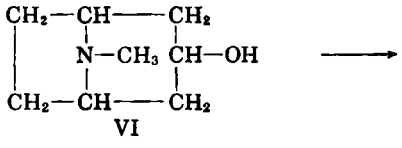
Compd.	Specific Activity, c.p.m./mM × 10^{4a}
Scopolamine hydrobromide	2.16
Atropic acid	Inactive
Tropic acid	Inactive
3,6-Tropanediol	1.88
Tropine picrate	2.01
N-Methylsuccinimide	1.80
1-Methyl-2,5-diphenylpyrrole	1.76 (after correction)[b]
Benzoic acid	0.88 (after correction)[b]

[a] All specific activities have been corrected for efficiency and self-absorption. [b] These are specific activities corrected for dilution with inactive *N*-methylsuccinimide.

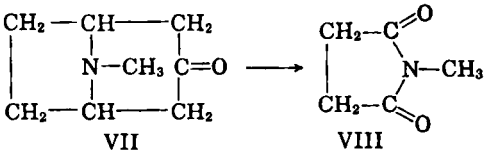
EXPERIMENTAL

General Procedure for Extraction and Separation of Alkaloids from D. Stramonium

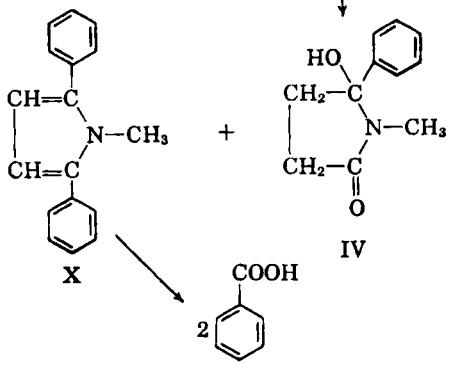
Extraction of the Alkaloids.—The method suggested by Evans and Partridge (7) for the extraction of the alkaloids of *D. stramonium* was employed in the isolation of hyoscyamine and scopolamine.



VI



VII VIII



Separation of the Alkaloids by Column Chromatography.—The hyoscyamine and scopolamine were separated by column chromatography using a Celite column. The procedure described by Schill and Agren (8) was employed. To determine the quantity of alkaloid present, the residue from each fraction was separately dissolved in 4 ml. of hydrochloric acid. Gold chloride solution (0.2 *M*) was added to the acidic solution to precipitate the aurichloride salts of the alkaloids. After cooling in the refrigerator for 1 day, the aurichlorides were collected by suction filtration, washed with cold water, dried, and weighed. The corresponding weights of the free bases were calculated from the weights of the aurichloride salts.

The weights of hyoscyamine and scopolamine in samples of *D. stramonium* which vary in age from 2 to 12 weeks are presented in Table I.

Isolation of the Radioactive Alkaloids

Administration of DL-Ornithine-2-C^{14} to D. stramonium Plants.—From 27-week-old *D. stramonium* plants, the 10 most vigorous plants of equal size were selected for the administration of ornithine-2-C^{14}. The flower pods were just beginning to emerge at this time. A solution was prepared containing 16.9 mg. of DL-ornithine hydrochloride-2-C^{14} with a specific activity of 1.0 mc./m*M* and a total activity of 0.1 mc. or 2.22×10^8 c.p.m. in 1 ml. of water. This solution was then injected into the plants by employing the following technique.

While holding the plant in a horizontal position, 1 drop of the active solution was placed at the base of the petiole of a large leaf. With a small sewing needle, a prick was made at the site of the water droplet. As soon as the solution had been absorbed into the plant, another drop was placed at the same spot. Several pin pricks were made in the same area to speed the uptake of the solution. From 50 to 100 μl. of the solution was allowed to be absorbed in each area. After the radioactive solution was absorbed, an additional 2–3 drops of water was allowed to be taken up to ensure that none of the ornithine was left clinging on the outside of the petiole. Using several leaves, a total of 0.1 ml. of the radioactive solution was injected into each plant.

One week after the administration of the radioactive ornithine, the plants were harvested. The leaves, roots, and stems were separated and dried in a circulating hot air oven at 50–55° for 2 days. They were then ground to a No. 40 powder.

Extraction of the Radioactive Alkaloids.—The 31.7 Gm. of combined plant powder was thoroughly moistened with 20 ml. of water and allowed to macerate for 1 hour. The moistened drug was triturated with 6.5 Gm. of calcium hydroxide and extracted with 300 ml. of ether for 6 hours. The alkaloids were then obtained from the ether extract as previously described (7).

Column Chromatographic Separation of the Alkaloids.—The hyoscyamine and scopolamine were separated on a Celite column moistened with 1.0 *N* hydrochloric acid in the manner previously described (8). To determine the purity of each fraction, the solvents were evaporated and the residues dissolved in 1 ml. of chloroform. Five microliters of each solution was chromatographed on paper according to the method of Drey and Foster (9). The developed chromatograms showed only one spot from each solution, an indication that the fractions were pure (Table IV).

TABLE IV.— CHROMATOGRAPHICALLY SEPARATED ALKALOIDS

	Alkaloid Wt., mg.	Specific Activity, c.p.m./m*M* × 10^6	% Incorporation
Hyoscyamine hydrochloride	29.00	8.2	0.37
Scopolamine	39.25	9.8	0.44

Preparation of the Hydrobromide Salts of the Alkaloids.—To the hyoscyamine and scopolamine fractions was added 25 ml. of chloroform saturated with hydrogen bromide gas, and the solvent was removed *in vacuo*. Inert hyoscyamine hydrobromide (318.0 mg.) and scopolamine hydrobromide (515.4 mg.) were added to their respective radioactive fractions and the salts recrystallized twice from absolute ethanol-ether to obtain a constant specific activity.

Degradation of the Radioactive Alkaloids

Saponification of Hyoscyamine Hydrobromide.—The 0.238 Gm. of recrystallized radioactive hyoscyamine hydrobromide was diluted with 1.757 Gm. of inactive alkaloid, and the mixture was saponified by refluxing for 45 minutes with 25 ml. of 10% sodium hydroxide solution. The aqueous solution was continuously extracted with ether for 20 hours.

After drying over anhydrous magnesium sulfate, the ether extract was evaporated to a light yellow syrup which crystallized after standing for several hours in a vacuum desiccator. The yield of light yellow crystals of tropine was 0.69 Gm. (90.8%), m.p. 62.5–63.5°.

The aqueous solution recovered from the ether extraction was acidified with 15 ml. of 6 *N* hydrochloric acid to precipitate the atropic acid. After cooling in an ice bath, the white solid was collected by suction filtration, washed with water, and dried. The weight of atropic acid was 0.34 Gm. (42.5%), m.p. 105–106°.

The aqueous mother liquor from the atropic acid was extracted with three 25-ml. portions of ether. The ether extracts were dried over anhydrous magnesium sulfate and evaporated to yield a light green oil which crystallized upon scratching. The yield of tropic acid was 0.42 Gm. (47%), m.p. 96–105°. After recrystallization from ether-petroleum ether (b.p. 30–60°), the melting point was 115–116°.

Hydrogenation of Scopolamine Hydrobromide.—The 0.385 Gm. of recrystallized radioactive scopolamine hydrobromide, after dilution with 10.612 Gm. of inactive alkaloid (26.8 mmoles), was dissolved in 100 ml. of water and hydrogenated for 4 days at room temperature and atmospheric pressure in a glass microhydrogenation apparatus. Eighteen grams of commercial W-28 Raney nickel (Raney Catalyst Co.), washed free of excess alkali, was used as the catalyst.

The catalyst was removed by suction filtration and thoroughly washed with water. The filtrate was concentrated to approximately 25 ml. and refluxed for 90 minutes with 30 ml. of 20% sodium hydroxide solution. The alkaline solution was continuously extracted with ether for 2 days. The

white crystals that had collected in the ether extract were removed by suction filtration, washed with ether, and dried. The yield of 3,6-tropanediol was 2.45 Gm. (54.6%), m.p. 177–179°.

The ether filtrate was evaporated to a yellow syrup which was placed in a vacuum desiccator to induce crystallization. The weight of tropine was 1.35 Gm. (33.6%), m.p. 59–61°.

The alkaline solution from the ether extraction was acidified with concentrated hydrochloric acid to precipitate atropic acid which was collected by suction filtration, washed with water, and dried; yield, 2.48 Gm. (58.5%), m.p. 97–101°. After recrystallization from ethanol-water, it melted at 105–106°.

The filtrate from the atropic acid isolation was extracted with four 25-ml. portions of ether which, after drying over magnesium sulfate, was evaporated to dryness. The light yellow oil crystallized to furnish 1.14 Gm. (24.0%) of tropic acid which, after recrystallization from ether-petroleum ether (30–60°), melted at 115–116°.

Tropinone.—A solution of 1.33 Gm. (9.45 mmoles) of tropine in 4 ml. of 25% sulfuric acid was cooled in an ice-bath. To this solution was added dropwise with constant swirling, 16 ml. of a solution composed of 100 ml. of 2 *M* potassium dichromate and 43 ml. of concentrated sulfuric acid (equivalent to 1.1 Gm. of potassium dichromate). The dark solution, kept cold in an ice bath, was treated with sufficient 20% sodium hydroxide solution to neutralize the sulfuric acid and dissolve the precipitate of chromium hydroxide. The solution was saturated with anhydrous potassium carbonate and extracted with ten 25-ml. portions of ether. The organic extracts were dried over anhydrous magnesium sulfate and the solvent evaporated to yield an oil which crystallized upon scratching. The yield of white crystalline tropinone was 1.08 Gm. (82.5%), m.p. 39–41°. [Lit. 41–42° (10).]

Oxidation of Tropinone.—To a cooled solution of 1.05 Gm. (7.6 mmoles) of tropinone in 15 ml. of 25% sulfuric acid was slowly added a solution of 7.0 Gm. of chromic acid in 20 ml. of 25% sulfuric acid. The resulting solution was heated in an oil bath at 140° for 4 hours. The acidic solution was poured over 30 Gm. of ice and the excess chromic acid decomposed by the addition of 5 Gm. of sodium bisulfite. The aqueous solution was continuously extracted with thiophene-free benzene for 2 days.

The benzene extract was dried over anhydrous magnesium sulfate, and the solvent was then evaporated at 30° by means of a flash evaporator. The residue, which consisted of white crystals in a yellow gum, was washed with several small portions of cold ether to remove the yellow oil and to yield 70.5 mg. (8.2%) of light brown crystals of *N*-methylsuccinimide, m.p. 64–66°.

1-Methyl-2,5-diphenylpyrrole.—To 1.5 Gm. (58 mmoles) of dried magnesium turnings was added 8 ml. (76 mmoles) of redistilled bromobenzene and 30 ml. of anhydrous ether. The reaction was protected from the atmosphere by means of soda-lime and calcium chloride. After the reaction had been initiated, an additional 15 ml. of ether was added, and the mixture was stirred until all of the magnesium had reacted.

A solution of 57.0 mg. (0.505 mmoles) of radioactive *N*-methylsuccinimide and 108.8 mg. (0.95 mmoles) of inactive *N*-methylsuccinimide in 40 ml. of dry benzene was prepared. This solution was added dropwise over a period of 30 minutes to the well-stirred Grignard solution. Stirring was continued at room temperature for an additional 22 hours.

The reaction mixture was poured over 50 Gm. of ice containing 3 ml. of sulfuric acid. This mixture was transferred to a separator, thoroughly mixed, and the layers separated. The aqueous layer was extracted with eight 30-ml. portions of ether and then set aside. The ether extracts were combined with the original organic layer, and the solvent was then removed *in vacuo*. The residue was steam distilled to remove excess bromobenzene and biphenyl.

The residue remaining after the steam distillation was cooled in an ice bath, the excess water decanted, and the brown tar dissolved in 15 ml. of absolute ethanol. The ethanol solution was then placed in the refrigerator for 2 days, during which time a crystalline product formed. The light tan crystals were collected and dried to yield 28.44 mg. (7.8%) of 1-methyl-2,5-diphenylpyrrole, m.p. 192–195°. [Lit. 192–196° (1).]

A solid which had crystallized after a few days from the aqueous layer was collected by suction filtration and dried. The weight of solid was 22.47 mg. (7.6%) and melted at 137–139°. [Lit. 139–141° (6).] The infrared spectrum was identical to 1-methyl-5-phenyl-5-hydroxy-2-pyrrolidone.

Oxidation of 1-Methyl-2,5-diphenylpyrrole to Benzoic Acid.—A mixture of 28.4 mg. (0.12 mmoles) of 1-methyl-2,5-diphenylpyrrole, 0.5 Gm. of potassium dichromate, and 25 ml. of 20% sulfuric acid was refluxed for 3 hours, during which time it was occasionally shaken. The small amount of unreacted starting material, removed by filtration, was again refluxed with 0.25 Gm. of fresh potassium dichromate in 15 ml. of 20% sulfuric acid for 2 hours. The solutions were combined, cooled, and the excess dichromate was decomposed with sodium bisulfite. The aqueous solution was then continuously extracted with ether for 18 hours.

The ether extract was dried over anhydrous magnesium sulfate, then evaporated to dryness *in vacuo* below 20° to afford 23.88 mg. of a light tan solid melting at 102–107°. The impure benzoic acid was recrystallized from water to yield 5.40 mg. (18.3%) of white benzoic acid melting at 119–120°. Evaporation of the mother liquor produced 6.03 mg. (20.4%) of additional impure benzoic acid, m.p. 110–113°.

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Determination of Micro Amounts of Estrogens in Anabolic Vitamin Tablets by Quantitative Paper Chromatography

By HAROLD KADIN, MARIARITA S. UGOLINI, and HENRY R. ROBERTS

A quantitative paper chromatographic method has been developed which is used routinely as a stability assay for estrogenic hormones in anabolic vitamin tablets. The method involves (*a*) extraction of the estrogenic hormone into ether from an alcohol-water suspension of the ground tablets, (*b*) clarification of the extract by chromatography on an alumina column, (*c*) paper chromatographic separation of the estrogenic hormone present in the column eluate from interfering degradation products, (*d*) location of the estrogenic hormone on the chromatogram by the guide strip technique employing a chromogenic agent, (*e*) elution of the estrogenic hormone from the chromatogram, and (*f*) quantitative spectrophotofluorometric analysis of the eluate. Recoveries in excess of 95 per cent are obtained by chromatographing simultaneously replicates of standard and sample solutions on the same chromatogram. A detailed account of the procedure is presented using the assay of ethinyl estradiol in concentrations of 0.0014 per cent in anabolic vitamin tablets as an example.

ANABOLIC VITAMIN tablets are oral preparations containing small amounts of anabolic steroid hormones combined with vitamins and minerals.

While the androgen–estrogen ratio in such tablets is proportioned to induce the desired anabolic action, the physiological potency of estrogens necessitates that they be present in micro amounts.

A quantitative paper chromatographic–spectrophotofluorometric method for the analysis of estrogens in castor oil and tablet formulations (1) serves as the basis for the quantitation of estrogens in anabolic vitamin tablets. However, the multiplicity of components present in such preparations requires extensive extraction and clarification prior to paper chromatographic quantitation.

The estrogen is extracted into ether from an alcohol-water suspension of the ground tablets, and is then adsorbed on an alumina column for separation from some interfering constituents. After elution from the column, the solvent is evaporated, and the estrogen residue is dissolved in a small volume of 95% ethanol. Aliquots of this extract are subjected to paper chromatography, followed by quantitation of the estrogen by spectrophotofluorometry.

The method is described in detail using, as an example, the determination of ethinyl estradiol in concentrations of 0.0014% in anabolic vitamin tablets[1] (Table I).

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[1] Oral preparation providing therapeutic amounts of ethinyl estradiol and methyltestosterone plus protective amounts of vitamins and added minerals. Marketed as Dumogran by E. R. Squibb & Sons, New Brunswick, N. J.

EXPERIMENTAL

Reagents and Equipment.—Analytical grade ether, chloroform (reagent grade), aqueous acetone solution (add 1% water to reagent grade acetone), 95% ethanol (redistilled for paper chromatography), toluene (reagent grade), and propylene glycol (reagent grade) were used. Aluminum oxide (Woelm, neutral activity, grade 1 for chromatography, Alupharm Chemicals, New Orleans, La.) was also employed. (Keep tightly stoppered and store in a desiccator to prevent moisture pickup.) Sea sand (Merck reagent No. 7478) was used. (Store in a dessicator.)

Chromatographic columns—the column body is approximately 9.5 mm. O.D. and 150 mm. long equipped with a capillary tip (approximately 6 mm. O.D. by 1 to 2 mm. I.D. and 40 mm. long) at one end and a solvent reservoir (approximately 38 mm. O.D. × 100 mm. long) at the other end. An electric mortar

TABLE I.—COMPOSITION OF ANABOLIC VITAMIN TABLET

	mg.
Anabolic Hormones	
Methyltestosterone	4.0
Ethinyl estradiol	0.008
Vitamins	
A	2,500 U.S.P. units
D	250 U.S.P. units
B_1	2.5
B_2	1.5
B_6	1.0
B_{12} as cobalamin concentrate	0.001
Folic acid	0.1
Niacinamide	10.0
Calcium pantothenate	2.5
C	37.5
E	2.5 Int. units
Minerals	
Iron	5.0
Iodine	0.05
Copper	0.5
Manganese	0.5
Magnesium	3.0
Zinc	0.5

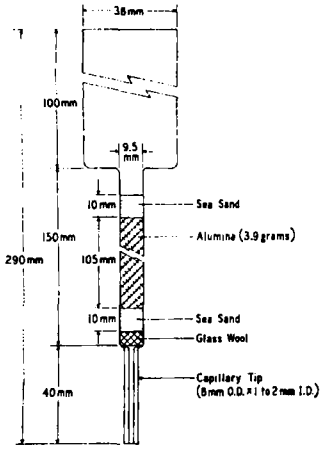


Fig. 1.—Column used in the purification of ethinyl estradiol.

grinder (similar to model MG-2, Torsion Balance Co., Clifton, N. J.), and glass syringes, 50 ml. (B & D Luer Lok 50 YL) with attachable 14 gauge, 5 in. long cannulae (B & D No. 1250 NR, Becton, Dickinson and Co., Rutherford, N. J.) were employed. A manometer bulb and release valve (No. 5000 BV, Becton, Dickinson and Co., Rutherford, N. J.) attached *via* rubber tubing to glass tubing in a single hole No. 7 rubber stopper was provided. The paper chromatographic equipment and reagents were previously described (1), except the filter paper was ethanol washed four times.

Preparation of Column.—Fill the column with the chloroform so that it reaches up into the reservoir about 5 cm., and seal the outlet with the finger tip. Tamp a small plug of glass wool into the bottom of the column. Add approximately 2 cm. of sea sand on top of the plug. Tap in some alumina, remove the finger seal, and stir the column contents as the alumina descends to insure the release of trapped air bubbles. Maintain the chloroform level at 5 cm. into the reservoir.

Perform the same operation with succeeding portions of alumina until an alumina column of about 10.5 cm. (3.9 Gm. of alumina) is obtained. Top off the column with 1 cm. of sea sand, and allow the chloroform to drain to about 2 cm. above the top of the column. Pressure is applied at the top of the column with the rubber stopper and manometer bulb to accelerate drainage and remove air bubbles.

Do not allow the column to run dry. Figure 1 shows a typical column.

Preparation of Standard.—The reference standard solution should contain 0.2 mg. of ethinyl estradiol per milliliter of solution. In a volumetric flask, the appropriate amount of the standard is dissolved in the smallest volume of *N,N*-dimethylformamide. Then methyl isobutyl ketone is added to the mark.

Preparation of Sample.—Grind 120 tablets in the mortar grinder for 5 to 10 minutes until a fine powder is obtained. An amount of powder which contains, based on theory, 400 mcg. of ethinyl estradiol is weighed and transferred to a 250-ml. tall form glass-stoppered bottle (2 in. diameter). Add, with swirling, 14 ml. of 95% ethanol followed by 40 ml. of water. Stopper and shake. Add 100 ml. of ether, stopper, invert twice, remove stopper to release pressure, then restopper, and shake vigorously for 15 to 20 seconds. Place bottle in centrifuge cup, and spin for 10 minutes at 2000 r.p.m.

Transfer the ether layer with a syringe and cannula to a 250-ml. separator containing 20 ml. of 0.1 *N* HCl. Extract the suspended tablet powder further with 2 × 50 ml. of ether, and transfer the ether layers (after centrifugation) to the separator.

Stopper the separator, and shake once. With the separator inverted, release ether pressure by opening the stopcock. Close the stopcock, and shake vigorously 10 to 15 times. Discard the lower aqueous layer. Wash the pooled ether extract with another 20 ml. of 0.1 *N* HCl, then with 1 × 5 ml. of distilled water.

Transfer the washed ether extract to a 500-ml. conical flask. Evaporate the ether at 60° under a stream of nitrogen. Redissolve the residue in 10 ml. of anhydrous ether, and take to dryness as before. Repeat the ether addition and evaporation a second time. Toward the end of the evaporation, insure a completely anhydrous and solvent-free residue by using a vigorous stream of nitrogen and rotating the flask to spread the oil layer. Remove the source of heat, and flush the flask with nitrogen for 5 minutes.

Column Chromatography.—Dissolve the oily residue obtained in the sample preparation in 10 ml. of chloroform, and transfer to the prepared alumina column. Allow this first solution to drain to within 2 cm. of the top of the column. Rinse the flask with two successive 10-ml. portions of chloroform, and transfer to the column, allowing each wash to descend to within 2 cm. of the top of the column. The manometer bulb and rubber stopper may be used to maintain a flow rate of 1–2 drops per second.

Elute the ethinyl estradiol from the column with 75 ml. of acetone containing 1% added water, and collect the eluate in a 200-ml. volumetric flask. Evaporate the acetone eluate to dryness at 60° with a current of nitrogen. Rinse down the sides of the flask with 5 ml. of acetone, and take down to dryness in the same manner. Allow the flask to cool under nitrogen. Add 4 ml. of 95% ethanol. Shake well to insure complete solution of the ethinyl estradiol. This solution is then assayed by paper chromatography.

Paper Chromatography.—Two chromatograms are run per sample. The slotted filter paper described previously (2) contains six 0.75-in. strips which are spotted as follows:

Origin: *1* *2* *3* *4* *5* *6*

1, Sample = 0.2 ml. *4*, Sample = 0.2 ml.
2, Standard = 0.1 ml. *5*, Sample = 0.2 ml.
3, Standard = 0.1 ml. *6*, Paper blank

Two-tenths-milliliter capacity blow-out pipets, graduated in 0.01 ml., are used to apply the standards and the sample to the paper. The spotting technique has been described in detail previously (2).

The spotted strips are impregnated with propylene glycol by dipping them into a 25% solution of propylene glycol in chloroform. The origin end of the paper is dipped first just up to the origin, and blotted. The remainder of the strip is then passed through the dipping solution with a rapid pass over the origin.

Following the dipping step, the strips are hung in a hood until the odor of chloroform can be detected no longer, then they are placed in the chromatography chamber, the bottom of which is covered with toluene. The tab ends of the strip are placed into the troughs containing the developing solvent—toluene saturated with propylene glycol.

TABLE II.—DATA FOR ETHINYL ESTRADIOL ASSAY[a]

Chromatogram	Flask No.	Cell No.	Spot, ml.	Photometer Readings: Uncorrected	Corrected for Blank	Av. St. = 21.0 mcg. Sa. = 0.2 ml.	Concn., mg./Tablet[b]
	1	1	St. = 0.10	69	63		
						63.5	
	2	2	St. = 0.10	70	64		0.0078
1	3	3	Sa. = 0.20	64	58		
						58.5	
	4	4	Sa. = 0.20	65	59		
	5	5	Paper blank	6	...		
	6	1	St. = 0.10	68	62		
						62.5	
	7	2	St. = 0.10	69	63		0.0078
2	8	3	Sa. = 0.20	64	58		
						58	
	9	4	Sa. = 0.20	64	58		
	10	5	Paper blank	6	...		

[a] Project: 22-654. Sample: anabolic vitamin tablets. Control: ZC73831. Assay: ethinyl estradiol. Sample weight: 27890 mg./4 ml. Av. tablet weight = 559 mg. Weight standard: 21.0 mg./100 ml. Filter paper: ethanol-washed finger strips. Impregnated with propylene glycol (25% in $CHCl_3$). Developing solvent: toluene saturated with propylene glycol. Developing time: 20 hours. Drying conditions: 20 minutes at 90° C. Eluting solution: 10 ml. redistilled 95% EtOH. Eluting time: 30 minutes. Spectrophotofluorometer specifications: instrument, Aminco-Bowman (catalog No. 4-8100); cells silica, 10 mm.; tube, 1P28; slit, No. 3; activate, 280 mμ; fluorescence, 310 mμ. [b] Av. 0.0078 mg./tablet.

After a 20-hour descending solvent development, the strips are removed from the chamber and dried in a mechanical convection oven for 20 minutes at 90°.

The positions of the ethinyl estradiol spots on the chromatograms are located by employing the guide strip technique. The strip corresponding to the *1* spot on each chromatogram is cut out, dipped into a solution consisting of 1 part of Folin-Ciocalteu phenol reagent and 4 parts of water (3), then placed into a chamber containing concentrated ammonium hydroxide for 5 minutes. Ethinyl estradiol appears on the chromatogram as a blue zone against a light gray background.

The color-developed guide strip is air-dried, then realigned with the untreated portion of the chromatogram. The portions of the ethinyl estradiol zones are marked off with a solder pencil, cut out and folded, and placed in 50-ml. conical flasks. A paper blank, equal in area to the standard and sample segments, is included for each chromatogram. Ten milliliters of redistilled 95% ethanol are added to each flask and the ethinyl estradiol eluted off the filter paper segment by shaking on a reciprocating shaker for 30 minutes (2).

The amount of ethinyl estradiol present in the eluates is determined by spectrophotofluorometry using an Aminco-Bowman spectrophotofluorometer (catalog No. 4-8100) as previously described (1).

Calculations.—The photometer readings are recorded on a data sheet having all information necessary to calculate the concentration of ethinyl estradiol in the sample. Table II gives the data obtained in a typical analysis.

For each of the two chromatograms, readings are obtained for the standard and sample at one level in duplicate. The readings of the two standards for each chromatogram are averaged as are the two sample readings.

The concentration of ethinyl estradiol per tablet is calculated from

$$\text{mg. Ethinyl Estradiol per Tablet} = \frac{A \times B \times C \times D}{E \times F \times G}$$

where A = average photometer reading of 10-ml. eluate of 0.2-ml. chromatographed sample extract, B = weight, in milligrams, of chromatographed 0.1-ml. standard, C = average weight, in milligrams, of one tablet, D = volume, in milliliters, of final sample extract, E = average photometer reading of 10-ml. eluate of 0.1-ml. chromatographed standard, F = weight, in milligrams, of sample, and G = volume, in milliliters, of chromatographed sample extract.

The final concentration of ethinyl estradiol per tablet is obtained by averaging the values obtained for each of the two chromatograms.

Sample Calculation.—Table II, chromatogram No. 1:

$$\text{mg. Ethinyl Estradiol per Tablet} = \frac{58.5 \times 0.021 \times 559 \times 4}{63.5 \times 27890 \times 0.2} = 0.0078$$

RESULTS AND DISCUSSION

Recoveries of 95% or better must be obtained if any analytical method is to serve as an effective stability assay. The assay procedure described here meets this requirement.

This was demonstrated by subjecting four known aliquots of ethinyl estradiol to the complete analytical procedure. The average of the four individual assays gave a figure deviating by 5% from the theoretical concentration of 103 mcg./ml. (Table III).

TABLE III.—ACCURACY AND PRECISION OF METHOD[a]

Aliquot No.	Chromatogram, mcg./ml. 1	2	Av., mcg./ml.	Deviation from Theory,[b] %
1	98.4	96.2	97.3	−5.5
2	96.3	99.5	97.9	−5.0
3	98.2	96.8	97.5	−5.4
4	96.7	100.3	98.5	−4.4
Av.			97.8	−5.0
S.D.			0.44	
Coefficient of variation, %			0.45	

[a] Known concentrations of ethinyl estradiol subjected to the complete analytical procedure. [b] Theoretical value = 103 mcg./ml.

The analysis of ethinyl estradiol in tablets gave even better results, as shown in the data presented in Table IV.

Recovery experiments where known amounts of ethinyl estradiol were added to the ground tablet powder confirm that the assay yields results of 95% or better (Table V).

The water content of the acetone used to elute the ethinyl estradiol from the column is critical. The data presented in Table VI indicate that a water content of 1 to 2% is necessary to achieve maximum recovery of the ethinyl estradiol from the column.

It is advisable not to perform the column chromatographic step in a very humid atmosphere. At high humidity, moisture pickup by the chloroform and alumina deactivates the alumina, so that some of the ethinyl estradiol is eluted with the chloroform used as a purification solvent.

To determine the effect of moisture on the recovery of ethinyl estradiol, a 400-mcg. quantity was subjected to the procedure using alumina to which 6% water had been added. Only 84% of the ethinyl estradiol was recovered in the acetone eluate (Table VII), while the remainder was found in the chloroform wash.

Glassware used in the chromatographic purification should be dried carefully before use. The chloroform used to prepare the alumina column and to transfer the partially clarified sample to the column should be free of moisture (less than 0.02% water). In a second experiment, the chloroform used in these steps was saturated with water. The subsequent elution of ethinyl estradiol off the column yielded only 92% of the total introduced onto the column (Table VII).

The 0.1 *N* HCl removes a considerable amount of yellow pigment from the ether extracts. The chromatographic purification separates the fat-soluble vitamins, methyltestosterone, and some yellow pigment from the estrogen. Several attempts to perform the paper chromatographic isolation without prior column chromatographic purification resulted in elongated smears unsuitable for stability analysis.

Attempts to perform a batching assay on the column chromatographic acetone eluate by colorimetry in ethanol–concentrated sulfuric acid (1:1) and by fluorimetry for the estrogen *per se* or by the Ittrich modification of the Kober reaction (4) gave erroneous results. Only the combination of column chromatography purification and paper chromatography quantitation gave a satisfactory assay.

TABLE IV.—ANALYSES OF ETHINYL ESTRADIOL IN ANABOLIC VITAMIN TABLETS

Sample No.	Found, mg./Tablet	Deviation from Theory,[a] %
1	0.0081	+1.2
2	0.0082	+2.5
3	0.0079	−1.2
4	0.0078	−2.5
5	0.0078	−2.5
6	0.0076	−5.0
7	0.0077	−3.8

[a] Theoretical concentration: 0.008 mg. per tablet.

TABLE V.—RECOVERY OF ETHINYL ESTRADIOL ADDED TO ANABOLIC VITAMIN TABLETS

Sample No.	Added, mcg./Tablet	Found, mcg./Tablet	Net Recovery, mcg./Tablet	Recovery, %
1	0	8.2	—	—
1	7.9	15.8	7.6	96.2
2	0	7.9	—	—
2	8.3	15.7	7.8	94.0
3	0	7.4	—	—
3	7.9	14.8	7.4	93.8
4	0	7.6	—	—
4	7.9	15.1	7.5	95.0
5	0	7.7	—	—
5	7.9	15.9	8.2	103.8

TABLE VI.—RECOVERY OF ETHINYL ESTRADIOL FOLLOWING CHROMATOGRAPHY ON ALUMINA AND THE EFFECT OF WATER CONTENT OF ACETONE USED AS ELUTING SOLUTION

Water Added to Acetone,[a] %	Recovery of Ethinyl Estradiol, %
0	67
0.25	90
0.5	92
1.0	98
2.0	98
4.0	93
6.0	89

[a] Acetone (Merck No. 7106) contained 0.28% water by gas chromatographic analysis.

TABLE VII.—RECOVERY OF ETHINYL ESTRADIOL FOLLOWING CHROMATOGRAPHY ON ALUMINA AND EFFECT OF WATER CONTENT OF ALUMINA

Water Content, %	Recovery of Ethinyl Estradiol, %
6	84
Zero, followed by wash with water-saturated chloroform	92

SUMMARY

1. A method combining quantitative paper chromatography and spectrophotofluorometry has been developed for the assay of micro amounts of estrogens in anabolic vitamin tablets.

2. The procedure is used routinely to measure the stability of ethinyl estradiol at a concentration of 8 mcg. per tablet or 0.0014%.

3. Prior to quantitative paper chromatography, the estrogen is extracted into ether from an alcohol–water suspension of the ground tablets, then adsorbed on an alumina column to separate it from some interfering constituents.

4. The estrogen is eluted off the column using acetone to which 1% water has been added. The concentration of water in the eluting solution is a critical factor in recovering the estrogen off the column.

5. Accuracies in excess of 95% are obtained by chromatographing simultaneously replicates of standard and sample solutions on the same chromatogram.

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Analogs of Tetrahydrofolic Acid XIV

Facile Synthetic Route to the 2-Amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol Type of Folic Reductase and Thymidylate Synthetase Inhibitor

By B. R. BAKER, DANIEL V. SANTI, and HOWARD S. SHAPIRO

A four-step synthesis of the title compound (X*b*) from *p*-tolylsulfonanilide has been described which proceeds in 35 per cent over-all yield *via* ethyl 2-acetyl-5-(*N*-tosylanilino)valerate (IV*b*) and the *N*-tosyl derivative of X*b* (VII*b*). That this synthetic route had considerable generality was shown with four other examples—namely, 2-amino-6-methyl-5-(*p*-nitroanilinopropyl)-4-pyrimidinol (X*c*), 2-amino-5-(*p*-carbethoxyanilinopropyl)-6-methyl-4-pyrimidinol (X*d*), 2-amino-5-(3-anilinopropyl)-6-benzyl-4-pyridiminol (XVII*b*), and 2-amino-5-(3-anilinopropyl)-6-phenyl-4-pyrimidinol (XVII*a*). XVII*a* and *b* were good inhibitors of rat liver folic reductase, being complexed five and two times better, respectively, than the substrate, folic acid. Since XVII*a* and *b* are 35 and 16 times better, respectively, than the 6-methylpyrimidine (X*b*), the phenyl ring at the 6-position is probably an additional point of binding to the enzyme.

INTRACELLULAR REDUCTION of folic acid by the enzyme, folic reductase, leads to the cofactor form, tetrahydrofolic acid. This cofactor form then participates in at least 14 known enzymatic reactions for acceptance and transfer of one-carbon fragments (1–4). The pyrimidyl analog (X*a*) was chosen as a likely candidate to be an inhibitor of enzymes utilizing tetrahydrofolic acid or its derivatives for reasons previously described (4). The original synthesis of X*a* was designed to have the largest possible scope for subsequent molecular manipulation so that the mode of binding of X*a* to a number of these 15 enzymes could be studied. The key step in this synthesis was the reductive condensation of the 5-pyrimidylpropionaldehyde (V) with substituted anilines (VIII), followed by base cleavage of the N^2-acetyl group of IX (5, 6).

That this synthesis was quite general was shown by synthesis of analogs of X where (*a*) the 4-hydroxyl group was replaced by 4-mercapto (7), which resulted in increased binding to folic reductase (7), 5,10-methylene-tetrahydrofolic dehydrogenase (7), and thymidylate synthetase (8); (*b*) replacement of the 6-methyl group with 6-phenyl (9), which also resulted in increased binding to the first two enzymes; (*c*) variation of the R group to determine the mode of binding of the carboxy-L-glutamate residue to folic reductase (10) and other enzymes; and (*d*) the variation of the 2-amino and 4-hydroxyl groups to assess their role in enzyme binding (8).

As a result of the study on the mode of binding of the carboxy-L-glutamate fragment to folic reductase, it was clear that its complete removal to give X*b* resulted in only a nominal decrease in binding to folic reductase (10) and thymidylate synthetase (8). Therefore, the authors considered it important to find a shorter synthesis of compounds of type X, even though it might not be so general in scope as the original synthesis (5, 6). This new synthesis of class X inhibitors and some aspects of its scope are the subjects of this paper.

DISCUSSION

The general approach undertaken was to combine a β-keto ester, a trimethylene fragment, and an aniline residue to give a key intermediate akin to VII. A number of variants of this approach were investigated, and the successful one will be described first.

Condensation of excess 1,3-dibromopropane (I) with *p*-toluenesulfonanilide (II*b*) in dimethylsulfoxide in the presence of potassium carbonate—a general method used for alkylation of other acidic NH compounds (11, 12)—gave pure *N*-bromopropyl-*p*-tolylsulfonanilide (III*b*), m.p. 64–65°, in 74% yield; the use of *N*,*N*-dimethylformamide as a solvent gave a 75% yield. The older alkylation method, using sodium ethoxide in ethanol, gave yields of 40–45% of intermediate purity product. The more recent sodium hydride-*N*,*N*-dimethylformamide method (13) afforded 60–65% of III*b*; its one advantage over the potassium carbonate method was that reaction was complete in 8 hours. However, the potassium carbonate method is considered the method of choice because of its simplicity.

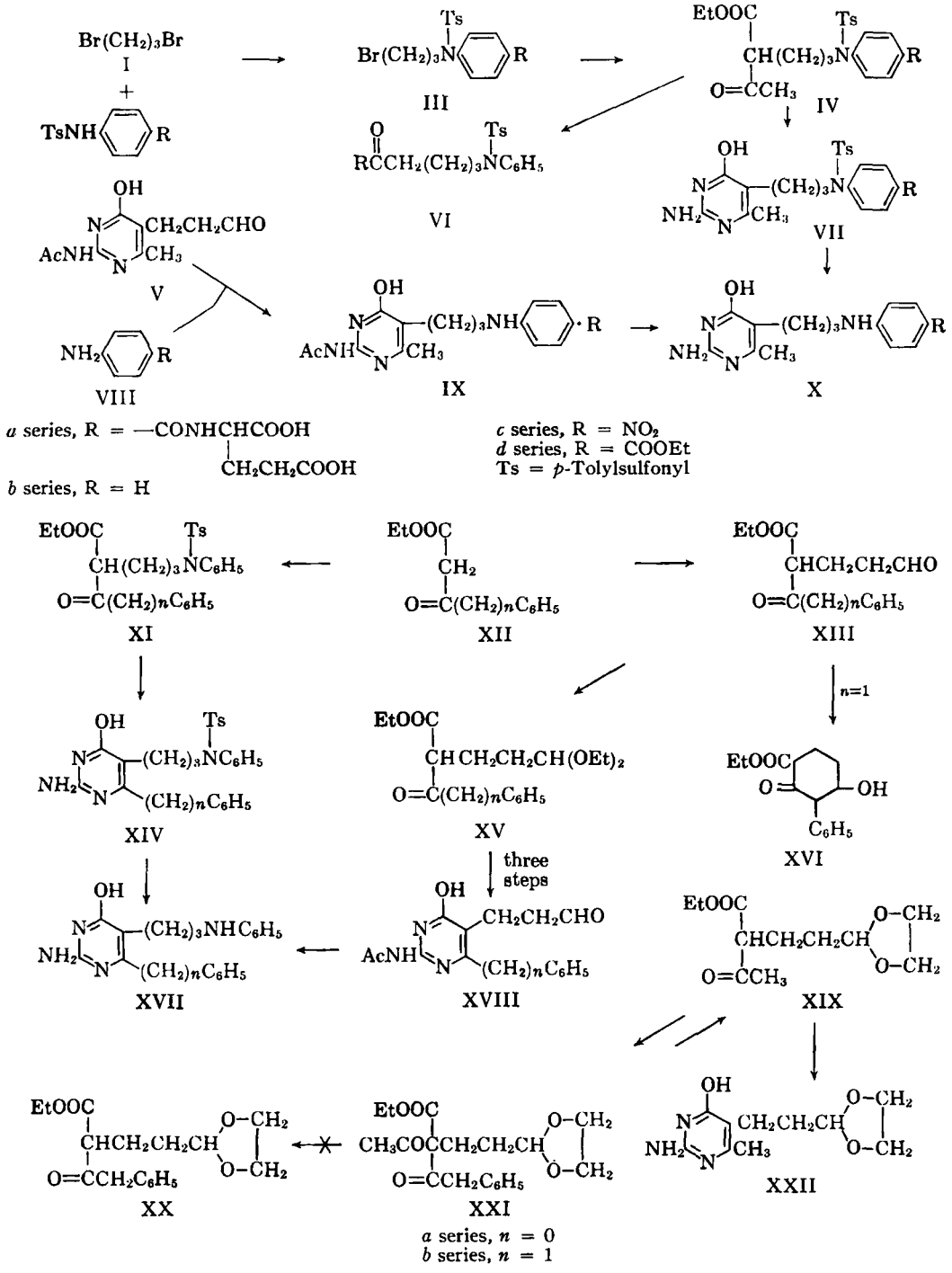
Condensation of the *N*-bromopropyl-*p*-toluenesulfonanilide (III*b*) with sodio ethyl acetoacetate in absolute ethanol proceeded poorly; cleavage of keto ester (IV*b*) to VI (R=CH_3 or C_2H_5O) appeared to be a serious side reaction. The cleavage was avoided by using *tert*-butanol as the solvent for alkylation of sodio ethyl acetoacetate with III*b*; the keto ester (IV*b*) was obtained as an oil in quantitative yield

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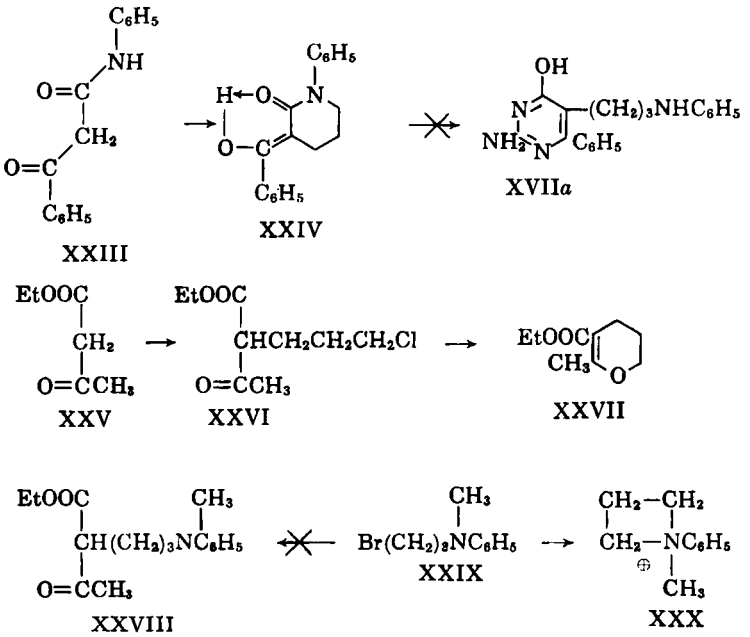
This investigation was supported by grant CA-06624 and training grant 2G-555 from the U. S. Public Health Service, Bethesda, Md.

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which was estimated to be 84% pure by ultraviolet analysis in 0.1 *N* methanolic sodium methoxide (see *Experimental*).

Since the keto ester (IV*b*) seemed to be labile to ethanol, containing a base, it was not surprising to find that condensation of IV*b* with guanidine carbonate proceeded poorly to the pyrimidine (VII*b*) in absolute ethanol. The product (VII*b*) could not be crystallized after processing the reaction mixture unless it was dissolved in hot 1 *N* sodium hydroxide, separated from insoluble material, then cooled, and the relatively insoluble sodium salt of VII*b* isolated; conversion of the sodium salt back to the free pyrimidine readily gave the crystalline pyrimidine (VII*b*) but in less than 50% yield, based on the quantity of pure keto ester (IV*b*) employed. To avoid alcoholysis, condensation of the keto ester (IV*b*) with guanidine carbonate was carried out in *tert*-butanol (9); the desired blocked pyrimidine (VII*b*) crystallized directly from the reaction solvent, and further



processing of the filtrate gave 80% yield of VII*b* based on the amount of pure keto ester (IV*b*) initially employed.

Attempts to remove the *N*-tosyl group from VII*b* by hydrolysis with 96% sulfuric acid at room temperature or with boiling concentrated hydrochloric acid (14) resulted in nearly quantitative recovery of starting material. However, reductive cleavage of the *N*-tosyl group with sodium and liquid ammonia (15) proceeded smoothly to give the pure, recrystallized anilinopropyl pyrimidine (X*b*) in 53% yield that was identical to the material prepared by the earlier synthetic route (10). The elegant *N*-tosyl cleavage method using hydrogen bromide in glacial acetic acid in the presence of phenol (16) gave a 71% conversion of VII*b* to recrystallized X*b*. The over-all yield of X*b* in this four-step sequence from *p*-toluenesulfonanilide was a satisfactory 35%, compared to 7% over-all yield in the earlier six-step sequence (6, 10).

The apparent generality of the method was then shown by synthesis of four more examples, two of which could not be synthesized by the earlier method (6, 9, 10). Two of the four examples were the synthesis of *p*-nitroanilinopropyl pyrimidine (X*c*) and the *p*-carbethoxyanilinopropyl pyrimidine (X*d*); the other two examples were variants in the 6-position of the pyrimidine ring (XVII*a* and XVII*b*).

Reaction of *p*-nitroaniline (VIII*c*) and ethyl *p*-aminobenzoate (VIII*d*) with a 10% excess of *p*-tolylsulfonyl chloride in pyridine at 90–100°, followed by direct crystallization by addition of 50% methanol, gave 95–97% yields of crystalline II*c* and II*d* suitable for further transformation. This hot pyridine method has been previously used for conversion of *p*-substituted anilines to sulfonanilides (14*a*) and gives higher yields of II*c* and II*d* than previously recorded in the literature. Alkylation of II*c* and II*d* with excess 1,3-dibromopropane (I) in dimethylsulfoxide was performed essentially as described for II*a*; the *p*-carbethoxy sulfonanilide (III*d*) required 2–3 days for completion, while III*c* required 5 days for completion, primarily because the more acidic NH group of II*c* rapidly formed an insoluble potassium salt with the potassium carbonate. After removal of the excess 1,3-dibromopropane, the remaining gummy *p*-carbethoxy sulfonanilide (III*d*) was analytically pure and was obtained in 84% yield; III*d* could be crystallized from petroleum ether, but such a step was considered unnecessary. The crystalline *p*-nitro sulfonanilide was obtained in 80% yield.

Conversion to the keto esters (IV*c* and IV*d*) proceeded in excellent yield in the same fashion as for IV*b*, except that the *p*-nitroanilino derivative (IV*c*) could be crystallized. Condensation of IV*c* with guanidine carbonate in *tert*-butanol gave the pyrimidine (VII*c*) which was best purified as its insoluble sodium salt; regeneration of the salt gave the crystalline pyrimidine (VII*c*) in 72% yield. The *p*-carbethoxyanilino pyrimidine (VII*d*) was obtained in quantitative crude yield but could not be purified through the sodium salt in 1 *N* sodium hydroxide due to concomitant saponification. Thin-layer chromatography of crude VII*d* showed several spots; but after the tosyl group was removed, the resultant X*d* could be purified.

When the *N*-tosyl-*p*-nitroanilino pyrimidine (VII*c*) was treated with sodium and ammonia, the characteristic blue which usually bleaches did not appear, but the solution immediately became dark brown and did not bleach. After 6 mole equivalents of sodium were added, the reaction mixture was processed to give a brown gummy product that was intractable; apparently the nitro group is not compatible with sodium-ammonia. Fortunately, the election-withdrawing *p*-nitro group activated the sulfonanilide linkage to cleavage by 96% sulfuric acid at room temperature (14); after 5 hours, the detosylated *p*-nitroanilino pyrimidine (X*c*) could be isolated in 77% yield as analytically pure yellow crystals.

The crude *N*-tosyl *p*-carbethoxyanilino pyrimidine (VII*d*) also appeared to react abnormally with sodium-ammonia leading to highly insoluble, intractable, amorphous powders. However, hydrolysis of

VII*d* with 96% sulfuric acid proceeded smoothly. After purification by solution in cold 3 *N* hydrochloric acid and neutralization with ammonium hydroxide, the desired detosylated *N*-carbethoxyanilino pyrimidine (X*d*) was isolated as an analytically pure, although amorphous, powder in 51% yield and was uniform on thin-layer chromatography. Saponification of X*d* gave the corresponding acid (X, R = COOH) in 77% yield, identical to that prepared by the earlier route (10).

The *N*-tosyl anilinopropylpyrimidine (VII*b*), which is obtained in 50% over-all yield for the three steps from *p*-toluenesulfonanilide, also proved to be a useful intermediate for further transformations on the pyrimidine ring where it was necessary to have the anilino function blocked—for example, synthesis of 2,4-diamino-5-(3-anilinopropyl)-6-methylpyrimidine (17). Since transformations on the 6-phenylpyrimidine moiety of XVII*a* also required blocking of the anilino group, the synthesis of the blocked 6-phenyl-5-(3-anilinopropyl) pyrimidine (XIV*a*) by the new route was investigated.

Alkylation of sodio ethyl benzoylacetate (XII*a*) in *tert*-butanol with *N*-(3-bromopropyl)-*p*-tolylsulfonanilide (III*b*) gave XI*a* as an oil. Condensation of the crude XI*a* with guanidine carbonate in boiling *tert*-butanol gave the crystalline blocked pyrimidine (XIV*a*) in 41% over-all yield for the two steps. Reductive cleavage of the *N*-tosyl group of XIV*a* with hydrogen bromide–acetic acid (16) afforded 90% yield of XVII*a*, identical to XVII*a* prepared *via* the 5-pyrimidylpropionaldehyde (XVIII*a*).

When the *N*-tosyl derivative (XIV*a*) was reduced with sodium in liquid ammonia, the *N*-tosyl group was removed. Surprisingly, the product appeared to be the 5,6-dihydro derivative of XVII*a*, since the product no longer contained the peak near 280 mμ characteristic of the 6-phenyl pyrimidine (XVII*a*) (18). Apparently the 6-phenyl group of XVII*a* activates the 5,6-double bond toward reduction; other marked differences between the chemistry of 5-alkyl-6-phenylpyrimidines and the corresponding 6-methyl derivatives have been noted in this laboratory. For example, a 5-alkyl-2-amino-4-chloro-6-methyl-pyrimidine failed to react with thiourea in boiling *tert*-butanol (7), whereas the 6-phenyl derivative readily formed the 4-mercapto derivative under these conditions (19). Furthermore, a 5-alkyl-2-amino-6-methyl-4-pyrimidinethiol was stable to hot hydrochloric acid, but the corresponding 6-phenyl derivative was rapidly hydrolyzed to the 4-pyrimidinol (19).

The 6-phenylpyrimidine (XVII*a*) was a good inhibitor[1] of rat liver folic reductase with $K_i = 1.8 \times 10^{-6}$, binding about five times better than the substrate, folate ($K_m = 10 \times 10^{-6}$), and 35 times better than the corresponding 6-methylpyrimidine (XI*b*) ($K_i = 63 \times 10^{-6}$) (10). Thus, the loss in inhibition when the carboxy-L-glutamate moiety is removed from X*a* ($K_i = 2.0 \times 10^{-6}$) (10) is compensated by increased binding of the 6-phenylpyrimidyl moiety of XVII*a*. The increased inhibition by XVII*a* compared to X*b* suggested that the 6-benzyl analog (XVII*b*) should be synthesized for evaluation as an inhibitor of folic reductase. The synthesis of XVII*b* by the new route *via* XI*b* was successful, whereas the synthesis of XVII*b* by the old route (9, 10) was not. XVII*b* had $K_i = 4 \times 10^{-6}$ with folic reductase,[1] which is 16 times better than the 6-methylpyrimidine (XI*b*).

One of the possible explanations (9) for the tighter binding of the 6-phenylpyrimidine (XVII*a*) and its *p*-carboxy-L-glutamate analog to folic reductase than to the corresponding 6-methylpyrimidines (*Xa* and *b*) was that the 6-phenyl group favorably influenced the possible tautomeric forms of the pyrimidyl moiety to increase binding. The binding of the 6-benzyl pyrimidine (XVII*b*) 16 times tighter than the 6-methylpyrimidine (XI*b*) to folic reductase is not tenable with a tautomeric shift increasing binding of the pyrimidyl moiety, since the phenyl group of XVII*b* is insulated from the pyrimidyl moiety by a methylene group. A more plausible explanation is that both the 6-phenyl and 6-benzyl groups also bind to some point on folic reductase to give a net tighter binding. Whether this binding is by charge-transfer complex (20), by hydrophobic binding (21), or by some other means (20, 22) is being investigated.

It is interesting that replacement of the 6-methyl group of X*a* by a 6-phenyl group (9) does not change the extent of binding to thymidylate synthetase, although this 6-phenyl derivative binds 20 times tighter than X*a* to folic reductase. Thus, this additional binding point, apparently present on folic reductase, is absent on thymidylate synthetase.

Condensation of ethyl γ-phenylacetoacetate (XII*b*) with acrolein under the base-catalyzed conditions previously used for XII*a* (9) did not give the expected adduct (XIII*b*). Apparently a further internal aldol condensation occurred to give XVI, as shown by the absence of aldehyde CH at about 2600 cm.$^{-1}$ in the infrared and the presence of hydroxyl at 3500 cm.$^{-1}$ in the infrared. When the acrolein condensation was performed under milder conditions, either starting material (XII*b*) was recovered, or mixtures of XIII*b* and XVI were obtained. Clearly, this route to XVII*b* *via* XIII*b*→XV*b*→XVIII*b* was unattractive, if not impossible.

A second route involved reaction of phenylacetyl chloride with the sodio derivative of the dioxolanyl-keto ester (XIX) (7), prepared with sodium hydride and XIX in benzene. The resultant product (XXI), on cleavage with methanolic sodium methoxide, did not give the expected XX by cleavage of the acetyl group (23), but mainly the phenylacetyl group was cleaved to regenerate XIX; the regenerated XIX was characterized by conversion to the previously synthesized (7) 6-methylpyrimidine (XXII).

The new route for synthesis of X *via* III*b* was successful when applied to the synthesis of XVII*b*, an indication that this new route probably has more diversity than the older route (6, 7, 9, 10). Condensation of sodio ethyl γ-phenylacetoacetate (XII*b*) with *N*-(3-bromopropyl)-*p*-tolylsulfonanilide (III*b*) in *tert*-butanol gave crude XI*b*, which was condensed with guanidine carbonate in *tert*-butanol, to give after purification a 46% over-all yield of analytically pure XIV*b*, which was converted to XVII*b*.

Four alternate routes for insertion of a 1,3-dihalopropane between a keto ester and an aniline residue were investigated before the fifth alternative *via* III→IV→VII→X*b* was established.

The dianion of benzoylacetanilide (XXIII) (24),

[1] The authors thank Dr. W. C. Werkheiser, Roswell Park Memorial Institute, for the assays on folic reductase from rat liver.

prepared from XXIII with sodium hydride in *N,N*-dimethylformamide, reacted with 1,3-dibromopropane to give the crystalline benzoyl piperidone (XXIV) in 25% yield. This benzoyl piperidone was fully enolic in neutral solution and apparently existed as the stable chelate, XXIV. That the piperidone was apparently fully enolized was shown by the broad acidic OH group at 3300–2000 cm.$^{-1}$, a bonded piperidone carbonyl at 1610 cm.$^{-1}$, the enolic peak at 345 mμ of nearly the same molecular extinction in neutral as basic solution, and the disappearance of this peak at pH 1, and finally a chelated hydrogen at δ 13.70 in the NMR in deuterochloroform that integrated for a full single proton (100% enolization).

Although the strong enolic chelate properties of XXIV were interesting, these properties stymied attempts to convert the highly stable chelate XXIV to a pyrimidine (XVII*a*). Reaction of XXIV with guanidine carbonate in ethanol, *tert*-butanol, or dimethylsulfoxide (9) was sluggish, and XXIV could be recovered unchanged. When conditions were forced, apparently C-benzoyl cleavage (9) of the molecule took place. With guanidine hydrochloride in polyphosphoric acid, decomposition occurred. Attempts to form an enol ether with boiling ethyl orthoformate or ethyl orthoacetate (25) with (9) or without acid catalysis gave complete recovery of unchanged XXIV. Attempts to form an enamine with ammonium formate (26) in *tert*-butanol or with piperidine in refluxing benzene (27) under a constant water separator were fruitless. An attempt to convert XXIV to an enol *O*-mesylate with methanesulfonyl chloride in pyridine also failed. Although it may have been possible to convert XXIV to an enol ether with diazomethane (28), thence to XVII*a*, this possibility was not investigated since the alternate route *via* XIV*a* was an obviously better preparative value than a route which would need large-scale use of the treacherous diazomethane.

Alkylation of ethyl acetoacetate with 1-bromo-3-chloropropane in ethanolic sodium methoxide has been reported to give a low yield of the chloropropyl keto ester (XXVI); the main by-product was the dihydropyran (XXVII), formed by cyclization of XXVI (29). If the chloropropyl ester (XXVI) could be obtained in better yield, if the chloropropyl ester could then be converted with guanidine to 2-amino-5-(3-chloropropyl)-6-methyl-4-pyrimidinol, and if the latter could be converted to the anilino derivative (X*b*) with aniline, this could be an attractive route to X*b*. Study of the effect of solvent on the initial alkylation reaction to form XXVI showed that yields were on the order of 5–10%, obviously unfeasible for a preparative route to X*b*.

Two alternate routes related to the sequence II→III→IV were investigated earlier. The anion of acetanilide, prepared with sodium hydride in dry *N,N*-dimethylformamide (13), rapidly reacted with 1,3-dibromopropane. However, the reaction was not clean-cut, and considerable acetanilide was regenerated, from which it was difficult to separate the desired *N*-(3-bromopropyl) acetanilide by simple means of preparative value.

Reaction of *N*-methylaniline with excess 1,3-dibromopropane gave XXIX, which was a distillable but unstable oil. On standing the distilled product soon deposited crystals that were presumably XXX bromide, and redistillation at 0.2 mm. always led to large losses, probably due to cyclization to XXX. Furthermore, attempted condensation of excess XXIX with ethyl sodioacetoacetate led to rapid formation of sodium bromide, but the solution remained strongly basic, and no XXVIII was formed. These results would be compatible with rapid cyclization to XXX as its methoxide salt. The four alternate routes to 5-(3-anilinopropyl) pyrimidines were not investigated further when the route *via* IV*a* was so successful.

EXPERIMENTAL

Melting points were determined in capillary tubes on a Mel-Temp block, and those below 230° are corrected. Infrared spectra were determined in KBr disk (unless otherwise indicated) with a Perkin-Elmer model 137B spectrophotometer; ultraviolet spectra were determined with a Perkin-Elmer model 202 spectrophotometer and NMR spectra with a Varian A 60 spectrophotometer. Thin-layer chromatograms (TLC) were run on silica gel G (Brinkmann) in the solvent systems specified, and spots were detected by iodine vapor.

N-(p-Nitrophenyl)-p-tolylsufonamide (IIc).—The tosylation procedure for 4-amino-4′-nitrodiphenylsulfide (14*a*) was readily adaptable to large-scale preparation of II*c*. To a stirred solution of 13.8 Gm. (0.1 mole) of *p*-nitroaniline in 50 ml. of reagent pyridine was added 21.0 Gm. (0.11 mole) of *p*-tolylsulfonyl chloride. The mixture was heated on a steam-bath for 30 minutes protected from moisture. Dilution of the hot reaction mixture with 150 ml. of 50% ethanol and chilling gave 27.7 Gm. (95%) of product, m.p. 186–187°, that was suitable for the next step [lit. m.p. 193° (30)]. Recrystallization of a sample from ethanol gave light yellow crystals, m.p. 193°.

N-(p-Carbethoxyphenyl)-p-tolylsulfonamide (IId).—Similarly, ethyl *p*-aminobenzoate gave this compound in 95% yield, m.p. 205–208°, that was suitable for further transformations. Recrystallization of a sample from ethanol gave white crystals, m.p. 206–207°; $\nu_{max.}$ 3200 (NH); 1675 (ester C=O); 1600 (C=C); 1325, 1150 (−SO_2N−); 807, 765, 695 cm.$^{-1}$ (phenyl CH). Although this compound is used several times in the literature, no physical constants for it could be found.

Anal.—Calcd. for $C_{16}H_{17}NO_4S$: C, 60.2; H, 5.37; N, 4.38. Found: C, 60.2; H, 5.19; N, 4.16.

N-(3-Bromopropyl)-p-tolylsulfonanilide (IIIb).—To a solution of 2.47 Gm. (0.01 mole) of *p*-tolylsulfonanilide in 10 ml. of practical dimethylsulfoxide was added 1.38 Gm. (0.01 mole) of anhydrous potassium carbonate and 8.07 Gm. (0.04 mole) of 1,3-dibromopropane. The mixture was magnetically stirred for 3 days at room temperature, then poured into 75 ml. of water. The mixture was extracted with 75 ml. of benzene. The benzene solution was washed with several portions of 1 *N* sodium hydroxide until acidification of the washing was clear. The extract was further washed with water (2 × 75 ml.), dried with magnesium sulfate, then spin-evaporated *in vacuo;* the last of the dimethylsulfoxide and 1,3-dibromopropane were removed at 1 mm. The residue was crystallized from 5 ml. of hot methanol by allowing the solution to cool to room

temperature undisturbed. (Fast cooling or mixing leads to a voluminous precipitate which is difficult to filter and is solvated.) The product was collected on a filter and washed with a small amount of ice-cold methanol; yield, 2.72 Gm. (74%), m.p. 64–65°; $\nu_{max.}$ 1580 (C=C); 1340, 1160 (−SO_2N−); 812 (*p*-C_6H_4−); 710, 700 (C_6H_5−); and no NH near 3400 cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{18}BrNO_2S$: C, 52.2; H, 4.93; Br, 21.7. Found: C, 52.2; H, 4.94; Br, 21.9.

N - (3 - Bromopropyl) - N - (p - nitrophenyl) - p-tolylsulfonamide (IIIc).—This was prepared from 2.92 Gm. (0.01 mole) of II*c* as described for *IIIb*, except the reaction time was 5 days. Recrystallization by solution in the minimum amount of hot ethyl acetate (about 5 ml.), then addition of 25 ml. of ethanol and chilling gave 3.29 Gm. (80%) of product, m.p. 111–113°, that was suitable for further transformation. Recrystallization from methanol gave yellow crystals, m.p. 119–120°; $\nu_{max.}$ 1590 (C=C); 1510 (NO_2); 1334 (broad, −SO_2N−, NO_2); 1160 (−SO_2N−); 727, 695 (*p*-C_6H_4); no NH near 3400 cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{17}BrN_2O_4S$: C, 46.6; H, 4.32; Br, 19.5. Found: C, 46.5; H, 4.15; Br, 19.3.

N - (3 - Bromopropyl) - N - (p - carbethoxyphenyl)-p-tolylsulfonamide (IIId).—This was prepared from 3.19 Gm. (0.01 mole) of II*d* as described for *IIIb*. After removal of the excess 1,3-dibromopropane *in vacuo*, a nearly white thick oil remained that was analytically pure and could be used for further transformations. This material could be crystallized from petroleum ether (b.p. 60–110°) with considerable loss: white crystals, m.p. 77–79°; $\nu_{max.}$ 1710 (C=O); 1600 (C=C); 1340, 1160 (−SO_2N−); 770, 812 (*p*-C_6H_4−), and no NH near 3200 cm.$^{-1}$.

Anal.—Calcd. for $C_{19}H_{22}BrNO_4S$: C, 51.8; H, 5.04; Br, 18.2. Found (oil): C, 52.0; H, 5.11; Br, 18.4. Found (crystals): C, 52.0; H, 5.15; Br, 18.2.

Ethyl 2-Acetyl-5-anilino-N-(p-tolylsulfonyl)valerate (IVb).—A mixture of 73 ml. of *tert*-butyl alcohol, 3.90 Gm. (0.03 mole) of ethyl acetoacetate, and 1.43 Gm. of sodium hydride (0.03 mole) (as a 53.5% suspension in mineral oil) was warmed with stirring and protection from moisture until the sodium hydride dissolved, and hydrogen evolution was complete. After the addition of 7.37 Gm. (0.02 mole) of III*b*, the mixture was refluxed for 20 hours with stirring, then spin-evaporated *in vacuo*. The residue was partitioned between water and chloroform. The chloroform layer was dried with magnesium sulfate, then spin-evaporated *in vacuo*. The residual ethyl acetoacetate was removed at 1 mm. (bath 80–100°); yield, 8.32 Gm. (100%) of a glass that had a maximum purity of 84% based on ultraviolet analysis. The glass had $\nu_{max.}^{film}$ 1725 (ester C=O); 1700 (ketone C=O); 1580 (C=C); 1340, 1160 (−SO_2N−); 815 (*p*-C_6H_4−); 710, 695 cm.$^{-1}$.

The ultraviolet analysis was based on (*a*) $\lambda_{max.}$ 284 (ε 12,000) in 0.1 *N* methanolic sodium methoxide for simple ethyl α-alkylacetoacetates, such as the α-allyl derivative; (*b*) the *N*-alkyl-*p*-toluenesulfonanilide moiety, such as III*b*, has $\lambda_{max.}$ 240 mμ (ε 10,900) in neutral or basic solution; and (*c*) the only impurities will be derived from II*b*. Thus, pure IV*b* should have an absorbance ratio of 1.09 for 284/240 mμ. Without weighing a sample, the per cent purity of IV*b* could be estimated as:

$$\% \text{ purity} = \frac{\text{observed O.D. ratio } 284/240}{1.09}$$

The preparation described above had an absorbance ratio (284/240) of 0.92 or a maximum purity of 84%. When the alkylation reaction was run in absolute ethanol, the purity was only 50%, indicating extensive ethanolysis of the β-keto ester to VI.

Ethyl 2-Acetyl-5-(p-nitroanilino)-N-(p-tolylsulfonyl)valerate (IVc).—This was prepared as described for *VIb*. Although the crude glassy product was best used for further transformations, this material could be obtained with some loss as nearly white crystals from petroleum ether (b.p. 60–110°), m.p. 107–108°; $\nu_{max.}$ 1730 (ester C=O); 1700 (ketone C=O); 1580 (C=C); 1500 (NO_2); 1340 (NO_2, −SO_2N−); 1150 (−SO_2N−); 810, 750, 720, 690 cm.$^{-1}$ (phenyl H).

Anal.—Calcd. for $C_{22}H_{26}O_7S$: C, 57.1; H, 5.63; N, 6.06. Found: C, 57.2; H, 5.80; N, 6.00.

2 - Amino - 6 - methyl - 5 - [N - (p - tolylsulfonyl anilino)propyl]-4-pyrimidinol (VIIb).—A mixture of 8.32 Gm. of crude IV*b* (0.017 mole, 84% pure), 2.0 Gm. of guanidine carbonate, and 50 ml. of *tert*-butyl alcohol was refluxed with stirring for 48 hours. The cooled mixture was filtered and the precipitate washed with *tert*-butyl alcohol. The solids were suspended in 50 ml. of water with stirring and the pH brought down to neutrality with dilute acetic acid. The solid was collected on a filter and dried; yield, 5.80 Gm., m.p. 220–224°. Recrystallization from hot 2-methoxyethanol by addition of water gave about 5.54 Gm. (66% based on III*b* or 80% based on crude IV*b*) of white crystals, m.p. 226–228°; $\nu_{max.}$ 3500, 3100; 2900, 2700 (broad NH, OH); 1650, 1600 (broad NH, pyrimidine, C=C); 1340, 1180 (SO_2); 805 (*p*-C_6H_4−); 710, 690 cm.$^{-1}$ (C_6H_5−).

Anal.—Calcd. for $C_{21}H_{24}N_4O_3S$: C, 61.1; H, 5.78; N, 13.3. Found: C, 61.1; H, 5.86; N, 13.6.

When ethanol was used as a solvent, the yield was about 30%. The product could not be directly crystallized; it was purified by solution in hot 1 *N* sodium hydroxide, decantation, then chilling to give the insoluble sodium salt of VII*b*. The sodium salt was dissolved in hot water, then acidified with acetic acid to give the crystalline product.

2 - Amino - 6 - methyl - 5 - [N - (p - tolylsulfonyl-p-nitroanilino)propyl]-4-pyrimidinol (VIIc).—A mixture of 1.156 Gm. (2.5 mmoles) of IV*c* and 0.255 Gm. (1.25 mmoles) of guanidine carbonate in 5 ml. of *tert*-butanol was refluxed for 48 hours. Since the product did not separate from the reaction mixture, the latter was neutralized with acetic acid, then spin-evaporated *in vacuo*. The residue was partitioned between chloroform and water. The separated chloroform layer, dried with magnesium sulfate, was spin-evaporated *in vacuo*. The residual glass was dissolved in 25 ml. of hot 1 *N* sodium hydroxide, the solution was decanted from a little insoluble gum, then cooled. The yellow sodium salt was collected on a filter and washed with a little 1 *N* sodium hydroxide. The sodium salt was dissolved in about 10 ml. of hot 50% aqueous ethanol, then added to about 50 ml. of 15% aqueous acetic acid with stirring. Recrystallization from absolute ethanol gave

0.825 Gm. (72% based on crude IV*c*, 69% for two steps based on III*c*) of buff-colored amorphous powder, m.p. 125–155° dec.; $\nu_{max.}$ 3600–3100 (broad NH, OH); 1650–1590 (broad NH, C=C, C=N); 1500 (NO_2); 1340 (NO_2, $-SO_2N-$); 1150 ($-SO_2-N-$); 810, 725, 695 cm.$^{-1}$ (phenyl H).

Anal.—Calcd. for $C_{21}H_{23}N_5O_5S$: C, 55.1; H, 5.09; N, 15.3. Found: C, 54.9; H, 5.29; N, 15.2.

2-Amino-6-methyl-5-[N-(p-tolylsulfonyl-p-carbethoxy anilino)propyl]-4-pyrimidinol (VIId).—This was prepared as described for *VIIc*. Evaporation of the chloroform solution *in vacuo* finally in high vacuum gave a buff-colored glass in 98% yield (97% yield based on III*d*) that could not be purified further; the sodium salt purification obviously could not be used because of concomitant saponification of the carbethoxy group. TLC in 3:1 benzene–methanol showed several spots.

2-Amino-6-methyl-5-(anilino propyl)-4-pyrimidinol (Xb).—*Preparation A.*—To a stirred solution of 1.03 Gm. (2.5 mmoles) of VII*b* in about 50 ml. of liquid ammonia was added small pieces (about 50 mg. each) of sodium. The blue color was allowed to bleach before the next piece was added. When the blue failed to fade in 15 minutes, a total of 340 mg. of sodium had been used. The mixture was acidified with 1 Gm. of ammonium chloride, then allowed to evaporate; the final traces of ammonia were removed *in vacuo*. The residue was suspended in 25 ml. of water, and the pH was adjusted to 1 with hydrochloric acid. The solution was filtered, then neutralized to pH 8 with sodium hydroxide. The product was collected on a filter and recrystallized from alcohol–water; yield, 0.34 Gm. (53%), m.p. 219°. This material was identical to X*b* prepared *via* V (10). No attempt was made to process the mother liquors for additional material.

Preparation B.—A mixture of 2.19 Gm. (5.3 mmoles) of VII*b*, 1.0 Gm. of phenol, and 11.5 Gm. of 30% hydrogen bromide in acetic acid (16) was magnetically stirred for 16 hours protected from moisture, then diluted with ether. The hydrobromide salt was collected on a filter, washed with ether, then dissolved in 20 ml. of 3 *N* hydrochloric acid. The acid solution was made slightly basic with ammonia water. The product was collected on a filter and recrystallized from aqueous ethanol; yield, 0.965 Gm. (71%), m.p. 219–220°, that was identical to *Preparation A*.

2-Amino-6-methyl-5-(p-nitroanilinopropyl)-4-pyrimidinol (Xc).—A solution of 1.00 Gm. of VII*c* in 2 ml. of 96% sulfuric acid was allowed to stand at room temperature for 5 hours, then poured into about 10 Gm. of ice. The resultant solution was poured in 25 ml. of a 1:1 mixture of concentrated ammonia-water and ice. The product was collected on a filter and washed with water. The solid was dissolved in warm 3 *N* hydrochloric acid, and the solution was poured into excess ice and ammonia-water. Recrystallization of the product from ethanol-water gave 0.513 Gm. (78%) of yellow crystals, m.p. 260–261°; $\lambda_{max.}^{EtOH}$ 230 (ε 17,300), 294 mμ (ε 10,600); $\lambda_{max.}^{pH\ 1}$ 230 (ε 17,300), 265 mμ (inflection, ε 11,400); $\lambda_{max.}^{pH\ 13}$ 280 mμ (ε 10,000).

Anal.—Calcd. for $C_{14}H_{17}N_5O_3$: C, 55.4; H, 5.65; N, 23.1. Found: C, 55.2; H, 5.76; N, 22.9.

If the acid solution of X*c* is gradually neutralized by the addition of base, the product is a mixture of the base and a base-salt.

2-Amino-6-methyl-5-(p-carbethoxyanilinopropyl)-4-pyrimidinol (Xd).—This was prepared as described for *Xc*, except that purification was effected by use of ice-cold 3 *N* hydrochloric acid. Final reprecipitation from ethanol by addition of water gave 0.84 Gm. (51%) of pure X*d* as a buff-colored powder, m.p. 194–196°; $\lambda_{max.}^{EtOH}$ 228 (ε 14,700), 308 mμ (ε 32,500); $\lambda_{max.}^{pH\ 1}$ 228 (ε 17,500), 271 mμ (ε 10,600); $\lambda_{max.}^{pH\ 13}$ 290–315 (broad, ε 7,950).

Anal.—Calcd. for $C_{17}H_{22}N_4O_3$: C, 61.8; H, 6.71; N, 17.0. Found: C, 61.7; H, 6.91; N, 16.6.

A solution of 150 mg. of X*d* in 2 ml. of 5% sodium hydroxide was heated on a steam-bath for 30 minutes. The hot solution was filtered, then adjusted to pH 4.5 with 3 *N* hydrochloric acid. The solid was collected by centrifugation and washed with water. A solution of the solid in 3 *N* ammonium hydroxide was filtered from a trace of insolubles, then the filtrate was adjusted to pH 4.5 with 3 *N* hydrochloric acid. The product was collected by centrifugation, then washed with hot water (2 × 2 ml.) and hot ethanol (2 × 2 ml.); yield, 105 mg. (77%) of the acid (X, R = COOH) that was identical to the sample previously prepared *via* V (10). Both samples showed an identical single spot on Whatman No. 1 paper with the upper phase of 4:1:5 *n*-butanol/acetic acid/water. The spot was detected under ultraviolet light and had an R_f value of 0.72. The same solvent system with silica gel G gave an R_f value of less than 0.1.

2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropanol.—To a magnetically stirred solution of 0.50 Gm. (1.7 mmoles) of XVIII*a* (9) in 10 ml. of *N,N*-dimethylformamide and 50 ml. of reagent methanol was added portionwise 1.0 Gm. of sodium borohydride. After being stirred for about 18 hours, the mixture was diluted with 25 ml. of 0.1 *N* sodium hydroxide, then spin-evaporated *in vacuo*. The residue was dissolved in 25 ml. of water, then acidified to pH 8–9 with 3 *N* hydrochloric acid. The product was collected on a filter, washed with water, and recrystallized from ethanol; yield, 0.349 Gm. (84%) of white crystals, m.p. 265–268°; $\nu_{max.}$ 3450, 3100 (NH, OH); 1670, 1640, 1610, 1570 (NH, pyrimidine, C=C); 765, 740, 695 cm.$^{-1}$ (phenyl CH).

Anal.—Calcd. for $C_{13}H_{15}N_3O_2$: C, 63.8; H, 6.22; N, 17.1; O, 13.1. Found: C, 63.7; H, 6.21; N, 17.4; O, 13.2.

The compound moved as a single spot TLC in 3:1 benzene–methanol or in methanol with a mobility slightly less than XVII*a*.

2-Amino-6-phenyl-[N-(p-tolylsulfonyl)-anilinopropyl]-4-pyrimidinol (XIVa).—To a solution of 0.294 Gm. of a 55.6% sodium hydride dispersion in mineral oil and 1.94 Gm. (10 mmoles) of ethyl benzoylacetate in 25 ml. of *tert*-butanol was added 2.50 Gm. (6.79 mmoles) of III*b*. After being refluxed with magnetic stirring for 20 hours protected from moisture, the solution had dropped to pH 9 when tested on moist pH paper. The mixture was spin-evaporated *in vacuo*. The residue was partitioned between 20 ml. of benzene and 10 ml. of water. The separated benzene layer was washed with ice-cold 3% sodium hydroxide (2 × 10 ml.) to remove unchanged ethyl benzoylacetate. After being washed further with water (2 × 10 ml.), the benzene solution was dried with magnesium sulfate, then spin-evaporated *in vacuo;* yield, 3.27 Gm. of

crude XI*a* as an oil; $\nu_{max.}^{film}$ 1620 (ester C=O); 1670 (ketone C=O); 1590 (C=C); 1340, 1150 (−SO_2-N−); 1040 (broad ester C—O—C); 813, 690 cm.$^{-1}$ (phenyl CH).

To 3.26 Gm. of the crude XI*a* was added 15 ml. of *tert*-butyl alcohol and 0.61 Gm. (3.39 mmoles) of guanidine carbonate. After being refluxed with magnetic stirring for 51 hours, the mixture was spin-evaporated *in vacuo*. The residue was triturated with hot ethyl acetate. The insoluble material was recrystallized twice from ethanol to give 0.327 Gm. of white crystals, m.p. 222–224°; $\nu_{max.}$ 3400, 3100–3030 (broad NH, OH); 1650 (broad NH, C=C, C=N); 1330, 1150 (−SO_2NH−); 815, 770, 695 cm.$^{-1}$ (phenyl CH); $\lambda_{max.}^{pH\ 1}$ 235 (ε 24,300), 275 mμ (ε 10,500); $\lambda_{max.}^{pH\ 7}$ 235 (ε 23,900), 297 mμ (ε 6530); $\lambda_{max.}^{pH\ 13}$ 288 mμ (ε 6300).

Anal.—Calcd. for $C_{26}H_{26}N_4O_3S \cdot {}^1/_2H_2O$: C, 64.8; H, 5.60; N, 11.6; O, 11.6. Found: C, 65.1; H, 6.13; N, 11.4; O, 11.2.

The ethyl acetate filtrate was spin-evaporated *in vacuo*, and the residue was crystallized from ethanol; yield, 0.804 Gm. (total 41% for two steps based on III*b*), m.p. 221–224°.

2-Amino-5-(3-anilinopropyl)-6-phenyl-4-pyrimidinol (XVIIa).—*Preparation A.*—A solution of 2.0 Gm. (7 mmoles) of XVIII*a* (9) and 6.5 Gm. of aniline (70 mmoles) in 40 ml. of *N,N*-dimethylformamide was magnetically stirred for 30 minutes. After addition of 300 ml. of reagent methanol, the solution was treated portionwise with 4.0 Gm. of sodium borohydride. After being stirred for about 18 hours, the mixture was diluted with 100 ml. of 0.1 *N* aqueous sodium hydroxide, then spin-evaporated *in vacuo* to near dryness. The residue was dissolved in 200 ml. of water and the solution acidified to about pH 8. The crude product was collected on a filter and washed with water. Two recrystallizations from 50% aqueous ethanol gave 1.52 Gm. (68%) of pure product as white crystals, m.p. 235–241° dec.; $\lambda_{max.}^{pH\ 1}$ 230 (ε 14,400), 276 mμ (ε 9200); $\lambda_{max.}^{pH\ 7}$ 236 (ε 22,600), 292 mμ (ε 8000); $\lambda_{max.}^{pH\ 13}$ 288 mμ (ε 8000); $\nu_{max.}$ 3500 (NH, OH); 1640, 1600, 1550, 1490 (NH, pyrimidine, C=C); 770, 745, 705, 696 cm.$^{-1}$ (phenyl CH).

Anal.—Calcd. for $C_{19}H_{20}N_4O$: C, 71.5; H, 6.27; N, 17.6. Found: C, 71.7; H, 6.36; N, 17.4.

Preparation B.—A solution of 500 mg. (1.06 mmoles) of XIV*a* and 200 mg. (2.12 mmoles) of phenol in 2.3 Gm. of 30% hydrogen bromide in acetic acid (16) was allowed to stand for 30 hours, then diluted with 20 ml. of ether. The hydrobromide salt was collected on a filter, dissolved in warm 70% ethanol, then neutralized to about pH 6 with 10% sodium hydroxide. After being chilled in an ice-bath, the product was collected on a filter and washed with water; yield, 300 mg. (89%) of white crystals that were identical to *Preparation A*, as shown by its ultraviolet and infrared spectra and by mixed melting point.

Ethyl γ-Phenylacetoacetate (XIIb).—Reaction of phenylacetyl chloride with ethyl ethoxymagnesiomalonate, as described by Ames and Davey (31), gave ethyl phenylacetylmalonate. After removal of unchanged ethyl malonate in high vacuum, the crude oil (74.3 Gm.) was refluxed with 400 ml. of water with stirring for 3 hours. This treatment removed one carbethoxy group (32). Distillation gave 15.7 Gm. (43% based on phenylacetyl chloride) of product, b.p. 80–107° (0.05 mm.), n_D^{22} 1.054–1.059.

2-Amino-6-benzyl-4-pyrimidinol.—A mixture of 4.0 Gm. of (XII*b*), 1.75 Gm. of guanidine carbonate, and 40 ml. of absolute ethanol was refluxed with stirring for 17 hours, during which time the product precipitated. After being kept at 3–5° (17 hours) overnight, the product was collected on a filter and washed with ethanol; yield, 1.72 Gm. (56%), m.p. 260–271°. The combined mother liquors and washings were evaporated *in vacuo* and gave 2.2 Gm. of a hard orange-red gum which was dissolved in hot 65% aqueous ethanol and neutralized to pH 7 with 3 *N* HCl and cooled at 3–5° to give an additional 0.34 Gm. (total 72%) of product, m.p. 275°. Recrystallization of the combined products from 2-methoxyethanol gave 1.64 Gm. (53%) of pure material, m.p. 278–280°; $\nu_{max.}$ 3400 (NH, OH); 1670, 1640 (NH, pyrimidine); 705 cm.$^{-1}$ (C_6H_5−).

Anal.—Calcd. for $C_{11}H_{11}N_3O$: C, 65.8; H, 5.48; N, 20.9. Found: C, 65.5; H, 5.60; N, 20.7.

2-Amino-6-benzyl-5-(N-tosylanilinopropyl)-4-pyrimidinol (XIVb).—This was prepared by alkylation of 20.3 Gm. (50 mmoles) of XII*b* with 12.5 Gm. (34 mmoles) of III*b*, followed by condensation with guanidine for 67 hours, as described for the preparation of XIV*a* from III*b*. Evaporation of the *tert*-butanol solution *in vacuo* gave a red-brown gum, which was purified by several recrystallizations from ethanol-water; yield, 7.4 Gm. (46%) of white crystals, m.p. 133–135°; $\lambda_{max.}^{pH\ 13}$ 235 (inflection, ε 23,500), 280 mμ (ε 7,800); $\nu_{max.}$ 3500 (NH, OH); 1660, 1630, 1600, 1490 (NH, pyrimidine, C=C); 1340, 1160 (−SO_2N−); 815, 770, 695 cm.$^{-1}$ (phenyl CH).

Anal.—Calcd. for $C_{27}H_{28}N_4O_3S$: C, 66.5; H, 5.75; N, 11.5; O, 9.85. Found: C, 66.6; H, 5.65; N, 11.3; O, 9.86.

2-Amino-5-(anilinopropyl)-6-benzyl-4-pyrimidinol (XVIIb).—Reduction of 450 mg. (0.92 mmole) of XIV*b*, as described for X*b*, gave 378 mg. of crude product on neutralization of the hydrochloric acid solution. Solution of the product in 10% sodium hydroxide, acidification of the solution to about pH 8 with hydrochloric acid, then two recrystallizations from ethanol-water gave an analytical sample that appeared amorphous. After being dried in high vacuum over P_2O_5 at room temperature, then at 80°, the sample weighed 133 mg. (44%), m.p. 181°, with sintering at 89°; $\nu_{max.}$ 3450, 3330, 3080 (NH, OH); 1650, 1640, 1600, 1490 (NH, pyrimidine, C=C); 745, 690 cm.$^{-1}$ (phenyl CH); $\lambda_{max.}^{pH\ 1}$ 233 (ε 14,500), 265 mμ (ε 10,200); $\lambda_{max.}^{pH\ 7}$ 237 (ε, 20,700), 295 mμ (ε 11,600); $\lambda_{max.}^{pH\ 13}$ 237 (ε 20,300), 283 mμ (ε 9,900).

Anal.—Calcd. for $C_{20}H_{22}N_4O$: C, 72.0; H, 6.61; N, 16.8. Found: C, 71.9; H, 6.48; N, 17.1.

No attempt was made to isolate additional material from the mother liquors.

3-Benzoyl-1-phenyl-2-piperidone (XXIV).—To a magnetically stirred suspension of 0.728 Gm. of 55% sodium hydride (16.7 mmoles) dispersed in mineral oil and 20 ml. of anhydrous *N,N*-dimethylformamide protected from moisture was added 2 Gm. of α-benzoylacetanilide (8.35 mmoles) (XXIII) (24). When evolution of hydrogen had ceased, the turbid yellow solution was treated with a solution of 1.69 Gm. (8.35 mmoles) of 1,3-dibromopropane in 15 ml. of *N,N*-dimethylformamide. After being stirred for 48 hours, the mixture was neutralized with

glacial acetic acid, then spin-evaporated *in vacuo* on a hot water bath. The syrupy residue was partitioned between 25 ml. each of chloroform and water. The separated aqueous phase was extracted with an additional 25 ml. of chloroform. The combined organic extracts, dried with magnesium sulfate, were spin-evaporated *in vacuo*. Crystallization from ethyl acetate gave 0.586 Gm. (25%) of product, m.p. 158–161°. Recrystallization gave white crystals, m.p. 160–162°; $\nu_{max.}$ 2800–1700 (broad acidic OH); 1620 (C=O); 1590, 1530, 1500, 1490 (C=C); 760, 725, 695, 690 cm.$^{-1}$ (phenyl CH); $\lambda_{max.}^{pH\ 1}$ 252 mμ (ε 12,800); $\lambda_{max.}^{pH\ 7}$ 235 (ε 10,100), 345 mμ (ε 17,100); $\lambda_{max.}^{pH\ 13}$ 240 (inflection), 345 mμ (ε 19,500); δ 13.70, one chelated hydrogen.

Anal.—Calcd. for $C_{18}H_{17}NO_2$: C, 77.5; H, 6.10; N, 5.01. Found: C, 77.2; H, 5.95; N, 4.90.

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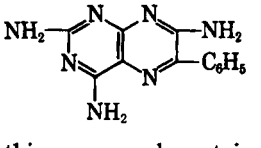
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By LEWIS W. DITTERT, TAKERU HIGUCHI, and DAVIS R. REESE

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SALT SPECIES, other than simple salts, are normally discovered accidentally or incidentally in other studies. Since these complex salts often have solubilities considerably different from normal salts, they may be important pharmaceutically from standpoints such as better *in vivo* availability, greater stability, ease of formulation, etc. In the present study, the phase solubility technique was used to detect complex salt species of triamterene in the presence of various acids. Although this study is limited to triamterene, it is apparent that the procedure is applicable to other systems.

Triamterene (2,4,7-triamino-6-phenylpteridine) is a pteridine diuretic with the structure



Although this compound contains three primary amine groups, it is only weakly monobasic, *e.g.*, toward perchloric acid in glacial acetic acid. Probably because of this weak basicity, most attempts to form classical salts have failed.

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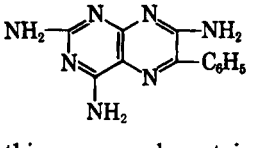
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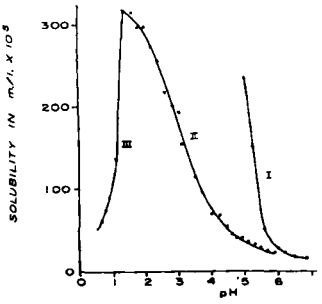


Fig. 1.—pH-solubility profile for triamterene in the presence of hydrochloric acid.

Triamterene is only slightly soluble in water (45 mcg./ml.). This study was initiated to determine to what degree the aqueous solubility of triamterene is increased in the presence of various acids.

EXPERIMENTAL

Analysis of Total Triamterene in Solution.—A clear portion of the solution in equilibrium with a suspension of triamterene at a given pH was obtained by withdrawing the solution into a 5 or 10 ml. Luer-Lok syringe[1] through a Swinney filter adapter[2] containing a type HA filter[2] and a pressed glass wool prefilter.[2] The Swinney adapter was removed, and the fluid was transferred to a small beaker. An aliquot of the solution was pipeted into a 100-ml. volumetric flask containing 4 ml. of concentrated reagent grade formic acid (88%). The contents of the flask were brought to volume with water, and the concentration of triamterene was determined using the absorbance maximum at 357.5 mμ.

pH-Solubility Profiles.—The experiments were conducted in a 600-ml. beaker suspended in a water bath maintained at 37°. The contents of the beaker were stirred by an overhead stirrer with a glass impeller. The pH was followed with a pH meter.

Two grams of triamterene and 400 ml. of water were stirred in the beaker. After approximately 15 minutes (the solution becomes saturated in less than 1 minute), the pH was recorded, and an aliquot of the saturated solution was withdrawn and analyzed as described above. Acid was added dropwise with stirring until the pH shifted about 0.3 units, and the stirring was continued for 15 minutes. A constant pH reading during this 15-minute period indicated that the system was at equilibrium. (Identical results were obtained when stirring times of 1 hour were allowed.) An aliquot was then removed and analyzed.

The pH was thus lowered stepwise and an aliquot removed and analyzed at each step, until the pH reached 1 or until all of the solid phase in the system dissolved. In some cases (*e.g.*, with hydrochloric acid) the pH initially fell, then rose upon further addition of acid. In these cases, sampling was discontinued until the pH remained constant with stirring. Sampling was then resumed at 0.3 pH unit intervals.

Elemental Analyses.—Suspensions of triamterene were adjusted to the proper pH with the acid under study and stirred at constant pH for 15 minutes. The suspensions were filtered through fine porosity sintered-glass Büchner funnels. The material remaining on the filter was dried over sulfuric acid for 3 days. The resulting solids were used for elemental analyses.

Solubility Product Constants.—A suspension of triamterene in a solution of a salt was adjusted to pH 1.3 with the appropriate acid. The suspension was stirred for 15 minutes at constant pH, then analyzed for triamterene in solution as previously described. The solubility product constants (K_{sp}) for four concentrations of each salt were calculated according to the K_{sp} equations shown in the tables below Figs. 2–5 and 7. (The concentration of anion in each case was calculated from the hydrogen ion, triamterene, and sodium salt concentrations.) The average of the four calculated constants is reported.

RESULTS AND DISCUSSION

Hydrochloric Acid.—The pH-solubility profile of triamterene in the presence of hydrochloric acid is shown in Fig. 1. After the solubility rose initially to a maximum at pH 5 (curve I), the pH became unsteady. Continued addition of a relatively large quantity of hydrochloric acid caused the pH to rise to about 6 and remain constant, while the solubility of triamterene fell near its initial level at pH 7. Further addition of acid resulted in the second rising curve (II), which ended in a precipitous drop in solubility (curve III) below pH 1.35.

The interaction of protons with triamterene in solution may be described by the equilibrium

$$\begin{matrix}\text{Triamterene} \\ \downarrow\ \uparrow \\ \text{Triamterene (s)}\end{matrix} + x\,H^+ \overset{K}{\rightleftarrows} \text{Triamterene}\cdot H_x^+ \qquad \text{(Eq. 1)}$$

where

$$K = \frac{[\text{Triamterene}\cdot H_x^+]}{[\text{Triamterene}]\,[H^+]^x} \qquad \text{(Eq. 2)}$$

K is the so-called stability constant of the tri-

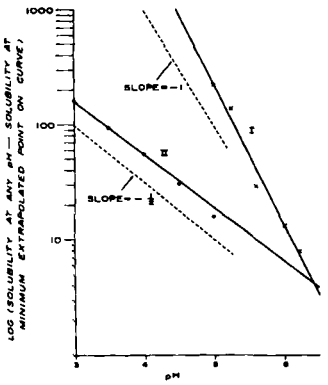


Fig. 2.—Log increase in solubility *vs.* pH plots for triamterene in the presence of hydrochloric acid. Key: x, high pH range (5.5–6.5); ●, medium pH range (3–5.5).

EQUILIBRIA IN THE HYDROCHLORIC ACID SYSTEM

pH Range	Equilibrium
5–7	Triamterene + $H^+ \rightleftharpoons$ triamterene$\cdot H^+$
About 5	Triamterene$\cdot H^+$ + triamterene + $Cl^- \rightleftharpoons$ (triamterene)$_2\cdot$HCl (ppt. 1)
2–5	(Triamterene)$_2\cdot$HCl + $H^+ \rightleftharpoons$ 2 triamterene$\cdot H^+$ + Cl^-
Below 1.35	Triamterene$\cdot H^+$ + $Cl^- \overset{K_{sp}}{\rightleftharpoons}$ triamterene$\cdot$HCl (ppt. 2)

$K_{sp} = 1.5 \times 10^{-4}$

[1] Becton-Dickenson and Co., Rutherford, N.J.
[2] Millipore Filter Corp., Bedford, Mass.

TABLE I.—ELEMENTAL ANALYSES OF TRIAMTERENE COMPLEXES

Complex	Structure	Element	Theoretical %	% Found[a]
Hydrochloric acid				
Precipitate 1	(Triamterene)$_2$·HCl	Cl	6.5	6.2
Precipitate 2	(Triamterene)·HCl	Cl	12.3	11.5*
Nitric acid				
Precipitate 1	(Triamterene)$_3$·HNO$_3$	N	37.5	38.1*
Precipitate 2	(Triamterene)·HNO$_3$	N	34.3	33.5*
Sulfuric acid				
Precipitate 1	(Triamterene)$_4$·H$_2$SO$_4$	S	2.9	2.8
Precipitate 2	(Triamterene)$_2$·H$_2$SO$_4$	S	5.3	5.1
Phosphoric acid				
Precipitate 1	(Triamterene)$_2$·H$_3$PO$_4$	P	5.1	5.0
Precipitate 2	(Triamterene)·H$_3$PO$_4$	P	8.8	9.9*

[a] When attempts were made to wash or recrystallize the complexes, they decomposed to triamterene base and the corresponding acid. The complexes were prepared for analysis without washing. Therefore, some of the elemental analyses (indicated by an asterisk) are outside the usual acceptable limits of error for well-defined compounds. The results are closer to the theoretical per cents shown that to those for any other triamterene:acid ratios.

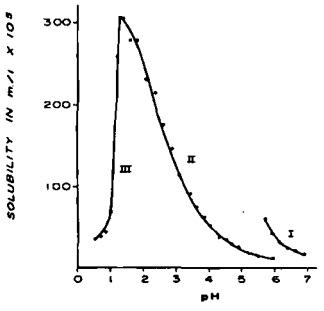


Fig. 3.—pH-solubility profile for triamterene in the presence of nitric acid.

EQUILIBRIA IN THE NITRIC ACID SYSTEM

pH Range	Equilibrium
5.7–7	Triamterene + H$^+$ $\rightleftharpoons$ triamterene·H$^+$
About 5.7	Triamterene·H$^+$ + 2 triamterene + NO$_3^-$ $\rightleftharpoons$ (triamterene)$_3$·HNO$_3$ (ppt. 1)
2–6	(Triamterene)$_3$·HNO$_3$ + 2H$^+$ $\rightleftharpoons$ 3 triamterene·H$^+$ + NO$_3^-$
Below 1.25	Triamterene·H$^+$ + NO$_3^-$ $\overset{K_{sp}}{\rightleftharpoons}$ triamterene·HNO$_3$ (ppt. 2)
	$K_{sp} = 7.6 \times 10^{-5}$

amterene·H$_x^+$ complex, and x is the number of protons required to solubilize one molecule of triamterene. In this expression, [triamterene] is the saturation solubility of the solid phase in the pH region covered by a particular curve. For example, triamterene base is the solid phase in the region of curve I, and the insoluble complex, (triamterene)$_2$·HCl, is the solid phase in the region of curve II. The solubility of the solid phase is a constant for each curve; the numerator term, [triamterene·H$_x^+$], is the increase in solubility of triamterene in the region of that curve. Equation 2 can be transformed into a form (Eq. 3) similar to that used by Higuchi *et al.* (1) to describe the solubility behavior of weak acids at various pH levels. In this equation, the term $(S - S_o)$ is the increase in solubility of triamterene as the pH is lowered. Plots of the hydrochloric acid data according to Eq. 3 are shown in Fig. 2. Values for S_o were determined by extrapolating curves I and II to their minima at the high pH ends. The slopes of the plots in Fig. 2 show that in the pH range of curve I, one proton solubilizes one molecule of triamterene; in the pH range of curve II, one proton solubilizes two molecules of triamterene.

$$\log (S - S_o) = -x\,\mathrm{pH} + \log C \quad \text{(Eq. 3)}$$

Between the two curves the new solid phase, (triamterene)$_2$·HCl, is formed. This complex first forms as a supersaturated solution (at pH 5) which nucleates as additional hydrochloric acid is added, causing the precipitous drop in solubility and the fluctuating pH observed. After all the triamterene in the system has been converted to the new complex, each molecule of the complex is solubilized by one proton, causing the log plot of curve II (Fig. 2) to have a slope of $-1/2$.

The equilibria controlling the solubility behavior of triamterene in hydrochloric acid solutions are summarized in the table below Fig. 2. The final reaction is the solubility product equilibrium for triamterene hydrochloride. The solubility product constant for this reaction was 1.5×10^{-4}. Apparently the final drop in solubility (curve III, Fig. 1) is due to common ion suppression of the solubility product equilibrium.

The results of the elemental analyses of precipitates 1 and 2 are shown in Table I. These results are in agreement with the proposed equilibria. Precipitates 1 and 2 were solid phases in equilibrium

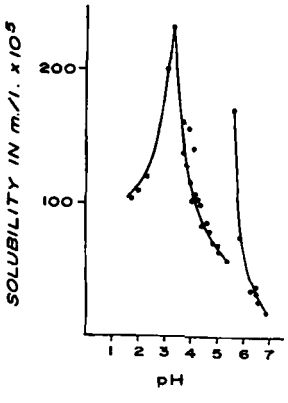


Fig. 4.—pH-solubility profile for triamterene in the presence of sulfuric acid.

EQUILIBRIA IN THE SULFURIC ACID SYSTEM

pH Range	Equilibrium
5.5–7	Triamterene + H$^+$ $\rightleftharpoons$ triamterene·H$^+$
About 5.5	2 Triamterene·H$^+$ + 2 triamterene + SO$_4^-$ $\rightleftharpoons$ (triamterene)$_4$·H$_2$SO$_4$ (ppt. 1)
3.2–5.5	(Triamterene)$_4$·H$_2$SO$_4$ + 2H$^+$ $\rightleftharpoons$ 4 triamterene·H$^+$ + SO$_4^-$
Below 3.2	2 Triamterene·H$^+$ + SO$_4^-$ $\overset{K_{sp}}{\rightleftharpoons}$ (triamterene)$_2$·H$_2$SO$_4$ (ppt. 2)
	$K_{sp} = 4.0 \times 10^{-5}$

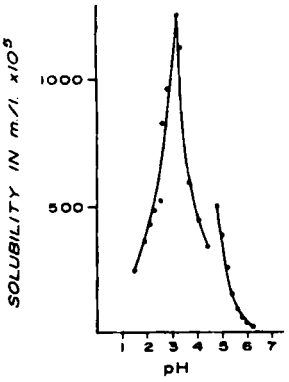


Fig. 5.—pH-solubility profile for triamterene in the presence of phosphoric acid.

EQUILIBRIA IN THE PHOSPHORIC ACID SYSTEM

pH Range	Equilibrium
4.8–7	Triamterene + $H^+ \rightleftharpoons$ triamterene·H^+
About 4.8	Triamterene·H^+ + triamterene + $H_2PO_4^- \rightleftharpoons$ (triamterene)$_2$·H_2PO_4 (ppt. 1)
3.2–4.8	(Triamterene)$_2$·H_2PO_4 + $H^+ \rightleftharpoons$ triamterene·H^+ + $H_2PO_4^-$
Below 3.2	Triamterene·H^+ + $H_2PO_4^- \overset{K_{sp}}{\rightleftharpoons}$ triamterene·H_2PO_4 (ppt. 2)

$K_{sp} = 8.7 \times 10^{-6}$

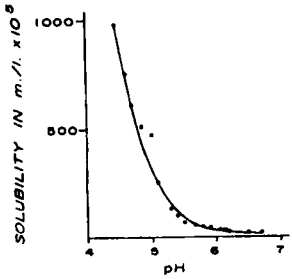


Fig. 6.—pH-solubility profile for triamterene in the presence of acetic acid.

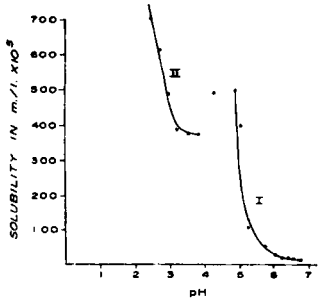


Fig. 7.—pH-solubility profile for triamterene in acetic acid solutions containing 6×10^{-3} *M* sodium acetate.

EQUILIBRIA IN THE ACETIC ACID–SODIUM ACETATE SYSTEM

pH Range	Equilibrium
4.8–7	Triamterene + $H^+ \rightleftharpoons$ triamterene·H^+
About 4.5	Triamterene·H^+ + triamterene + $OAc^- \rightleftharpoons$ (triamterene)$_2$·HOAc
2–4	(Triamterene)$_2$·HOAc + $H^+ \rightleftharpoons$ 2 triamterene·H^+ + OAc^-

with solutions at pH values 4 and 1, respectively. They are assumed to be composed of one molecular specie.

Nitric, Sulfuric, and Phosphoric Acids.—The pH solubility profiles and equilibria governing the behavior of triamterene in nitric, sulfuric, and phosphoric acid systems are shown in Figs. 3–5. In the nitric acid case, the slope of the log plot of curve II is $-2/3$, suggesting that the insoluble complex that precipitates near pH 5.7 has the composition triamterene$_2$·HNO_3. The elemental analysis of precipitate 1 (Table I) is in agreement with this hypothesis.

The sulfuric and phosphoric acid profiles differ from the nitric and hydrochloric acid profiles in that the final drops in solubility due to common ion suppression of the solubility product equilibrium occur at somewhat higher pH's in the former two systems. Also, the solubilities achieved with phosphoric acid are somewhat higher than those achieved with the other inorganic acids. With both sulfuric and phosphoric acid, elemental analyses of the precipitates removed from the systems agree with the proposed equilibria.

Acetic Acid.—The pH-solubility profile for triamterene in the presence of acetic acid is shown in Fig. 6. In this system the solubility of triamterene rises continuously following a single curve. Solubilities higher than those observed with any of the inorganic acids were achieved with acetic acid. The experiment was discontinued at pH 4.5 when all the triamterene in the system dissolved.

When the acetic acid experiment was repeated in the presence of 6×10^{-3} *M* sodium acetate, the resulting pH-solubility profile (Fig. 7) showed the familiar two-curve plot. Log $(S - S_o)$ plots of the data have slopes of -1 (curve I) and $-1/2$ (curve II), as in the hydrochloric acid case. There is no final drop in solubility due to solubility product effects at lower pH's with acetic acid, probably because the low pH's required cannot be reached with acetic acid and also because at low pH's acetic acid exists largely in the undissociated form. The equilibria which describe the solubility behavior of triamterene in solutions of acetic acid are shown in the table below Fig. 7.

CONCLUSIONS

The phase solubility technique has been used to determine the solubility behavior of triamterene in the presence of several acids. The pH-solubility profiles for triamterene revealed (*a*) the formation and stoichiometries of complex salt species in solution, (*b*) the formation and stoichiometries of insoluble complex salts, and (*c*) the controlling equilibria in each pH range. Phosphoric acid and acetic acid produced solubilities of triamterene higher than hydrochloric, nitric, or sulfuric acid. Acetic acid formed complexes more soluble than the inorganic acids.

The experimental method employed in studying triamterene might also be used for the following types of studies:

(*a*) Determination of the pH-solubility characteristics of salts of a drug without actual synthesis and and isolation.

(*b*) Detection and study of complexes of a drug under pharmaceutically significant conditions, *i.e.*, saturated solutions.

(*c*) Detection and isolation of pharmaceutically interesting complexes.

REFERENCE

(1) Higuchi, T., Gupta, M., and Busse, L. W., THIS JOURNAL, 42, 157(1953).

β-Pyridylacrylophenones and Analogs

New Class of Active Coronary Dilating Agents

By JOHN KOO*

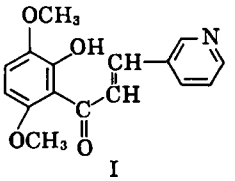
A series of β-pyridylacrylophenones and closely related analogs was synthesized by the condensation of pyridinecarboxaldehydes and other heterocyclic aldehydes with appropriate acetophenones in the presence of various catalysts. The proportions of various reaction products depended upon catalyst and reaction conditions. Several compounds of this type have been found pharmacologically to exhibit remarkable coronary dilating activity.

MANY CHROMONE derivatives exhibit interesting pharmacological activites. Khellin has long been used as a vasodilator (1, 2); recently, some 5,8-dimethoxychromone derivatives were found to exert a powerful coronary dilating (3) or central nervous system depressing action (4). Also, it has been reported (5, 6) that flavonoids possess the ability to form chalcones under certain biological conditions.

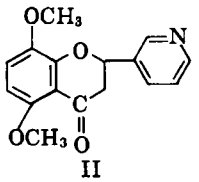
Our investigations in the chromone series (4, 7) therefore were extended to chalcone-type compounds—namely, heterocyclic acrylophenone, whose structures corresponded to some active chromones. Desirable structural features for pharmacological activity were a β-substituted heterocyclic radical, the presence of methoxy-groups, and a 2-hydroxy group in the acrylophenone structure.

The β-heterocyclic-acrylophenones reported here were synthesized according to the usual route for chalcones by condensation of a pyridyl or other heterocyclic carboxaldehyde with the appropriate acetophenone. Several synthetic methods employing various catalysts, solvents, and conditions were evaluated. The catalysts employed were sodium hydroxide in ethanol-water (8, 9), piperidine in absolute ethanol, piperidine in pyridine (10), and piperidine-acetic acid in benzene (11). Depending upon the catalyst and conditions used, similar reactants might form products different from those expected. Also, the reaction of the same acetophenone with different isomeric pyridine-carboxaldehydes using the same catalyst system under identical reaction conditions frequently produced different results. Two factors might be responsible for some difficulties encountered by us. First, the electronic effects of the heterocyclic moiety appeared to influence the course of reaction. Second, the presence of the hydroxy-group on the 2-position of the phenyl ring added the complication of possible ring closure.

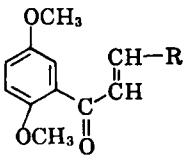
Condensation of 3,6-dimethoxy-2-hydroxyacetophenone[1] with 3-pyridinecarboxaldehyde in ethanol-water with sodium hydroxide as catalyst at 5° yielded the expected 3,6-dimethoxy-2-hydroxy-β-(3-pyridyl)-acrylophenone (I). Its hydrochloride was isolated as cherry-red needles. However, by using absolute ethanol as solvent in the presence of piperidine as catalyst under prolonged heating, the isomeric colorless 5,8-dimethoxy-2-(3-pyridyl)-chromanone (II) was obtained in addition to I.



I



II



III

IIIa, R = 

IIIb, R = 

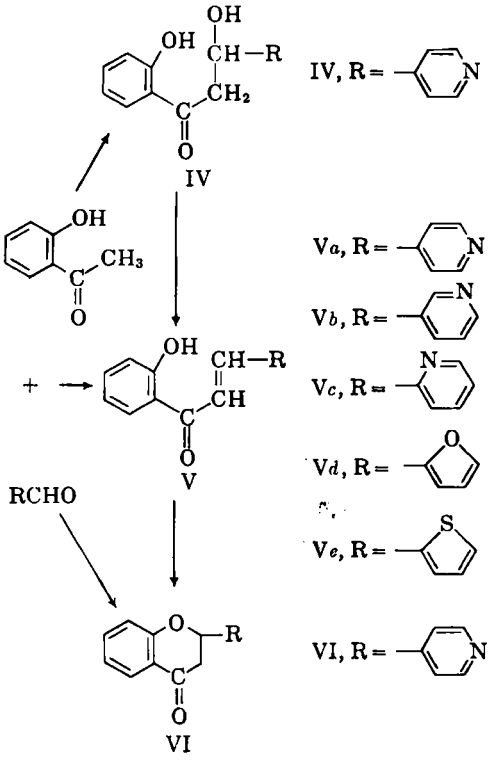
IIIc, R = 

To study the relationship between the 2-hydroxy-group and the pharmacological activity of I and also to shorten the synthetic route, 2,5-dimethoxyacetophenone was employed in place of 3,6-dimethoxy-2-hydroxyacetophenone. By condensation of this compound with 4-pyridinecarboxaldehyde in pyridine or absolute ethanol with piperidine as catalyst, 2,5-dimethoxy-β-(4-pyridyl)-acrylophenone (III*a*) was then obtained.

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* Present address: Geigy Research Laboratories, Ardsley, N. Y.

[1] This compound was prepared according to the general route of Baker (12) in four steps from 2,6-dihydroxyacetophenone, which in turn was obtained in another four steps from resorcinol by following the method of Frye (13). However, the product of the first step, 4-methyl-7-hydroxy-coumarin, was produced more conveniently by using polyphosphoric acid instead of sulfuric acid as the condensing agent (14) and the last compound, 3,6-dimethoxy-2-hydroxyacetophenone, was obtained by an improved method of catalytic debenzylation of the corresponding benzyloxy-analog, as described under *Experimental*.

By an analogous reaction in absolute ethanol, using pyridine or benzene with piperidine or piperidine-acetic acid as catalyst, the 3-pyridyl isomer (III*b*) was obtained in better yields. Similarly, the 2-pyridyl isomer (III*c*) was obtained in absolute ethanol or pyridine in the presence of piperidine as catalyst.



For further pharmacological study, work was then extended to 2-hydroxy-β-heterocyclic-acrylophenones containing no methoxy-groups. Condensation of 2-hydroxyacetophenone and 4-pyridinecarboxaldehyde in ethanol-water with sodium hydroxide as catalyst gave both the colorless 2′,3-dihydroxy-3-(4-pyridyl)-propiophenone (IV) and its dehydration product, the yellow 2-hydroxy-β-(4-pyridyl)-acrylphenone (V*a*). When heated mildly with dilute hydrochloric or sulfuric acid or polyphosphoric acid, IV was promptly dehydrated to the yellow compound V*a*. However, the same condensation carried out in absolute ethanol or pyridine catalyzed with piperidine gave the colorless ring-closed product 2-(4-pyridyl)-chromanone (VI). Alternatively, when piperidine-acetic acid was used as catalyst in benzene solution for the same reaction, IV was the chief product which gradually changed to the yellow compound V*a* upon standing in air or in solution. The results of these experiments support the view that the mechanism of the reaction between a 2-hydroxyacetophenone and a heterocyclic aldehyde is first to form the ketol IV which then dehydrates to an acrylophenone V and finally undergoes ring closure to a chromanone VI. The state at which the reaction stops is strongly conditioned and catalyst dependent.

However, when 3- or 2-pyridylcarboxaldehyde was employed with sodium hydroxide at catalyst for the analogous condensation, the only product isolated was V*b* or V*c*. The preparation of the 2-furyl (V*d*) and the 2-thiophenyl (V*e*) analogs were similarly achieved using the usual aqueous ethanolic sodium hydroxide procedure for the condensation.

Pharmacology.—Pharmacological study[2] of these compounds showed that a few of them exhibited remarkable coronary dilating action with relatively low toxicity. Both compounds I and III*b* increased similarly the coronary flow in the Langendorf preparation of the excised cat heart, when 5 to 100 mcg. were introduced into the perfusion system at the aortic cannula. Quantitatively, the coronary dilating action compared favorably with published data by Khellin on similar action. The effect usually reached its maximum over approximately a 30-minute period. Doses of 5 to 10 mg./Kg. of these compounds given intravenously to the dog lowered the arterial pressure moderately, accompanied by a brief apnea, which was later followed by respiratory stimulation. Fighting of the Siamese fighting fish (*Beta splendens*) was suppressed in concentrations of 0.1 to 0.5 mcg./ml. of solution. Evipal sleeping time in mice was significantly prolonged by 250 mg./Kg. of III*b* orally, while compound I exhibited no significant action.

Compound III*a* also exerted coronary dilating action inconsistently at doses of about 2 to 6 mcg.; Evipal sleeping time was considerably potentiated. No consistent action on coronary flow was shown by IV. It was moderately hypotensive at about 5 to 20 mg./Kg. i.v. in dogs and potentiated Evipal sleeping time in mice considerably.

Compounds V*a*, V*b*, and V*c* increased coronary flow moderately at doses of about 50 to 100 mcg. They showed mild hypotensive action at about 2 to 10 mg./Kg. i.v. in dogs. Also, 2-hydroxy-5-chloro-β-(2-pyridyl)-acrylophenone, which was one of several previously known compounds synthesized for pharmacological study, exhibited moderate coronary dilating action by Langendorf preparation at doses of about 2 to 10 mcg. and was moderately hypotensive at levels of 2 mg./Kg. i.v. in dogs. The acute oral LD_{50} of I in mice was approximately 600 mg./Kg., and its acute intravenous LD_{50} was 52 mg./Kg. The acute oral and intravenous LD_{50} of III*b* were 1000 mg./Kg. and 55–68 mg./Kg.

A preliminary study of the correlation between structure and activity of many newly synthesized and some known heterocyclic-substituted acrylophenones seems to indicate that the presence of the 3,6-dimethoxy and 3-pyridyl groups are essential to insure a higher degree and greater consistency of coronary dilating action. The 2-hydroxy group does not appear to be necessary for the activity; its omission even reduces the toxicity moderately.

[2] The pharmacological study was made by Dr. S. Krop and associates.

EXPERIMENTAL[3]

3,6-Dimethoxy-2-hydroxyacetophenone.—To a solution of 14.3 Gm. of 2,5-dimethoxy-6-benzyloxyacetophenone (see *Footnote 1*) in 100 ml. of absolute ethanol was added 4 Gm. of 5% Pd on charcoal catalyst, and the mixture was hydrogenated at room temperature under 40 lb. pressure for 1.5 hours. It was then filtered, and the solution was evaporated to give 8.3 Gm. (95%) of a yellow crystalline solid, m.p. 60–61° [reported (12) m.p. 61°]. In our investigation the original hydrochloric-acetic acid procedure of debenzylation gave only about 70% yield of less pure material.

3,6-Dimethoxy-2-hydroxy-β-(3-pyridyl)-acrylophenone Hydrochloride (I).—(*a*) To a stirred solution of 1.8 Gm. of 3,6-dimethoxy-2-hydroxyacetophenone in 50 ml. of 10% NaOH aqueous ethanol (2:1) solution at 10° there was added 1.1 Gm. of 3-pyridinecarboxaldehyde. Stirring was continued for 5 hours while the solution was permitted to come to room temperature. The solution was acidified with dilute hydrochloric acid to pH 7, and the yellow oil which separated was extracted with ether. After drying over magnesium sulfate, the ether solution was treated with hydrogen chloride gas, causing a heavy precipitate to form. It was recrystallized first from ethanol-ether and then from aqueous ethanol to yield 0.2 Gm. (7%) of cherry-red needles, m.p. 233–234° dec.

Anal.—Calcd. for $C_{16}H_{16}ClNO_4$: C, 59.54; H, 5.31; N, 4.34. Found: C, 59.47; H, 5.09; N, 4.41.

(*b*) A solution of 3.92 Gm. of 3,6-dimethoxy-2-hydroxyacetophenone and 2.14 Gm. of 3-pyridinecarboxaldehyde in 60 ml. of absolute ethanol containing 16 drops of piperidine was gently refluxed for 3 hours. The reaction mixture was concentrated to a small volume, diluted with cold water, and repeatedly extracted with ether. After drying, the combined ether extracts were concentrated to a small volume. Some colorless material which separated was filtered off, and the ether solution was saturated with hydrogen chloride gas. The reddish paste which separated was recrystallized from absolute ethanol twice to give 0.75 Gm. (11%) of red needles. The melting point and mixed melting point with the material obtained from method (*a*) was 233–234° dec.

5,8-Dimethoxy-2-(3-pyridyl)-chromanone (II).—The colorless material obtained from the above experiment (*b*) weighed 0.5 Gm. (8%), m.p. 162–166°. It was recrystallized from dilute ethanol as long colorless needles, m.p. 170°. It was not soluble in dilute sodium hydroxide solution, and the infrared spectrum exhibited a strong carbonyl band at 5.9 μ.

Anal.—Calcd. for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91. Found: C, 67.55; H, 5.44; N, 4.80.

2,5-Dimethoxy-β-(4-pyridyl)-acrylophenone Hydrochloride (III*a*).—(*a*) A solution of 82 Gm. of 2,5-dimethoxyacetophenone, 50 Gm. of 4-pyridinecarboxaldehyde, and 14 ml. of piperidine in 200 ml. of absolute ethanol was refluxed gently for 55 hours. The dark solution was diluted with cold water and extracted with ether. The ether solution was washed with sodium bisulfite solution, dried over magnesium sulfate, and filtered. Introduction of hydrogen chloride gas to the ether solution yielded 75 Gm. (53%) of a yellow precipitate, m.p. 190–195°. It was recrystallized from absolute ethanol three times to give 17.5 Gm. (12%) of long bright-yellow needles, m.p. 229–230° dec.

Anal.—Calcd. for $C_{16}H_{16}ClNO_3$: C, 62.85; H, 5.24; N, 4.58. Found: C, 62.63; H, 5.40; N, 4.62.

(*b*) A solution of 27 Gm. of 2,5-dimethoxyacetophenone, 16 Gm. of 4-pyridinecarboxaldehyde, 100 ml. of pyridine, and 15 drops of piperidine was heated on the steam bath for 8 hours. The pyridine was evaporated under reduced pressure, and the residue was diluted with water. The oily material was extracted with ether; the ether solution was washed with saturated sodium bisulfite, cold water, then extracted with 15% hydrochloric acid. On cooling, the acid solution 3.2 Gm. (7%) of yellow product separated, m.p. 220–225°. It was recrystallized from absolute ethanol twice to give bright fluffy needles, m.p. 228–230°. The mixed melting point with the sample obtained from method (*a*) showed no depression.

2,5-Dimethoxy-β-(3-pyridyl)-acrylophenone Hydrochloride (III*b*).—(*a*) A solution of 11 Gm. of 3-pyridinecarboxaldehyde and 18 Gm. of 2,5-dimethoxyacetophenone in 200 ml. of absolute ethanol was heated for 72 hours in the presence of 20 drops of piperidine. It was then worked up by the method (*b*) for compound III*a*. There was obtained 15.5 Gm. (49%) of bright-yellow needles, m.p. 204–205° dec.

Anal.—Calcd. for $C_{16}H_{16}ClNO_3$: C, 62.85; H, 5.24; N, 4.58. Found: C, 62.71; H, 5.53; N, 4.65.

(*b*) By method (*b*) of III*a*, heating a solution of 11 Gm. of 3-pyridinecarboxaldehyde, 18 Gm. of 2,5-dimethoxyacetophenone in 150 ml. of pyridine, and 20 drops of piperidine for 5 hours yielded 11 Gm. (34%) of bright-yellow product. The melting point and mixed melting point with product from (*a*) were 204–205°.

(*c*) To a solution of 9 Gm. of 5.5 Gm. of 3-pyridinecarboxaldehyde and 9 Gm. of 2,5-dimethoxyacetophenone in 50 ml. of absolute ethanol was added 1 ml. of piperidine and 3 ml. of acetic acid. It was gently refluxed for 25 hours, then worked up as in method (*b*) of III*a*. The yield of product was 7.7 Gm. (49%), and the mixed melting points with sample from above experiments were 204–205° dec.

2,5-Dimethoxy-β-(2-pyridyl)-acrylophenone Hydrochloride (III*c*).—(*a*) A solution of 18 Gm. of 2,5-dimethoxyacetophenone, 10.7 Gm. of 2-pyridinecarboxaldehyde, and 20 drops of piperidine in 60 ml. of absolute ethanol was refluxed for 50 hours and then worked up as method (*a*) of III*b*. The yield of bright-yellow needles was 6.5 Gm. (41%), m.p. 189–190°.

Anal.—Calcd. for $C_{16}H_{16}ClNO_3$: C, 62.85; H, 5.24; N, 4.58. Found: C, 62.82; H, 5.54; N, 4.86.

(*b*) The above experiment was repeated with pyridine instead of ethanol as solvent, yielding 2 Gm. (12%) of pure product.

2-Hydroxy-β-(4-pyridyl)-acrylophenone (V*a*).—To a stirred solution of 21.5 Gm. of 4-pyridinecarboxaldehyde in 300 ml. of 10% NaOH aqueous ethanol (2:1) solution was added dropwise 27.2 Gm. of 2-hydroxyacetophenone over a 1-hour

[3] Experiments were carried out during 1956 and early 1957. Melting points are uncorrected. Analyses were performed by Mr. E. R. Hoffman and staff, Research Division, Ethicon, Inc.

period. The stirring was continued at 5–10° for 8 hours and then at room temperature for 1 hour more. The reaction mixture was diluted with cold water, extracted with ether, and acidified with acetic acid to pH 6. The oily material which separated was shaken with ether. Some pale-yellow solid, which was not soluble in ether, was filtered out. The ether solution was first washed with sodium bisulfite solution, then with 10% hydrochloric acid solution. This acidic solution was made weakly basic with sodium carbonate solution. The heavy yellow precipitate that formed was filtered off, washed, and dried, yielded 25 Gm. (55%), m.p. 110–116°. It was recrystallized once from 70% ethanol to yield 3.5 Gm. (8%) pure product as long yellow needles, m.p. 124–125°. Concentration of the above filtrate yielded only a dark oil. It seemed likely that most of the crude product was decomposed during the recrystallization.

Anal.—Calcd. for $C_{14}H_{14}NO_2$: C, 74.65; H, 4.92; N, 6.22. Found: C, 74.68; H, 4.92; N, 6.08.

2',3-Dihydroxy-3-(4-pyridyl)-propionphenone (IV).—(*a*) The pale-yellow material separated from the above experiment weighed 3.5 Gm. (7.5%), m.p. 141–145°. It was recrystallized twice from ethanol to give colorless needles, m.p. 147–149°.

Anal.—Calcd. for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76. Found: C, 69.36; H, 5.54; N, 5.85.

When this compound was warmed with dilute hydrochloric acid, sulfuric acid, or polyphosphoric acid, it was readily dehydrated to the yellow acrylophenone (V*a*).

(*b*) To a solution of 13.6 Gm. of 2-hydroxyacetophenone and 11 Gm. of 4-pyridinecarboxaldehyde in 200 ml. of benzene in a 500-ml. round-bottom flask fitted with a water-benzene separator, was added 4 ml. of acetic acid and 2 ml. of piperidine. The solution was refluxed for 20 hours and then washed with cold water, 30% sodium bisulfite solution, and again with water. The benzene was extracted with two 50-ml. portions of 20% hydrochloric acid solution. Upon standing at room temperature, some of the hydrochloride of IV separated and was filtered, weighing 2.1 Gm. (8%), m.p. 202–204°. It was recrystallized from ethanol-ether to provide cream-colored crystals, m.p. 205–206°.

Anal.—Calcd. for $C_{14}H_{14}ClNO_3$: N, 5.01; Cl, 12.67. Found: N, 4.78; Cl, 12.48.

The acid filtrate was neutralized with potassium carbonate solution, causing 4.5 Gm. (18%) of the free base of IV to precipitate, m.p. 145–148°. It was recrystallized once from benzene-petroleum ether to yield off-white material, m.p. 148–149° (deep-yellow melt). The mixed melting point with a sample from (*a*) was not depressed.

2-Hydroxy-β-(3-pyridyl)-acrylophenone (V*b*).—This compound was prepared from 22 Gm. of 3-pyridinecarboxaldehyde and 28 Gm. of 2-hydroxyacetophenone in 300 ml. of sodium hydroxide solution by the method of V*a*, giving 4.5 Gm. (10%) of yellow needles, m.p. 149–150° [reported (13) m.p. 161–162°].

Anal.—Calcd. for $C_{14}H_{11}NO_2$: C, 74.65; H, 4.92; N, 6.22. Found: C, 74.40; H, 5.22; N, 6.15.

2-Hydroxy-β-(2-pyridyl)-acrylophenone (V*c*).—This compound was obtained from 10.7 Gm. of 2-pyridinecarboxaldehyde and 13.6 Gm. of 2-hydroxyacetophenone similarly by the method of V*a*, giving 14.5 Gm. (60%) of yellow product, m.p. 98–99° [reported (15) m.p. 101–102°].

Anal.—Calcd. for $C_{14}H_{11}NO_2$: C, 74.65; H, 4.92; N, 6.22. Found: C, 74.86; H, 5.19; N, 6.05.

2-Hydroxy-β-(2-furyl)-acrylophenone (V*d*).[4]—The condensation of 14 Gm. of 2-hydroxyacetophenone and 10 Gm. of 2-furaldehyde in 150 ml. of 15% NaOH aqueous ethanol (2:1) gave 9 Gm. (42%) of long yellow needles, m.p. 109–110°.

Anal.—Calcd. for $C_{13}H_{10}O_3$: C, 72.98; H, 4.71. Found: C, 73.40; H, 5.01.

2-Hydroxy-β-(2-thiophenyl)-acrylophenone (V*e*).—This compound was obtained according to the above method from 4.6 Gm. of 2-hydroxyacetophenone and 3.8 Gm. of 2-thiophenecarboxaldehyde, giving 2.5 Gm. (37%) of yellow needles, m.p. 99–100°.

Anal.—Calcd. for $C_{13}H_{10}O_2S$: C, 67.80; H, 4.38; S, 13.92. Found: C, 67.97; H, 4.49; S, 13.60.

2-(4-Pyridyl)-chromanone (VI).—(*a*) To a solution of 10.7 Gm. of 4-pyridinecarboxaldehyde and 13.6 Gm. of 2-hydroxyacetophenone in 100 ml. of absolute ethanol was added 3 ml. of piperidine, and the solution was heated on a steam bath for 3 hours. The dark reaction mixture was then allowed to stand at room temperature for 1 week, during which time a yellow solid separated. It was filtered off, washed with a little cold water, and dried, 5.5 Gm. (24%), m.p. 210–215°. The crude material was recrystallized from 95% ethanol to give colorless needles, m.p. 224–225°.

Anal.—Calcd. for $C_{14}H_{11}NO_2$: C, 74.65; H, 4.93; N, 6.22. Found: C, 74.68; H, 4.83; N, 5.95.

(*b*) To a solution of 13.6 Gm. of 2-hydroxyacetophenone and 10.6 Gm. of 3-pyridinecarboxaldehyde in 150 ml. of pyridine was added 1 ml. of piperidine. The solution was gently refluxed on a steam bath for 3 hours, then diluted with cold water. The dark-red paste which separated was recrystallized from ethanol to give 3.5 Gm. (15%) of colorless needles, m.p. 225°. Neither of the products from the above experiments was soluble in dilute sodium hydroxide solution, and the mixed melting point was 224–225°. The infrared spectra of both materials were identical with a strong carbonyl band at 5.9 μ.

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[4] The preparation of this compound was mentioned in the earlier literature (16), but the material was neither characterized nor were experimental details given.

Thyromimetics IV

Synthesis of Some Derivatives of 3,3′,5-Triiodo-D and L-Thyronine

By BENJAMIN BLANK

Three pathways leading to the methyl ethers of 3,3′,5-triiodo-D and L-thyronine were explored. In these syntheses the acetyl, benzoyl, and trifluoroacetyl groups were studied as suitable amine-protecting groups, thus providing a number of hitherto unreported thyronine derivatives. Consideration of the over-all yields and the quality of the products from the various reaction sequences showed that the acetyl group was best suited for the protection of the amino group in these syntheses.

DURING A STUDY in which the thyromimetic activities of several ethers of thyromimetic substances were determined (1) it became desirable to prepare the methyl ethers of 3,3′,5-triiodo-D and L-thyronine (VII-D and L). The synthesis of the L-isomer has been described by Tomita *et al.* (2). The other reports dealing with VII-L contained biological data only (3, 4).

In the synthesis reported by Tomita *et al.* (2), 3,3′,5-triiodo-L-thyronine methyl ester (II*a*-L) was treated with diazomethane to give the methyl ether of II*a*-L. Basic hydrolysis followed by neutralization yielded VII-L in about 43% overall yield.

An alternate synthesis of VII-L using *N*-benzoyl-3,3′,5-triiodo-L-thyronine (IV*a*-L) and dimethyl sulfate was also explored by Tomita (2). However, difficulty in obtaining IV*a*-L caused the abandonment of this route. This route has since been used successfully in our laboratories.

DISCUSSION

Except for the work described by Tomita (2), the chemical preparation of derivatives of 3,3′,5-triiodo-D and L-thyronine (I-D and L) has not been described adequately in the literature. Consequently, two reaction sequences, in addition to the one using IV*a*, were studied in order to describe more fully such chemical work and to investigate alternate syntheses of VII-D and L. It was possible as a result of these studies to have available for biological and physical chemical examination a number of previously unavailable and unknown derivatives of I-D and L and methods for their preparation.

The syntheses varied since different acyl groups were used to protect the amine function in I during methylation of the 4′-phenolic group. Most of the reactions that were carried out are shown in Scheme I.

In the sequence leading to IV*a*, I was converted to its methyl or ethyl ester according to the method of Ashley and Harington (5). The methyl ester II*a* was purified and characterized as the hydrochloride, while the ethyl ester II*b* was isolated and purified as the free base.

The addition of benzoyl chloride to a suspension of either II*a* or II*b* in a mixture of tetrahydrofuran and 5% sodium bicarbonate resulted in the formation of the *N,O*-dibenzoyl-3,3′,5-triiodothyronine esters, III*a* and *b*. These esters were usually contaminated with a small amount of incompletely benzoylated material. This was removed conveniently either by triturating the crude reaction product with methanol or by extracting a chloroform solution of the reaction product with dilute base.

Basic hydrolysis of III*a* or *b* yielded IV*a* in what appeared to be a solvated form. Analyses of IV*a*-D and L have varied, despite numerous manipulations to prepare pure IV*a*-D and L. The elemental analyses have supported more consistently the structure indicated for IV*a* plus an additional mole of methanol than they have any other reasonable structure.

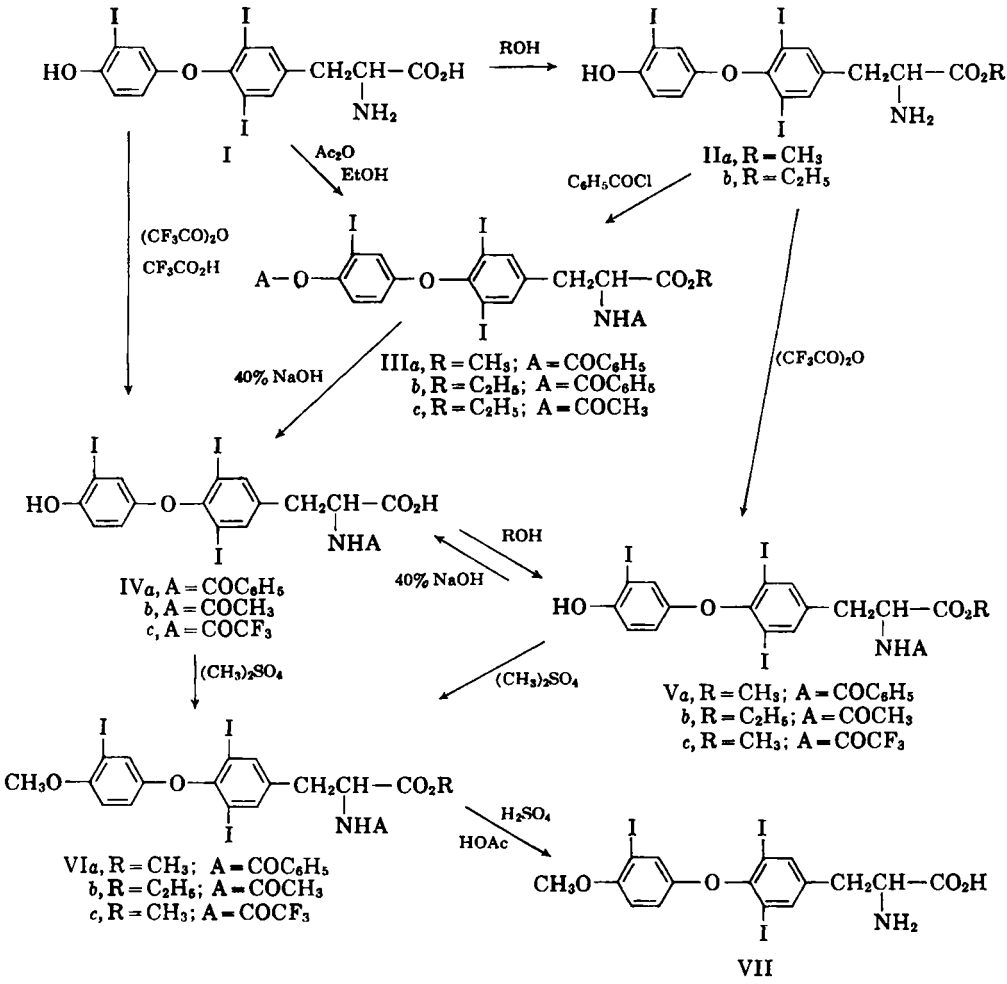
The structures of IV*a*-D and L have been substantiated by their conversion with methanolic hydrogen chloride to the esters V*a*-D and L, by appropriate infrared spectra, and by their conversion to *N*-benzoyl-3-[3,5-diiodo-4-(3-iodo-4-methoxyphenoxy)phenyl]-D and L-alanine methyl esters (VI*a*-D and L). V*a*-D and L and VI*a*-D and L have expected infrared spectra and give satisfactory elemental analyses. VI*a*-D and L prepared by complete methylation of IV*a*-D and L were identical to the products from the methylation of V*a*-D and L.

To demonstrate that IV*a*-D and L were indeed homogeneous, paper chromatograms were prepared in an isoamyl alcohol–*tert*-amyl alcohol–6 *N* ammonia system (1:1:2) and in an *n*-butanol–aqueous ammonia system (6). The components were detected by spraying with ninhydrin and Emerson's reagent (turns pink in the presence of *o*-iodophenols). The chromatograms showed IV*a*-D and L to consist of one major component contaminated with a trace amount of an unknown impurity. The major component was ninhydrin-negative and gave a positive reaction with Emerson's reagent.

Hydrolysis of VI*a* with a 1:1 mixture of acetic and concentrated hydrochloric acids for 4 hours, as reported by Tomita (2), or for 2 hours, as carried out subsequently by Blank, caused some cleavage of the ether. Evidence of this was obtained from paper chromatograms of the purified products using previously described systems and sprays (6). When the hydrolysis was performed for 18–24 hours with a 1:1 mixture of acetic acid and 6 *N* sulfuric acid, pure 3-[3,5-diiodo-4-(3-iodo-4-methoxyphenoxy)phenyl]-alanine (VII) was obtained.

In the route to VII, using *N*-acetyl-3,3′,5-triiodothyronine (IV*b*) as an intermediate, numerous

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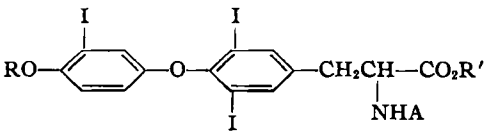
Scheme I

attempts were made to prepare IV*b* directly from I using conditions which were successful for the preparation of *N*-acetyl-3,5-diiodotyrosine (7) and *N*-acetylthyroxine (8). These attempts led to the recovery of unreacted impure I. The addition of ethanol to the reaction solution of I in acetic anhydride and aqueous sodium hydroxide, either during the acetylation or just prior to acidification of the acetylation solution, resulted in the isolation of an acetylated product.[1] That an *N*-acetyl derivative had been formed was shown by the facts that the isolated material was ninhydrin-negative, was acid insoluble, and had an amide peak in its infrared spectrum. In addition to the amide absorption at 6.09 μ, there were two additional carbonyl peaks in the infrared spectrum at 5.68 and 5.77μ which could correspond to a phenolic acetate and carboxylic acid ester, respectively. Moreover, the phenolic absorptions at 7.05–7.08 and 8.07–8.10 μ, which were present in IV*b* and V*b*, were not present in this material. Thus, this material was considered to be chiefly *N*,*O*-diacetyl-3,3',5-triiodothyronine ethyl ester (III*c*). Such a structure was in keeping with the report by Ashley and Harington (8) that *N*,*O*-diacetylthyroxine ethyl ester was formed under comparable conditions. Unfortunately, the isolation of pure III*c* was never accomplished, although several attempts were made. In one instance a sample was obtained which melted sharply and which showed only one spot on thin-layer and paper chromatography. Drying this material *in vacuo* for analysis caused the melting point to be lowered, and subsequent elemental analyses were unsatisfactory. Crude III*c*-D and L could, however, be converted with ethanolic hydrogen chloride to the readily purified and identified *N*-acetyl-3,3',5-triiodo-D and L-thyronine ethyl esters (V*b*-D and L).

Treatment of V*b* or the crude acetylation product with 40% sodium hydroxide yielded, after recrystallization from aqueous methanol, what seems to be a methanolate of *N*-acetyl-3,3',5-triiodothyronine (IV*b*) (on the basis of melting point and elemental analyses). This material behaved much like the *N*-benzoyl analog IV*a* since treatment of IV*b* with ethanolic hydrogen chloride gave V*b* comparable to the formation of V*a* from IV*a*. Then, too, both IV*a* and IV*b* were soluble in dilute aqueous sodium carbonate, both were ninhydrin-negative, and on paper chromatograms both showed a single spot which was positive to Emerson's reagent and negative to ninhydrin.

[1] The need for alcohol in such acetylations has been reported previously for the preparation of *N*-acetylthyroxine (9).

TABLE I.—DERIVATIVES OF 3,3′,5-TRIIODO-D AND L-THYRONINE



No.	R	R′	A	M.p., °C.	Recrystal. Solvent	Optical Rotation in degrees $[\alpha]_D^{25}$[a]	Formula	Anal., % Calcd. C	Calcd. H	Calcd. I	Found C	Found H	Found I
IIa-D	H	CH_3	H	213–215[b]	CH_3OH-$(C_2H_5)_2O$	−9.4[c]	$C_{16}H_{15}ClNO_4$[d]	27.40	2.16	54.27	27.77	2.30	54.01
a-L	H	CH_3	H	215[b]	CH_3OH-$(C_2H_5)_2O$	+8.9[c]					27.66	1.97	54.49
b-D	H	C_2H_5	H	149–150	C_2H_5OH-H_2O	−28.2[e]	$C_{17}H_{16}I_2NO_4$	30.07	2.38	56.07	30.32	2.25	56.14
b-L	H	C_2H_5	H	148–150	C_2H_5OH-H_2O	+27.7[e]					30.44	2.51	55.62
IIIa-D	C_6H_5CO	CH_3	COC_6H_5	182–183	EtOAc-pet. ether	+34.4[f]	$C_{30}H_{22}I_2NO_6$	41.26	2.54	43.60	41.54	2.48	43.56
a-L	C_6H_5CO	CH_3	COC_6H_5	184–185	CH_3OH	−37.8[f]	[g]				41.20	2.47	43.89
b-D	C_6H_5CO	C_2H_5	COC_6H_5	165–167	EtOAc-pet. ether	+28.6[f] (c,1.2)	$C_{31}H_{24}I_2NO_6$	41.96	2.73	42.91	42.33	2.78	43.16
b-L	C_6H_5CO	C_2H_5	COC_6H_5	169–171	EtOAc-pet. ether	−28.1[f]					42.08	2.72	42.84
IVa-D	H	H	COC_6H_5	148	CH_3OH-H_2O	+15.6[e]	$C_{23}H_{20}I_2NO_6$[h]	35.09	2.56	48.37	35.46	2.60	49.61
a-L	H	H	COC_6H_5	148	CH_3OH-H_2O	−14.8[e]					35.34	2.49	48.43
b-D	H	H	$COCH_3$	140	CH_3OH-H_2O	−16.6[i]	$C_{18}H_{16}I_2NO_6$[h]	29.81	2.52	52.51	29.86	2.51	52.95
b-L	H	H	$COCH_3$	136–138	CH_3OH-H_2O	+14.1[i]					29.78	2.23	52.94
c-D	H	H	$COCF_3$	199–201	EtOAc-pet. ether	−4.6[f]	$C_{17}H_{11}F_3I_2NO_5$	27.33	1.48	50.97	27.58	1.57	51.18
c-L	H	H	$COCF_3$	197–199	EtOAc-pet. ether	+4.4[f]					27.57	1.68	51.19
Va-D	H	CH_3	COC_6H_5	112–114	CH_3OH-H_2O	+39.8[e]	$C_{23}H_{18}I_2NO_5$	35.92	2.36	49.50	36.24	2.46	49.37
a-L	H	CH_3	COC_6H_5	113–115	CH_3OH-H_2O	−33.6[e]					36.12	2.41	49.60
b-D	H	C_2H_5	$COCH_3$	150–152	C_2H_5OH-H_2O	−9.5[i]	$C_{19}H_{18}I_2NO_5$	31.65	2.52	52.80	31.87	2.45	52.85
b-L	H	C_2H_5	$COCH_3$	147–148	50% Aq.C_2H_5OH	+8.0[i]					31.69	2.43	52.59
c-D	H	CH_3	$COCF_3$	137–139	CH_3OH-H_2O	+5.9[e] (c,0.9)	$C_{18}H_{12}F_3I_2NO_5$	28.41	1.72	50.03	28.57	1.85	50.01
c-L	H	CH_3	$COCF_3$	139–141	CH_3OH-H_2O	−6.5[e] (c,0.9)					28.52	1.77	50.25
VIa-D	CH_3	CH_3	COC_6H_5	100–103	C_2H_5OH-H_2O	+44.2[i] (c,0.8)	$C_{24}H_{20}I_2NO_5$	36.81	2.57	48.61	36.96	2.48	48.54
a-L	CH_3	CH_3	COC_6H_5	102–105	C_2H_5OH-H_2O	−45.8[i]					37.00	2.69	48.55
b-D	CH_3	C_2H_5	$COCH_3$	125–128	EtOAc-pet. ether	−9.3[i]	$C_{20}H_{20}I_2NO_5$	32.68	2.74	51.79	32.61	2.82	51.85
b-L	CH_3	C_2H_5	$COCH_3$	127–129	EtOAc-pet. ether	+8.8[i]					32.52	2.79	51.33
c-D	CH_3	CH_3	$COCF_3$	120–122	70% Aq.C_2H_5OH	−8.6[i]	$C_{19}H_{15}F_3I_2NO_5$	29.44	1.95	49.12	29.70	2.04	49.08
c-L	CH_3	CH_3	$COCF_3$	115–117	70% Aq.C_2H_5OH	+7.3[i]					29.60	2.02	48.98
VII-D	CH_3	H	H	234–236[b]	C_2H_5OH-H_2O[j]	−21.6[e] (c,1.1)	$C_{16}H_{14}I_2NO_4$		[k]				
-L	CH_3	H	H	229–230[b]	C_2H_5OH-H_2O[j]	+18.0[e]	$C_{16}H_{16}I_2NO_5$[l]		[k]				
VIII-L	CH_3	C_2H_5	H	145[b]	$(CH_3)_2CO$	+17.2[i]	$C_{18}H_{19}ClI_2NO_4$[d]	29.63	2.63	52.19	29.78	2.65	51.94

[a] c = 1 unless indicated. [b] With decomposition. [c] Determined in methanol. [d] Hydrochloride. [e] Determined in ethanol–1 *N* hydrochloric acid (3:1 by volume). [f] Determined in ethyl acetate. [g] Also reported in *Reference 2*. [h] Methanolate. (See *text*.) [i] Determined in ethanol. [j] Recrystallized as described under *Experimental*. [k] See *Reference 1*. [l] Hydrate.

Unfortunately, NMR studies of IV*b*-L in deuteroacetone showed no evidence of methanol. No explanation can be presented currently to explain these anomalous data.

Methylation of V*b* to VI*b* and hydrolysis of VI*b* with acetic and sulfuric acids to VII-D and L proceeded in a straightforward manner.

In an effort to circumvent the *O*-acylation which occurred when I was acetylated or benzoylated and which necessitated at least an additional hydrolysis step, the use of the *N*-trifluoroacetyl group was investigated. The preparation of *N*-trifluoroacetyl-3,3',5-triiodo-D and L-thyronines (IV*c*-D and L) was accomplished conveniently in good yield by adding trifluoroacetic acid and anhydride to I. The methyl ester methyl ether VI*c* was obtained with difficulty by treating IV*c* with dimethyl sulfate. This conversion required two treatments with dimethyl sulfate since the first treatment gave mainly the impure methyl ester V*c*. Further treatment of impure V*c* with dimethyl sulfate gave VI*c*.

Pure V*c* was prepared by stirring IV*c* with methanolic hydrogen chloride or by adding trifluoroacetic anhydride to II*a* hydrochloride. Hydrolysis of analytically pure samples of VI*c*-D and L with 10% sodium hydroxide yielded impure VII-D and L. These samples of VII were grayish-purple in color, had lower melting points, lower optical rotations, and poorer paper chromatograms than VII made using VI*b*. Re-examination of VI*c*-D and L by paper chromatography showed these materials to contain significant amounts of unetherified material. Since the intermediates IV*c* and V*c* can withstand only limited exposure to base without loss of the *N*-trifluoroacetyl group it was apparent from these results that the etherification did not go to completion under the conditions of these experiments.

A comparison of the over-all percentage yields of VII and the purity of the VII from the three routes showed that the pathway using IV*b* was best—45% *versus* 15% *via* IV*a* *versus* 30% *via* IV*c*. Thus, the best route described here compares quite favorably with that described by Tomita (2), particularly if it is felt that the preparation and use of diazomethane is a problem.

To determine whether over-all retention of configuration occurred in these syntheses, VII-L (prepared *via* IV*b*) was hydrolyzed with a mixture of hydriodic and acetic acids to give I-L with $[\alpha]_D^{25} = +25.5°$, c = 1 in ethanol − 1 *N* hydrochloric acid (3:1 by volume). The starting material had $[\alpha]_D^{25} = +22.6°$, c = 2 in the above solvent system at a dilution of 4:1.

The ethyl ester of VII-L (VIII-L) was prepared simply by stirring VII-L with ethanolic hydrogen chloride.

Table I lists the compounds prepared, appropriate physical constants, and analytical data.

EXPERIMENTAL[2]

3,3',5-Triiodo-D and L-Thyronine Methyl Esters (IIa-D and L).—II*a*-D and L hydrochlorides were prepared according to the method of Ashley and Harington (5). The hydrochlorides were dissolved in aqueous methanol and neutralized with 5% sodium carbonate. The precipitated II*a* was cooled, filtered, washed with water, and recrystallized from tetrahydrofuran-water to give 80–85% of product, m.p. 195–197°.[3]

3,3',5-Triiodo-D and L-Triiodothyronine Ethyl Esters (IIb-D and L).—These esters were prepared in 45–55% yield using the method employed for II*a* with ethanol instead of methanol.

N,O-Dibenzoyl-3,3',5-Triiodo-D and L-Thyronine Methyl and Ethyl Esters (IIIa-D and L and IIIb-D and L).—A solution of 2.5 mmoles of II*a* or II*b* in 50 ml. of tetrahydrofuran was treated with 12 ml. of 5% sodium bicarbonate, 1 Gm. of solid sodium bicarbonate, and 1 ml. of benzoyl chloride, and stirred at room temperature for 2 hours. The mixture was poured into 3–4 vol. of ice-water. The resulting gum was dissolved in chloroform, and the chloroform solution was washed three times with 10% sodium hydroxide, three times with water, and dried over sodium sulfate. The dried solution was concentrated, and the syrupy residue was triturated with aqueous methanol or petroleum ether to produce impure solid II*a* or *b*. After recrystallization, the yields were 45–65%.

Acidification of the aqueous alkaline washes from above gave a small amount of impure IV*a*.

N-Benzoyl-3,3',5-triiodo-D and L-Thyronines (IVa-D and L).—A solution of 1 mmole of III*a* or *b* in 25 ml. of methanol and 5 ml. of 40% sodium hydroxide was stirred 1 hour at room temperature, diluted with water, cooled, and acidified. The precipitate was filtered, washed with water and ether, and recrystallized. Yield 75–90%.

N-Benzoyl-3,3',5-triiodo-D and L-Thyronine Methyl Esters (Va-D and L).—These were prepared in 55–80% yield using the method described for V*b*.

N-Benzoyl-3-[3,5-diiodo-4-(3-iodo-4-methoxyphenoxy)phenyl]-D and L-Alanine Methyl Esters (VIa-D and L).—These compounds could be prepared from either IV*a* or V*a* under similar conditions. However, the product from V*a* was purer and more easily purified.

To a solution of 1 mmole of V*a* in 25 ml. of 5% sodium carbonate and 25 ml. of tetrahydrofuran was added 1.3 ml. of dimethyl sulfate. The mixture was stirred at room temperature for 2.5 hours. The pH of the mixture was checked periodically to insure the basicity of the reaction mixture. More base was added if needed. The mixture was diluted with cold water to precipitate a gum. The gum was dissolved in chloroform; the chloroform was washed once with base and several times with water. The chloroform was dried over sodium sulfate and evaporated to produce an oil. Trituration of the oil with petroleum ether gave a solid which was filtered, washed with petroleum ether, and recrystallized. Yield, 55–70%.

Acetylation of 3,3',5-Triiodo-D and L-Thyronines (IIIc?).—To a cooled stirred solution of 3.25 Gm. (5 mmoles) of I in 100 ml. of 2 *N* sodium hydroxide was added slowly 1.5 ml. of acetic anhydride. Stirring was continued for 2 hours at room temperature when 50 ml. of ethanol was added. The solution was diluted with water, cooled, and acidified with dilute hydrochloric acid. The white precipitate was filtered, washed with water, and dried to give 3.4 Gm. of material with an indefinite melting point.

[2] All melting points were taken in a Thomas-Hoover capillary melting point apparatus and are corrected. Microanalyses were performed by Mrs. Doris Rolston and coworkers, and optical rotations were determined by Mr. J. Walter Hamill and staff, Analytical and Physical Chemistry Section, Smith Kline and French Laboratories, Philadelphia, Pa.

[3] The DL-isomer has been reported to melt at 178.5–179° dec. (10) and the L-isomer at 174–181° dec. (2).

Recrystallization of 2 Gm. of this material from boiling toluene gave 1.2 Gm. of a product, melting about 140° after washing with petroleum ether. A small sample (300 mg.) was then suspended in 40 ml. of ether and stirred for 30 minutes. Most of the solid dissolved. The insoluble portion was filtered, and the filtrate was added to 100 ml. of petroleum ether to precipitate 200 mg. of solid, m.p. 189°. This material showed one spot on thin-layer (silica gel plates developed with methanol) and paper chromatograms (ethanol–water–mineral oil, 2:1:10). After drying at 80° *in vacuo*, the material melted at 179–181° and gave unsatisfactory elemental analyses.

N-Acetyl-3,3′,5-Triiodo-D and L-Thyronines (IVb-D and L).—*Preparation A.*—A solution of 3.4 Gm. of crude acetylation product in 100 ml. of ethanol and 20 ml. of 40% sodium hydroxide was stirred for 1 hour at room temperature. The solution was diluted with water and acidified with dilute hydrochloric acid. The resulting solid was filtered, washed with water, and dried to give 3 Gm. of crude product.

Preparation B.—A solution of 2.5 mmoles of V*b* in 50 ml. of ethanol and 10 ml. of 40% sodium hydroxide was stirred 1 hour at room temperature, and the product was isolated as described under *Preparation A*. Yield, 84–98%.

N-Acetyl-3,3′,5-triiodo-D and L-Thyronine Ethyl Esters (Vb-D and L).—*Preparation A.*—A suspension of 4.2 Gm. of crude acetylation material in 50 ml. of absolute ethanol was cooled and saturated with dry hydrogen chloride. The resulting solution was evaporated, and the solid residue was evaporated twice again with fresh portions of ethanol. The residue was triturated with water, filtered, washed with water, and recrystallized.

Preparation B.—A suspension of 0.8 mmole of IV*b* in 35 ml. of absolute ethanol was saturated with dry hydrogen chloride and was isolated and purified as indicated in *Preparation A*. Yield, 67–93%.

N - Acetyl - 3 - [3,5 - diiodo - 4 - (3 - iodo - 4 - methoxyphenoxy)phenyl]-D and L-Alanine Ethyl Esters (VIb-D and L).—A solution of 4.2 mmoles of V*b* in 50 ml. of tetrahydrofuran and 50 ml. of 5% sodium carbonate was treated with 3.5 ml. of dimethyl sulfate. The reaction was performed, and the crude product was isolated as described in the preparation of VI*a*. The crude gummy product was solidified by stirring, cooling, and scratching with water. Yield, 65–70%.

On some occasions the gum was dissolved in ethyl acetate, and the organic solution was washed with water, dried (Na_2SO_4), and distilled. The residue was triturated with petroleum ether to produce solid.

N-Trifluoroacetyl-3,3′,5-triiodo-D and L-Thyronines (IVc-D and L).—To a cooled stirred suspension of 5 mmoles of I in 45 ml. of ethyl acetate was added 9 ml. of trifluoroacetic acid and 1.5 ml. of trifluoroacetic anhydride. Stirring and cooling were continued for 1 hour. The solution was washed with water, once with 5% sodium bicarbonate, and twice with saturated saline solution. The combined aqueous phases were extracted twice with ethyl acetate. The combined ethyl acetate layers were washed once with water and dried over sodium sulfate. Removal of the ethyl acetate left a solid which was dissolved in ether and reprecipitated with petroleum ether. This solid melted at 180–185° and represented a 91% yield of product.

N-Trifluoroacetyl-3,3′,5-triiodo-D and L-Thyronine Methyl Esters (Vc-D and L).—*Preparation A.*—The esters were prepared in 60–65% yield using IV*c* and methanolic hydrogen chloride in a manner analogous to that described for the preparation of V*b*.

Preparation B.—To a suspension of 1 mmole of II*a* hydrochloride in 5 ml. of ethyl acetate and 5 ml. of chloroform was added with stirring 0.5 ml. of trifluoroacetic anhydride. Stirring was continued for an additional 10 minutes, and the resulting clear solution was diluted with an equal volume of ethyl acetate. The solution was washed once with 5% sodium bicarbonate and twice with water. The combined aqueous phases were washed twice with ethyl acetate, and the combined organic phases were dried (Na_2SO_4). The solid residue obtained upon removal of the solvents melted at 147° after recrystallization.[4] Yield, 600 mg. (79%).

N - Trifluoroacetyl - 3 - [3,5 - diiodo - 4 - (3-iodo-4-methoxyphenoxy)phenyl]-D and L-Alanine Methyl Esters (VIc-D and L).—To a stirred suspension of 2.5 mmoles of IV*c* and 3 Gm. of sodium carbonate in 50 ml. of tetrahydrofuran was added 1.5 ml. of dimethyl sulfate. The product was isolated as were VI*a* and *b*. In this way an impure product was obtained which appeared to be predominantly V*c*.

Further treatment of this material in tetrahydrofuran with 1 ml. of dimethyl sulfate and 2 Gm. of sodium carbonate in 5 ml. of water produced the desired methyl ester methyl ether in 40–45% yield.

Treatment of V*c* with dimethyl sulfate under these conditions gave only unreacted starting material.

3 - [3,5 - Diiodo - 4 - (3 - iodo - 4 - methoxyphenoxy)phenyl]-D and L-Alanines (VII-D and L).—*Preparation A.*—A mixture of 1 mmole of VI*a* or *b* in 35 ml. of acetic acid and 35 ml. of 6 *N* sulfuric acid was refluxed for 18–24 hours. The resulting solution was diluted with water and cooled to give a precipitate of what was probably the sulfate of VII. This was not isolated; the suspension was adjusted to pH 5–6 with 10% sodium hydroxide. This precipitate was filtered, washed with water, and purified by several isoelectric precipitations from hot ethanolic hydrochloric acid by the addition of hot water and hot 2 *N* sodium acetate. Yield, 75–90%.

Preparation B.—A solution of 500 mg. (0.645 mmole) of VI*c* in 25 ml. of methanol and 2.5 ml. of 10% sodium hydroxide was allowed to stand at room temperature overnight. The solution was diluted with water, cooled, and adjusted to pH 4–5 with acetic acid. The product was isolated and purified as above. Yield, 82%.

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[4] The hydrate of the DL-isomer of V*c* is reported to melt at 173–174.5° dec. 10).

8-Alkoxyquinolinium and 8-Alkoxy-*N*-alkylquinolinium Salts and Their Antibacterial Activity

By WILLIAM O. FOYE and JAMES R. MARSHALL*

A series of 8-alkoxyquinolinium hydrobromides and 8-alkoxy-*N*-alkylquinolinium quaternary iodides has been synthesized. Quaternization of the 8-alkoxy ethers was difficult or impossible with alkyl groups larger than ethyl. The quaternary salts showed characteristic antibacterial activities lower than that from 8-hydroxyquinoline itself. The lower ethers had antibacterial activities which showed more variation with liposolubility and may be attributed to metal ion complexation.

ALBERT'S PROOF that 8-hydroxyquinoline (oxine) exerts its antibacterial activity as a result of its metal-binding ability (1) may now be considered as classical, although several investigators have disagreed, at least in part, with this postulation (2*a*,*b*). One of the more convincing bits of evidence advanced by Albert in favor of a metal-binding mechanism was the lack of antibacterial activity of oxine derivatives unable to chelate metal ions, notably the *O*-methyl and the *N*-methyl derivatives. Other reports from the literature, however, state that 8-alkoxy derivatives and *N*-alkyl quaternary salts of oxine do possess antibacterial activity (3). To resolve this apparent discrepancy, a series of 8-alkoxy ethers and *N*-alkyl quaternary derivatives of oxine covering a range of liposolubilities has been synthesized and tested for activity against several microorganisms. It was believed that any activity found should vary widely with liposolubility if the derivatives were acting as metal-binding agents, as already shown by Albert (4) for oxine derivatives but would show only small changes with varying liposolubilities if the derivatives were behaving as quaternaries.

The formation of an 8-alkoxyquinoline was first reported in 1881 by Bedall and Fischer (5) by reaction of oxine with methyl iodide in alkaline solution. Other short chain 8-alkoxyquinolines have since been prepared by this procedure or by the Skraup synthesis (6), although Kuhn and Westphal (7) have reported 8-dodecoxyquinoline and its methyl methosulfate. The first reported quaternization of oxine was by Claus and Howitz (8), who prepared the *N*-methyl and *N*-methyl-8-methoxy iodides in a sealed tube at 100°. Vis (9) also reported the 8-ethoxy ether and its ethiodide at the same time. Dialkyl sulfates have also been used to prepare both the ether quaternary salts and the 8-alkoxy ethers (10). The quaternary derivatives of oxine reported have generally been the methyl, ethyl, or allyl, although Ioffe and Selezneva (11) recently reported the octyl and hexadecyl iodides of oxine from reaction in sealed tubes at 110°. However, the latter derivative was impure.

DISCUSSION

The 8-alkoxyquinolines reported here were prepared by refluxing the sodium salt of oxine and the appropriate alkyl halide, generally the iodide, in absolute ethanol. Exclusion of water from the reaction was necessary. After neutralization and distillation, the resulting oils were converted to stable hydrobromides. Crystallization of the hydrobromides was accomplished by a variety of methods, including repeated crystallization attempts from anhydrous ether or other anhydrous solvents, removal of moisture by azeotropic distillation, or freezing. Recrystallization could then be carried out in absolute ethanol.

Quaternization of oxine with alkyl iodides was attempted under pressure at 100°. The methyl and ethyl derivatives were obtained in quantitative yield, but the reaction failed with longer chain iodides. Albert (12) has claimed that quaternization of pyridine and quinoline occurs readily with methyl, allyl, or benzyl iodides but more reluctantly with ethyl or higher iodides. He stated further that difficult quaternizations may sometimes take place in refluxing nitrobenzene. When this solvent was utilized in the reaction with butyl iodide, a solid product was obtained which gradually decomposed over a 10-day period. The solid was found to be the hydroiodide of oxine by comparing the melting point and R_f value to a freshly prepared sample. Reaction of oxine and butyl iodide in a Parr bomb at temperatures up to 250° produced 8-butoxyquinoline hydroiodide which was characterized by conversion to the hydrobromide.

Further identification of the two possible derivatives, the ether and the quaternary salt, was made by treatment of each with silver oxide. The ethers were converted to the oils already identified, whereas the suspected quaternaries gave solid hygroscopic hydroxides. In addition, infrared absorption spectra in KBr wafer gave clear evidence of both *N*- and *O*-alkylation. A diffuse H-bonding absorption

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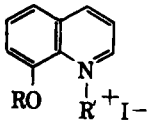
TABLE I.—OXINE ETHERS AND ETHER QUATERNARIES

A. 8-Alkoxyquinoline Hydrobromides



No.	R	M.p., °C.	Purification Procedure[a]	Yield, %	Formula	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found
1	CH_3	183(s)[b]	A	40	$C_{10}H_{10}BrNO$	50.02	49.52	4.20	4.74
2	C_2H_5	199(s)	A	48	$C_{11}H_{12}BrNO$	51.99	51.99	4.76	4.90
3	n-C_3H_7	200–202	A	54	$C_{12}H_{14}BrNO$	53.74	53.82	5.26	5.63
4	n-C_4H_9	178–180	B	60	$C_{13}H_{16}BrNO$	55.33	54.56	5.72	5.84
5	n-C_6H_{13}	137–139	B	75	$C_{15}H_{20}BrNO$	58.07	58.05	6.50	6.81
6	n-C_8H_{17}	105–107	B	71	$C_{17}H_{24}BrNO$	60.37	60.09	7.15	7.08
7	n-$C_{10}H_{21}$	70–72	C	68	$C_{19}H_{28}BrNO$	62.29	61.88	7.70	7.88

B. 8-Hydroxy- and 8-Alkoxy-N-alkylquinolinium Iodides



No.	R	R′	M.p., °C.	Yield, %	Formula	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found
8	H	CH_3	182–184[c]	100	$C_{10}H_{10}INO$	41.83	41.86	3.51	4.22
9	H	C_2H_5	134–136[d]	100	$C_{11}H_{12}INO$	43.88	44.22	4.03	4.23
10	CH_3	CH_3	166(s)[e]	100	$C_{11}H_{12}INO$	43.88	43.21	4.03	4.69
11	C_2H_5	C_2H_5	168–170[f]	100	$C_{13}H_{16}INO$	47.43	46.48	4.90	4.87
12	n-C_3H_7	n-C_3H_7	133–135	25	$C_{15}H_{20}INO$	50.43	51.55	5.64	5.61
13	n-C_4H_9	CH_3	165(s)	100	$C_{14}H_{18}INO$	48.99	47.94	5.29	5.01
14	n-C_6H_{13}	CH_3	137–139	100	$C_{16}H_{22}INO$	51.76	52.02	5.97	6.06
15	n-C_8H_{17}	CH_3	117–119	100	$C_{18}H_{26}INO$	54.16	53.36	6.56	6.38
16	n-$C_{10}H_{21}$	CH_3	119–121	100	$C_{20}H_{30}INO$	56.20	56.11	7.08	7.04

[a] A, repeated washing with hot anhydrous ether and cooling to 5°. B, repeated washing with hot ethyl acetate and cooling to 5°. C, azeotropic distillation with toluene and cooling to 5°. [b] s = sublimed. [c] A hydrate of this compound has been reported (8), m.p. 143° dec. [d] Reported (11) m.p. 129–132°. [e] Reported (8) m.p. 160° dec. [f] Reported (9) m.p. 168–169°

band at 3000 cm.$^{-1}$ was observed in both oxine hydrobromide and the *N*-methiodide of oxine but was absent in the methyl ether of oxine. Absorption due to N—CH_3 was found at 1350 cm.$^{-1}$ in the methiodide but was missing in both oxine hydrobromide and the methyl ether.

Since the longer chain quaternary salts were difficult or impossible to obtain in good yield or purity, a series of ether quaternaries of varying liposolubility was prepared. The length of the ether alkyl varied from methyl to decyl, while the quaternary alkyl was methyl in all cases. These compounds were easily obtained from the ethers described above. Attempts to obtain ether quaternaries with longer chain alkyls on the quaternary nitrogen gave the methyl methoxy iodide and the ethyl ethoxy iodide in quantitative yield but the propyl propoxy iodide in only 25% yield. Higher homologs of this type could not be obtained, probably because of steric hindrance. Construction of the Stuart-Briegleb molecular model of the 8-propoxy-*N*-propyl derivative showed that quaternization of an oxine ether with an alkyl chain longer than two carbon atoms was attended with considerable steric strain. The various ethers, quaternary salts, and ether quaternaries prepared are described in Table I.

Antimicrobial Activity—Generally, nonchelateable derivatives of oxine of the ether or quaternary type have shown antibacterial activity of a lower degree than oxine itself (3). A series of methyl methosulfates of 8-alkanoyl esters of oxine reported by Vogt (3*d*) particularly did not show a high degree of antibacterial activity *versus E. coli* or staphylococci. Dorier *et al.* (13) have stated that *N*-methyl-8-hydroxyquinolinium sulfate possessed no antibacterial activity, and Bahal *et al.* (3*e*) reported that the methyl ether of oxine was inactive.

The serial dilution procedure of McKee (14) was followed in determining the minimum inhibitory dilutions of the oxine derivatives prepared using five common microorganisms. These included Gram-positive cocci, a Gram-positive rod, Gram-variable rod, and Gram-negative rod. As shown in Table II, the oxine derivatives were less effective than oxine itself. The best activity among the ethers was shown by the ethyl derivative, and the best activity among the quaternaries was shown by the *N*-methyl-8-octoxy derivative. Increase in alkyl chain length, either on the oxygen or nitrogen, generally appeared to lower the activity, except for the hexyl and octyl ethers and the *N*-methyl 8-octoxy quaternary salt. A greater variation in activity with chain length is evident among the ethers, compared to the quaternary salts, and may be expected where a metal-binding mechanism of antibacterial activity is involved.

The activity of the quaternary salts may be due to the cationic group, known to be antibacterial in a variety of compounds. The activity of the ether derivatives cannot immediately be explained as

TABLE II.—ANTIBACTERIAL ACTIVITIES OF THE OXINE DERIVATIVES

Compd.	Min. Inhibitory Concn., 1/M: *S. faecalis*	*B. subtilis*	*B. stearothermophile*	*B. circulans*	*S. aureus*
Oxine·HBr	42,500	42,500	42,500	42,500	42,500
1	2,125	2,125	1,063	1,063	1,063
2	8,500	8,500	4,250	4,250	2,833
3	850	850	850	850	850
4	850	850	850	850	850
5	2,833	2,125	2,125	2,125	1,063
6	2,833	2,833	4,250	2,833	1,214
7	850	850	850	850	850
8	850	850	850	850	850
9	850	850	850	850	850
10	850	850	850	850	850
11	1,417	1,417	1,417	1,417	1,063
12	850	850	850	850	850
13	1,063	1,063	1,063	1,063	1,063
14	1,063	1,063	1,063	1,063	1,063
15	8,500	8,500	8,500	8,500	8,500
16	850	850	850	850	850

due to either metal ion chelation or cationic activity. Pershchin and Vichkanova (*3b*) claimed that their higher 8-alkoxyquinolines possessed antibacterial activity because of the membrane-disrupting effect of substances with long aliphatic chains. However, the methoxy and ethoxy ethers hardly qualify for this type of action, and a more likely explanation may be derived from a statement of Thilo and Demant (15). They claimed that residual valences were present in metal-oxine chelates, as shown by their ability to add hydrogen chloride. A corollary to this postulation is that the oxine ethers may undergo weak metal binding analogous to oxonium salt formation.

Accordingly, the ethers were tested with ferric chloride test solution, and a weak positive test for complexation was given in each case. In addition, 1% ferric chloride solution was added to 0.1 *N* sodium hydroxide solution saturated with 8-ethoxyquinoline and compared to its reaction with 0.1 *N* sodium hydroxide solution alone. The latter solution gave a copious precipitate of ferric hydroxide, whereas the former produced a dark, blue-green solution. Acidification discharged the color. This evidence may indicate that the lower oxine ethers may still undergo metal binding or complexation of a weak nature, and their antibacterial activity may be associated with this ability.

The conclusion is that the antibacterial activities exerted by the oxine ether quaternaries are due to the positively charged nitrogen rather than to a metal-binding effect. However, the latter mechanism is a possibility with the oxine ethers because of their demonstrated ability to prevent precipitation of ferric hydroxide from solutions of ferric ion and their variation in antibacterial activity with variation in alkyl chain length.

EXPERIMENTAL

Melting points were taken on a Fisher-Johns or Mel-Temp block and are uncorrected. Carbon-hydrogen analyses were done by Weiler and Strauss, Oxford, England.

Sodium 8-Quinolate.—One mole (145.15 Gm.) of 8-hydroxyquinoline was dissolved in 500 ml. of acetone. While the solution was stirred, a hot solution of 40.01 Gm. (1.0 mole) of sodium hydroxide in 50 ml. of water was added slowly. Bright yellow crystals of sodium 8-quinolate precipitated immediately and were filtered, washed with acetone, and recrystallized from hot water. A yield of 160.2 Gm. (90%) was obtained, m.p. above 300°.

8-Alkoxyquinoline Hydrobromides.—The following procedure is representative. A solution of sodium 8-quinolate (50.1 Gm., 0.3 mole), absolute ethanol (200 ml.), and iodomethane (42.6 Gm., 0.3 mole) was stirred and refluxed for 6 hours. The alcohol was then removed by distillation through a short Vigreux column. The oily residue was treated with 20 ml. of 10% sodium hydroxide solution, and the resultant two-phase system was extracted with diethyl ether for 6 hours using a liquid-liquid continuous extractor. The ether extract was distilled *in vacuo* to remove the solvent, and the remaining viscous yellow oil was distilled *in vacuo* with an acetone–dry ice bath surrounding the receiver. The clear yellow oil was redissolved in diethyl ether and dried over anhydrous sodium sulfate for 24 hours. Anhydrous hydrogen bromide was passed into the filtered solution cooled in an ice-salt bath, and an ether-insoluble oil was isolated. The oil was crystallized by three washings with anhydrous diethyl ether and cooling to 5° and recrystallized from absolute ethanol. The yield of yellow-green solid was 28.8 Gm. (40%), m.p. 183° with sublimation.

Anal.—Calcd. for $C_{10}H_{10}BrNO$: C, 50.02; H, 4.20. Found: C, 49.52; H, 4.74.

8-Hydroxy- and 8-Alkoxy-N-alkylquinolinium Iodides.—The following procedure is representative. 8-Hydroxyquinoline (14.5 Gm., 0.1 mole) and 21.3 Gm. (0.15 mole) of iodomethane were placed in a Parr bomb, capped and heated in a water bath at 100° for 1 hour. After the contents were cooled, precipitation of golden crystals occurred and was increased by the addition of 100 ml. of diethyl ether. The crystals were isolated, extracted with diethyl ether in a Soxhlet extractor for 2 hours, and recrystallized from hot acetone-ethanol solution. A quantitative yield (28.5 Gm.) of product was obtained, m.p. 182–184°.

Anal.—Calcd. for $C_{10}H_{10}INO$: C, 41.83; H, 3.51. Found: C, 41.86; H, 4.22.

Antimicrobial Testing

Each compound was placed in a separate sterile 100-ml. volumetric flask, and sterile nutrient broth

was added to the mark, giving $^1/_{850}$ M solutions. A 10.0-ml. portion of this solution was placed in each of five sterile test tubes inoculated with 0.5 ml. each of a 24-hour nutrient broth culture of the following organisms: *Streptococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus stearothermophile*, and *Bacillus circulans*. The seeded test tubes were incubated for 24 hours at 37°. If growth was observed at the initial concentration of compound, no dilutions were made. If growth was not observed at this concentration, additional dilutions were tested by taking appropriate quantities of the stock solution of inhibiting compounds and adding sterile broth in the required amount. The tubes were examined visually for growth. Minimum inhibitory concentrations, expressed as 1/molarity are recorded in Table II.

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Effect of Procainesterase Levels on Duration of Procaine Local Anesthesia

By H. B. DANIELL*, A. E. WADE, and F. FORD MILLIKAN

This study originated with the observation that rabbit serums consistently exhibited low levels of cholinesterase activity while having variable levels of procainesterase activity. This finding enabled the authors to determine the effects of procainesterase on the duration of procaine anesthesia using a single species. On conducting double blind duration studies, an inverse correlation between serum procainesterase activity and duration of conduction anesthesia existed which was highly significant ($p < .001$). Atropinesterase activity was present in the serums of those rabbits that contained intermediate or high procainesterase activity but was not evident in the serums of rabbits exhibiting low procainesterase levels. This, coupled with the fact that atropine inhibited procaine hydrolysis, suggested that in rabbit serum procaine and atropine are hydrolyzed by the same enzyme.

WHILE THE LIVER is the chief site of enzymatic detoxication of drugs, other tissues in the body are capable of drug inactivation. Human blood serum contains an enzyme capable of hydrolyzing acetylcholine and certain other choline esters (1). This enzyme is also responsible for the hydrolysis of the local anesthetic procaine (2) and other noncholine esters (3). Serums of other species appear to contain esterases that differ from those in human serum (4–8). Sawyer (9) reported the presence of an esterase in guinea pig and rabbit liver which hydrolyzes benzoylcholine but is not concerned with acetylcholine hydrolysis. A similar enzyme has been found in the plasma of rabbits (6).

The serum of certain rabbits exhibits atropinesterase activity, while that of others does not (10). This enzyme is also capable of deacetylating some derivatives of morphine (11) and hydrolyzing several alkyl and aryl esters (12). It appears to be different from cholinesterase (12, 13).

Kalow (2) suggested that an inverse correlation existed between the duration of action of certain local anesthetics and their speed of hydrolysis *in vitro* by human serum cholinesterase. It was the purpose of this investigation to determine the correlation between serum procainesterase activity *in vitro* and duration of procaine local anesthesia *in vivo* and to determine if any relationship exists between the activities of procainesterase, acetylcholinesterase, and atropinesterase in the serum of rabbits.

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was added to the mark, giving $^1/_{850}$ M solutions. A 10.0-ml. portion of this solution was placed in each of five sterile test tubes inoculated with 0.5 ml. each of a 24-hour nutrient broth culture of the following organisms: *Streptococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus stearothermophile*, and *Bacillus circulans*. The seeded test tubes were incubated for 24 hours at 37°. If growth was observed at the initial concentration of compound, no dilutions were made. If growth was not observed at this concentration, additional dilutions were tested by taking appropriate quantities of the stock solution of inhibiting compounds and adding sterile broth in the required amount. The tubes were examined visually for growth. Minimum inhibitory concentrations, expressed as 1/molarity are recorded in Table II.

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This study originated with the observation that rabbit serums consistently exhibited low levels of cholinesterase activity while having variable levels of procainesterase activity. This finding enabled the authors to determine the effects of procainesterase on the duration of procaine anesthesia using a single species. On conducting double blind duration studies, an inverse correlation between serum procainesterase activity and duration of conduction anesthesia existed which was highly significant ($p < .001$). Atropinesterase activity was present in the serums of those rabbits that contained intermediate or high procainesterase activity but was not evident in the serums of rabbits exhibiting low procainesterase levels. This, coupled with the fact that atropine inhibited procaine hydrolysis, suggested that in rabbit serum procaine and atropine are hydrolyzed by the same enzyme.

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EXPERIMENTAL

Serum Cholinesterase Activity.—Serum cholinesterase activity at 25±1° was determined by the procedure of Michel (14) using a Beckman model G pH meter. The acetylcholine chloride substrate (0.165 *M*) was prepared daily by dissolving 100 mg. acetylcholine chloride (Merck & Co.) in 3.33 ml. of distilled water. The cholinesterase activity was expressed as corrected pH change per hour.

Blood was taken by heart puncture from guinea pigs (mixed breed) and rabbits (New Zealand) and from the cephalic vein in humans (Caucasian). The serums were removed from the clot, clarified by centrifugation at room temperature, and stored at −20°. Before use, the serums were diluted with distilled water so that each milliliter of the solution contained 0.02 ml. of serum.

Serum Procainesterase Activity.—The enzymatic hydrolysis of procaine was conducted in 1-cm. quartz cells at 25±1° by the method of Kalow (2). Two milliliters each of a 1:4 dilution of rabbit serum and 1×10^{-4} *M* procaine solution were mixed and added to the cell. The absorbance at 313 mμ was determined within 45 seconds of mixing the two solutions and at 5-minute intervals thereafter for 1 hour using the Beckman model DU spectrophotometer. The *M*/15 phosphate buffer of Kalow and Lindsay (15) was used as the diluent and the blank. Procaine hydrolysis at timed intervals was calculated from the decrease in absorbance and reported as micromoles of procaine hydrolyzed per hour per milliliter of serum.

Duration of Procaine Local Anesthesia.—The duration of procaine local anesthesia in the rabbit and guinea pig was determined by a modification of the intracutaneous wheal method of McIntyre and Sievers (16) and by other investigators (17–19). The animal's hair was clipped from the lumbar region of the back, and the sensitivity of the area was tested by six jabs with a dissecting needle at intervals of not less than 3 seconds. In all animals used, the six jabs elicited six flinches of the skin prior to drug administration. One-half milliliter of a 1.0% solution of procaine hydrochloride in isotonic sodium chloride solution was injected intracutaneously in the area predetermined to be sensitive, and the slight elevation in the skin produced by the injected solution was marked off. The duration of anesthesia was measured by testing the marked area in the manner described at 5-minute intervals until the end point was reached. The end point chosen for the duration of anesthesia was the reappearance of response to at least four of the six jabs in two successive trials. Since this procedure involved some subjective judgment on the part of the researcher, it was conducted "double blind."

Atropinesterase Activity.—Ing *et al.* (20) described a procedure for determining the presence of atropine involving the intraperitoneal injection of atropine into albino mice with the subsequent observation of the mydriatic effect. A modification of this method was used in this study to determine the presence of atropinesterase activity of serum.

Groups of five male albino mice (BALB/c strain) were used. A solution of 250 mcg. atropine sulfate in 5.0 ml. of rabbit serum was incubated in a water bath for 1 hour at 37°. One-half milliliter of this incubated solution (equivalent to 25 mcg. atropine sulfate) was injected intraperitoneally into each of five mice, and 0.25 ml. of the same solution was injected into five additional mice. This procedure was repeated using rabbit serums of different procainesterase activity. In addition, two groups of mice received intraperitoneal injections of 25 and 12.5 mcg. of atropine sulfate in normal saline solution, respectively. The diameter of the pupil of each mouse was measured prior to and 15 minutes after injection.

Inhibition of Procaine Hydrolysis by Atropine.—Rabbit serums with high procainesterase activity were used in this study. The effect of atropine sulfate on the rate of procaine hydrolysis by this serum was investigated by comparing the absorbances at 313 mμ of solutions of procaine in serum with those of a mixture of procaine and atropine in serum.

In assessing the rate of uninhibited procaine hydrolysis by the serum, an 8.4×10^{-5} *M* concentration of procaine hydrochloride was prepared. One milliliter of this solution diluted with 1.0 ml. of buffer was mixed with 2.0 ml. of a 1:4 dilution of rabbit serum. The rate of hydrolysis was measured as described previously. The rate of hydrolysis of the same dilution of procaine hydrochloride in the presence of atropine sulfate was measured by mixing 1.0 ml. of 8.4×10^{-5} *M* procaine hydrochloride with 1.0 ml. of 1×10^{-4} *M* atropine sulfate solution and allowing the mixture to equilibrate for 5 minutes. This mixture was then added to 2.0 ml. of 1:4 rabbit serum, and the procaine hydrolysis observed. This procedure was repeated using 1.0 ml. of 1×10^{-2} *M* atropine sulfate. The percentage of inhibition of procaine hydrolysis by atropine was calculated by use of the formula provided by Varley (21).

$$\%\ \text{Inhibition of Procaine} = 1 - \frac{\text{Change in O.D./hr. with Atropine}}{\text{Change in O.D./hr. without Atropine}} \times 100$$

Statistical Analyses.—All analyses were conducted by the University of Georgia Bureau of Statistics on standard I.B.M. electronic data processing equipment using standard statistical formulas. Levels of significance, where applicable, were determined using $n-2$ degrees of freedom (22).

Table I.—Serum Cholinesterase Activity

Species	Subjects Tested, No.	ChoE Activity[a] ± S. D.
Human	5	0.966 ± .12
Guinea pig	10	0.635 ± .10
Rabbit	22	0.256 ± .05

[a] Expressed as corrected pH change/hour.

RESULTS

Cholinesterase Activity.—The cholinesterase activity of human, guinea pig, and rabbit serums was relatively constant within each species tested, with human serum having the greatest activity and rabbit serum the least (Table I). This agrees with earlier findings of Mendel *et al.* (23). The human serum cholinesterase activity reported here falls within normal established limits (24).

Procaine Hydrolysis.—The ultraviolet absorption spectra of a 5×10^{-5} *M* solution of procaine hydro-

chloride in phosphate buffer and of a 5×10^{-5} M solution of p-aminobenzoic acid in buffer are shown in Fig. 1. Since the absorbance of p-aminobenzoic acid was negligible at 313 mμ, this wavelength was chosen for enzymatic hydrolysis studies. Although a greater difference in the absorbance of the substrate and its metabolized product exists at wavelength 300, this point could not be used because of the absorbance of serum at this wavelength.

The relationship between absorbance at 313 mμ and molar concentration of procaine is shown in Fig. 2. Since Beer's law is obeyed, a factor was calculated which allowed the conversion of decrease in absorbance to micromoles of procaine hydrolyzed. At this wavelength a change in absorbance of 0.078 occurred for each change of 1×10^{-5} M concentration. Since each milliliter of a 1×10^{-5} M concentration contains .01 μm. of procaine, a drop in absorbance of 0.078 would be the equivalent of .01 μm. of procaine hydrolyzed per milliliter of the solution.

Typical hydrolyses of procaine at timed intervals by human and guinea pig serums are shown in Fig.

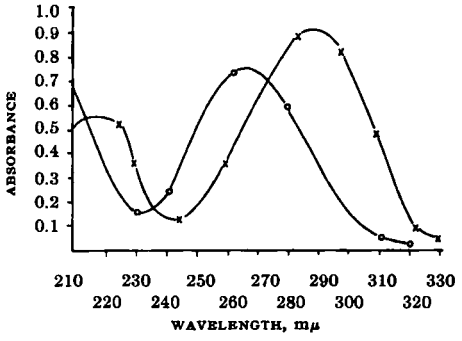


Fig. 1.—The ultraviolet absorption spectra of a 5×10^{-5} M solution of procaine hydrochloride in phosphate buffer (×) and a 5×10^{-5} M solution of p-aminobenzoic acid in buffer (○).

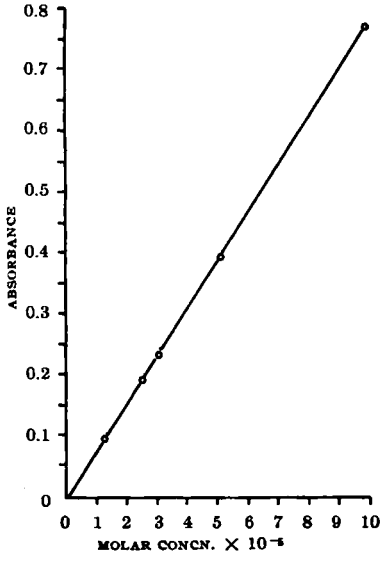


Fig. 2.—The relationship between absorbance at 313 mμ and molar concentration of procaine hydrochloride in phosphate buffer.

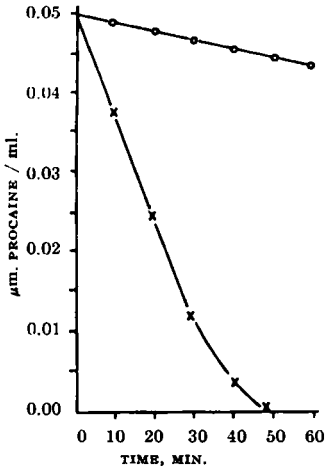


Fig. 3.—Typical hydrolyses of procaine at timed intervals by guinea pig serum (○) and human serum (×) as calculated from absorbance measurements at 313 mμ.

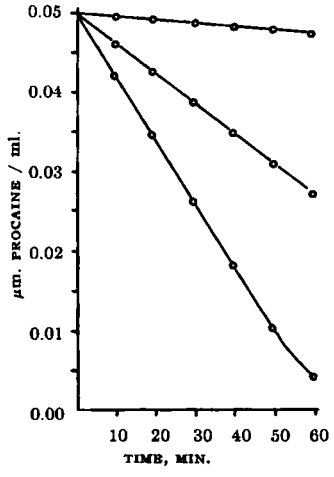


Fig. 4.—Typical hydrolyses of procaine at timed intervals by different rabbit serums as calculated from absorbance measurements at 313 mμ.

3. Human serum hydrolyzed procaine at a rate more rapid than that of either of the other two test species. In the presence of excess substrate, this hydrolysis was relatively constant until substrate concentrations became low, at which time the rate of hydrolysis decreased. Guinea pig serum, while fairly rich in cholinesterase activity, failed to hydrolyze procaine to an appreciable extent.

The amounts of procaine hydrolyzed by three different but typical rabbit serums is shown in Fig. 4; unlike the human and guinea pig serums tested, rabbit serums exhibit wide variations in procainesterase activity. The procainesterase activities of the serums from test rabbits were grouped at three levels of activity: low (0.0152 to 0.0592 μm./hour/ml. serum), intermediate (0.1232 to 0.1824 μm./hour/ml. serum), and high (0.248 to 0.368 μm./hour/ml. serum).

Duration of Procaine Local Anesthesia.—The duration of local anesthetic action due to the intracutaneous injection of 0.5 ml. of 1% procaine hydro-

TABLE II.—RELATIONSHIP BETWEEN CHOLINESTERASE AND PROCAINESTERASE ACTIVITY IN RABBIT SERUM TO DURATION OF ACTION OF PROCAINE *In Vivo*

Rabbit No.	ChoE Activity pH Change/Hr.	Procainesterase Activity Drop in O.D./Hr.	Procainesterase Activity μm. Hydrolyzed/Hr./ml. Serum	Duration of Anesthesia,[a] Min.
12	0.21	0.015	0.0152	95
20	0.23	0.016	0.0168	96
15	0.23	0.017	0.0176	120
18	0.31	0.020	0.0208	93
10	0.20	0.026	0.0264	120
2	0.26	0.027	0.0280	118
1	0.23	0.037	0.0376	100
6	0.35	0.040	0.0408	90
14	0.20	0.047	0.0480	115
16	0.23	0.050	0.0512	110
8	0.31	0.050	0.0512	80
7	0.16	0.057	0.0584	115
5	0.31	0.058	0.0592	126
17	0.21	0.120	0.1232	62
21	0.28	0.144	0.1480	62
3	0.24	0.175	0.1792	50
11	0.23	0.178	0.1824	60
19	0.24	0.242	0.2480	55
13	0.24	0.275	0.2824	86
22	0.26	0.334	0.3424	40
9	0.35	0.359	0.3680	45

[a] Due to intracutaneous injection of 0.5 ml. of 1% procaine hydrochloride in isotonic sodium chloride solution.

chloride in guinea pigs was 42.9±4.1 minutes. This was shorter than that observed in those rabbits having the highest procainesterase levels. Thus, it became apparent that the duration of local anesthesia in different species could not be explained on the basis of procainesterase levels in the serum.

Due to the wide variation of serum procainesterase activity between individual rabbits, this animal was used to compare procainesterase activity of serum *in vitro* to duration of procaine anesthesia *in vivo*. Table II illustrates this correlation. The correlation coefficient of procainesterase activity *versus* the reciprocal of duration of procaine local anesthesia was calculated to be 0.8539 ($p < .001$).

Atropinesterase Activity.—Ing *et al.* (20) reported that the extent of dilatation of the mouse pupil by atropine is directly correlated with the logarithm of the dose. In this study mice having pupil diameters of 0.43 mm. prior to atropine injection had pupil diameters of 1.1 and 1.9 mm. 15 minutes following 12.5 and 25 mcg. of atropine sulfate, respectively.

The comparison of procainesterase activity to atropinesterase activity of the individual rabbit serums is shown in Table III. Serum atropinesterase activity is indicated if atropine mydriasis is destroyed upon incubation of the drug with the serum. Each animal received an intraperitoneal injection of 0.5 ml. of rabbit serum that had been incubated for 1 hour with 25 mcg. of atropine sulfate. Rabbit serums having little procainesterase activity had little atropinesterase activity; however, rabbit serums having appreciable procainesterase activity were able to destroy 50 mcg. of atropine sulfate per milliliter of serum in 1 hour.

The inhibitory effects of atropine upon procaine hydrolysis by a rabbit serum are illustrated in Fig. 5. Approximately equal molar concentrations of the two drugs resulted in only a slight inhibition of procaine hydrolysis; but when 2.5×10^{-3} *M* (final dilution) atropine sulfate was incubated with 2.1×10^{-5} *M* (final dilution) procaine hydrochloride, procaine hydrolysis was inhibited 85.2% at the end of 1 hour.

TABLE III.—PROCAINESTERASE AND ATROPINESTERASE ACTIVITIES OF RABBIT SERUMS

Rabbit No.	Procainesterase Activity μm. Procaine Hydrolyzed/Hr./ml. Serum	Atropinesterase Activity[a] Mouse Pupil Size Before and After I. P. Injection Incubated Serum, mm. Before	After
20	0.0168	0.43	1.74
20	0.0168	0.43	2.17
20	0.0168	0.43	1.74
2	0.0280	0.43	1.96
2	0.0280	0.43	1.74
6	0.0408	0.43	1.74
6	0.0408	0.43	1.74
6	0.0408	0.43	1.74
3	0.1792	0.43	0.43
3	0.1792	0.43	0.43
3	0.1792	0.43	0.43
22	0.3424	0.43	0.43
22	0.3424	0.43	0.43
9	0.3680	0.43	0.43
9	0.3680	0.43	0.43

[a] Indicated by absence of atropine mydriasis after incubation of serum with 50 mcg. of atropine sulfate/milliliter for 1 hour.

DISCUSSION

The serum cholinesterase activity of humans, guinea pigs, and rabbits varies widely. As previously reported (23), human serum exhibits the greatest amount of cholinesterase activity, followed by guinea pig and then by rabbit serum. Optimum substrate concentrations exist for both pseudocholinesterase and true cholinesterase (23). The method employed for cholinesterase determinations in this study requires the use of high acetylcholine substrate concentrations. The inability of rabbit serum to hydrolyze acetylcholine to any appreciable extent at these high substrate concentrations lends support to the findings of Levine *et al.* (6) that acetylcholine hydrolysis by rabbit serum is largely a function of true cholinesterase. Similarly, the high activity of human serum toward acetylcholine in high substrate concentrations indicates that acetylcholine hydrolysis by human serum is a function of pseudocholinesterase.

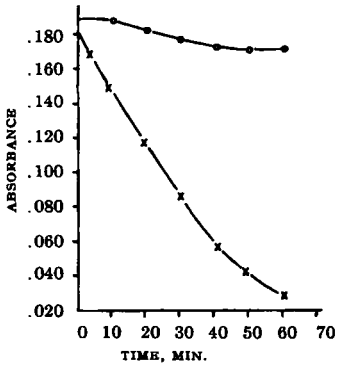


Fig. 5.—The hydrolysis of procaine by a rabbit serum when uninhibited (×) and when inhibited (○) by atropine.

Guinea pig serum, while exhibiting the characteristic action of pseudocholinesterase toward acetylcholine, failed to hydrolyze procaine at an appreciable rate. This finding indicates that acetylcholine hydrolysis is accomplished in the serum of guinea pigs in the same manner as it is in the liver, by true cholinesterase (9), or by a pseudocholinesterase which is distinct from the nonspecific enzyme found in human serum.

The human serum enzyme exerted the same high activity for the hydrolysis of procaine as toward the hydrolysis of acetylcholine. This was anticipated since it has been shown that in man both hydrolyses are accomplished by the same enzyme (2). This hydrolysis *in vitro* clearly shows that human serum cholinesterase has the ability to destroy considerable amounts of procaine when injected *in vivo*.

Rabbit serums varied widely in their ability to hydrolyze procaine *in vitro*. Three distinct levels of procainesterase activity were observed in the serums of individual rabbits. Procainesterase activity *in vitro* in the first group was essentially the same as that found in the serums of guinea pigs. This slow hydrolysis of procaine might be due to the action of some enzyme system not primarily concerned with substrates of the general molecular configuration of procaine. Procaine hydrolysis by the second and third groups is accomplished by an enzyme capable of actively hydrolyzing procaine, differing only in the level of enzymatic activity. No relationship was found between *in vitro* cholinesterase activity and *in vitro* procainesterase activity in rabbit serum; however, both are inhibited by diisopropyl phosphorofluoridate.

The duration of local anesthesia in guinea pigs was generally shorter than in rabbits. Since the serum of guinea pigs failed to hydrolyze procaine *in vitro*, it might be assumed that this short duration of action was due to a more rapid diffusion from the site of injection with or without subsequent destruction by the enzymes of some other organ of the body.

Many variables exist between different species of animals, such as rates of diffusion, skin sensitivity, and the presence of different enzymes in the serums. Therefore, attempts to correlate serum enzyme activity and duration of local anesthetic action between different species may easily lead to erroneous conclusions. Consequently, duration of anesthesia determinations *in vivo* were not conducted in humans. However, since rabbits have several procainesterase levels, they appeared to be the ideal test animal to study the relationship between quantity of serum procainesterase and duration of anesthetic action of procaine *in vivo*.

Studies in the rabbit imply that the duration of local anesthetic activity of procaine is indeed affected by the level of procainesterase activity in the serum. A correlation ($r = 0.8539$) existed between the procainesterase activity of serum and the reciprocal of anesthetic duration which was highly significant statistically ($p < 0.001$). Based on the results of these experiments in rabbits one may assume, therefore, that if an enzyme is found in the serum of a given species capable of destroying a given drug actively, the duration of action of that drug will be affected directly by the enzymatic level of the serum.

It was not within the scope of this investigation to quantitate the amount of atropinesterase activity but to determine if atropinesterase activity was present in the serum of those rabbits that had displayed procainesterase activity. Qualitative tests employing the mouse pupil as an indicator for the presence of atropine showed that atropinesterase activity occurred in rabbit serums that had procainesterase activity; conversely, this activity was absent in the serum of rabbits that did not hydrolyze procaine extensively.

Inhibition studies provided additional evidence that procaine and atropine are hydrolyzed by the same enzyme. Low substrate concentrations of atropine produced only a transient effect on procaine hydrolysis, while higher concentrations of atropine were able to inhibit hydrolysis of procaine by rabbit serum by as much as 85% for 1 hour.

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Solubilization of Weakly Acidic and Basic Drugs by Aqueous Solutions of Polysorbate 80

By E. G. RIPPIE*, D. J. LAMB, and P. W. ROMIG†

The relationship of apparent solubility of weakly acidic and basic drugs to pH and surfactant concentration has been established. Equations are presented which enable calculation of the total solubility of such drugs as represented by methylprednisolone hemiesters and procaine. The fraction of drug in the various solvated states can be computed readily.

NONIONIC SURFACTANTS have been studied by various workers (1–7) and found to solubilize effectively drugs which possess low water solubilities. In particular, Guttman *et al.* (8) and Hall (9) have presented data on the relationship of surfactant concentration to the degree of solubilization for anti inflammatory steroids and salicylic acid, respectively. Solubilization may take place by several mechanisms, *i.e.*, shallow or deep penetration of the micelles or simply by surface adsorption.

The present study was undertaken to define the relationship of apparent solubility of weakly acidic and basic drugs in aqueous systems to both pH and surfactant concentration. Accordingly, equations are presented which enable calculation of total solubility at any given pH and surfactant concentration and of the fraction of material present in the various solvated states. Three methylprednisolone-21-hemiesters and procaine have been investigated in aqueous solutions of polysorbate 80.

THEORY

Consider the case of a sparingly soluble drug possessing a single acidic functional group capable of salt formation. The total solubility of drug can be expressed as the sum of the various species present.

$$(D)_T = (D) + (D^-) + [D] + [D^-] \quad \text{(Eq. 1)}$$

where $(D)_T$ = total drug concentration, (D) = free acid not in micelle, (D^-) = ionized acid not in micelle, $[D]$ = free acid in micelle, $[D^-]$ = ionized acid in micelle, and concentration is expressed in terms of total volume.

If it is assumed that solubilization occurs by partitioning of the drug into micelles formed by the surfactant, partition coefficients can be defined for the free and ionized acid as $K' = \frac{[D]_0}{(D)_0}$ = partition coefficient of free acid into micelles and $K'' = \frac{[D^-]_0}{(D^-)_0}$ = partition coefficient of ionized acid into micelles. The subscript 0 denotes concentrations expressed in terms of the individual phase volumes rather than total volume. These constants may also be expressed in terms of total volume for convenience in computation and measurement.

$$K' = \frac{[D][1-(M)]}{(D)(M)} \qquad K'' = \frac{[D^-][1-(M)]}{(D^-)(M)}$$

Micelle concentration (M) is considered equal to volume fraction surfactant, neglecting that which is present in true solution. Where (M) is relatively small, the term $1-(M)$ can be neglected and the following equations used as a good approximation:

$$[D] = K'(D)(M) \quad \text{(Eq. 2)}$$

$$[D^-] = K''(D^-)(M) \quad \text{(Eq. 3)}$$

Substituting Eqs. 2 and 3 into Eq. 1

$$(D)_T = (D)[1 + K'(M)] + (D^-)[1 + K''(M)] \quad \text{(Eq. 4)}$$

The fraction, X, of drug unionized in the aqueous phase is

$$X = \frac{(D)}{(D^-) + (D)} = \frac{(H^+)}{Ka + (H^+)} \quad \text{(Eq. 5)}$$

where Ka is the acid dissociation constant of the drug.

Let $(D)_T^* = (D) + (D^-)$ = total drug solubility at a given pH in the absence of surfactant. Since from Eq. 5

$$(D) = (D)_T^*\left[\frac{(H^+)}{Ka + (H^+)}\right] \text{ and}$$

$$(D^-) = (D)_T^*\left[\frac{Ka}{Ka + (H^+)}\right]$$

then

$$(D)_T = (D)_T^*\left\{\left[\frac{H^+}{Ka + (H^+)}\right][1 + K'(M)] + \left[\frac{Ka}{Ka + (H^+)}\right][1 + K''(M)]\right\} \therefore$$

$$\frac{(D)_T}{(D)_T^*} = 1 + (M)\left[\frac{(H^+)K' + KaK''}{Ka + (H^+)}\right] \quad \text{(Eq. 6)}$$

A plot of $(D)_T/(D)_T^*$ *versus* (M) should give a straight line with slope equal to $[(H^+)K' + KaK'']/[Ka + (H^+)]$. From Eq. 5

$$\text{Slope} = \frac{(H^+)K' + KaK''}{Ka + (H^+)} = XK' + (1 - X)K''$$

If the slope of $(D)_T/(D)_T^*$ *versus* (M) is plotted

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versus X, the intercept at $X = 0$ is K'', and at $X = 1$ is K'. K' and K'' as previously defined are the partition coefficients of the free and ionized acid, respectively. Given (D), Ka, K', and K''

$$(D)_T = (D)\left\{1 + K'(M) + \frac{Ka}{(H^+)}[1 + K''(M)]\right\}$$

may be used to calculate total drug solubility at any pH and surfactant concentration.

The following equations derived from preceding considerations are listed for information and hold at all concentrations of drug, at saturation or below, subject to the assumptions made previously.

$(D)/(D)_T$ = fraction of drug present as free acid

$$= \left\{1 + K'(M) + \frac{Ka}{(H^+)}[1 + K''(M)]\right\}^{-1}$$

$(D^-)/(D)_T$ = fraction of drug present as ionized acid

$$= \left\{1 + K''(M) + \frac{(H^+)}{Ka}[1 + K'(M)]\right\}^{-1}$$

$[D]/(D)_T$ = fraction of drug present as free acid in the micelles

$$= \left[1 + \frac{1}{K'(M)} + \frac{Ka}{K'(H^+)(M)} + \frac{K''Ka}{K'(H^+)}\right]^{-1}$$

$[D^-]/(D)_T$ = fraction of drug present as ionized acid in the micelles

$$= \left[1 + \frac{1}{K''(M)} + \frac{(H^+)}{K''Ka(M)} + \frac{K'(H^+)}{K''Ka}\right]^{-1}$$

The forms of the equations given above will remain essentially the same for the case of a drug having a single basic functional group. Consider the protonated base as analogous to the ionized acid discussed earlier and define K' and K'' as the partition coefficients of the free and protonated forms of the base, respectively. By substituting Kw/Kb for (H^+), and (H^+) for Ka in the previous equations, one obtains expressions peculiar to a basic drug with dissociation constant Kb.

EXPERIMENTAL

Materials.—Methylprednisolone hemi-β,β'-dimethylglutarate, methylprednisolone hemiglutarate, and methylprednisolone hemisuccinate were recrystallized from water-alcohol and micronized. Procaine hydrochloride U.S.P., procaine base (B. L. Lemke & Co., Inc.), and polysorbate 80 (Atlas Powder Co.) were used without purification.

Equilibration.—The apparent solubilities of three methylprednisolone hemiesters and procaine base in buffered aqueous solutions of polysorbate 80 have been studied by equilibration of the drugs with the solvent and subsequent analysis of total drug in solution. Solubilities of the steroids were determined by shaking an excess of the hemiesters with 5 ml. of buffer containing the surfactant. Equilibration was carried out in 6-ml. vials with butyl closures for periods of at least 48 hours. Procaine solubilities were determined in a similar manner, except that a 3-hour equilibration time was sufficient.

Temperatures were maintained ±0.01° in all cases. Solutions of steroid were buffered between pH's of 3 and 7 using a combination of 0.1 *M* tri-(hydroxy methyl)-aminomethane and 0.1 *M* β,β'-dimethylglutaric acid titrated to the desired pH with concentrated HCl or NaOH solution. Procaine solutions were studied in the pH range 8.5 to 9.4 with 0.5 *M* ammonium hydroxide - HCl buffers. Hydrolysis of polysorbate 80 was negligible under these conditions over the time intervals involved.

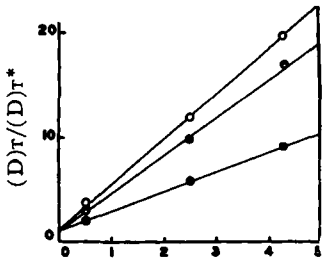


Fig. 1.—Plot of $(D)_T/(D)_T^*$ *vs.* per cent w/v polysorbate 80 for aqueous solutions of methylprednisolone DMG at 25°C. Key: ○, pH 3.0; ⊖, pH 4.8; ●, pH 6.9 in 0.1 *M* tris buffers.

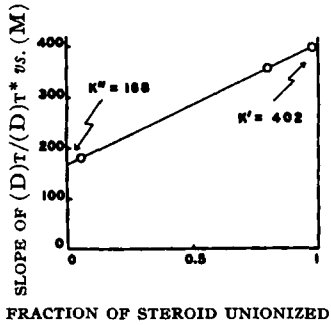


Fig. 2.—Graph showing the slopes of the lines of Fig. 1 plotted *vs.* the fraction of methylprednisolone DMG unionized. The intercepts are the partition coefficients of the free acid (K') and that of the ionized acid (K'').

Assay Procedure.—Filtered aliquots of the steroid solutions were diluted with 95% alcohol and analyzed for steroid content spectrophotometrically at 243 mμ. Procaine solutions were treated in the same way, except that absorption measurements were made at 271.5 and 288 mμ and the procaine content calculated by the method of Higuchi *et al.* (10). This method was employed since it was desired to avoid interference by *p*-aminobenzoic acid which is formed upon hydrolysis of procaine. Vials containing only the buffer and surfactant solutions, treated in the same way as the samples, served as blanks for both the steroid and procaine analyses. In no case was a shift in absorption maxima observed as a result of the presence of surfactant. All spectrophotometric determinations were made with a Beckman model DU spectrophotometer.

RESULTS AND DISCUSSION

Data were plotted in accordance with theory for methylprednisolone hemi-β,β'-dimethylglutarate, methylprednisolone hemiglutarate, methylprednisolone hemisuccinate, and procaine. Good agreement with theory was found in all cases. A typical plot of $(D)_T/(D)_T^*$ *versus* per cent w/v polysorbate 80 is shown in Fig. 1 for methylprednisolone hemi-β,β'-dimethylglutarate. Good linearity is evident over the pH range 3.0 to 6.9.

Figure 2 plots the slope of $[(D)_T/(D)_T^*$ *versus*

TABLE I.—PARTITION COEFFICIENTS IN BUFFERED AQUEOUS SOLUTIONS OF POLYSORBATE 80

Compd.	Temp., °C.	K'	K''
Methylprednisolone hemi-β,β'-dimethylglutarate	13.8	534	158
	25.0	402	168
	34.8	390	125
	45.2	358	102
	55.3	312	77
Methylprednisolone hemi-succinate[a]	25.0	400	22
Methylprednisolone hemi-glutarate[a]	25.0	680	43
Procaine	30.0	53	11

[a] Polysorbate 80 purified by the method of Malkemus and Swan (11).

TABLE II.—DISSOCIATION CONSTANTS IN AQUEOUS SOLUTIONS AT SATURATION

Compd.	Temp., °C.	Dissociation Constant
Methylprednisolone hemi-β,β'-dimethylglutarate[a]	25.0	4.0×10^{-6}
Methylprednisolone hemi-glutarate	25.0	2.7×10^{-5}
Methylprednisolone hemi-succinate	25.0	1.5×10^{-5}
Procaine	30.0	1.6×10^{-5}

[a] No change in the value observed at 25° was noted over the temperature range studied.

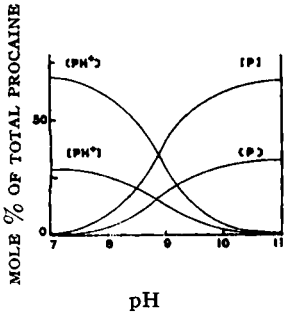


Fig. 3.—Graph showing the fraction of procaine present in various solvated states in 4% aqueous solutions of polysorbate 80. Calculations are for systems at 30°C. buffered with 0.5 *M* ammonium hydroxide–ammonium chloride. Key: (PH^+), protonated procaine in the bulk of the solution; (P), free procaine in the bulk of the solution; [PH^+], protonated procaine in the micelles; [P], free procaine in the micelles.

(M)] *versus* X, the fraction of drug unionized. Again, good linearity indicates close conformity with theory. Extrapolation of the curve to $X = 0$ and $X = 1$ gives the partition coefficients of ionized and free drug. K'' represents the partition coefficient of ionized acid for an acidic drug or protonated base in the case of a basic drug. Table I presents partition coefficients of procaine and the selected steroids between polysorbate 80 micelles and the bulk of the solutions.

In methylprednisolone hemi-β,β'-dimethylglutarate, determinations were made at several temperatures and the enthalpy of partitioning calculated. ΔH for the over-all process was −2.2 Kcal. per mole for the undissociated acid and −4.7 Kcal. per mole for the acid anion. The greater energy required for partitioning of the ionized acid may be due in part to the ion-dipole interaction with water which does not occur in the free acid. The lower consolute temperature of the water-polysorbate 80 system limits the temperatures over which these studies can be made. It has been reported (12) that the micelle weight increases exponentially with temperature at temperatures below the cloud point. This would indicate that the observed changes in the partition coefficients with temperature change may be partially due to the expected change in micelle size.

Acid dissociation constants of the steroid hemiesters and the base constant for procaine are given in Table II. Constants were derived from the pH dependency of solubility in the absence of surfactant. These values were used in calculation of the data presented in Table I.

A graphical representation of the procaine-polysorbate 80 system is shown in Fig. 3. The fraction of total procaine present in the indicated solvated states over the pH range 7 to 11 (4% polysorbate 80) was computed from data in Tables I and II. Similar plots can be made for systems comprising other combinations of drugs and surfactants. Such information can be useful in the determination of the relative stability of drugs within micelles and in product formulation.

CONCLUSIONS

The micellar solubilization of weakly basic and acidic compounds, represented by procaine and methylprednisolone hemiesters, can be described by equations based on the assumption that the drugs partition into the micelles.

Calculation of the concentrations of the several solvated species in solution is possible.

Given the partition coefficients and dissociation constants, total solubility can be predicted for a range of pH and surfactant concentrations.

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Solubility of Salicylic Acid as a Function of Dielectric Constant

By A. N. PARUTA*, B. J. SCIARRONE, and N. G. LORDI

The solubility of salicylic acid was determined in various pure solvents and binary solvent mixtures having known or measured dielectric constants. When the solubility was plotted *versus* the dielectric constant, a peak solubility or "dielectric requirement" occurred at a value independent of the nature of the solvent or solvent mixture. Salicylic acid showed two rather close solubility peaks occurring at $\epsilon = 15$ and $\epsilon = 25$ which may be due to different molecular species present in solution. The magnitude of the peak solubility varied for different binary systems, but a family of curves was observed having a common shape and a common peak dielectric requirement.

IN PREDICTING solubility of solutes, Hildebrand's solubility parameter theory (1) applies quite well when both components are nonpolar. The application of this theory is most striking in "regular" or near-ideal solution (2). The theoretical solubility in these systems is maximized when the solubility parameter of both the solute and solvent mixtures are the same. Accordingly, the solubility of the solute at solubility parameter values differing from that of the solute results in diminished solubility, and a solubility distribution curve is observed.

In extending this principle to semipolar systems with its greater pharmaceutical utility, Martin and Chertkoff (3) found that the shape of the theoretical solubility curve was approximately maintained, but that the prediction of the actual solubility failed. Since this is the case, it was felt that the dielectric constant of solvents would be a more advantageous property of solutions upon which one may qualitatively predict a solubility distribution curve. This is based on the relationship of polarity to dielectric constants (4, 5).

An empirical equation linking the dielectric constant and solubility parameter which has been reported (6) is given by

$$\delta = 7.5 + 0.2\epsilon \qquad \text{(Eq. 1)}$$

The validity and utility of Eq. 1 depends upon observation of a solubility distribution curve as a function of the dielectric constant rather than the solubility parameter. A maximum solubility of a given solute should occur within a particular narrow dielectric constant range, regardless of whether this dielectric constant is of a pure solvent or from an appropriate mixture of two solvents.

To test this proposition, the solubility of salicylic acid was studied in pure solvents and binary mixtures. The solvent mixtures tested were prepared so that a number of binary mixtures could be made which would cover the dielectric constant at peak solubility, and a number of binary mixtures of various chemical types could be obtained. Thus, solubility was studied as a function of the dielectric constant by "bridging" the gap between nonpolar and polar solvents through the use of semipolar solvents which were miscible with both the nonpolar and polar solvents.

EXPERIMENTAL

The solubility of salicylic acid was determined in pure solvents and solvent mixtures using 15-ml. screw-capped vials fitted with Teflon liners. The vials were attached to a rotating wheel in a water bath maintained at 30.6 ± 0.2°. The equilibrium time of 24 hours was sufficient in all cases. The solvents were used as such or purified as previously described (7). After 24 hours, samples were withdrawn from the reaction vials using a pipet fitted with a glass wool filtering plug. The solubility of salicylic acid was determined by base titration using freshly prepared 0.1 *N* sodium hydroxide as titrant and phenolphthalein as the end-point indicator. The sodium hydroxide was standardized against recrystallized salicylic acid. Each solvent or solvent mixture was titrated with sodium hydroxide solution to determine the base consuming capacity of the solvent.

Although the solubility of salicylic acid was studied at 30.6 ± 0.2° and the dielectric constants were measured at 25 ± 1.0°, the latter values could be used directly due to the relatively small temperature effect. Typical temperature coefficients (8) for benzene and cyclohexane are 0.002 and 0.0016, respectively. A 5° change causes an approximate change of 0.01 units in the dielectric constant for nonpolar solvents which is far below the accuracy of the experimental method.

For polar liquids such as water and methanol, a 5° change can result in a large change in the dielectric constant. For example, the dielectric constant of water is reduced to 76.3 at 30° from 78.5 at 25°. This change can result in a shift of about 0.5 dielectric constant units at peak solubility.

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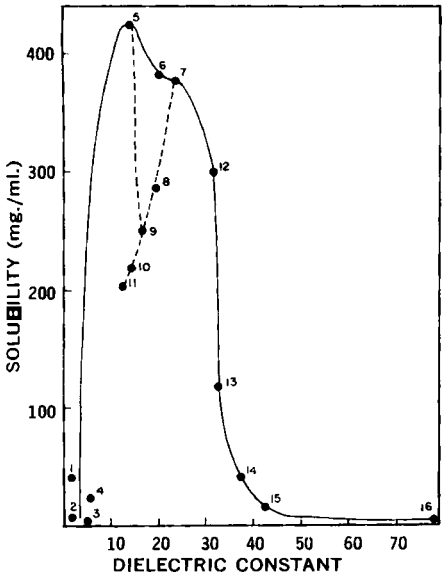


Fig. 1.—A composite plot of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of 16 pure solvents: Key: 1, dioxane; 2, benzene; 3, chloroform; 4, ethyl acetate; 5, ethyl cellosolve; 6, acetone; 7, ethanol; 8, propanol; 9, *n*-butanol; 10, cyclohexanol; 11, benzyl alcohol; 12, methanol; 13, propylene glycol; 14, ethylene glycol; 15, glycerin; 16, water.

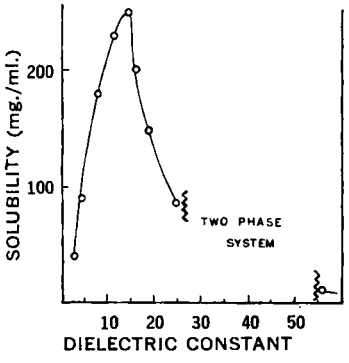


Fig. 2.—A plot of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant in the binary mixture, dioxane–water.

However, it is felt that this shift could be neglected since the dielectric constant at peak solubility apparently varied about 3 dielectric constant units for all the binary systems studied.

The dielectric constants of all solvent systems were determined by a resonance method. The general approach was to utilize and measure the resonance peaks at 550 micromicrofarads and 200 micromicrofarads at a fixed frequency of 300 kc./second for a cell containing air. A coupled, tunable, capacitance bridge was dialed until a maximum voltage was obtained on a vacuum tube voltmeter. At the maximum voltage, the apparent capacitance was noted for a given sample.

The calibration curves were obtained by measuring the relative capacitances of a pure anhydrous solvent at the two resonance peaks and plotting these values *versus* their known dielectric constants (8).

The dielectric constant was obtained by reading the dielectric constant scale at a point perpendicular to the average value of the capacitance from the calibration curves. All binary solvent mixtures were checked against both calibration curves, and average values were obtained.

RESULTS AND DISCUSSIONS

The solubility of salicylic acid was first determined in pure solvents of known dielectric constant for the purpose of scanning the dielectric constant spectrum for maximum solubility. The results are shown in Fig. 1. The dashed lines are drawn to indicate that the full line may be the sum of at least two solubility distribution curves when plotted as a function of the dielectric constant. When the peak solubility of a solute occurs at a given dielectric constant for pure solvents or solvent mix-

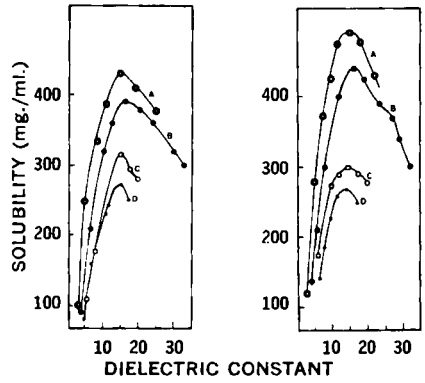


Fig. 3 (left).—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of binary mixtures of benzene and either A, ethanol; B, methanol; C, *n*-propanol; or D, *n*-butanol. Fig. 4 (right).—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of binary mixtures of dioxane and either A, ethanol; B, methanol; C, *n*-propanol; or D, *n*-butanol.

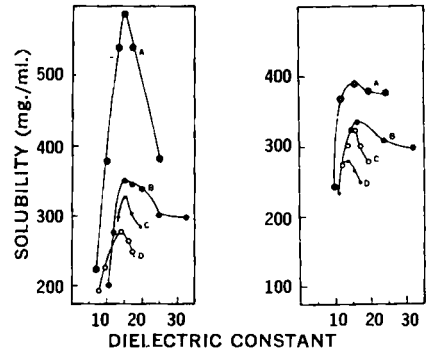


Fig. 5 (left).—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of binary mixtures of chloroform and either A, ethanol; B, methanol; C, *n*-propanol; or D, *n*-butanol. Fig. 6 (right).—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of binary mixtures of ethyl acetate and either A, ethanol; B, methanol; C, *n*-propanol; or D, *n*-butanol.

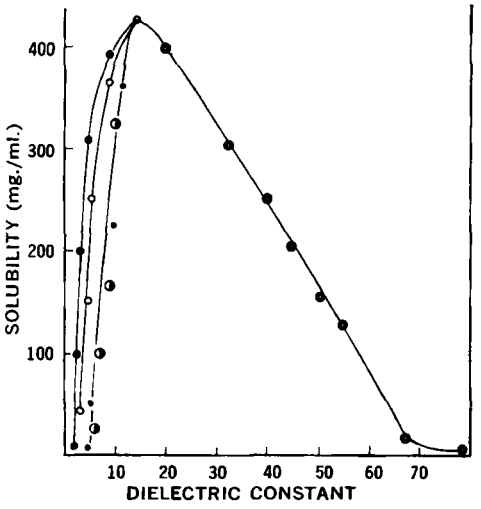


Fig. 7.—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of binary mixtures of ethyl cellosolve and either benzene (●), dioxane (○), chloroform (◉), ethyl acetate (◑), or water (◎). The peak occurs in pure ethyl cellosolve.

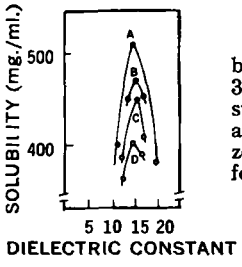


Fig. 8.—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of binary mixtures of acetone and either A, benzene; B, dioxane; C, chloroform; or D, ethyl acetate.

tures, this dielectric constant value is defined as the "dielectric requirement" of the solute. It would appear that the dielectric requirement for salicylic acid may be species dependent. Under these conditions, the first peak at a value of about 15 may be due in part to a certain degree of solute association. The prediction of the species present is complex in view of another possible peak at a value of 25. Whether the first peak is due to some association and the possible second peak due entirely to the monomeric species is difficult to interpret, since these values are rather close to each other. It is further possible that if a solvent of the correct chemical nature and dielectric constant were tested, these two solubility curves may be superseded by a major peak, the dielectric requirement being a value intermediate between these two solubility curves.

Since dioxane–water mixtures can be made to cover the dielectric constant range from 2 to about 80, it was deemed advisable to scan the solubility curve in these mixtures. In Fig. 2, the solubility of salicylic acid is plotted *versus* the dielectric constant for the binary mixtures of dioxane and water. It is evident that only one peak is present at a value of about 15. A mixture of dioxane and water with a dielectric constant value of above 25 formed a two-phase immiscible system in the presence of salicylic acid; therefore, if a second peak did exist, it could not be determined for these binary mixtures.

In Figs. 3–6, the solubility of salicylic acid is plotted *versus* the dielectric constant of the binary mixtures noted. Since the binary mixtures described below contained the same four alcohols, it was thought necessary to vary the nonpolar component with respect to chemical type and to provide a narrow range of dielectric constants. The four nonpolar components chosen as one component of the binary mixtures were benzene, dioxane, chloroform, and ethyl acetate, which have dielectric constant values of 2.2, 2.2, 4.8, and 6.0, respectively. The purpose of using these four solvents was to test the assumption of a fairly constant dielectric requirement, irrespective of the nature of the solvents used to prepare the binary mixtures.

In Fig. 3, the solubility of salicylic acid is plotted *versus* the dielectric constant of the binary mixtures composed of benzene and *n*-alkyl alcohols ranging from methanol to butanol. The apparent dielectric requirement for salicylic acid in these binary mixtures is about 15. It is observed further that solubility distribution curves exist when the solubility of salicylic acid for these binary mixtures is plotted on common axes. The difference in solubility at the peak observed for the different binary mixtures is probably related to the magnitude of the particular solute-solvent interaction.

Figure 4 shows a plot of the solubility of salicylic acid *versus* the dielectric constant of the binary mixtures composed of dioxane and *n*-alkyl alcohols

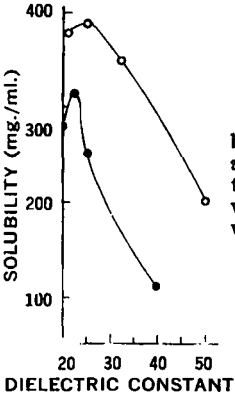


Fig. 9.—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of the binary mixtures acetone–water (○), and *n*-propanol–water (●).

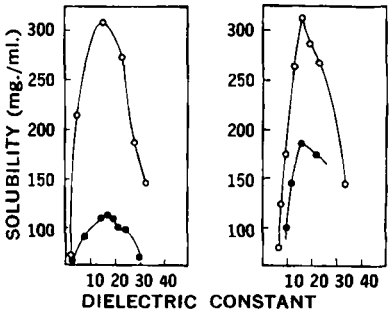


Fig. 10 (left).—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of the binary mixtures propylene glycol–dioxane (○) and ethylene glycol–dioxane (●). Fig. 11 (right).—Plots of the solubility of salicylic acid at 30.6° *vs.* the dielectric constant of the binary mixtures propylene glycol–chloroform (○) and ethyl acetate–propylene glycol (●).

ranging from methanol to butanol. The same remarks apply here as discussed under Fig. 1. It is interesting to note a shoulder in the methanol–dioxane binary mixture at a value of about 25. This experimental point and points close to it were determined several times.

Again, in each solubility distribution curve, the peak solubility for salicylic acid occurred at a dielectric constant of about 15. A shoulder occurred at a value of 25 when methanol and dioxane mixtures were used, but could not be duplicated in mixtures of benzene and other alcohols since this dielectric constant value cannot be attained in these mixtures. The decreasing magnitude of the solubility at the peak values is in the order: ethanol, methanol, propanol, and butanol.

In Fig. 5, the solubility of salicylic acid is plotted *versus* the dielectric constant for the binary mixtures composed of chloroform and *n*-alkyl alcohols ranging from methanol to butanol. As with the previous mixtures with these alcohols, these binary mixtures show the same solubility behavior, having a peak at about 15. Chloroform–methanol mixtures also show a shoulder at a value of about 25.

The results for the binary mixtures formed between ethyl acetate and *n*-alkyl alcohols are shown in Fig. 6.

Figure 7 gives the solubility curve for salicylic acid as a function of the dielectric constants obtained from binary mixtures using ethyl cellosolve as a common solvent. Since the dielectric constant of this solvent is approximately 15, which is also the dielectric requirement for salicylic acid, greater solubility should be expected in this solvent alone. Adding solvents with a greater or lesser dielectric constant than 15 should result in a binary solvent with decreased solvent power for salicylic acid. This was true when the solubility of salicylic acid was determined in ethyl cellosolve–water mixtures or mixtures with either benzene, dioxane, chloroform, or ethyl acetate. Figure 7 illustrates these distribution curves which all show a peak occurring at the value for pure ethyl cellosolve.

In Fig. 8, the solubility of salicylic acid is plotted for the binary mixtures of acetone with either benzene, dioxane, chloroform, or ethyl acetate. Again, the dielectric requirement for salicylic acid in these binary mixtures have a common value of about 15. When acetone is the common solvent, the magnitudes of the solubility in these binary mixtures are in the decreasing order benzene, dioxane, chloroform, and ethyl acetate. It is interesting to note that this is also in the order of increasing dielectric constant of the pure solvent.

Figure 9 shows a plot of the solubility of salicylic acid in the usual manner for the binary mixtures composed of acetone and water and *n*-propanol and water. In both of these binary mixtures, a solubility distribution curve is observed, but in these cases the dielectric requirement for salicylic acid is about 25. This is a confirmation that a second peak for this solute exists, although only a shoulder is found in the pure solvent scan.

To test the existence of this second peak in other binary mixtures, the solubility of salicylic acid was determined in propylene glycol–dioxane and ethylene glycol–dioxane mixtures. The glycol solvents were chosen to broaden the dielectric constant range so that the second peak at a value of 25 would be a value intermediate between the extremes of the pure solvents (Fig. 10). Both of these solvent systems give a dielectric requirement for salicylic acid at a value of 15. Enough experimental points were obtained only for the dioxane–ethylene glycol mixtures; the resultant solubility curve showed a shoulder at a value of 25.

Since propylene glycol was miscible with both chloroform and ethyl acetate, it was deemed advisable that data with a glycol as one component in binary mixtures should provide a further basis for the dielectric requirement being essentially independent of the nature of the components (Fig. 11).

In a previous communication (6), a correlation between solubility parameters and dielectric constants was presented. This study illustrates the utility of studying solubility phenomena in terms of dielectric constants.

The data obtained for the dielectric requirement for the binary mixtures tested have been summarized in Table I.

The binary mixtures studied are listed in Table II and give the peak solubility for the first peak of salicylic acid. Also given in this table are the maximum solubilities of salicylic acid for the first peak along with a solubility ratio. In this case, the solubility ratio is defined as the experimentally determined solubility compared to an arbitrary standard solubility of 500 mg./ml. The purpose of this table is to illustrate further the relatively constant dielectric requirement for the first salicylic acid peak, but the magnitude of the solubility at this dielectric requirement varies widely. From these results, it can be stated qualitatively that the magnitude of the peak solubility depends mainly

TABLE I.—SUMMARY OF THE EXPERIMENTALLY DETERMINED DIELECTRIC REQUIREMENT OF SALICYLIC ACID IN BINARY MIXTURES

Solvent	Benzene $\epsilon_{req.}$	Dioxane $\epsilon_{req.}$	Chloroform $\epsilon_{req.}$	Ethyl Acetate $\epsilon_{req.}$	Water $\epsilon_{req.}$
Ethyl cellosolve	14.5[a]	14.5[a]	14.5[a]	14.5[a]	14.5[a]
Acetone	15.6	15.5	16.1	15.0	25.0[b]
n-Butanol	15.4	14.5	14.0	15.0	...
n-Propanol	15.3	14.8	15.3	15.3	22.3[b]
Ethanol	15.8	15.0	15.7	16.0	...
Methanol	16.0	16.3[c]	16.0	16.0	...
Ethylene glycol	...	17.5[c]	...	...	...
Propylene glycol	...	15.1	16.2	16.3	...
Water	...	14.5	...	...	...

[a] Dielectric requirement of salicylic acid and dielectric constant of ethyl cellosolve have about equal values; thus "peak" exists at the dielectric constant. [b] Dielectric requirement for the second peak. [c] Shouldering in these systems exists at about 25.

TABLE II.—SUMMARY OF THE SOLUBILITY OF SALICYLIC ACID AND SOLUBILITY RATIO AT THE PEAK VALUES FOR THE BINARY MIXTURES INDICATED

Solvent Mixture	Solubility mg./ml.	Solubility Ratio[a]
Ethanol–chloroform	595	1.19
Benzene–acetone	510	1.02
Dioxane–ethanol	495	0.99
Dioxane–acetone	475	0.95
Chloroform–acetone	445	0.89
Dioxane–methanol	440	0.88
Benzene–ethanol	430	0.86
Benzene–ethyl cellosolve[b]	425	0.85
Dioxane–ethyl cellosolve[b]	425	0.85
Chloroform–ethyl cellosolve[b]	425	0.85
Ethyl acetate–ethyl cellosolve[b]	425	0.85
Ethyl acetate–acetone	415	0.83
Ethyl acetate–ethanol	390	0.78
Benzene–methanol	390	0.78
Ethyl acetate–*n*-propanol	355	0.71
Chloroform–methanol	348	0.70
Ethyl acetate–methanol	335	0.67
Ethyl acetate–*n*-butanol	330	0.66
Chloroform–*n*-propanol	315	0.63
Benzene–*n*-propanol	315	0.63
Dioxane–*n*-propanol	305	0.61
Chloroform–propylene glycol	292	0.58
Dioxane–propylene glycol	290	0.58
Chloroform–*n*-butanol	282	0.56
Dioxane–*n*-butanol	268	0.54
Dioxane–water	250	0.50
Ethyl acetate–propylene glycol	160	0.32
Dioxane–ethylene glycol	89	0.18

[a] Solubility ratio based on 500 mg./ml. = 1. [b] In these mixtures curves continuously rise to a maximum solubility corresponding to the solubility in ethyl cellosolve alone.

TABLE III.—SUMMARY OF THE EXPERIMENTALLY DETERMINED DIELECTRIC REQUIREMENT OF SALICYLIC ACID FOR THE SECOND PEAK AND SOLUBILITY RATIO

Solvent Mixture	Exptl. $\epsilon_{req.}$	mg./ml.	Ratio[a]
Acetone–water	25.0	387	0.77
Water–*n*-propanol	22.3	320	0.64

[a] Ratio based on 500 mg./ml. = 1.

TABLE IV.—SUMMARY OF THE SOLUBILITY AND SOLUBILITY RATIO FOR SALICYLIC ACID IN PURE SOLVENTS

Solvent	ϵ[a]	mg./ml.	Ratio[b]
Benzene	2.2	7	0.014
Dioxane	2.2	40	0.080
Chloroform	4.8	2	0.004
Ethyl acetate	6.0	23	0.040
Benzyl alcohol	13.00	203	0.406
Ethyl cellosolve	14.5	425	0.850
Cyclohexanol	15.0	217	0.430
n-Butanol	17.1	251	0.500
n-Propanol	20.1	287	0.560
Acetone	20.7	381	0.760
Ethanol	24.3	377	0.740
Methanol	32.6	299	0.600
Propylene glycol	33.0	118	0.230
Ethylene glycol	37.7	42	0.084
Glycerin	42.5	15	0.030
Water	78.5	3	0.006

[a] Dielectric constant of pure solvent. [b] Solubility ratio based on 500 mg./ml. = 1.

upon the degree of the solute-solvent mixture interactions. However, the prediction of the degree of these interactions cannot be deduced from the nature of the binary mixtures tested. Furthermore, there appears to be no apparent order in the magnitude of solubility for solvent mixtures containing a common liquid or functional group.

The only important qualitative observation made is that the maximum solubility is a function of the difference of the dielectric constant of the individual solvents with respect to the value of the dielectric requirement.

The data concerning the only two binary mixtures which exhibited the second peak are given in Table III. Both the solubility and solubility ratio are also given. Systems showing shouldering at the dielectric requirement of 25 are not included.

All the pure solvents used in this study with their respective dielectric constants are given in Table IV. Also listed are the respective salicylic acid solubilities in these pure solvents and the solubility ratio as defined above. Table IV is presented to illustrate the cosolvent effect by comparison with solubility ratio values in Table III. For example, the solubility ratios for salicylic acid in pure ethanol and pure chloroform are 0.74 and approximately 0.004, respectively. Yet an appropriate mixture of ethanol and chloroform gives a solubility ratio of 1.2 at peak solubility. Thus, salicylic acid shows a greater solubility in mixtures of solvents than in either one alone. By comparing the solubility ratio of any pure solvent to the solubility ratio of any binary mixture formed from that solvent, the binary mixture always shows greater solvent power.

That a reproducible peak solubility occurs within a narrow range of dielectric constant of the solvent and apparently independently of its chemical nature, strongly indicates a close tie to Hildebrand's solubility parameter theory. Possibly the more rigorous link to the dielectric constant and solubility parameter lies in a closer examination of the "cohesive energy density" concept. This, of course, requires a more exact knowledge of the liquid state which is yet unresolved.

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Cardiovascular Pharmacology of *N,N'*-Bis-[α-(2-pyridyl)ethyl]-ethylenediamine Dimaleate

By ROBERT P. HALLIDAY*, WILLIAM J. KINNARD, and JOSEPH P. BUCKLEY

N,N'-Bis-[α-(2-pyridyl)ethyl]-ethylenediamine dimaleate (JB 5058), a substituted polymethylene diamine with hypotensive action, was noted to have alpha adrenergic blocking activity, as demonstrated in the cat superior cervical ganglion nictitating membrane preparation. It did not produce a centrally mediated hypotensive response in the dog cross-circulation preparation. JB 5058 produced only a transient decrease in cardiac output while increasing femoral blood flow. The hypotensive activity of JB 5058 appears to be caused by a decrease in peripheral vascular resistance primarily due to alpha adrenergic blockade. It also appears to produce a modest antagonism of angiotensin II on vascular smooth muscle.

HALLIDAY ET AL. (1) have reported that *N,N'*-bis-[α-(2-pyridyl)ethyl]ethylenediamine dimaleate (JB 5058) was the most active hypotensive compound in a series of substituted polymethylene diamines. The compound produced marked hypotensive effects of long duration in anesthetized rats and dogs, and preliminary data suggested that the compound blocked alpha adrenergic receptors. This present study was undertaken to define more clearly the effects of JB-5058[1] on the cardiovascular system and to elucidate more fully the mechanisms of action of this compound.

METHODS

Dog Cross-Circulation Preparation.—The central and peripheral hypotensive activity of JB 5058 was investigated in the dog cross-circulation preparation, as described by Bickerton and Buckley (2). Mongrel dogs were anesthetized by an intravenous injection of 35 mg./Kg. of pentobarbital sodium and the vertebral venous sinuses and vertebral arteries occluded between C-2 and C-3 or C-3 and C-4 utilizing a 21-gauge stainless steel wire. Circulation was established between the left common carotid artery of the anesthetized donor dog, and the two common carotid arteries of the recipient, and the two jugular veins of the recipient, and the left jugular vein of the donor. Circulatory leakage between the head and trunk of the recipient was determined periodically utilizing the I^{131} method. Blood pressure was recorded from a femoral artery of each dog *via* a Statham transducer onto a Grass polygraph. Only those experiments in which no leakage was observed were utilized for this study. JB 5058, 35 mg./Kg., was administered into the arterial inflow to the recipient's head in three such preparations.

Cat Nictitating Membrane.—The cat superior cervical ganglion-nictitating membrane preparation was utilized to investigate the effects of JB 5058 on ganglionic transmission. Cats of either sex were anesthetized with pentobarbital sodium, 35 mg./Kg., by intraperitoneal injection. The head was rigidly secured and a nictitating membrane connected to an isotonic lever system which recorded on a kymograph. Responses of the membrane to submaximal electrical stimulation of the pre- and postganglionic fibers and to exogenous epinephrine, 10 mcg., i.v., were recorded prior to and following the administration of JB 5058, 35 mg./Kg., i.v.

Cardiac Output.—The effects of JB 5058 on cardiac output were investigated in anesthetized dogs using the method described by Olmsted (3). Dogs, 12 to 15 Kg., were anesthetized with pentobarbital sodium, 35 mg./Kg., i.v. The dogs were placed on intermittent positive-pressure respiration utilizing a Mine Safety Appliance respirator and the left thoracic wall opened at the fourth intercostal space using electrocautery. The lungs were retracted to expose the pericardium, which was then incised, retracted, and fastened to the chest wall with wound clips. The ascending aorta was carefully stripped of surrounding tissue and a 14-mm. i.d. probe placed around the vessel. Negative pressure was created in the thoracic cavity by overinflation of the lungs while the chest was tightly closed with sutures. Artificial respiration was then discontinued and the animal permitted to breathe voluntarily. Cardiac output (ascending aortic blood flow) was recorded *via* an electromagnetic flowmeter (Medicon FM-6) and femoral blood pressure *via* a Statham transducer onto a Grass polygraph. After femoral blood pressure and cardiac output had stabilized, JB 5058 was administered intravenously. In two of the experiments, the effects of epinephrine, 1 to 2 mcg./Kg., i.v., on cardiac output, prior to and following JB 5058, also were determined.

Peripheral Blood Flow.—The effects of the compounds on renal and hind-limb blood flow and coronary perfusion pressure were investigated in anesthetized dogs. Dogs were anesthetized with pentobarbital sodium, 35 mg./Kg., i.v. Renal and femoral blood flows were determined utilizing a Medicon electromagnetic flowmeter in which a

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[1] Kindly supplied by Lakeside Laboratories, Milwaukee, Wis.

TABLE I.—EFFECT OF JB 5058, 35 mg./Kg., ON DOG CROSS-CIRCULATION PREPARATION

Expt. No.	Animal	Route	Original Blood Pressure, mm. Hg	Max. Change, %	Duration, Min.
1	Donor		210/120	−47/46	80+
	Recipient	IA-R[a]	150/100	+33/25	7
2	Donor		200/125	−40/44	60+
	Recipient	IA-R	115/ 70	+26/28	20
3	Donor		160/100	−38/35	420+
	Recipient	IA-R	150/ 90	+84/94	5

[a] IA-R = Intra-arterially *via* the carotid inflow to the recipient's head.

suitable probe was placed around the respective artery. Vascular resistance was calculated using

$$\frac{\text{Mean Blood Pressure (mm.Hg)}}{\text{Flow (ml./min.)}} = \text{Vascular Resistance Units}$$

The left descending coronary arteries were perfused in the following manner. A carotid artery was cannulated and flow established between the carotid artery and the anterior descending branch of the left coronary artery. Flow was held constant utilizing a Sigmamotor pump and perfusion pressure recorded *via* a T-tube on the outflow side of the pump with a Statham transducer. Heparin, 1000 units/Kg., i.v., was administered to prevent clotting. In each experiment, femoral blood pressure and either renal blood flow, hind limb blood flow, or coronary perfusion pressure were recorded on a model 5 Grass polygraph.

Pithed Rat.—Rats were anesthetized with urethan, 1.2 Gm./Kg., i.p., and placed on positive-pressure respiration. A rod (2.2 mm. in diameter and 25 cm. long) was inserted obliquely into and through the eye socket and passed down the length of the spinal canal. The vagi were cut and a carotid artery cannulated for blood pressure recording. Blood pressure responses to 2 mcg. of epinephrine HCl and synthetic angiotensin II,[2] 1 mcg., were obtained prior to and after the administration of JB 5058, 10 mg./Kg., i.v.

Rabbit Aortic Spirals.—The method described by Furchgott and Bhadrakoni (4) was employed for this study. Albino rabbits, 2 to 4 Kg., were sacrificed by cervical dislocation. A segment of the descending aorta between the arch and the diaphragm was removed, pulled over polyethylene tubing (PE 360), and rotated against a scalpel blade so that spiral strips approximately 0.5 cm. in width were cut. A strip then was suspended in a magnus bath containing Tyrode's solution maintained at 38° and oxygenated with 95% O_2 and 5% CO_2. Contractions were recorded onto a Grass polygraph *via* a Grass force displacement transducer adjusted to apply 5-Gm. tension to the strip. Responses induced by epinephrine, 0.1 mcg./ml., of bathing solution or angiotensin II, 0.1 mcg./ml., were obtained prior to and after the addition of JB 5058 in doses ranging from 25 to 800 mcg./ml.

Denervated Dog Hind Limb.—Mongrel dogs were anesthetized with pentobarbital sodium, 35 mg./Kg., i.v., the right femoral artery and vein isolated, and the right sciatic and femoral nerves cut, as described by Clonninger and Green (5). A wire tourniquet was placed beneath the exposed vessels and tightened around the limb utilizing a Schiffrin wire tightener. Carotid blood was perfused into the cannulated femoral artery of the isolated limb by means of a constant output pump (Sigmamotor pump, model TM-10) and perfusion pressure recorded on a Grass polygraph *via* a force displacement transducer. Arterial blood pressure was recorded from the left femoral artery. Responses to epinephrine HCl, 5 mcg., and angiotensin II, 0.5 mcg./Kg., injected into the arterial inflow to the limb were recorded prior to and after administration of JB 5058, 35 mg./Kg., i.v.

[2] Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

RESULTS

Dog Cross-Circulation Preparation.—The effects of JB 5058, administered *via* the arterial inflow to the recipient's head, on the blood pressure of the recipient and donor animals are summarized in Table I. JB 5058 produced marked hypotensive effects in the donor animal only, and the donor's blood pressure remained depressed throughout the remainder of the experiment. The compound produced pressor responses of relatively short duration in the recipient's trunk. It would appear that these effects in the recipient animal were of a reflex nature.

Cat Nictitating Membrane.—JB 5058, 35 mg./Kg., i.v., reduced the response of the nictitating membrane to stimulation of both pre- and postganglionic fibers of the superior cervical sympathetic nerve approximately 60% in three cats. Response of the membrane to exogenous epinephrine was completely abolished; in one experiment, four times the control dose of epinephrine failed to stimulate the nictitating membrane.

Cardiac Output.—JB 5058, 35 mg./Kg., i.v., produced a transient (1 to 2 minutes) decrease in cardiac output ranging from 10 to 43% in three of four dogs. Cardiac output then returned to predrug levels and remained constant for the duration of the experiments (2 to 3 hours). The compound did not alter the increase in cardiac output produced by epinephrine in two additional experiments.

Renal and Hind-Limb Blood Flow and Coronary Perfusion Pressure.—Effects of JB 5058, 35 mg./Kg., i.v., on renal blood flow and vascular resistance are summarized in Table II. Renal vascular resistance was reduced approximately 35%, even though only a slight change in actual renal blood flow occurred. JB 5058, 35 mg./Kg. administered intravenously reduced vascular resistance in the hind limb and produced an increase in blood flow in three experiments (Table III). JB 5058, 35 mg./Kg., i.v., reduced coronary perfusion pressure 28% in two dogs (Table IV). Perfusion pressure returned to predrug levels approximately 30 minutes following administration of the compound, even

TABLE II.—EFFECT OF JB 5058, 35 mg./Kg., I.V., ON RENAL VASCULAR RESISTANCE

Time, Min.	Control Responses,[a] % Mean Blood Pressure	Renal Blood Flow	Vascular Resistance Units
Control	100	100	100
5	59 ± 11	83 ± 30	74 ± 17
15	62 ± 12	102 ± 24	73 ± 8
30	70 ± 7	105 ± 17	74 ± 6
60	70 ± 6	113 ± 18	66 ± 9
120	62 ± 5	109 ± 16	65 ± 8

[a] $\bar{X}$ ± S. E. $N = 3$.

TABLE III.—EFFECT OF JB 5058, 35 mg./Kg., I.V., ON VASCULAR RESISTANCE IN HIND LEG OF THE DOG

Time, Min.	Control Responses,[a] % Mean Blood Pressure	Femoral Blood Flow	Vascular Resistance Units
Control	100	100	100
5	58 ± 9	155 ± 23	43 ± 8
15	68 ± 3	156 ± 8	47 ± 3
30	69 ± 1	140 ± 13	50 ± 6
60	67 ± 5	123 ± 13	59 + 6
120	75 ± 3	140 ± 13	52 ± 4

[a] $\bar{X}$ ± S. E. $N = 3$.

TABLE IV.—EFFECT OF JB 5058, 35 mg./Kg., I.V., ON CORONARY PERFUSION PRESSURE IN THE DOG

Expt. No.	Time, Min.	Mean Blood Pressure, mm. Hg	Coronary[a] Perfusion Pressure, mm. Hg
1	Control	120	125
	5	50	90
	15	60	100
	30	60	120
2	Control	140	175
	5	70	125
	15	80	150
	30	85	170

[a] A Sigmamotor pump was used to perfuse the anterior descending coronary artery.

TABLE V.—EFFECT OF JB 5058, 10 mg./Kg., I.V., ON PRESSOR RESPONSE TO EPINEPHRINE AND ANGIOTENSIN II IN THE PITHED RAT

Expt. No.	Control Response, mm. Hg Epi.	Ang.	Response after JB 5058, mm. Hg Epi.	Ang.
1	115	28	60	12
2	100	96	40	65
3	115	32	61	17
4	140	..	60	..

though arterial pressure was still depressed approximately 50%.

Pithed Rats.—The effects of JB 5058 on the epinephrine and angiotensin II pressor responses in the pithed rat are summarized in Table V. The compound depressed the pressor response to both epinephrine and angiotensin II 48 and 42%, respectively. Administration of the compound itself produced a mild transient pressor effect. Injections of normal saline solution, equal to the volume of drug solution injected, produced similar pressor responses. However, the saline solution did not alter the responses to epinephrine and angiotensin II.

Rabbit Aortic Strips.—JB 5058 blocked or diminished contractions of the rabbit aorta induced by epinephrine and angiotensin II. The inhibitory $dose_{50}$ for JB 5058 against epinephrine-induced contractions was approximately 70 mcg./ml. of bath, whereas the inhibitory $dose_{50}$ against angiotensin II-induced contractions was approximately 350 mcg./ml.

Dog Denervated Limb.—JB 5058, 35 mg./Kg., produced a mean decrease in perfusion pressure of 38% in three preparations. Hind-limb perfusion pressure returned to predrug levels within 20 minutes, even though the hypotensive effect was still at its maximum. The compound inhibited the vasoconstricting effects of epinephrine and angiotensin administered directly into the perfused femoral artery 75 and 23%, respectively.

DISCUSSION

JB 5058 has been reported to produce hypotensive effects of long duration in anesthetized rats and dogs, to reverse the pressor response to epinephrine, and to decrease the pressor response to *l*-norepinephrine, angiotensin II, and bilateral carotid occlusion in anesthetized dogs (1). In the present study, the compound decreased the response of the nictitating membrane in the cat to both pre- and postganglionic stimulation by the same magnitude, thus indicating no interference with the impulse transmission across the ganglion. The response of the nictitating membrane to injected epinephrine was abolished, further substantiating the previously observed alpha adrenergic blocking activity of JB 5058. The compound appeared to produce its hypotensive effects through purely peripheral mechanisms when investigated in the dog cross-circulation preparation. Administration of the compound into the arterial inflow to the isolated head of the recipient failed to produce a centrally induced depressor response.

Page and Bumpus (6) have reported that other adrenolytic agents have relatively little effect on the pressor response to angiotensin II. JB 5058 appears to inhibit angiotensin II directly, independent of its alpha adrenergic blocking activity and mild vascular smooth muscle depressing action. JB 5058 inhibited the constricting effects of angiotensin II *in vitro* (rabbit aortic spirals) and *in vivo* (pithed rat and perfused denervated dog hind limb). In the denervated hind limb, the compound produced only transient direct relaxation of arterial smooth muscle, whereas the inhibitory effects of angiotensin II could be demonstrated long after this brief dilatory effect of JB 5058.

JB 5058 produced only a transient decrease in cardiac output, an indication that the hypotensive activity of the compound was due to the marked decrease in peripheral resistance. Blood flow studies suggest that this decreased resistance was, at least in part, due to increased blood flow to skeletal muscle and cutaneous tissue. The renal blood flow remained approximately unchanged, even though systemic blood pressure was greatly reduced. In conclusion, the hypotensive activity of JB 5058 results in a decrease in peripheral vascular resistance, primarily due to alpha adrenergic blockade. The compound also appears to produce a

mild inhibitory effect on the direct vasoconstrictor activity of angiotensin II on vascular smooth muscle.

SUMMARY

No centrally induced hypotensive effect could be demonstrated with JB 5058 in the dog cross-circulation preparation.

Alpha adrenergic blocking activity in the absence of ganglionic blockage was demonstrated with JB 5058 in the cat superior cervical ganglion nictitating membrane preparation.

Cardiac output, except for a brief (1–2 minutes) reduction, was not altered by the administration of JB 5058.

Following the administration of JB 5058, femoral blood flow was increased, coronary vascular resistance was transiently decreased, and renal blood flow was unchanged.

A modest antagonism of angiotensin II was observed with JB 5058 both *in vivo* and *in vitro*.

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Effect of the Site of Release on the Absorption of Trimeprazine-S^{35} and Penicillin G in Dogs

By T. H. LIN*, J. R. GUARINI, S. P. ERIKSEN†, and J. V. SWINTOSKY

A radiofrequency generator described in a previous paper was applied to release trimeprazine-S^{35} and potassium penicillin G at selected sites in the gastrointestinal tract of dogs. Plasma and urinary excretion data indicate that trimeprazine-S^{35} is readily and efficiently absorbed when released in the stomach and the upper and middle small intestine. Potassium penicillin G in its biologically active form is more available for absorption when released just beyond the pylorus than when released in either the stomach or more than 3 ft. beyond the pylorus.

THE EFFECT of drug release site on the rates and efficiency of drug absorption has been a subject of great interest to pharmaceutical investigators. A technique developed in our laboratories using a radiofrequency generator for opening specially designed capsules at selected sites in the gastrointestinal tract offers a useful tool for studying the effect in intact animals under normal physiologic conditions. Useful information thus can be gathered for the design and evaluation of oral dosage forms. This report describes the application of this technique to a study of the effect of release site on the absorption of a phenothiazine antipruritic, trimeprazine, and an antibiotic, potassium penicillin G.

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The authors thank Mrs. J. Abrams, I. M. Juliani, and Messrs. F. M. Cosmi, F. C. Tye, and Mr. C. Chong for their technical assistance during various phases of the study.

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EXPERIMENTAL

The design and the filling of the capsule and the release of drugs from the capsule by induction heating from a radiofrequency generator have been described in detail in a previous report (1).

Adult mongrel dogs were fasted (18–20 hours) prior to the oral administration of a capsule. Induction heating (approximately 5 minutes) was applied either 15–30 minutes after capsule administration to release the drug in the stomach or after sufficient time had been allowed for the capsule to reach certain sites in the intestine, determined by a marked thread attached to the capsule or by fluoroscopy (1). Usually the dog had been fasted for 21–25 hours prior to drug release. Urine was collected up to 24 hours following drug release by catheterizing the dogs and for longer periods by housing the animals in a metabolic cage. Blood samples were drawn from the jugular vein.

Absorption of Trimeprazine-S^{35}.—Each capsule was filled with 0.3–0.4 ml. of an aqueous solution of the drug to provide the dose of 2.5 mg. of trimeprazine-S^{35} tartrate/Kg. body weight. To help provide adequate urine collection, each dog was given 20 ml./Kg. of water 1 hour before drug release. Blood was drawn prior to induction heating and at 0.5, 1, 2, 3, 6, 9, 12, and 24 hours thereafter. Urine was collected for the periods: 0–0.5, 0.5–1,

mild inhibitory effect on the direct vasoconstrictor activity of angiotensin II on vascular smooth muscle.

SUMMARY

No centrally induced hypotensive effect could be demonstrated with JB 5058 in the dog cross-circulation preparation.

Alpha adrenergic blocking activity in the absence of ganglionic blockage was demonstrated with JB 5058 in the cat superior cervical ganglion nictitating membrane preparation.

Cardiac output, except for a brief (1–2 minutes) reduction, was not altered by the administration of JB 5058.

Following the administration of JB 5058, femoral blood flow was increased, coronary vascular resistance was transiently decreased, and renal blood flow was unchanged.

A modest antagonism of angiotensin II was observed with JB 5058 both *in vivo* and *in vitro*.

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Effect of the Site of Release on the Absorption of Trimeprazine-S^{35} and Penicillin G in Dogs

By T. H. LIN*, J. R. GUARINI, S. P. ERIKSEN†, and J. V. SWINTOSKY

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1–3, 3–6, 6–9, 9–24, 24–48, 48–72, and 72–96 hours. Fecal samples were collected every 24 hours for 4 days. The animals were fed following the ninth hour sampling and allowed drinking water at all times.

Assay of S^{35} Activity.—The specific activity of the trimeprazine-S^{35} tartrate was 0.15–0.25 mcg./mg. Sulfur-35 activity in samples was determined by liquid scintillation counting with a Tricarb liquid scintillation spectrometer. Four-tenths of a milliliter of urine was counted in 20 ml. of a POPOP[1] solvent system and 0.2–0.5 ml. of plasma in 20 ml. of the solvent containing 5% w/v of Cab-O-Sil. Fecal samples were dissolved in concentrated nitric acid. A 10-ml. aliquot was oxidized with a HNO_3-$HClO_4$ mixture (v/v, 1 part of concentrated HNO_3, 1 part of water, and 2 parts of 60% $HClO_4$). The clear colorless digest was adjusted to 10.0 ml., and a 0.2-ml. aliquot was counted in 20 ml. of POPOP solvent system.

Absorption of Penicillin G.—The potassium salt of penicillin G (about 1600 units/mg.) was formulated into a tablet which disintegrated readily in an aqueous medium. This tablet was placed in a capsule. Each dog received approximately 20,000 units (12.5 mg.) of the antibiotic per kilogram body weight. To help maintain adequate urine flow rate, each dog received water *via* stomach tube: 20 ml./Kg. 1 hour before drug release and 10 ml./Kg., 1, 3, and 5 hours after drug release. Blood samples were drawn at 0 time and 0.5, 1, 2, 4, 6, 8, and 24 hours after drug release. Urine was collected at 0, 0.5, 1, 2, 4, 6, 8, and 24 hours. An aliquot of each collection was diluted with an equal volume of a pH 6.0 buffer. The animals were fed after the eighth hour collections. The penicillin activity in the plasma and urine was determined by a standard *B. subtilis* agar-diffusion plate assay.

RESULTS AND DISCUSSION

Trimeprazine-S^{35} Absorption.—Figures 1 and 2 show that trimeprazine-S^{35} was absorbed rapidly and attained a peak level in plasma usually within 1 hour following its release. As shown in Fig. 2, the plasma S^{35} patterns were quite similar, whether the drug was released in the stomach, in the proximal portion (1.5 ft. past the pylorus sphincter), or in the distal portion (5 ft. past the pylorus) of the small intestine,[2] except that drug release in the small intestine appeared to give rise to slightly higher peak levels and faster absorption than did drug release in the stomach.

The bulk of S^{35} excretion occurred within 24 hours. As shown in Table I, 4-day urinary excretion of S^{35} activity appeared similar for each of several experiments (Dogs 250 and 300) and irrespective of the site of drug release (Dog 300). The total urinary and fecal excretion also appeared similar in these experiments. Although fecal excretion of the drug varied between 20 and 36% of the dose administered, this variation is not unusual. Previous studies with dogs in our laboratories have shown that phenothiazine tranquilizers are actively metabolized in the biliary system. A considerable portion of the fecal S^{35} activity appears to be derived from trimeprazine which was absorbed and metabolized and returned to the intestinal tract *via* the biliary route.

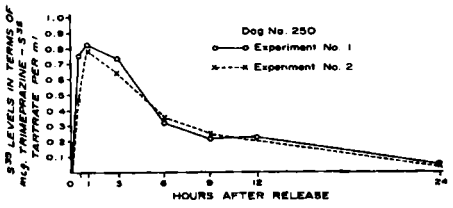


Fig. 1.—Plasma S^{35} levels after the release of 2.5 mg./Kg. trimeprazine-S^{35} tartrate in the stomach of a dog.

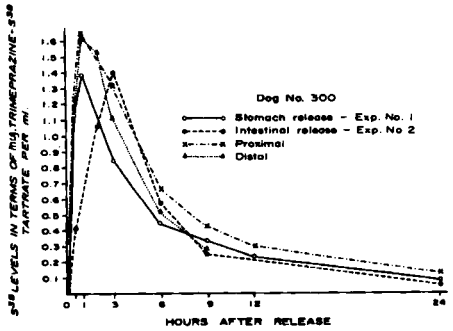


Fig. 2.—Plasma S^{35} levels following the release of 2.5 mg./Kg. trimeprazine-S^{35} tartrate at different sites in the dog gastrointestinal tract.

TABLE I.—TOTAL 4-DAY URINARY AND FECAL EXCRETION OF S^{35} ACTIVITY FOLLOWING ORAL ADMINISTRATION AND RELEASE OF TRIMEPRAZINE-S^{35} AT VARIOUS SITES IN THE GASTROINTESTINAL TRACT[a]

	Dog 250		Dog 300			
Site of Release	Stomach Exp. 1	Stomach Exp. 2	Stomach Exp. 1	Stomach Exp. 2	Intestine Proximal	Intestine Distal
Urinary	42.06	37.56	43.99	52.70	54.59	50.04
Fecal	36.34	34.15	36.08	29.63	20.90	24.06

[a] Expressed as % of total administered.

Inspection of the plasma and excretion data tends to show that trimeprazine was absorbed rapidly and to the same order when it was released at different sites in the gastrointestinal tract, even though the apparent increase in pH from the stomach to the distal portion of the small intestine might have been expected to affect the ionization of trimeprazine, which decreases as pH increases.

Studies in humans (2–4) corroborate the view that trimeprazine is absorbed well through a reasonable length of the gastrointestinal tract, evidenced by good plasma levels and urinary recovery of the drug that result when it is given in a sustained-release dosage form.

A valuable technique for measuring absorption efficiency as a function of drug release site is illustrated in this study. Whether a drug is absorbed efficiently from different regions of the small intestine is important because it is a criterion which determines whether the drug may be used successfully in a sustained-release dosage form.

Penicillin G Absorption.—The antibiotic activity in the plasma and urine indicated an absorption

[1] POPOP liquid scintillation solvent system for aqueous samples: 1,4-dioxane, 400 ml.; toluene, 400 ml.; absolute alcohol, 240 ml.; *p*-bis [2-(5-phenyl-oxazolyl)] benzene, 5 Gm.; and 2,5-diphenyloxazole, 5 Gm.

[2] Length of small intestine of these dogs is approximately 10 ft.

capability for penicillin G when it was released in the stomach or as far as 3 or 4 ft. beyond the pylorus. Considerable variability in results was encountered when the drug was released in the stomach. This may be explained as follows. When potassium penicillin G was released in the stomach, it might have been wholly absorbed at this site or only partly absorbed there and partly absorbed in the intestine, following its passage through the pylorus. Plasma levels could have been influenced by gastric acidity and by the time required for the penicillin to pass through the stomach. Wide fluctuations in these levels could have occurred because the penicillin was inactivated to a different degree, depending on the pH of the stomach and the rate of stomach emptying.

A comparison of the plasma and urinary data obtained after penicillin release at different sites in the gastrointestinal tract (Table II and Fig. 3) shows that absorption was most efficient in the small intestine, approximately 1 to 2 ft. beyond the pylorus. These data also show that while penicillin appeared rapidly in the plasma, it was reduced to trace amounts by the end of 5 hours in all instances.

Penicillin, when released 3 or 4 ft. beyond the pylorus, might be expected to remain stable because of the high pH in this area. But since penicillin has a pKa near 2.8, its high degree of dissociation may impede its absorption. In addition, its inactivation by penicillinase probably becomes greater in the lower intestinal tract. Thus, urinary recoveries of penicillin released 3 to 4 ft. beyond the pylorus were consistently much lower than the recoveries when penicillin was released immediately beyond the pylorus. A comparison of the urinary recoveries resulting from the release of penicillin at three distinct sites is shown in Table II.

The capacity of the various sections of the human gastrointestinal tract to absorb penicillin has been studied by introducing penicillin into these sections using capsules with enteric coatings of different thicknesses (5), laparotomy (6), a probe (7), and sustained-release coated pellets (8). The observations made in dogs in the present study generally agree with those made in humans by several investigators—namely, penicillin is available for absorption most readily in the upper small intestine and is considerably less available in the stomach and lower intestine. The lesser availability may be due not to the poor absorption of the drug, but rather to its rapid destruction at around pH 2, where its chemical half-life is in the order of only a few minutes (9).

TABLE II.—PENICILLIN RECOVERY IN DOG URINE FOLLOWING RELEASE OF POTASSIUM PENICILLIN G (20,000 u./Kg.) AT VARIOUS SITES IN THE GASTROINTESTINAL TRACT

	% Total Penicillin Recovered in Urine in 6 Hr.		
Dog	Release in Stomach	Released in Small Intestine Distance Past Pylorus, <2 Ft.	Distance Past Pylorus, 3–4 Ft.
201	6%	13% (20)[a]	
215	8	24 (18)[a]	16% (36)[a]
265	22	26 (10)[a]	10 (36)[a] (run 1)
			14 (40)[a] (run 2)
			9 (>36)[a] (run 3)

[a] Number in parentheses is the distance (in inches) past the pylorus where the drug was released.

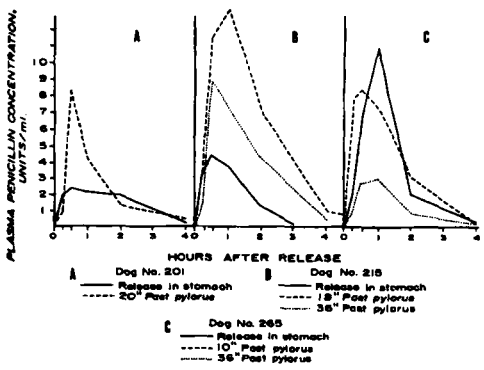


Fig. 3.—Plasma penicillin concentrations following the release of 20,000 U.S.P. units potassium penicillin G per Kg. at different sites in the gastrointestinal tract.

Penicillin G's short biologic half-life (about 0.5 hour), coupled with its reduced absorption efficiency as it is released further and further beyond the pylorus, makes it a poor candidate for a sustained-release product that would give uniformly high plasma blood levels for 10 to 12 hours. Furthermore, our data suggest that the drug's availability for absorption would be decreased. Studies in our laboratories (but not reported here) indicate poor availability to humans of penicillin G from lipid-coated pellets.

This part of the present study again illustrates the applicability of the radiofrequency method for the study of absorption as a function of release sites under near physiologic conditions in intact experimental subjects.

SUMMARY

The study describes how the site of drug release in the gastrointestinal tract affects the absorption of trimeprazine-S^{35} and penicillin G in dogs, and how the drug release at selected sites is accomplished with a radiofrequency generator. Using data on drug blood levels and urine recoveries, we show how the technique employed can be applied to study, under near physiologic condition, absorption efficiency in relation to drug release site. These data are useful in considering the design of oral dosage forms. The data obtained on trimeprazine are consistent with the view that this drug is available for efficient absorption, whether released in the stomach, in the intestine near the pylorus, or midway in the small intestine. The data obtained on penicillin G show it to be readily available when released in the intestine just beyond the pylorus, and not so available when released in the stomach or deeper in the small intestine.

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Segregation Kinetics of Particulate Solids Systems II

Particle Density–Size Interactions and Wall Effects

By EDWARD G. RIPPIE, JAMES L. OLSEN*, and MORRIS D. FAIMAN

Segregation in particulate mixtures is not observed in systems subjected to vertical sine wave motion when the particles differ only in density. However, a marked nonlinear interaction is found between the variables of density and size where the spheres differ in both density and size. The influence of the over-all dimensions of the systems and the mode of agitation on the observed rates of unmixing has been studied and found to be a function of particle size.

PARTICULATE DENSITY has been studied by several workers (1–3) as a factor involved in the mixing of solids. It is also an important consideration in the segregation which accompanies most mixing operations. Pharmaceutically, mixtures of solid particles may contain components which vary widely in density and therefore can be expected to separate when subjected to agitation. Hardy and Gard (1) found that in mixtures of powders 100 mesh or finer surface energy prevented or retarded segregation; in some systems no separation was noted even with great differences in density of the components. Recent studies on the rate of mixing in horizontal drum mixers show the marked effect of a density differential between components (2, 3). In certain cases, a differential in density between particles resulted in a greater final degree of mixing than would be found otherwise. Much of the previously reported information is difficult to interpret since the results are obtained in systems where variables other than density are allowed to fluctuate. The present work is intended to give quantitative information on the extent and nature of the effect of density and interparticulate density differences on the segregation rate of idealized systems composed of spheres.

Volume of the mixer relative to the mean particle size is a factor which has received little consideration in the literature. It can be expected that interactions which occur between particle and container wall may influence the rate of mixing and also that of segregation. In the present studies we are limited to small containers, and the spheres used are relatively large. The wall effects which occur have been studied as a function of the size of the cylinder, depth of fill in the cylinder, mode of agitation, and size of the spheres. Results indicate that the observed wall effects should not alter significantly the relationships between particulate variables and segregation rate or the interpretation of the data.

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EXPERIMENTAL

Materials.—Chrome steel balls of the grade and type commonly employed in manufacture of commercial ball bearings were used. Glass spheres were of the common type used as laboratory boiling beads but were selected for uniformity of size and spheroidicity. Cylinders of 0.75- and 1.00-in. internal diameter were made of brass tubing and fitted with plungers for the removal of samples.

Procedures.—Systems consisting of binary mixtures of balls which varied as to size, density, or both were subjected to vertical sine wave motion of 1025 ± 5 c.p.m. frequency and an amplitude of 0.100 in. Studies were carried out in both 0.75- and 1.00-in. diameter cylinders with general procedure, particle size analysis, calculation of standard deviation, and computation of confidence limits carried out as previously reported (4). Additional studies were done with a variation in the mode of agitation. A 0.75-in. internal diameter brass cylinder was split longitudinally and arranged to allow movement of one side relative to the other which was held fixed. The resulting squeeze-type motion was adjusted to a frequency of 1025 ± 5 c.p.m. and an amplitude of 0.035 in. Horizontal motion of the movable half of the cylinder was approximately sine wave. However, since many of the systems studied in the present work contained spheres which were of different density and size, this fact should be considered in the interpretation of the computed standard deviations. Equivalence of the systems was maintained by using equal weights of spheres where densities were equal but differed in size, and equal volumes were taken in cases where the components varied in density. The standard deviation, on the basis of weight, of a completely unmixed system of this type and of a system in any other state of separation, is a function of the density differential between components and the state of mixedness. Since the effect of density difference on standard deviation is a constant factor in any given system and because the function $(S_\infty - S)$ is plotted as the logarithm, there is no net effect on the computed rate constant. For this reason, rate constants for monodensity systems can be compared directly with those where the components vary in density.

TABLE I.—SEGREGATION RATE CONSTANTS IN BINARY SYSTEMS OF GLASS SPHERES

Sphere Diameters,[a] mm.	$\frac{V_l - V_s}{V_s}$	k (Min.$^{-1}$) 95% C.L.
3–6	7.00	0.100 ± 0.015
3–5	3.63	0.050 ± 0.011
3–4	1.37	0.014 ± 0.003
4–6	2.38	0.033 ± 0.009
5–6	0.73	0.012 ± 0.001

[a] Equal weights of both components were used (28.5 Gm.).

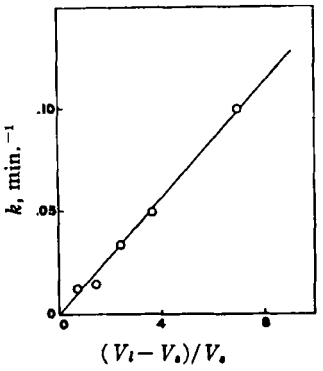


Fig. 1.—First-order rate constant, k, *versus* the ratio of the difference in particulate volumes, $(V_l - V_s)$, to the particulate volume of the smaller component, V_s. Glass balls were used in all runs at a 1:1 ratio (w/w) of the two components. The solid line represents the least-square regression.

RESULTS AND DISCUSSION

Density Effects.—Earlier work (4) has shown that systems of steel balls segregate as a function of the ratio of their particulate volumes. Plots of apparent rate constants *versus* particulate volume ratios resulted in straight lines for systems where the large balls were held constant and the size of the smaller component varied and also where the reverse was true. As noted previously, the slopes of these lines were similar and passed through the point (1,0).

Rate constants are presented in Table I for systems of glass balls occupying the same volume within the 0.75-in. cylinder and subjected to the same vertical agitation as the steel balls studied previously—1025-c.p.m. frequency, 0.100-in. amplitude. The rate constants have been plotted in Fig. 1 against $(V_l - V_s)/V_s$, where V_l and V_s are the particulate volumes of the large and small spheres, respectively. The function corresponds to the particulate volume ratio, V_r, minus 1. This method of plotting results in a straight line as before (4) but transposes the line so that it passes through the origin rather than the point (1,0). The least-square slope of this line through the origin was 0.014. The composite data reported previously for systems of steel spheres yield a least-square slope of 0.017. Confidence limits of the points from which the slopes were determined indicate that no significance can be given to the observed difference. These results for systems whose densities were in the ratio of 3.14 (7.79 density for steel, 2.48 density for glass) suggest that density is not an important factor in segregation where it is the same for all particles in the system.

In an attempt to obtain data on systems of higher density, experiments were carried out with lead shot. Here no segregation could be detected in mixtures with particulate volume ratios as high as 111. It is apparent from these results that a factor or factors other than size and density must be taken into account. In this case, it is likely that elasticity plays an important role.

The coefficient of restitution, C, is defined as $(V_4 - V_3)/(V_2 - V_1)$, where two bodies moving in a straight line with velocities V_1 and V_2, respectively, collide and after impact move with velocities V_3 and V_4. As a rough indication of C, balls of the material to be studied were dropped onto a glass plate and the height to which they bounced was noted. The ratio of the initial height to the bounce height gave a measure of the elasticity as we were concerned with it. No attempt was made to use a plate of the same material as the ball since plates of the same composition and hardness could not be obtained. The chrome steel balls gave a bounce height ratio of 0.95, glass balls 0.87, and lead shot 0.05. While these results are quite approximate and not done for a degree of accuracy, they correlate well with the aforementioned segregation rates.

Segregation was anticipated in binary systems in which components of the same particle size differed in density. A decrease in free energy would occur with a displacement of lighter balls to the top of the cylinder with a resultant drop in the center of gravity. This was not the case experimentally. Lead shot and glass balls of the same diameter (0.110 in.) showed no unmixing when subjected to vertical sine wave motion for a prolonged period (1 to 5 hours) of time. This could have been due possibly to the low elasticity of the lead. A system of glass balls (0.118 in.) and steel balls (0.094 in.), where elasticity should not be a critical factor was also agitated for several hours; again no segregation could be detected. It should be emphasized that these results have been found for a specific mode of vibration only (sine wave at 1025 c.p.m. and amplitude 0.100 in.) and may not hold where void space created by shaking is greater, or where other modes of vibration become dominant.

Combined Density and Size Effects.—Studies on density effects in systems with no size differential fail to give data relative to the combined effect of these two variables. Extrapolation of results to cases where both density and size differences exist would require an assumption that these variables are independent. Therefore, the apparent first-order rate constants for binary systems comprising equal volumes of various sized glass and steel balls were determined. The results, which are presented in Table II, fall into two groups on the basis of the more

TABLE II.—SEGREGATION RATE CONSTANTS IN SYSTEMS OF GLASS AND STEEL SPHERES

—Sphere Diameters— Glass, mm.	Steel, 32nds of an in.	$\frac{V_l - V_s}{V_s}$	k, Min.$^{-1}$ 95% C.L.
6	3	15.64	0.621 ± 0.047
6	4	6.08	0.190 ± 0.050
6	5	2.64	0.071 ± 0.015
5	3	8.20	0.284 ± 0.063
3	8	8.51	0.087 ± 0.010
4	8	2.96	0.043 ± 0.004
5	8	1.04	0.021 ± 0.003
3	7	5.39	0.073 ± 0.025
3	6	3.04	0.065 ± 0.008
3	5	1.31	0.037 ± 0.008

dense component being either larger or smaller than the less dense component. Dimensions of the balls will be given in thirty-seconds of an inch and millimeters for the steel and glass balls, respectively, so that previously reported data (4) may be compared more easily with the present results. Figure 2 shows plots of the apparent rate constants *versus* $(V_l - V_s)/V_s$ for the two cases. It is apparent from this graph that a distinctly nonlinear interaction occurs between size and density. When the larger particles are more dense, the segregation rate falls off at higher particulate volume ratios, and the reverse is noted in the case of more dense–smaller systems. In general, the effect of density becomes more pronounced as the size difference increases.

The solid curves through the points in Fig. 2 were drawn from Eq. 1. Coefficients were determined by

$$k = B\left(\frac{V_l - V_s}{V_s}\right) + C\left(\frac{V_l - V_s}{V_s}\right)^2 \quad \text{(Eq. 1)}$$

the method of least squares for the curves passing through the origin. For the more dense–smaller curve, $B = 0.027$ and $C = 0.0008$; for the more dense–larger curve, $B = 0.022$ and $C = -0.0014$. While no theoretical significance should be attached to this equation, it is useful in analysis of the data. It is interesting that the linear coefficients, B, are higher in both cases than those in the monodensity systems. The reasonably close numerical correspondence of the coefficient of the square term, C, for the two curves is significant since this coefficient largely represents the influence of the density differential as it interacts with the difference in particle size. Obviously, the equation breaks down at higher particulate volume ratios than those studied and would require the addition of higher-order terms. Attempts to extend these studies to glass–lead systems were unsuccessful, presumably due to the low elasticity of the lead. No detectable segregation occurred regardless of particulate size ratio.

Particle–Wall Interactions.—A study designed to yield fundamental information on the behavior of mixtures of solid particles must necessarily allow control or evaluation of the interactions between

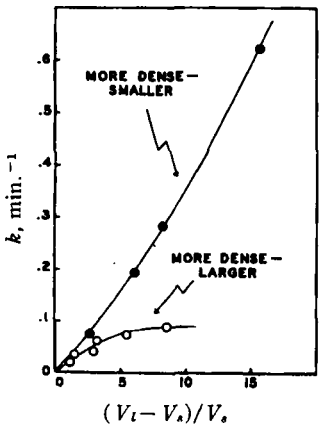


Fig. 2.—First-order rate constant, k, *versus* the ratio of the difference in particulate volumes, $(V_l - V_s)$, to the particulate volume of the smaller component, V_s. Glass and steel balls were used in all runs at a 1:1 total volume ratio of the two components. The solid lines represent the least-square regression.

TABLE III.—SEGREGATION RATE CONSTANTS IN BINARY SYSTEMS OF STEEL SPHERES

Particle Diam., 32nds of an in.	Cylinder Diam., in.	Fill Wt., Gm.	$\frac{V_l - V_s}{V_s}$	k (Min.$^{-1}$) 95% C.L.
3–5	1.00	304	3.63	0.031 ± 0.006
3–6	1.00	304	7.00	0.124 ± 0.027
3–7	1.00	304	11.18	0.283 ± 0.038
3–8	1.00	304	17.96	0.401 ± 0.067
3–5	1.00	171	3.63	0.030 ± 0.010
3–6	1.00	171	7.00	0.280 ± 0.044
3–7	1.00	171	11.18	0.407 ± 0.100
3–8	1.00	171	17.96	0.538 ± 0.121
3–5	0.75	171	3.63	0.036 ± 0.003[a]
3–6	0.75	171	7.00	0.161 ± 0.017[a]
3–7	0.75	171	11.18	0.183 ± 0.025[a]
3–8	0.75	171	17.96	0.314 ± 0.023[a]

[a] Reported previously (4).

container and particle. Vertical agitation was selected for the bulk of this work since the system of spheres is accelerated only by the bottom surface of the cylinder, and the results will correspond more closely to those expected of a large body of particles. Vertical agitation also can be expected to reduce wall effects since primary particulate motion is parallel to the sides of the container, and inhibition of motion of those particles contacting the sides is minimized. The nature and magnitude of the wall effects occurring in these studies has been evaluated by varying the dimensions of the systems and the mode of shaking and noting the changes in over-all rate of segregation and in the composition profiles within the agitation cylinder. Precise control of agitation requires that the total weight of particles be limited to a range which can be accommodated by the apparatus. For this reason, cylinders of internal diameter larger than 1 in. were not used.

Cylinders of 0.75- and 1.00-in. internal diameter were filled to various levels, and the effects of fill weight and cylinder diameter on the rate constants of several systems were determined. Results of these studies are reported in Table III. Comparison of data obtained in 1-in. cylinders for fill weights of 304 and 171 Gm. shows differences in rate constants with the higher particulate volume ratios. While this may indicate increased inhibition of separation by particle–wall interactions for the larger spheres, a second factor should be taken into account. Individual particles in 171-Gm. systems have a proportionately shorter distance to travel before becoming separated regarding the total system. This effect, however, should be independent of particle size. A further comparison of data is possible since column height is the same (about 5.5 in.) for 304-Gm. fill in the 1-in. cylinder and 171-Gm. fill in the 0.75-in. cylinder. Lower segregation rates are observed in the smaller tube, but again only with the larger particles. Good conformity to the linear relationship between particulate volume ratio and segregation rate constant was found in all systems, regardless of column diameter or fill weight.

Composition profiles at various time intervals indicate the effects of particle–wall interactions within the segregating systems. Figure 3 shows the per cent of large balls within a 1.00-in. diameter column for binary systems of $^3/_{32}$ and $^5/_{32}$ in. steel spheres. A rather symmetrical concentration curve is obtained for these systems up to infinite time (times in excess of seven half-lives). This type

curve is typical of the systems composed of smaller balls and indicates little or no wall effect.

A composition profile is shown in Fig. 4 for a 304-Gm. system of $^{3}/_{32}$ and $^{8}/_{32}$ in. steel balls in a 1.00-in. cylinder. These curves, representing particle size distribution within the column at various times, are typical of systems composed of the larger sized balls, although deviation from symmetry becomes much less marked with the intermediate sized balls studied. However, asymmetry of the composition profiles extends to smaller sized balls in the 0.75-in. cylinder than in the larger tube. Decreased mobility of the balls at the lower levels is manifested by the slower rate of segregation here compared with the upper regions of the column. However, after shaking to equilibrium for periods in excess of seven half-lives, symmetrical curves are obtained in all cases. It should be mentioned that no deviation from linearity of log $(S_\infty - S)$ *versus* time plots was evident in any case, regardless of wall effect. This evaluation of the particle–wall interactions as they occur in this work allows an appraisal of the phenomenon and its influence on the observed rate constants.

Composition profiles of the segregation of binary systems of $^{3}/_{32}$ in. *versus* $^{5}/_{32}$ in. and $^{3}/_{32}$ in. *versus* $^{8}/_{32}$ in. steel balls in 0.75-in. cylinders were compared for two modes of agitation. When these systems were run in the vertically split, squeeze-type cylinder previously described, curves which were similar to Fig. 4 were obtained with both systems. Comparison of these results with those obtained with the usual vertical agitation indicates that the latter mode of shaking produces less particle–wall interaction as would be expected.

SUMMARY

While the work presented here has been carried out on idealized systems under closely controlled conditions, several conclusions can be drawn which may be applied in principle to granulations or other mixtures of solids as they are encountered in pharmacy.

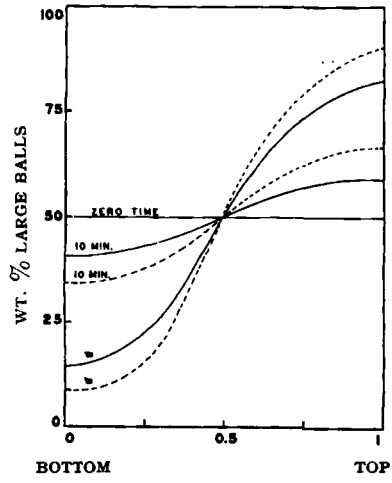


Fig. 3.—Composition profile curves in 1-in. diameter cylinders after various periods of vertical sine wave agitation for a binary system of $^{3}/_{32}$ and $^{5}/_{32}$ in. steel spheres at a 1:1 ratio (w/w). Solid lines represent a total system weight of 171 Gm., and dotted lines are for a 304-Gm. system.

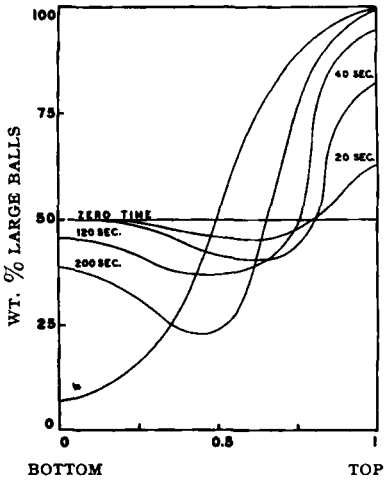


Fig. 4.—Composition profile curves in a 1-in. diameter cylinder after various periods of vertical sine wave agitation for a binary system of $^{3}/_{32}$ and $^{8}/_{32}$ in. steel spheres at a 1:1 ratio (w/w). Total weight of the balls was 304 Gm., and the curves shown are typical of those where detectable particle–wall interactions occur.

In general, segregation is not greatly influenced by density when all the particles within a given system have the same density. No detectable unmixing occurs in binary systems of equal sized spheres in which the components have a threefold difference in density. However, segregation is rapid in systems in which the small particles are more dense, but a marked retardation is evident when the larger particles are more dense. Moreover, the over-all effect of combined differences in density and size indicates a marked nonlinear interaction between these two variables.

Elasticity of the particles, reflected by the coefficient of restitution, plays a large part in segregation as observed in these studies. This is not unexpected since interparticulate transfer of kinetic energy is dependent on the degree of elasticity of the collisions.

Wall effects resulting from particle–cylinder contact cause no apparent change in the relationship between segregation rate and particle size. Linearity of the segregation rate constant as a function of particulate volume ratio is maintained within a series in which cylinder diameter and fill weight are held constant.

In application to real systems, it should be emphasized that variables other than those studied here may play a critical role in determining the segregation rate. For this reason, the conditions under which these results were obtained should be considered when using them to predict or explain granulation stability.

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Potential Antineoplastic Agents Derived from 1,2-Epoxyindan

By JOSEPH SAM and THOMAS C. SNAPP, JR.*

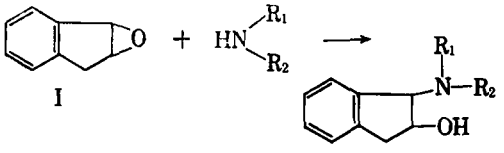
The reaction of a number of amines and aminoacid derivatives with 1,2-epoxyindan provided 1-substituted amino-2-indanols. Preliminary tests, which are not complete, have not demonstrated pronounced antineoplastic activity in the compounds.

As part of a broad program involving the investigation of potential antineoplastic agents it was desirable to investigate the preparation of a number of ring opening derivatives of epoxyindan.

Indan compounds recently have become of interest as antineoplastic agents. Connors and associates (1) observed that 1-amino-1-indancarboxylic acid possesses antitumor activity, while Leushina and co-workers (2) noted similar activity in 5-[bis(2-chloroethyl)amino]indan hydrochloride.

Although extensive investigations have been reported on cyclopentane-, cyclohexane-, and tetralin-1,2-epoxides, only a limited amount of information is available for 1,2-epoxyindan (3–6).

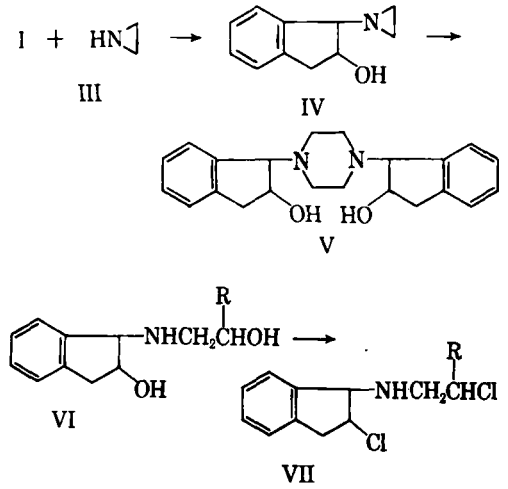
The work described in this paper involves the reaction of 1,2-epoxyindan (I) with amines to yield compounds (II) which may be considered either as potential alkylating agents and antimetabolites *per se* or which may be readily converted to potential alkylating agents.



I II

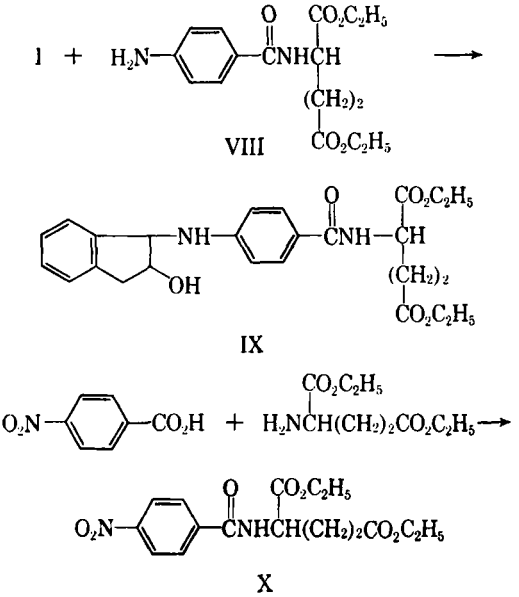
For example, the reaction of 1,2-epoxyindan (I) with aziridine (III) afforded 1-aziridinyl-2-indanol (IV) which was readily converted to 1,4-bis(2-hydroxy-1-indanyl)piperazine (V).

The latter also was prepared by the direct action of piperazine with 1,2-epoxyindan as described by Mousseron (6). The indanamines derived from 1,2-epoxyindan are listed in Table I. The nitrogen mustards (VII) were prepared by the chlorination of the corresponding alcohol (VI)



III IV V VI VII

whereas the potential antimetabolites were prepared from 1,2-epxoyindan by the reaction with amino acid derivatives. The folic acid relative (IX) was prepared in this manner. The intermediate, ethyl *N*-(*p*-nitrobenzoyl)glutamate (X), was prepared in good yield from *p*-nitrobenzoic acid and ethyl glutamate *via* the mixed anhydride reaction utilized for the preparation of peptides. The nitro group was readily reduced with platinum oxide catalyst at low hydrogenation pressures.



VIII IX X

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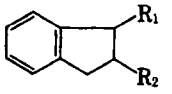
Presented to the Scientific Section, A.Ph.A., New York City meeting, August 1964.

Abstracted in part from a thesis submitted by Thomas C. Snapp, Jr., to the Graduate School, University of Mississippi, in partial fulfillment of Doctor of Philosophy degree requirements.

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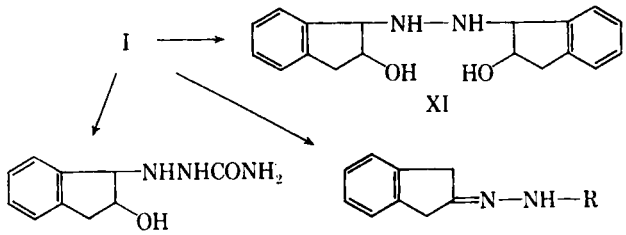
* Present address: Texas Eastman Co., Division of Eastman Kodak Co., Longview, Tex.

TABLE I.—INDANAMINE DERIVATIVES



No.	R_1	R_2	Method	Recryst. Solvent	M.p., °C.	Yield, %	Formula	Anal. C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	$N(CH_3)_2$	OH	*A*	Toluene	149–150	61	$C_{11}H_{13}NO$	75.40	75.41	7.48	7.69	8.00	8.12
2	*p*-NHC_6H_4CN	OH	*F*	Ethanol	196–197	54	$C_{16}H_{14}N_2O$	76.77	76.59	5.64	5.56	...	...
3	$NHCH_2CHOHCH_3$	OH	*A*(*B*)	Ethyl acetate	111–112	64(27)	$C_{12}H_{17}NO_2$	69.53	69.67	8.27	8.26	6.76	6.66
4	*p*-NHC_6H_4COOH	OH	*C*	Acetic acid	238–239	85	$C_{16}H_{15}NO_3$	71.36	71.26	5.61	5.38	...	...
5	*p*-$NHC_6H_4CO_2CH_2CH_3$	OH	*F*	Ethanol-water	146–147	56	$C_{18}H_{19}NO_3$	72.71	72.77	6.44	6.20	...	...
6	*p*-$NHC_6H_4CON(CH_3)_2$	OH	*C*	Ethanol-water	177–178	46	$C_{18}H_{20}N_2O_2$	72.95	73.18	6.80	6.64	9.45	9.71
7	$NHCH_2CH_2OH$	OH	*E*	Ethanol-acetone	183–184	41	$C_{11}H_{16}ClNO_2$[a]	57.51	57.70	7.02	6.94	6.10	5.98
8	*p*-$NHC_6H_4CONHCH(CH_3)_2$	OH	*F*	Ethanol	254–255	44	$C_{19}H_{22}N_2O_2$	73.52	73.51	7.15	6.97	9.03	9.04
9	*p*-$NHC_6H_4COC_9H_{16}NO_4$[b]	OH	*E*	Ethanol-water	184–185	32	$C_{25}H_{30}N_2O_6$	66.05	66.60	6.65	6.73	6.16	6.15
10	*m*-$NHC_6H_4CF_3$	OH	*C*	Ethanol	108–109	53	$C_{16}H_{14}F_3NO$	65.52	65.94	4.81	4.91	...	...
11	$NHCH_2CO_2CH_2CH_3$	OH	*C*	Toluene	113–114	62	$C_{13}H_{17}NO_3$	66.36	66.05	7.28	7.10	5.95	6.11
12	$N(CH_2CH_2OH)_2$	OH	*D*	Ethanol-ethyl acetate	154–155	42	$C_{19}H_{22}N_4O_{10}$[c]	48.93	48.90	4.76	4.53	12.01	12.32
13	$NHNHC_9H_9O$[d]	OH	*D*	Ethanol	194–196	58	$C_{18}H_{20}N_2O_2$	72.95	72.57	6.80	6.75	9.45	9.39
14	$NHCH_2CHClCH_3$	OH	*H*	Ethanol-ethyl acetate	203–204	70	$C_{12}H_{17}Cl_2NO$[a]	54.97	54.78	6.54	6.57	...	...
15	$N(CH_2)_4NCH_2CH_2Cl$[e]	Cl	*D*	Ethanol-ethyl acetate	188–189	83	$C_{15}H_{22}Cl_4N_2$[e]	48.47	48.24	5.96	6.26	7.53	7.25
16	*p*-NHC_6H_4CN	H	*J*	Ethanol-cyclohexane	87–88	41	$C_{16}H_{15}ClN_2$[a]	70.98	71.11	5.58	5.48	...	...
17	*p*-NHC_6H_4COOH	Cl	*H*	Acetic acid	202–203	64	$C_{16}H_{14}ClNO_2$	66.78	67.45	4.90	5.15	...	...
18	$NHCH_2CH_2Cl$	Cl	*H*	Ethanol-ethyl acetate	192–193	62	$C_{11}H_{14}Cl_2N$[a]	49.56	49.62	5.29	5.30	5.26	5.41
19	$NHCH_2COOH$	OH	*K*	Ethanol	233–234	64	$C_{11}H_{13}NO_3$	63.75	64.38	6.32	6.42	6.76	6.74
20	NCH_2CHCH_2[f]	OH	*K*	Toluene	136–137	53	$C_{12}H_{15}NO$	76.15	76.60	7.99	8.12	7.40	7.32
21	H[g]	O	*M*	Isopentanol	218–219	77	$C_{10}H_{11}N_2O$[h]	...	...	...	...	...	...
22	H[i]	O	*M*	Ethanol	203–204	70	$C_{15}H_{12}N_4O_4$[j]	...	...	...	...	...	...
23	$N(CH_2)_4C_9H_9O$[k]	OH	*C*(*L*)	Isopentanol	252–253	72(80)	$C_{22}H_{26}N_2O_2$	...	...	...	...	...	...
24	$NHNHCONH_2$	OH	*C*	Methanol-water	206–208	60	$C_{10}H_{13}N_3O_2$	57.96	58.67	6.32	6.86	20.28	19.62

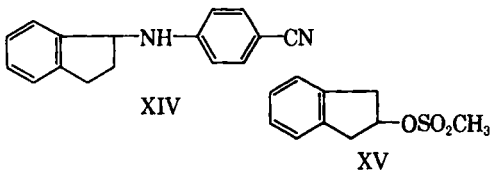
[a] Hydrochloride. [b] Ethyl *N*-[4-(2-hydroxy-1-indanylamino)benzoyl]-*dl*-glutamate. [c] Picrate. [d] Bis-(2-hydroxy-1-indanyl)hydrazine. [e] Dihydrochloride. [f] 1-(2-Methylaziridinyl)-2-indanol. [g] *Reference 14*. [h] Semicarbazone. [i] *Reference 15*. [j] 2,4-Dinitrophenylhydrazone. [k] 1,4-Di-(2-hydroxy-1-indanyl)piperazine. [l] *Reference 6*.



Several attempts were unsuccessful in the synthesis of the folic acid relative (IX) from 4-(2-hydroxy-1-indanylamino)benzoic acid and ethyl glutamate *via* the mixed anhydride reaction. Glutamic acid, morpholine, and piperidine were substituted for ethyl glutamate in this reaction, but the desired product was not obtained. In each reaction the starting materials were recovered.

The reaction of 1,2-epoxyindan with hydrazine, semicarbazide, and 2,4- dinitrophenylhydrazine also was investigated. Hydrazine and semicarbazide gave normal reaction products (XI, XII, respectively). However, 2,4-dinitrophenylhydrazine did not yield a product which could be identified. Both semicarbazide and 2,4-dinitrophenylhydrazine in the presence of 20% alcoholic sulfuric acid solution containing 30% water gave derivatives (XIII*a*, XIII*b*, respectively) of 2-indanone. The latter two products were identified by comparing melting points and infrared spectra to known samples derived from 2-indanone.

Included also in this paper is a description of the preparation of 4-(1-indanylamino)benzonitrile (XIV) and 2-indanylmethanesulfonate (XV).



EXPERIMENTAL[1]

Syntheses

The methods utilized for the preparation of the compounds in Table I are described below. Most of the amines were purchased from commercial sources; 4-amino-*N*,*N*-diethylbenzamide (7) was prepared according to the procedure described in the literature.

Indanamines.—*Method A.*—A solution of 13.2 Gm. (0.1 mole) of 1,2-epoxyindan (8) and 0.2 mole of amine in 80 ml. of dry benzene was refluxed for 48 hours. The benzene was evaporated *in vacuo*. The residue was dissolved in ether and washed with five 100-ml. portions of water. The ether portion was dried over anhydrous sulfate and then evaporated to dryness. (In the preparation of similar compounds, the extraction in ether, washing with water, and drying may be omitted.) The residue was recrystallized from a suitable solvent.

Method B.—A mixture of 0.2 mole of amine, 0.1 mole of 2-bromo-1-indanol (8), and 80 ml. of dry benzene was refluxed for 24 hours. The solution was cooled, washed with water, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to dryness. The residual solid was recrystallized from a suitable solvent

Method C.—The procedure described in *Method A* was followed, except that ethanol was used as the solvent; the reflux period was 5–10 hours.

Method D.—The procedure described in *Method A* was followed, except that no solvent was used; the mixture was heated at reflux or at 120–125° for 4 hours.

Method E.—The procedure described in *Method A* was followed, except that *n*-amyl alcohol was used as the solvent; the reflux period was 5–10 hours.

Method F.—The procedure described in *Method C* was followed, except that 0.2 Gm. of phenylacetylene /0.1 mole of epoxide was added to catalyze the reaction.

4-(2-Hydroxy-1-indanylamino)benzoic Acid.—*Method G.*—To a solution containing 75 ml. of ethanol and 30 ml. of 12 *N* aqueous sodium hydroxide was added 8.5 Gm. (0.03 mole) of 4-(2-hydroxy-1-indanylamino)benzonitrile. The mixture was refluxed until a negative test for evolution of ammonia was observed. The ethanol was evaporated *in vacuo;* the residual sodium salt was acidified with dilute hydrochloric acid. The solid was removed by filtration and recrystallized from glacial acetic acid to give 6.8 Gm. (86%) of product, m.p. 238–239°.

4-(2-Chloro-1-indanylamino)benzoic Acid.—*Method H.*—A solution of 12 Gm. (0.1 mole) of purified thionyl chloride in 50 ml. of dry chloroform was gradually added to a cooled mixture of 2 Gm. (0.1 mole) of 4-(2-hydroxy-1-indanylamino)benzoic acid and 50 ml. of dry chloroform. The mixture was refluxed for 4 hours. The solvent was distilled *in vacuo,* and the residue was neutralized with 10% aqueous sodium carbonate. Removal of the solid by filtration and recrystallization from glacial acetic acid yielded 1.7 Gm. (80%) of product, m.p. 202–203°.

1-(2-Chloroethyl)amino-2-chloroindan Hydrochloride.—*Method I.*—The procedure in *Method H* was followed, except that the reaction mixture was not neutralized; the product was isolated as the hydrochloride

4-(1-Indanylamino)benzonitrile Hydrochloride.—*Method J.*—A solution of 15.3 Gm. (0.1 mole) of 1-chloroindan (9) and 12.0 Gm. (0.1 mole) of 4-aminobenzonitrile in 100 ml. of dioxane was refluxed for 1.5 hours in an oil bath. The solution was cooled; the tan solid was isolated by filtration and recrystal-

[1] All melting points were taken on a Fisher-Johns apparatus and are uncorrected; boiling points are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 infracord spectrophotometer.

lized from an ethanol-cyclohexane mixture to yield 4 Gm. (15%) of product, m.p. 87–88°.

N-(2-Hydroxy-1-indanyl)aminoacetic Acid.—*Method K.*—A mixture of 2 Gm. (0.01 mole) of ethyl *N*-(2-hydroxy-1-indanyl)aminoacetate and 25 ml. of 0.1 *N* aqueous sodium hydroxide was stirred at room temperature for 24 hours. The resulting solution was chilled and acidified with concentrated hydrochloric acid to pH 2.5. The solid was isolated by filtration and recrystallized from ethanol to yield 1.3 Gm. (64%) of product, m.p. 233–234°.

2-Indanyl Methanesulfonate.—The general method described by Berti (10) was followed. A mixture of 4 Gm. (0.03 mole) of 2-indanol (8) and 4 Gm. (0.05 mole) of pyridine was placed in a dry 100-ml. three-necked flask fitted with a thermometer, stirrer, and dropping funnel. The mixture was cooled to 0° in an ice-salt bath, and treated with 4.6 Gm. (0.04 mole) of methanesulfonyl chloride over a 1-hour period. After stirring for 3 hours at 0°, the mixture was allowed to warm to room temperature with stirring. On addition of 50 ml. of water, a brown solid precipitated which was recrystallized from either hexane or an ethanol-water mixture to yield 4.4 Gm. (70%) of 2-indanyl methanesulfonate, m.p. 74–75°.

Ethyl N-(4-nitrobenzoyl)-dl-glutamate.—The mixed anhydride method generally utilized for the preparation of peptides was followed (11). To a stirred and cooled solution of 33.4 Gm. (0.2 mole) of *p*-nitrobenzoic acid in 250 ml. of diglyme, 20.2 Gm. (0.2 mole) of triethylamine was added dropwise. The yellow solution was maintained at 0° during the addition of 27.3 Gm. (0.22 mole) of ethyl chloroformate. The mixture was stirred at 0° for an additional hour and maintained at this temperature while a solution of 40.6 Gm. (0.2 mole) of ethyl *dl*-glutamate in 150 ml. of diglyme was added. The mixture was allowed to warm to room temperature and stirred for 12 hours. The triethylamine hydrochloride was removed by filtration, and the filtrate diluted with 1.5 L. of ice water. After refrigeration for 24 hours, the product was isolated by filtration. Recrystallization from an ethanol-water mixture gave 45 Gm. (56%) of product, m.p. 96–97° [lit. m.p. 97–98° (12)].

Ethyl N-(4-aminobenzoyl)-dl-glutamate.—Seventeen grams (0.05 mole) of ethyl 4-nitrobenzoyl-*dl*-glutamate in 200 ml. of ethanol was hydrogenated over 0.2 Gm. of platinum oxide catalyst at 40–50 p.s.i. at room temperature. Hydrogenation required 1 hour. The catalyst was removed by filtration, and the solvent evaporated *in vacuo*. Recrystallization of the residual solid from dilute ethanol yielded 13.6 Gm. (83%) of product, m.p. 142–143° [lit. m.p. 139–141° (12)].

1,4-Di-(2-hydroxy-1-indanyl)piperazine.—*Method L.*—A mixture of 25 ml. of 50% aqueous ethanol, 2.4 Gm. (0.015 mole) of sodium iodide, and 1 Gm. (0.006 mole) of 1-aziridinyl-2-indanol was refluxed on a steam bath for 24 hours. After cooling, the solid was isolated by filtration and recrystallized from isoamyl alcohol to yield 0.75 Gm. (80%) of product, m.p. 252–253° [lit. m.p. > 220° dec.(6)].

The infrared spectra of the products from *Methods C* and *L* were identical; a mixed melting point determination showed no depression.

4-Amino-N-isopropylbenzamide.—A solution of 10.4 Gm. (0.05 mole) of 4-nitro-*N*-isopropylbenzamide (13) in 200 ml. of ethanol was treated with 0.3 Gm. of 5% palladium-on-carbon catalyst and reduced in a Parr hydrogenator at 3 atm. The catalyst was removed by filtration, and the solvent was distilled *in vacuo*. Recrystallization of the residue from dilute ethanol yielded 8.1 Gm. (90%) of product, m.p. 95–96°.

Anal.—Calcd. for $C_{10}H_{14}N_2O$: C, 67.38; H, 7.92; N, 15.72. Found: C, 67.19; H, 8.21; N, 15.43.

2-Indanone Semicarbazone and 2,4-Dinitrophenylhydrazone.—*Method M.*—The procedure described in *Method A* was followed, with the exception that 20% ethanolic sulfuric acid solution containing 30% water was used as the solvent.

Pharmacological Results[2]

Compounds 1–8, 10, 11, 14, 15, 16, 18, 19, 20, and 23 of Table I have been submitted for testing for antineoplastic activity. Although the tests are not complete, pronounced antineoplastic activity has not been observed in the compounds tested so far.

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[2] Screening for antineoplastic activity was coordinated by the Cancer Chemotherapy National Service Center.

Enzyme Inhibitors I

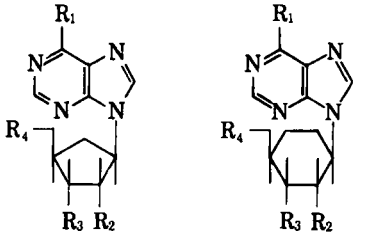
Inhibition of Adenosine Deaminase by Isosteric Nucleosides

By HOWARD J. SCHAEFFER, S. MARATHE, and VITAUTS ALKS

A series of isosteric nucleosides has been evaluated as inhibitors of adenosine deaminase in an attempt to determine which sites of adenosine are important for binding to the enzyme. The 6-amino group in the purine nucleus makes a strong contribution to binding, and the enzyme appears to be specific for amino since other groups (SH, OH, $NHNH_2$, Cl, H) at the 6-position were noninhibitory. For effective inhibition the purine must be substituted in the 9-position; the effect of the stereochemistry of a 2′-hydroxyl group on certain cyclic substituents at the 9-position of the purine nucleus has been measured.

THE CHEMISTRY and biochemistry of purine nucleosides and purine nucleotides have been studied extensively during the past 20 years, with the result that certain compounds have been prepared which are capable of inhibiting the growth of malignant cells (1). These studies have also resulted in an understanding of the *de novo* synthesis of purine nucleotides and of certain other purine transformations (2, 3). Little is known, however, about which of the atoms or functional groups in purine nucleosides and tides are required for binding to the active site of the various enzymes. Since it is well known that purine ribonucleosides and purine ribonucleotides are easily cleaved hydrolytically or enzymatically, certain purine nucleosides and nucleotides which may be effective agents in inhibiting the growth of malignant cells become ineffective *in vivo* because they are rapidly destroyed by cleavage into a purine and a carbohydrate moiety (4). In an attempt to circumvent this difficulty, we have synthesized a novel class of compounds which are hydrolytically and presumably enzymatically stable, but which should be capable of binding to an enzyme that normally employs a purine ribonucleoside as the substrate. These compounds, the isosteric nucleosides, are purines that contain at the 9-position a cyclopentyl or a cyclohexyl ring which is substituted in such a manner that it simulates sterically the sugar moiety of a nucleoside (I and II) (5, 6).

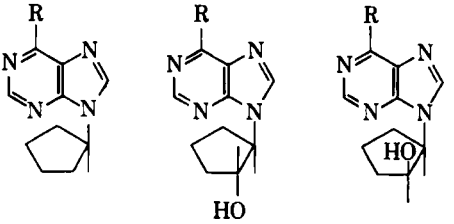
To obtain information about how a purine nucleoside binds to an enzyme, the authors have initiated a study of the effect of certain isosteric nucleosides on the enzyme, adenosine deaminase. This paper describes the effect on binding of certain substituents at the 6-position and at the 9-position of I and II. The compounds



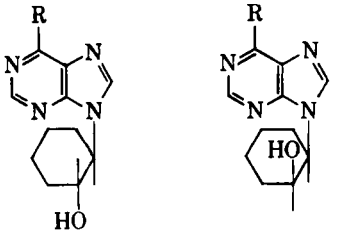
I II

[where R_1 may be NH_2, NHMe, NMe_2, SH, $NHNH_2$, H, or Cl; and R_2, R_3, and R_4 may be various combinations of H, OH, or NH_2.]

which were employed in this investigation fall into five types, depending upon the substituent at the 9-position of the purine nucleus, as shown by structures III, IV, V, VI, and VII.



III IV V



VI VII

[where the R group in each case = NH_2, SH, $NHNH_2$, H, and Cl.]

EXPERIMENTAL[1]

Reagents and Isosteric Nucleosides.—Adenosine and adenosine deaminase were purchased from the

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[1]The ultraviolet recordings were made on a Perkin-Elmer model 4000 A spectrophotometer.

TABLE I.—K_m OF ADENOSINE AND K_i OF ISOSTERIC NUCLEOSIDES WITH ADENOSINE DEAMINASE[a]

Compd.	$K_m \times 10^5 M$	$K_i \times 10^5 M$
Adenosine	7.3	
III (R = NH_2)		49.3
IV (R = NH_2)		13.5
V (R = NH_2)		17.4
VI (R = NH_2)		13.9
VII (R = NH_2)		21.1

[a] The values reported are at pH 7.6 and at 25° in 0.05 *M* phosphate buffer.

Sigma Chemical Co. Compounds of type III, where R = NH_2, SH, $NHNH_2$, H, and Cl, were prepared according to the procedure of Montgomery and Temple (7). Compounds of type IV, V, VI, and VII, where R = NH_2, SH, $NHNH_2$, H, and Cl, were prepared as previously described (5, 6).

trans - 2 - (6 - Methylamino - 9 - purinyl)cyclopentanol.—A 0.288-Gm. (1.23 mmole) sample of *trans*-2-(6-chloro-9-purinyl)cyclopentanol (6) was dissolved in 10 ml. of ethanol, and 10 ml. of 40% methylamine in water was added. The mixture was heated under reflux for 3 hours, and the volatile materials were removed *in vacuo;* the residue was extracted with hot benzene (4 × 30 ml.). The benzene extract on cooling gave 0.160 Gm. (59.3%) of the crude product, m.p. 194–195°. Three recrystallizations of the crude product from benzene gave the pure sample, m.p. 194–195°; ν in cm.$^{-1}$ (KBr); 3300 and 3150 (NH); 1640 (C=N).

Anal.[2]—Calcd. for $C_{11}H_{15}N_5O$: C, 56.63; H, 6.48; N, 30.03. Found: C, 56.88; H, 6.51; N, 29.92.

Assay Procedure

The general method of assay which was employed is described in Colowick and Kaplan (8) and involves measuring the rate of disappearance of the absorption band of adenosine at 265 mμ. All reactions were run in 0.05 *M* phosphate buffer at pH 7.6 at 25°. The stock solutions of all reagents were prepared in 0.05 *M* phosphate buffer at pH 7.6.

RESULTS

The initial testing for the ability of the isosteric nucleosides to inhibit adenosine deaminase was carried out at substrate concentrations which were approximately 20% below that concentration which was required to saturate the enzyme. The concentration of adenosine which was employed for the initial testing was 0.061 m*M*. Compounds that did not show significant inhibition of the enzymatic reaction at concentrations three to four times the concentration of the substrate were classified as noninhibitory. For those compounds which were significantly inhibitory, K_i's were determined by the method of Lineweaver and Burk (9). For each determination of K_i, two different concentrations of inhibitor were used, and each point on the plot was an average of at least two determinations. Finally, the total procedure was repeated, so that each K_i that is reported is an average of four evaluations. Table I gives the results of the determination of K_i for five different isosteric nucleosides.

[2] Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Compounds III–VII, with R = SH, $NHNH_2$, H, or Cl, did not exhibit significant inhibition of adenosine deaminase when they were present at a concentration three to four times that of the substrate. *cis-* and *trans*-2-Aminocyclopentanol and *cis-* and *trans*-2-aminocyclohexanol did not inhibit the enzymatic reaction even when they were present in a concentration 30 times greater than adenosine. Adenine, inosine, and 5′-adenylic acid were neither substrates nor inhibitors at three to four times substrate concentration, whereas 2′-deoxyadenylic acid was a substrate.

The results of a typical experiment which was used to evaluate K_m for adenosine and K_i for VI (R = NH_2) are given in Table II.

DISCUSSION

In 1956, Feigelson and Davidson (10) studied the inhibition of adenosine deaminase by 8-azaguanine in an attempt to explain the carcinostatic effects of this compound and found that 8-azaguanine was a noncompetitive inhibitor with regard to substrate. They obtained indirect evidence that the inhibitor exhibits a higher affinity for the enzyme-substrate complex than for the free enzyme.

Our data give information on the effect on binding to adenosine deaminase by various substituents at the 6-position and at the 9-position of the purine nucleus. Concerning the 6-position, it is apparent that an amino group must be present in order for a reversible complex to be formed, since compounds III–VII, with R = SH, $NHNH_2$, H, or Cl, were essentially noninhibitory. However, when a 6-amino group was present, all of the compounds were inhibitors of adenosine deaminase, and they exhibited competitive inhibition as shown by the reciprocal plot method of Lineweaver-Burk. In addition, it should be noted that binding by the substituent at the 6-position appears to be specific for an amino group and not just a basic group since the 6-hydrazino compounds (III–VII) were not inhibitory. It might be suggested that the 6-hydrazino compounds did not bind to the enzyme because the hydrazino group is larger than an amino group, and if a group smaller than amino were present, inhibition would occur. This reasoning, however, was incorrect since those compounds with a 6-hydrogen substituent were also noninhibitory. In addition, the 6-amino group may be secondary, but cannot be tertiary and still cause inhibition.

TABLE II.—INHIBITION OF ADENOSINE DEAMINASE BY *trans*-2-(6-AMINO-9-PURINYL)CYCLOHEXANOL (VI, R = NH_2)

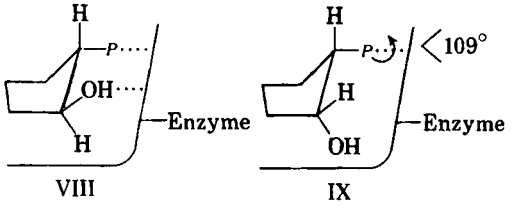
Run No.	m*M* Concn. of Adenosine	Rate[a,b]	m*M* Concn. of VI (R = NH_2)	Rate[c]	m*M* Concn. of VI (R = NH_2)	Rate[d]
1	0.085	0.98	0.082	0.78	0.123	0.69
2	0.077	0.94	0.082	0.74	0.123	0.65
3	0.070	0.90	0.082	0.68	0.123	0.61
4	0.062	0.84	0.082	0.63	0.123	0.55
5	0.054	0.77				

[a] All rates that are given are for the initial portion of the reaction in which the change in absorbance is linear with respect to time. [b] Change in O.D./minute for the various substrate concentrations shown in column 2. [c] Change in O.D./minute for the various substrate concentrations shown in column 2 in the presence of 0.082 m*M* concentration of VI (R = NH_2). [d] Same as *footnote c*, but in the presence of 0.123 m*M* concentration of VI (R = NH_2).

For example, IV and V (R = NMe_2) were both noninhibitory, whereas IV (R = NHMe) did inhibit the enzymatic reaction, but it was approximately one-tenth as effective an inhibitor as IV (R = NH_2). It appears, therefore, that steric factors may play a critical role in binding by the 6-position; but additional work is necessary before this question can be answered unambiguously. The result of such a study will be the subject of a future paper.

To assess the importance of substituents at the 9-position of the purine nucleus and of hydroxyl groups on the 9-substituent as binding points to adenosine deaminase, we determined the K_i of each compound (III–VII) where the 6-substituent was held constant (see Table I). It was anticipated that the hydroxyl groups in the ribose moiety of adenosine would make a contribution in binding to the enzyme; by studying those compounds in which the stereochemistry of the substituents at the 9-position is known, it should be possible to evaluate the approximate contribution by the 2′-hydroxyl group. Not unexpectedly, the unsubstituted cyclopentyl compound (III, R = NH_2) exhibited the largest K_i, thus, the enzyme-inhibitor complex is dissociated to a larger extent than with the other inhibitors. The *trans*-2-hydroxycyclopentyl (IV, R = NH_2) and the *trans*-2-hydroxycyclohexyl analogs (VI, R = NH_2) exhibited the smallest K_i's. and the two derivatives with the *cis* hydroxyl groups (V and VII, R = NH_2) exhibited K_i's which were similar and only slightly larger than the *trans* compounds. Since adenine is not inhibitory, it is apparent that a substituent is necessary at the 9-position of the purine for binding to the enzyme. The hydroxyl group at the 2-position of the cycloaliphatic ring contributes to binding, evidenced by the reduction in K_i in IV–VII (R = NH_2) relative to III (R = NH_2). That the K_i's of IV, V, VI, and VII (R = NH_2) are rather similar reveals that the stereochemistry of the hydroxyl group at the 2′-position of the cycloaliphatic ring does not appear to be so critical as might be presumed.

The authors believe that this lack of steric requirements for the 2′-hydroxy group may be explained in the following way. Because the *trans* compound (IV, R = NH_2) is a close structural analog of adenosine which has the same stereochemistry of the 2′-hydroxyl group and because IV (R = NH_2) is a competitive inhibitor of adenosine deaminase it is reasonable to assume that the inhibitor and substrate possess a similar conformation and that they bind at essentially the same site on the enzyme. Thus one can postulate that the *trans* compound IV (R = NH_2) binds to the enzyme through the 2′-hydroxyl group and 6-aminopurine nucleus by means of the conformation represented by VIII. The *cis* derivative (V, R = NH_2) may be represented by the conformation shown in IX. Since the K_i's of the two compounds are nearly equal, it follows that the binding through the 2′-hydroxyl groups is almost equal. From a stereochemical viewpoint this result can be explained by assuming that the 6-aminopurinyl nucleus of the *cis* compound binds to the enzyme in exactly the same manner as it does in VIII. However in the case of IX a rotation about the C-N bond connecting the 1′-carbon of the cyclopentyl ring and the 9-position of the purine nucleus must occur. The result of this rotation in IX in which the purine nucleus is not rotated would allow the *cis*-2′-hydroxyl to bind to the same place on the enzyme that it does in the *trans* compound (VIII). However when the rotation does occur in IX the cyclopentyl ring must occupy a plane different from when VIII is bound to the enzyme. A similar argument can be presented to explain the binding of the *trans*- and *cis*-2′-hydroxycyclohexyl analogs (VI and VII, R = NH_2). Thus it appears that the active site of adenosine deaminase has a large bulk tolerance in the area where the 9-substituent of the adenosine analogs bind.[3] The study of the bulk tolerance and the steric requirements for binding to adenosine deaminase is continuing in this laboratory.



P = 6-Aminopurinyl

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[3] A different argument to explain that the binding of the compounds with *cis*- and *trans*-2′-hydroxy groups is nearly equal can be advanced if it is assumed that the *trans* compounds bind as in VIII by means of a hydrogen bond to a point on the enzyme that is close to the plane of the 5-membered ring. The *cis* compound (IX) could also form a hydrogen bond to the same place on the enzyme, even though the oxygen atom is above the plane of the 5-membered ring because of the free rotation of the C—O—H bonds. At present, it is impossible to differentiate between the two types of binding; however, studies on other compounds are planned to determine which of the modes of binding is important.

Enzyme Inhibitors II

Synthesis of *trans*-3-(6-Substituted-9-purinyl)cyclohexanols as Adenosine Deaminase Inhibitors

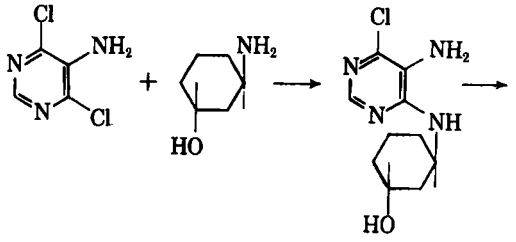
By HOWARD J. SCHAEFFER, K. K. KAISTHA, and S. K. CHAKRABORTI

The syntheses of *trans*- and *cis*-3-[9-(6-chloropurinyl)] cyclohexanols (IV and XI) have been accomplished by condensation of 5-amino-4,6-dichloropyrimidine with *trans*- and *cis*-3-aminocyclohexanol followed by ring closure of the resultant pyrimidine to the desired purines. Several 6-substituted analogs of IV and XI were prepared by nucleophilic displacement of the 6-chloro group. Evaluation of the resultant isosteric nucleosides as inhibitors of adenosine deaminase revealed that those compounds with a 6-amino group were inhibitory and that the 3′-hydroxyl group, whether *trans* or *cis*, is not involved in binding to the enzyme.

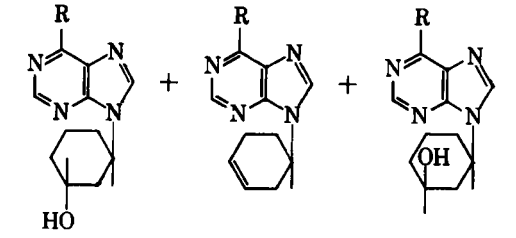
PURINE NUCLEOSIDES are utilized as substrates by a variety of enzymes, but few studies have been made to determine which of the atoms and functional groups of the nucleoside are important for binding to the enzyme. Recently, a number of nucleoside antibiotics has been discovered which are capable of inhibiting certain enzymatic reactions. For example, psicofuranine has been shown to be an inhibitor of the enzyme, xanthosine-5′-phosphate aminase (1, 2). In addition, the enzyme, adenosine phosphorylase, has been shown to be inhibited by the antibiotic, tubercidin (3). Both of these antibiotics are 6-aminopurines in which an unusual sugar is present at the 9-position.

The authors have been interested in the chemistry and biochemistry of purine nucleosides but have been concerned with the ease by which the ribose moiety can be removed hydrolytically and enzymatically from the purine nucleus. Consequently, we have been interested in preparing compounds which resemble nucleosides sterically but which are presumably stable to hydrolytic or enzymatic cleavage. These compounds, the isosteric nucleosides, are purines which are substituted at the 9-position by a cycloaliphatic group, which in turn may be hydroxylated so that it resembles the carbohydrate moiety of a nucleoside (4, 5). We have previously described the results of the enzymatic evaluation using adenosine deaminase with those isosteric nucleosides which were substituted at the 9-position of the purine nucleus with cyclopentyl, *cis*- and *trans*-2-hydroxycyclopentyl and *cis*- and *trans*-2-hydroxycyclohexyl groups (6). The present paper describes the synthesis and evaluation of *trans*-3-(6-substituted-9-purinyl)-cyclohexanols as inhibitors of adenosine deaminase.

Chemistry.—The method which was selected for the synthesis of the desired purines is a modification of the elegant method of Montgomery and Temple (7). This method involves the condensation of 5-amino-4,6-dichloropyrimidine (I) with an appropriate amine, followed by ring closure of the condensation product to give the appropriate purine. This method is particularly valuable when it is



I II III



IV, R=Cl
V, R=NH_2
VI, R=NHMe
VII, R=NMe_2
VIII, R=$NHNH_2$
IX, R=SH

X, R=Cl

XI, R=Cl
XII, R=NH_2

desired to synthesize 6-chloro-9-substituted purines uncontaminated by the corresponding 6-chloro-7-substituted isomer, which would result by alkylation of 6-chloropurine. Thus, catalytic hydrogenation of *m*-acetamidophenol with a rhodium-on-alumina catalyst gave as the major product *trans*-3-acetamidocyclohexanol, which was separated from the *cis* isomer by fractional crystallization (8, 9).

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Saponification of the *trans* isomer gave the desired *trans*-3-aminocyclohexanol (II) (9), which on condensation with I in the presence of triethylamine gave the substituted pyrimidine (III) in good yield. Attempted cyclization of III with diethoxymethyl acetate resulted in considerable decomposition, even though this appears to be the reagent of choice for related purine syntheses (7). Finally, when III was allowed to react with ethyl orthoformate and the crude reaction product chromatographed on neutral alumina, a 35% yield of IV was obtained along with a 20% yield of the dehydrated product X. The identity of X was established by its conversion into 9-cyclohexylpurine (10). Furthermore, since 6-chloro-9-(2-cyclohexenyl) purine is a known compound (4) and is different from X, the double bond in X must be at the 3-position of the cyclohexenyl ring. In a subsequent preparation of IV, in which a crude sample of II had been employed in addition to the two products previously isolated (IV and X), a third product (XI) was obtained which, on the basis of its elemental analysis, was isomeric with IV. Consequently, it became necessary to establish the stereochemistry of the two products (IV and XI) since they were to be used to evaluate the mode of binding to adenosine deaminase. The assignment of stereochemistry was done by both I.R. and NMR analyses. It was observed that the infrared spectrum of XI exhibited strong absorption at 1060 cm.$^{-1}$, whereas IV was essentially free of absorption in this region. It is known that compounds with an equatorial hydroxyl absorb near 1050 cm.$^{-1}$, whereas compounds with axial hydroxyl exhibit little or no absorption in that region (11). It is reasonable to assume that the *cis* purine would have its substituents diequatorial; whereas in the *trans* compound the bulky purine nucleus would occupy the equatorial position which would force the hydroxyl group to occupy the axial position. Therefore, the purine which exhibits absorption at 1060 cm.$^{-1}$ (XI) is *cis*, and the compound which is free of absorption in that region (IV) is *trans*. An identical assignment of configuration was made on the basis of the NMR spectra. Several investigators have found that the NMR signal of the hydrogen on the carbon atom bearing the hydroxyl group in a cyclohexanol exhibits a half-width of 7 cps. if the hydrogen is equatorial, but exhibits a half-width of 22 cps. if the hydrogen is axial (12–14) and that the signal from an equatorial hydrogen is approximately 0.5 p.p.m. downfield from the axial hydrogen (15). In our investigation, one compound (IV) exhibited a mutiplet at δ4.51 with a half-width of 8 cps. for the hydrogen on the carbon atom bearing the hydroxyl group (C_3'), whereas the other compound (XI) exhibited a multiplet at δ3.92 with a half-width of 23 cps. for the corresponding hydrogen. Clearly this establishes that the hydrogen at C_3' in IV is equatorial, whereas the hydrogen at C_3' in XI is axial. In addition, in both compounds the NMR signal for the hydrogen at C_1' was a multiplet with a half-width of 22 cps. These results establish that in both IV and XI the hydrogen at C_1' is axial (12–14); consequently, the purine nucleus must occupy the equatorial position. These results establish that in IV the hydroxyl and purine groups are *trans*, whereas in XI these groups are *cis*.

The key intermediates, IV and XI, were individually converted into their corresponding 6-substituted derivatives by modification of the known procedures; the experimental details of these transformations are described under *Experimental*.

EXPERIMENTAL[1]

trans-3-(5-Amino-6-chloro-4-pyrimidinylamino)cyclohexanol (III).—A solution of 5.05 Gm. (31.0 mmoles) of 5-amino-4,6-dichloropyrimidine, 3.58 Gm. (31.1 mmoles) of *trans*-3-aminocyclohexanol,[2] and 3.10 Gm. (31.1 mmoles) of triethylamine in 46 ml. of *n*-butyl alcohol was heated under reflux for 6.5 hours, then the volatile materials were removed *in vacuo*. The residue was triturated with 20 ml. of water, and the resultant solid was collected by filtration. Recrystallization of the crude product from aqueous methanol gave 5.8 Gm. (77%) of the desired product, m.p. 228–230°. For analysis, a small sample was recrystallized from aqueous methanol, m.p. 229–231°.

Anal.[3]—Calcd. for $C_{10}H_{15}ClN_4O$: C, 49.50; H, 6.23; N, 23.08. Found: C, 49.39; H, 6.41; N, 23.01.

Cyclization of trans-3-(5-Amino-6-chloro-4-pyrimidinylamino)cyclohexanol with Triethyl Orthoformate.—A mixture of 2.0 Gm. (8.2 mmoles) of III and 25 ml. of triethyl orthoformate was heated under reflux for 48 hours, then the volatile materials were removed *in vacuo*. The residue was allowed to react at 0° with 25 ml. of a 20% solution of ammonia in methanol for 20 hours. Concentration of the mixture gave a viscous residue which could not be crystallized. Consequently, it was chromatographed on neutral alumina (Grade I, 40 Gm.). Elution of the column with 100 ml. of benzene gave a fraction which crystallized from ethyl acetate; yield, 0.42 Gm. (20%), m.p. 152–155°. Two recrystallizations of the crude material from ethyl acetate gave the analytical sample, which has been identified as 6-chloro-9-(3-cyclohexenyl)-purine (X), m.p. 162°; $\lambda_{max.}^{pH1}$ 265 (ϵ, 7,820); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3150 (C=C); 1590 and 1560 (C=C and C=N).

Anal.—Calcd. for $C_{11}H_{11}ClN_4$: C, 56.29; H, 4.73; N, 23.87. Found: C, 56.17; H, 4.80; N, 23.80.

Elution of the alumina column with an additional 100 ml. of benzene followed by 75 ml. of chloroform gave a small amount of resinous substance which was discarded. Subsequent elution of the column with chloroform (300 ml.) and evaporation of the eluate gave a residue which, on trituration with ethyl acetate, gave a colorless crystalline solid; yield, 0.70 Gm. (35%), m.p. 150–155°. Several recrystallizations of the crude product from benzene gave the analytical sample of *trans*-3-(6-chloro-9-purinyl)cyclohexanol (IV), m.p. 159–160°; $\lambda_{max.}^{pH1}$ 264 (ϵ, 9,760); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3350(OH); 1590 and 1560 (C=C and C=N).

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[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra and enzyme rates were determined on a Perkin-Elmer model 4000 A spectrophotometer. The melting points were determined on a Kofler Heizbank and are corrected.

[2] This compound was prepared by the catalytic hydrogenation of *m*-acetamidophenol essentially by the procedure described in the literature (9), except that a 5% rhodium-on-alumina catalyst was employed.

[3] Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Anal.—Calcd. for $C_{11}H_{13}ClN_4O$: C, 52.28; H, 5.18; N, 22.17. Found: C, 52.46; H, 5.30; N, 21.93.

In a larger experiment which employed crude II, a third compound was eluted in a 10% yield from the alumina column after IV and X were removed. Recrystallization of this crude product gave *cis*-3-(6-chloro-9-purinyl)cyclohexanol (XI), m.p. 190°; $\lambda_{max.}^{pHl}$ 265 (ϵ, 12,400); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3350 (OH); 1590 and 1560 (C=C and C=N); 1060 (C—O—H).

Anal.—Calcd. for $C_{11}H_{13}ClNO$: C, 52.28; H, 5.18; N, 22.17. Found: C, 52.39; H, 5.29; N, 22.32.

trans-3-(6-Amino-9-purinyl)cyclohexanol (V).—A solution of 247 mg. (0.98 mmole) of IV in 5 ml. of liquid ammonia was heated in a stainless steel bomb at 55° for 16 hours. The ammonia was allowed to evaporate at room temperature, and the residue was extracted with chloroform. Evaporation of the chloroform solution gave 224 mg. (98%) of crystalline solid, m.p. 238°. Recrystallization of the crude product from aqueous ethanol, then from absolute ethanol, gave the analytical material, m.p. 241–243°; $\lambda_{max.}^{pHl}$ 259 (ϵ, 15,550); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3400 (OH and NH_2); 1655 (NH_2); 1590 and 1550 (C=C and C=N).

Anal.—Calcd. for $C_{11}H_{15}N_5O$: C, 56.63; H, 6.48; N, 30.03. Found: C, 56.57; H, 6.60; N, 30.08.

trans-3-(6-Methylamino-9-purinyl)cyclohexanol (VI).—A solution of 200 mg. (0.79 mmole) of IV in 5 ml. of ethanol and 5 ml. of 40% aqueous methylamine was heated under reflux for 2 hours, then evaporated to dryness *in vacuo*. The residue was extracted with chloroform; the insoluble methylamine hydrochloride, m.p. 228°, was removed by filtration. The residue, obtained after evaporation of the filtrate, on crystallization from benzene gave 165 mg. (84.6%) of the product, m.p. 173–175°. Recrystallization of the crude product from chloroform–benzene mixture gave pure VI, m.p. 175°; $\lambda_{max.}^{pHl}$ 264 (ϵ, 15,300); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (OH and NH); 1650 (NH); 1575 and 1530 (C=C and C=N).

Anal.—Calcd. for $C_{12}H_{17}N_5O$: C, 58.27; H, 6.92; N, 27.58. Found: C, 58.12; H, 7.04; N, 27.45.

trans-3-(6-Dimethylamino-9-purinyl)cyclohexanol (VII).—A solution of 150 mg. (0.62 mmole) of IV in 5 ml. of ethanol and an equal volume of aqueous 25% dimethylamine was heated under reflux for 2 hours, and the mixture was evaporated to dryness *in vacuo*. An aqueous solution (5 ml.) of the residue was stirred with 2 Gm. of Dowex 1-X2 resin in CO_3 form for 2 hours. The resin was removed by filtration, and the aqueous filtrate was evaporated *in vacuo* to give a solid residue which, after several crystallizations from benzene, gave pure VII, m.p. 156°; yield, 78 mg. (48%); $\lambda_{max.}^{pHl}$ 269 (ϵ, 15,660); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (OH); 1590 and 1550 (C=C and C=N).

Anal.—Calcd. for $C_{13}H_{19}N_5O$: C, 59.75; H, 7.33; N, 26.80. Found: C, 59.65; H, 7.35; N, 26.90.

trans-3-(6-Hydrazino-9-purinyl)cyclohexanol (VIII).—To 1.5 ml. of hydrazine (95%) was added over a period of 2 minutes 0.17 Gm. (0.67 mmole) of IV. Initially, the mixture was cooled in an ice-bath, then stirred at room temperature for 2 hours. The residue, obtained after removing the volatile materials *in vacuo*, was repeatedly digested with small volumes of chloroform to extract the desired product from the insoluble hydrazine hydrochloride. The combined chloroform extracts on evaporation yielded 100 mg. (60.2%) of the crude product, which after two crystallizations from a methanol–ethyl acetate mixture gave pure VIII, m.p. 175° dec. $\lambda_{max.}^{pHl}$ 263 (ϵ, 12,500); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (OH); 1625 (NH_2); 1575 and 1525 (C=C and C=N).

Anal.—Calcd for $C_{11}H_{16}N_6O$: C, 52.26; H, 6.58; N, 33.25. Found: C, 52.50; H, 6.64; N, 33.37.

trans-3-(6-Mercapto-9-purinyl)cyclohexanol (IX).—A solution of 252 mg. (1.00 mmole) of IV and 76.1 mg. (1.00 mmole) of thiourea in 5 ml. of *n*-propyl alcohol was heated under reflux for 1 hour. The product precipitated from the reaction mixture after a short time of refluxing. After cooling, the solid was collected by filtration, washed with cold *n*-propyl alcohol, and dried, m.p. 340–350°; yield, 230 mg. (95%). A portion of the product was crystallized from a large amount of a cellosolve and methanol mixture to give pure IX, m.p. 345–350°; $\lambda_{max.}^{pHl}$ 227 (ϵ, 8,750) and 325 (ϵ, 17,500); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3450 (OH); 1595 and 1530 (C=C and C=N).

Anal.—Calcd for $C_{11}H_{14}N_4OS$: C, 52.78; H, 5.64; N, 22.4. Found: C, 52.46; H, 5.50; N, 22.47.

cis-3-(6-Amino-9-purinyl)-cyclohexanol (XII).—A solution of 200 mg. (0.79 mmole) of XI in 10 ml. of methanol saturated with ammonia was heated in a stainless steel bomb at 55° for 20 hours. The volatile materials were removed *in vacuo*, and the crystalline residue was then extracted with hot chloroform (5 × 10 ml.). The solid residue, obtained on evaporating the chloroform solution, was crystallized from methanol to yield 100 mg. (54%) of the desired product, m.p. 215–218°. Two recrystallizations from methanol gave the analytically pure *cis*-3-(6-amino-9-purinyl)-cyclohexanol, m.p. 222°; $\lambda_{max.}^{pHl}$ 258.5 (ϵ, 16,050); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3375, 3225 (OH and NH_2); 1655 (NH_2); 1600 and 1560 (C=C and C=N).

Anal.—Calcd for $C_{11}H_{15}N_6O$: C, 56.63; H, 6.48; N, 30.03. Found: C, 56.70; H, 6.56; N, 29.83.

9-Cyclohexylpurine.—A small sample of X was hydrogenated with a palladium-on-charcoal catalyst in ethanol in the presence of magnesium oxide. The product which was obtained, m.p. 92°, was identical to 9-cyclohexyl-purine, which has previously been reported in the literature (10).

Reagents and Assay Procedure

Adenosine and adenosine deaminase were purchased from the Sigma Chemical Co. The general method of assay employed is described in Colowick and Kaplan (16) and involves measuring the rate of disappearance of the absorption band of adenosine at 265 mμ. All reactions were run in 0.05 *M* phosphate buffer at pH 7.6 at 25°. The stock solutions of all reagents were prepared in 0.05 *M* phosphate buffer at pH 7.6. For the assay, the cell contained a total volume of 3.1 ml., which was 0.066 m*M* with respect to adenosine. In those cases where inhibition was studied, varying amounts of the buffer were replaced by the appropriate volume of a solution of the inhibitor in buffer. A sufficient amount of enzyme was used, so that the initial rate of reaction gave a change of approximately 0.8–1.0 absorbance units per minute.

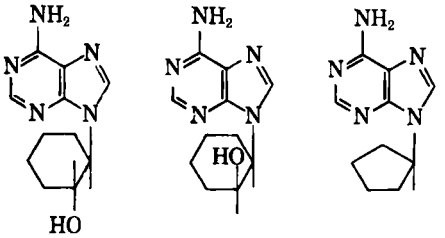
TABLE I.—PARTIAL INHIBITION OF ADENOSINE DEAMINASE BY ISOSTERIC NUCLEOSIDES

Compd.	Concn., m*M*[a]	*Vo/V*	% Inhibition
V	0.12	1.14	13
XIII	0.12	1.64	39
XII	0.12	1.14	13
XIV	0.12	1.28	22
XV	0.12	1.16	14

[a] The concentration of adenosine in all experiments was 0.066 m*M*.

RESULTS

When the isosteric nucleosides were tested as inhibitors of adenosine deaminase, two of the compounds were inhibitory (V and XII); the remaining compounds, when tested at concentrations three to four times that of the substrate, were essentially noninhibitory. A plot of *Vo/V versus I*, where *Vo* = initial velocity of the enzymic reaction in the absence of inhibitor, *V* = initial velocity of the enzymic reaction in the presence of inhibitor, and *I* = concentration of inhibitor (17) for V and XII, revealed a nonlinear relationship of *Vo/V* to the concentration of inhibitor. Consequently, V and XII were not evaluated by this technique; rather the percentage inhibition of a 0.12 m*M* solution of inhibitor was compared to the percentage inhibition of a 0.12 m*M* solution of a close structural analog. Thus, V and XII were compared to *trans*-2-(6-amino-9-purinyl) cyclohexanol (XIII), *cis*-2-(6-amino-9-purinyl) cyclohexanol (XIV), and (6-amino-9-purinyl)-cyclopentane (XV). The results of such a comparison are given in Table I.



XIII XIV XV

DISCUSSION

In some earlier work on isosteric nucleosides as inhibitors of adenosine deaminase, in which the purine nucleus was substituted at the 9-position by cyclopentyl, *cis*- and *trans*-2-hydroxycyclopentyl, and *cis*- and *trans*-2-hydroxycyclohexyl groups, those compounds with a 6-amino group were the most effective inhibitors of the enzymatic reaction (6). In addition, the 2′-hydroxyl group of the isosteric nucleosides makes a contribution to binding since the 2′-hydroxy compounds were more effective inhibitors than the cyclopentyl compound. The isosteric nucleosides with a *trans*-2′-hydroxyl group were slightly more effective inhibitors than those compounds with a *cis*-2′-hydroxyl group (6).

In the present work, we have also observed that inhibition of adenosine deaminase occurred only with those compounds that contained an amino group at the 6-position of the purine nucleus (V and XII). It should be noted, however, that V and XII are considerably less potent as inhibitors of adenosine deaminase than XIII and XIV (see Table I). A comparison of the data shows that the compound with the *trans*-2′-hydroxyl group (XIII) is a better inhibitor than the compound with the *cis*-2′-hydroxyl group (XIV) and that both are better than the compounds with the *cis*- and *trans*-3′-hydroxyl groups (V and XII). Furthermore, it can be seen that V and XII are essentially identical to the 9-cyclopentyl compound (XV) in their inhibitory ability. Therefore, these results establish that there is little or no contribution of the 3′-hydroxyl group to the binding of isosteric nucleosides to the enzyme. If one assumes that the inhibitor binds at the same place on the enzyme to which the substrate binds, then the 3′-hydroxyl group of adenosine offers little to the binding of substrate to the enzyme.

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Critical *In Vitro* Factors in Evaluation of Gastric Antacids II

Inhibition of Neutralization Rate of Dried Aluminum Hydroxide Gel

By MILO GIBALDI, JOSEPH L. KANIG, and LEWIS AMSEL

It was determined that the *in vitro* neutralization rates of dried aluminum hydroxide gel are dependent on the amount of antacid used. Neutralization rates of aluminum hydroxide were inhibited by a variety of materials, including proteins, enzymes, polypeptides, amino acids, and certain organic acids. Several possible inhibition mechanisms are explored.

THE MARKED INHIBITORY effect of peptone and pepsin on the *in vitro* activity of noneffervescent antacids has recently been reported (1). The significance of these findings is that similar inhibition might occur in the stomach. Since antacids are most frequently administered after meals, it is conceivable that a nonspecific, protein-induced inhibition of certain alkalizing agents could result in considerable loss of antacid activity. In view of the importance of gastric antacids in modern therapeutics, it was decided to continue the investigation of this phenomenon.

Previous work has indicated that aluminum hydroxide, of all antacids tested, demonstrates the highest susceptibility to the inhibitory effect of polypeptides. Therefore, the possible inhibitory effect of various proteins, polypeptides, and amino acids on the *in vitro* activity of dried aluminum hydroxide gel U.S.P. is considered in this report. Certain aspects of the inhibition mechanism are also explored.

EXPERIMENTAL

Rate of neutralization and buffering capacity studies were carried out in accordance with the specifications reported by Desai *et al.* (1). All experiments, except where noted, were conducted using 1.0 Gm. of dried aluminum hydroxide gel U.S.P.

Relationship of Rate of Neutralization to Buffering Capacity.—It has been proposed that the polypeptide inhibitory effect on the buffering capacity of noneffervescent antacids indicates a kinetic or reversible phenomenon rather than an irreversible state (1). This conclusion was based on the observation that the polypeptides had no effect on the total volume of acid neutralized but reduced the maximum pH attained during the course of the experiments. Therefore, it was reasoned that the over-all effect of polypeptides on the antacid buffering capacity could be ascribed to an inhibitory influence on the rate of neutralization. This reasoning is supported by the results depicted in Figs. 1 and 2. A definite relationship exists between these two evaluatory procedures. A more prolonged neutralization time results in a lower maximum pH value in an analogous buffering capacity determination.

Past reports have considered the buffering capacity of a given antacid as involving an equilibrium state. Therefore, if antacid compound A is capable of neutralizing X ml. of 0.1 N HCl, and antacid compound B consumes an equal quantity, these compounds have been considered comparable. Since the rate of secretion of HCl *in vivo* is a significant variable, it must be appreciated that the integral, $d\,\mathrm{pH}/d$ ml. acid, is a more valid criterion of antacid activity. Thus, if one were to consider the plots in Fig. 2 as representative of hypothetical compounds A, B, and C, it may be noted that although all antacids have the same buffering capacity (*i.e.*, consume the same quantity of 0.1 N HCl), they are far from equivalent. Compound A would most likely be the more effective antacid. If the total area under the curve is a more valid indication of antacid activity, then the situation is no longer equilibrium dependent. Rather it becomes kinetic in nature, and the rate of reaction of the antacid with HCl is the determining factor.

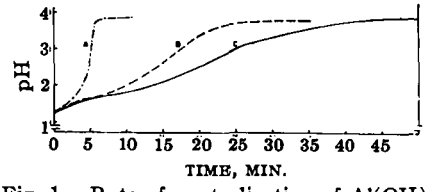


Fig. 1.—Rate of neutralization of $Al(OH)_3$ in 50 ml. 0.1 N HCl. Key: A, 1.0 Gm. $Al(OH)_3$; B, 1.0 Gm. $Al(OH)_3$ + 250 mg. pepsin; C, 1.0 Gm. $Al(OH)_3$ + 250 mg. peptone.

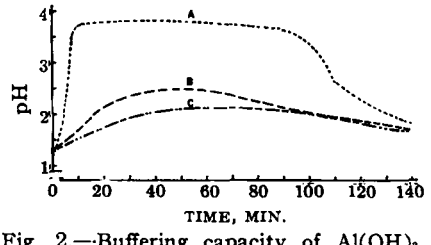


Fig. 2.—Buffering capacity of $Al(OH)_3$ in an initial quantity of 50 ml. 0.1 N HCl + 0.1 N HCl added at rate of 2.0 ml./min. Key: A, 1.0 Gm. $Al(OH)_3$; B, 1.0 Gm. $Al(OH)_3$ + 250 mg. pepsin; C, 1.0 Gm. $Al(OH)_3$ + 250 mg. peptone.

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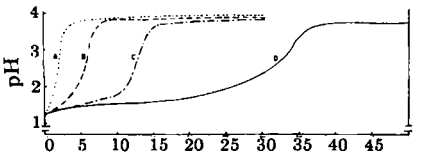


Fig. 3.—Rate of neutralization of various doses of $Al(OH)_3$ in 50 ml. 0.1 *N* HCl. Key: A, 2.0 Gm.; B, 1.0 Gm.; C, 0.5 Gm.; D, 0.25 Gm.

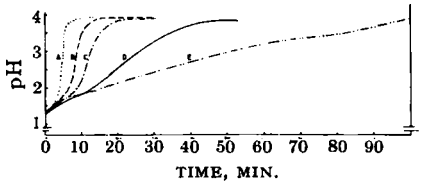


Fig. 4.—Effect of peptone on neutralization rate of 1.0 Gm. $Al(OH)_3$ in 50 ml. 0.1 *N* HCl. Key: A, control; B, 50 mg. peptone; C, 100 mg.; D, 250 mg.; E, 500 mg.

RESULTS AND DISCUSSION

Effect of Antacid Dose on Neutralization Rate.—The neutralization of aluminum hydroxide by HCl is an extremely complex process which has not been elucidated completely. A schematic representation of this reaction may be

a, aluminum hydroxide (solid, excess) → aluminum hydroxide (solution)

b, aluminum hydroxide (solution) + H^+ → aluminum salts + H_2O

The demonstration has been made in our laboratories that both the K_{sp} and rate of step *a* is pH dependent. In addition, step *b* is instantaneous. Therefore, although the kinetics of such a reaction are extremely involved, it would appear that the over-all process is rate-limited by step *a*. However, exploratory experiments have indicated that the neutralization rate is independent of rate of agitation above a given minimum, which is considerably lower than the stirring rate employed throughout this investigation. It would appear, therefore, that this model is oversimplified. Considering the hydrophobic nature of aluminum hydroxide, an alternate scheme would assign the rate-limiting step to the wetting of the solid surface by the test fluid. Stated in another way, the rate-limiting step would involve the diffusion of protons to the surface of the poorly soluble antacid. This proposition seems to be the more valid.

In either case, the rate of neutralization would be dependent on the total surface area of the antacid and in turn on the total amount of antacid employed. This concept is contrary to conclusions in a recent report (2) which evaluated aluminum salts of water-insoluble aliphatic acids. These authors stated, (with respect to *in vitro* testing) "the exact amount (of antacid) added is not important provided an excess is present; however, it must be in an immediately dispersable form." Although the conclusions reached may be valid for the antacids tested in the cited study, it was believed that this was not the case for the more commonly used antacids. To verify this belief, the rate of neutralization of various excess amounts of aluminum hydroxide was studied. The results depicted in Fig. 3 clearly reveal the strong dependence of neutralization rate on the amount of antacid employed. Experiments using magnesium trisilicate and calcium carbonate yield equivalent results.

These data indicate that, under similar *in vivo* conditions, the onset of action of noneffervescent antacids will be dependent on the dose administered. According to these findings, it is quite plausible that a double dose of an antacid would have considerably more than twice the antacid activity of a single dose. Additional experiments established that the rate of neutralization of effervescent antacids are independent of the amount used.

Effect of Amount of Inhibitor on Rate of Neutralization.—As indicated in Fig. 4, the extent of inhibition increases proportionately with the amount of peptone[1] employed. This relationship held for all inhibitors tested. It is clear that even relatively small amounts of polypeptide would essentially negate the antacid activity of aluminum hydroxide by depressing the neutalization rate.

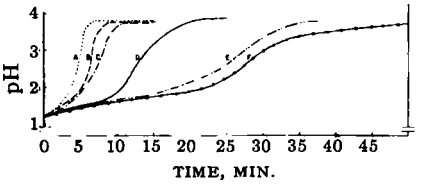


Fig. 5.—Effect of various protein inhibitors on the reaction rate of 1.0 Gm. $Al(OH)_3$ in 50 ml. 0.1 *N* HCl. Key: A, control; B, 100 mg. amylase; C, 250 mg. gelatin; D, 100 mg. cellulase; E, 100 mg. protease; F, 100 mg. Tryptone, a polypeptide prepared by Difco Laboratories, Detroit, Mich.

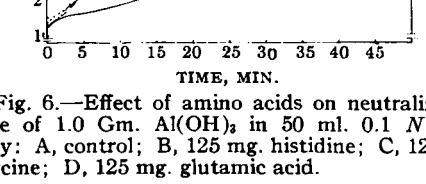


Fig. 6.—Effect of amino acids on neutralization rate of 1.0 Gm. $Al(OH)_3$ in 50 ml. 0.1 *N* HCl. Key: A, control; B, 125 mg. histidine; C, 125 mg. glycine; D, 125 mg. glutamic acid.

Protein Inhibition.—Newey (3) was the first investigator to point out that a combination of pepsin and peptone incorporated in the test fluid acted to inhibit the *in vitro* buffering capacity of aluminum hydroxide. The present study reveals that a combination of these two materials is not a necessary condition for the inhibitory process but that each can independently exert an inhibitory effect (Fig. 1). The polypeptide, peptone, appears to be an inhibitor more effective than pepsin when the two are compared at equal concentrations.

To determine the specificity of this inhibition, a number of protein materials, including undegraded proteins, enzymes, and polypeptides, was tested. The inhibition was not limited to pepsin and peptone but was a rather general phenomenon. Each of the

[1] Bacto-Peptone, Difco Laboratories, Detroit, Mich.

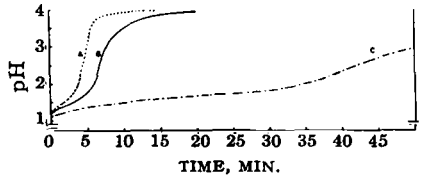


Fig. 7.—Effect of organic acids on neutralization rate of 1.0 Gm. $Al(OH)_3$ in 50 ml. 0.1 *N* HCl. Key: A, control; B, 1 meq. acetic acid; C, 1 meq. citric acid.

TABLE I.—GLUTAMIC ACID IN SOLUTION (PER CENT NITROGEN) DURING THE COURSE OF NEUTRALIZATION

Sample Time, Min.	pH	N, %
Control	1.25	0.022
15	2.17	0.022
30	3.10	0.022
45	3.40	0.022
60	3.66	0.022

protein materials tested was observed to exhibit some degree of inhibitory activity. The results of some of these tests are plotted in Fig. 5. It would appear likely that the existence of any protein or degraded protein in the stomach could result in the inhibition of the *in vivo* antacid activity of aluminum hydroxide.

Amino Acid Inhibition.—Experimental results indicated that partially degraded proteins were inhibitors generally more effective than intact proteins at equal concentrations. This aspect of the study was enlarged to examine the effect of amino acids on the neutralization rate. The results of this phase, summarized in Fig. 6, demonstrate that all three amino acids investigated have inhibitory activity at the concentration level employed.

The dicarboxylic compound, glutamic acid, was the most potent inhibitor of the three. A more imposing aspect is obtained when the inhibitory activities of the amino acids are compared on a molar basis. When 2 m*M* of each compound was tested, glycine and histidine each inhibited neutralization of aluminum hydroxide for 3 to 4 minutes. Glutamic acid under the same test conditions inhibited equilibrium to the extent of about 100 minutes.

Organic Acid Inhibition.—In view of the high inhibitory activity manifested by glutamic acid, a dicarboxylic amino acid, it was decided to determine the possible role of the carboxyl moiety in the inhibition process. Exploratory studies conducted with either acetic or citric acids demonstrated significant inhibition of the neutralization rate of aluminum hydroxide. Figure 7 indicates that, when compared on an equivalent basis the tricarboxylic citric acid was the more effective inhibitor.

It is important to note that neither organic acid had a perceptible effect on the initial pH of the test solution or on the equilibrium pH. Thus, a simple pH effect cannot be invoked in explaining the results.

Existence of Insoluble Interaction Product.—Among the possible explanations for the observed inhibition effects are (*a*) the formation of an insoluble aluminum hydroxide-inhibitor complex or (*b*) the adsorption of the inhibitor on the surface of the dispersed aluminum hydroxide particles. To check these hypotheses, the decision was made to determine accurately the amount of glutamic acid remaining in solution during the course of neutralization. Glutamic acid, 125.0 mg., was added to each of a series of flasks containing 50 ml. of 0.10 *N* HCl. Dried aluminum hydroxide gel, 1.000 Gm., was added to each; neutralization was allowed to proceed. After intervals of 15, 30, 45, and 60 minutes, the contents of successive flasks were rapidly filtered *in vacuo*, and the filtrate was analyzed for nitrogen content by a micro-Kjeldahl technique.[1] The results, summarized in Table I, show that the glutamic acid content in the solution had remained constant during the course of the reaction.

These experiments would seem to rule out the occurrence of an insoluble complex or the existence of inhibitor adsorption as possible mechanisms for the inhibitory process. However, there remains the possibility of a soluble complex formation between the aluminum hydroxide and a free carboxyl group, a moiety which has been present in each of the inhibitors tested. This interesting consideration is presently under investigation.

SUMMARY

The inhibitory effect of pepsin and peptone on the buffering capacity of noneffervescent antacids may be attributed to a depression of the rate of neutralization of the antacid.

The dose of noneffervescent antacids was critical in determining the neutralization rate. The larger the dose employed, the faster the neutralization process.

The pepsin-peptone inhibition of aluminum hydroxide was observed to be a rather general phenomenon. Inhibition was noted with various proteins, enzymes, polypeptides, and amino acids.

Preliminary studies showed that the carboxylic acids, acetic and citric, were inhibitors of aluminum hydroxide neutralization.

The existence of an insoluble product of antacid and inhibitor, either *via* complex formation or inhibitor adsorption, was eliminated as a potential mechanism in the inhibition phenomenon.

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[1] The authors thank Dr. T. Cayle, Wallerstein Co., Staten Island, N. Y., for conducting these analyses.

Relationship Between Rate of Dissolution, Disintegration Time, and Physiological Availability of Riboflavin in Sugar-Coated Tablets

By E. J. MIDDLETON, J. M. DAVIES, and A. B. MORRISON

Studies were conducted on the relationship between dissolution rate, disintegration time, and physiological availability of seven sugar-coated multivitamin products. Dissolution rates and disintegration times were determined with the U.S.P. disintegration apparatus. A close relationship was found between *in vitro* disintegration time and the dissolution rate ($T_{50\ per\ cent}$) of riboflavin for the various products. Both procedures correlated reasonably well with physiological availability as measured by urinary riboflavin excretion. It was concluded that either *in vitro* disintegration time or dissolution rate can provide a useful estimate of the availability to the body of riboflavin in sugar-coated tablets.

PREVIOUS STUDIES from this laboratory (1–3) demonstrated that sugar-coated tablets which did not disintegrate in 1 hour by a specified *in vitro* test were not fully available to the body, judged by urinary excretion of riboflavin or *p*-aminosalicylate. Morrison *et al.* (4) concluded that measurement of riboflavin excretion provided a valid indication of physiological availability of vitamin preparations, and that limits for *in vitro* disintegration time based on riboflavin excretion were applicable to other water-soluble vitamins.

The validity of the relationship between disintegration time and physiological availability has been questioned recently. Levy (5) showed that disintegration times for five acetylsalicylic acid tablets were not correlated with the rate of absorption or biological availability of the drug in humans. He noted, however, a direct relationship between the rate at which the tablets went into solution (the dissolution rate) and the amount of salicylate excreted in the urine. Campagna *et al.* (6) reported that the disintegration time for prednisone tablets was not a valid indicator of the availability of this drug to the body. Schroeter *et al.* (7) examined relationships between rate of dissolution and disintegration time for tablets containing an anti-inflammatory steroid, sulfonamide, antidiabetic agent, acetylsalicylic acid-phenacetin-caffeine, or sodium *p*-aminosalicylate. They concluded there was a specificity in the presence or absence of a relationship between rate of dissolution and disintegration time and questioned the validity of uniform disintegration time limits applied to large groups of drugs in compressed tablets.

The experiments described here were conducted to study relationships between *in vitro* disintegration time, dissolution rate, and physiological availability of riboflavin in sugar-coated multivitamin products. The results indicated that either of the two *in vitro* methods can provide a valid indication of availability of riboflavin to the body.

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METHODS

In Vitro Disintegration Times and Dissolution Rates.—The apparatus and fluids described in U.S.P. XVI (8) were used to determine disintegration time. The tablets were immersed for 30 minutes in simulated gastric fluid and the remainder of the time in simulated intestinal fluid. Disintegration times reported were mean values for at least two separate tests on six tablets each.

Without stopping the agitation of the disintegration apparatus, aliquots of the simulated digestive fluids were withdrawn at various time intervals using pipets wound with glass wool at their tips. The aliquots were then analyzed for riboflavin by the U.S.P. XVI fluorometric procedure (8).

Physiological Availability.—The method used to determine physiological availability of riboflavin was essentially that of Melnick *et al.* (9). Seven sugar-coated multivitamin products were tested. Five normal males known to be receiving nutritionally adequate diets were used in the experiment. While on test, they consumed regulated meals containing a relatively constant amount of riboflavin. The subjects were given 6 mg. of riboflavin in a gelatin capsule as the standard and 2–10 mg. of riboflavin as commercial preparations. A single level of standard was chosen because of previous findings that riboflavin excretion expressed as percentage of the dose was linearly related to doses up to 20 mg. (10). The doses were taken at 8:30 a.m. after breakfast. Urine was collected in opaque bottles containing 2 ml. of 3.5 N H_2SO_4 at 2, 4, 6, 8, 14, and 24-hour intervals after dosing. Riboflavin in urine was determined by U.S.P. XVI fluorometric procedure (8). Excretion values were corrected by subtracting the appropriate blank determined on the same individuals without dosing. As in previous studies (1–3), physiological availability was calculated as

$$\% \text{ Physiological Availability} = \frac{\% \text{ Excretion From Preparation}}{\% \text{ Excretion From Standard}} \times 100$$

TABLE I.—DESCRIPTION OF TABLETS STUDIED

Sample	Medication	Labelled Riboflavin Content/Unit, mg.	Riboflavin Unit Found, mg.	*In Vitro* Disintegration Time, Min.	*In Vitro* Dissolution Rate $T_{50\%}$, Min.	Physiological Availability, %
A	Multivitamin and minerals	3	3	151	114	58 ± 10[a]
B	Multivitamin and minerals	10	10	45	25	87 ± 4
C	Multivitamin	6	6	101	83	36 ± 10
D	Multivitamin and minerals	3	3	45	38	88 ± 11
E	Multivitamin	10	11	20	11	94 ± 7
F	Multivitamin and minerals	3	3	98	124	12 ± 5
G	Multivitamin	2	2	20	11	98 ± 5

[a] Standard error of the mean.

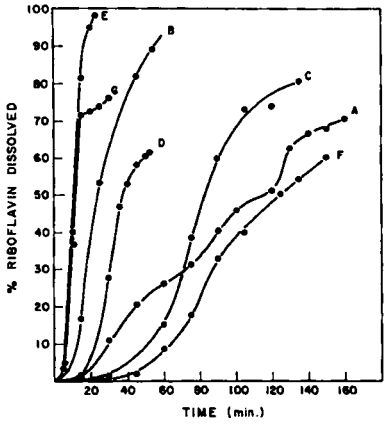


Fig. 1.—*In vitro* dissolution curves of products A to G plotted as per cent riboflavin dissolved in simulated gastrointestinal fluids against time in minutes.

RESULTS

Data describing the products used are shown in Table I. All products contained vitamins; products A, B, D, and F also contained minerals. *In vitro* disintegration times varied from 20 minutes (products E and G) to 151 minutes (product A). *In vitro* dissolution rates were expressed as the time for 50% of the riboflavin to be dissolved in simulated digestive fluids ($T_{50\%}$). Dissolution curves are given in Fig. 1. The $T_{50\%}$ values varied from 11 minutes (products E and G) to 124 minutes (product F).

The relationship between dissolution rate ($T_{50\%}$) and disintegration time is shown in Fig. 2. A significant relationship was found (r = 0.923). Product F, which was only partially available to the body, had a longer dissolution rate than would have been predicted from the disintegration time, suggesting that the product disintegrated but did not go into solution.

Results of the physiological availability studies also are summarized in Table I, and the relationship between disintegration time and physiological availability is illustrated in Fig. 3. Products A, C, and F, which did not disintegrate within 60 minutes *in vitro*, were not fully available to the body.

Products C and F gave lower physiological availability values than predicted from their *in vitro* disintegration times.

A significant relationship (r = 0.906) was observed between dissolution rate ($T_{50\%}$) and physiological availability (Fig. 4). Tablets F and C, however, which displayed abnormal disintegration times gave results quite different from those of the other products tested.

DISCUSSION

The results of these studies confirm the previously established relationship between *in vitro* disintegration time and physiological availability. Tablets which did not disintegrate within 60 minutes (30 minutes in simulated gastric juice and 30 minutes in simulated intestinal juice) were not fully available to the body. Similarly, tablets with $T_{50\%}$ values of greater than 60 minutes were not fully available.

It is of interest that two products gave results for disintegration time and dissolution rate which did not correlate closely with physiological availability. This discrepancy may be related, in part, to the fact that these tablets formed a viscous mass in the simulated digestive fluids, making it difficult to determine disintegration time accurately. It is possible also that the *in vivo* availability of riboflavin in these products may have been lower than expected be-

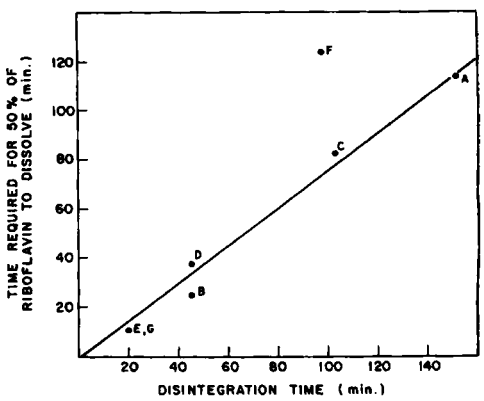


Fig. 2.—Correlation of time required for 50% of riboflavin to dissolve in simulated gastrointestinal fluids with disintegration time for products A to G.

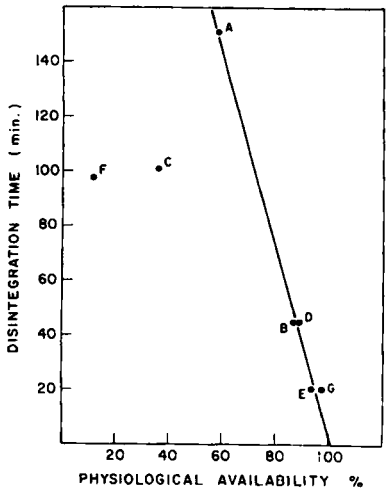


Fig. 3.—Relationship between disintegration time for products A to G and physiological availability of the products measured by urinary riboflavin excretion data.

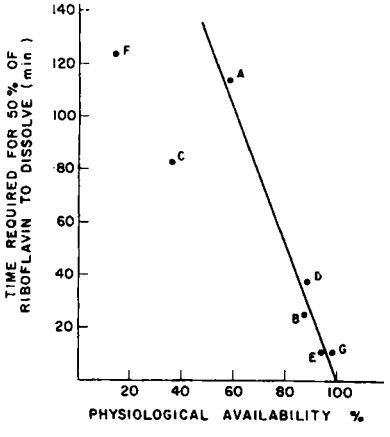


Fig. 4.—Correlation between time required for 50% of the riboflavin from products A to G to dissolve in simulated gastrointestinal fluids with physiological availability of the products measured by urinary riboflavin excretion data.

cause the vitamin was bound or adsorbed to tablet excipients and hence rendered partially unavailable. For example, it is well known that thiamine bound to Fuller's earth is only partially available to man (11).

A close relationship was found between *in vitro* disintegration time and dissolution rate ($T_{50\%}$) values. Results obtained by both *in vitro* procedures correlated reasonably well with those for physiological availability. The relationship between $T_{50\%}$ values and physiological availability was particularly good. This finding indicates that the rate of solution of riboflavin from sugar-coated tablets is an important factor in determining *in vivo* availability of the vitamin. From other studies (12) it is known that riboflavin is absorbed mainly from the upper part of the small intestine. Therefore, it would appear likely that tablets from which riboflavin dissolves only slowly may be beyond the portion of the gut in which efficient absorption can take place before dissolution occurs, and hence show reduced availability of riboflavin.

The results indicate that *in vitro* disintegration time provides a valid indication of the rate at which riboflavin goes into solution from sugar-coated tablets. Thus, either *in vitro* disintegration time or dissolution rate can provide a useful estimate of the availability to the body of riboflavin in sugar-coated tablets. For other drugs disintegration time and dissolution rate may not be so closely related (5, 6). There is a need for further studies on methods for determining dissolution rate and on the relationship between dissolution rate and physiological availability.

Previous studies have shown that limits for *in vitro* disintegration time based on riboflavin also are applicable to other water-soluble vitamins. Therefore, it would be expected that the rate of solution of riboflavin would provide useful information about the availability of other water soluble vitamins to the body. Studies now are underway to investigate this possibility. Relationships between *in vitro* disintegration time and dissolution rate for enteric coated tablets with varying degrees of *in vivo* availability also are being investigated.

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Kinetic Study on Hydrolysis of a Mannich Base Compound

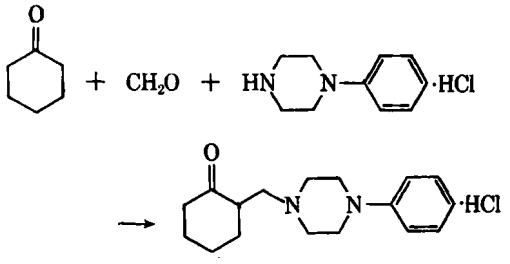
By K. THOMAS KOSHY and H. MITCHNER*

The hydrolysis of 2-(4-phenyl-1-piperazinylmethyl)cyclohexanone hydrochloride, a Mannich condensation product, was studied in buffered aqueous solutions in the pH range of 1.1 to 5.5. The hydrolytic products, *N*-phenylpiperazine and a rearranged dimer of 2-methylenecyclohexanone, were isolated and characterized. The hydrolysis was specific acid and general base catalyzed with maximum stability around pH 2. The hydrolysis was pseudo first order in nature with an energy of activation, uncorrected for the heat of ionization of water, of 27.3 Kcal./mole.

THE MANNICH condensation reaction is frequently used in the synthesis of pharmaceutical compounds. In 1922, Mannich and Heilner (1) observed that ω-dimethylaminopropiophenone hydrochloride prepared from acetophenone, formaldehyde, and dimethylamine hydrochloride decomposed on steam distillation to give acrylophenone and dimethylamine hydrochloride. Similar results were noted by Blicke and Burckhalter (2). Mannich (3) and Mannich and Hönig (4) also had reported that 2-(piperidinomethyl)cyclohexanone on fusion decomposed to piperidine hydrochloride and 2-methylenecyclohexanone.

References on the breakdown of Mannich bases have been essentially qualitative. Stability studies in this laboratory on a pharmaceutical product formed by a Mannich base reaction showed an unexpected hydrolysis and afforded an opportunity to investigate quantitatively the kinetics of the hydrolysis of this compound. Factors affecting hydrolysis which could be utilized as guidelines for pharmaceutical formulation were of particular interest.

2-(4-Phenyl-1-piperazinylmethyl)cyclohexanone hydrochloride (compound MA1050) was prepared by the Miles Chemical Therapeutics Research Laboratory by the condensation of cyclohexanone, formaldehyde, and *N*-phenylpiperazine hydrochloride as shown in Scheme I.



Scheme I

This compound was stable to moderate amounts of dry heat, but broke down under accelerated conditions of combined heat and humidity. In accordance with findings on similar compounds (3, 4), the methylene-nitrogen bond was considered the most probable point of hydrolytic attack. This was confirmed by the identification of the breakdown products. Analytical procedures were adapted to follow the appearance of one of the degradation components, which introduced into the system a new functional group, a secondary amine.

EXPERIMENTAL

Isolation and Characterization of Degradation Compounds.—Approximately 2.0 Gm. of compound MA1050 was dissolved in about 100 ml. of 1.0 *N*-hydrochloric acid and digested on a steam bath for a few hours. The solution was cooled and extracted with several portions of ethyl ether. The ether extract was discarded. The aqueous phase was made alkaline with sodium hydroxide solution and extracted with ethyl ether. The ether extract was washed free of alkali, dried with anhydrous sodium sulfate, and the ether evaporated. The residue was identified as *N*-phenylpiperazine by its infrared spectrum and by the melting point of its hydrochloride.

A second nonnitrogenous product was isolated by heating a 6% aqueous solution of MA1050 in a closed system for a few hours on a steam bath. A white crystalline material separated. This was recrystallized from aqueous ethanol and dried *in vacuo* at 60° for 3 hours, m.p. 156–159°. This material was characterized as the hydrated dimer of 2-methylenecyclohexanone which was previously reported by Warnhoff and Johnson (5).[1] The melting point checked more closely with that reported by Mannich (3) for the same compound.

Analytical Method for Determining the Rate of Hydrolysis.—The rate of hydrolysis was followed by determining the rate of appearance of *N*-phenylpiperazine. A color reaction of secondary amines with carbon disulfide and cupric ion reported by Umbreit (6) was adapted for this purpose. The copper dithiocarbamate of the amine formed in the reaction was extracted into benzene and determined colorimetrically. This reaction was suitable for

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[1] The authors thank Dr. O. E. Fancher, Director of the Chemical Therapeutics Research Laboratory, Miles Laboratories, Inc., for characterizing this degradation product.

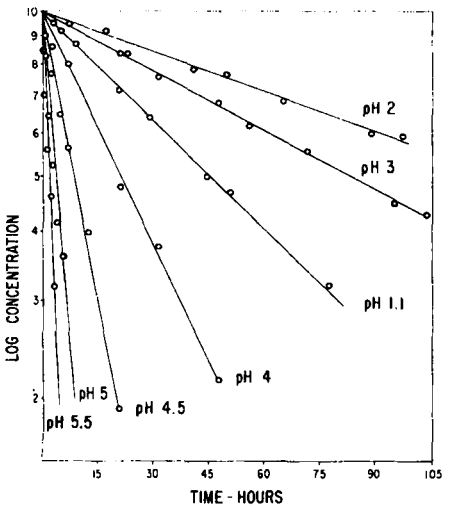


Fig. 1.—First-order plot of the hydrolysis of compound MA1050 in buffered aqueous solutions at 60°.

TABLE I.—OBSERVED FIRST-ORDER SPECIFIC REACTION CONSTANTS (k IN HOUR^{-1}) FOR THE HYDROLYSIS OF COMPOUND MA1050 IN BUFFERED AQUEOUS SOLUTIONS AT 60°[a]

pH	k, hr.$^{-1}$ × 10^3
1.1	15.30
2.0	5.63
3.0	8.46
4.0	31.72
4.5	78.70
5.0	205.70
5.5	382.70

[a] The ionic strength of all solutions was 0.15.

determining *N*-phenylpiperazine with no interference from compound MA1050 or its nonnitrogenous breakdown product. *N*-Phenylpiperazine was stable under the experimental conditions of hydrolysis.

Procedure.—The following general procedure was used for studying the rate of hydrolysis. Samples of 0.2 Gm. of compound MA1050 were weighed accurately into 200-ml. volumetric flasks containing an amount of potassium chloride to adjust the ionic strength to a predetermined value. The contents were dissolved in the appropriate buffer solution preheated to the particular temperature at which the hydrolysis was studied. More of the hot buffer solution was added to volume, the contents mixed, and the flask placed in a constant temperature bath. Fifteen-milliliter aliquots were withdrawn after 5 minutes equilibration and thereafter at periodic intervals. These solutions were cooled to room temperature, the pH adjusted to 6 with 0.2 *N* NaOH, transferred to a 100-ml. volumetric flask, and water added to volume. A 2.0-ml. aliquot of each was assayed for *N*-phenylpiperazine. The comparative color developed simultaneously on a 2.0-ml. aliquot of a suitable standard solution of *N*-phenylpiperazine hydrochloride was used in calculating the concentration of the sample.

pH and Temperature Effect.—The effect of pH and temperature on the rate was determined by the hydrolysis at three different temperatures in buffer solutions as follows: pH 1.1, 2, 3, 4, 4.5, 5, and 5.5 at 60°, and pH 3, 4, and 5 at 50° and 70°. Hydrochloric acid was used for pH 1.1 solution; phosphoric acid and monobasic sodium phosphate were used for solutions of pH 2 and 3, and acetic acid and sodium acetate for pH 4, 4.5, 5, and 5.5. The ionic strength of all solutions was adjusted to 0.15 with potassium chloride. Buffers of pH higher than 5.5 could not be employed in this study due to precipitation of free base of the compound.

Effect of Initial Concentration on the Order and Rate of Hydrolysis.—The procedure, as outlined, was used for studies in acetate buffer at pH 5 at 60° using two different initial concentrations of compound MA1050.

Effect of Ionic Strength.—The effect of ionic strength on the rate was investigated by the hydrolysis of compound MA1050 at 60° in solutions of pH 1.1, 2, 3, 4, and 5. The ionic strengths were adjusted to different levels with potassium chloride.

Buffers as General Acid or General Base Catalysts.—The effect of buffer constituents as general acid or general base catalysts was investigated by hydrolyzing compound MA1050 in solutions of pH 2, 3, 4, and 5 at constant ionic strength but different concentrations of the buffer species at each pH.

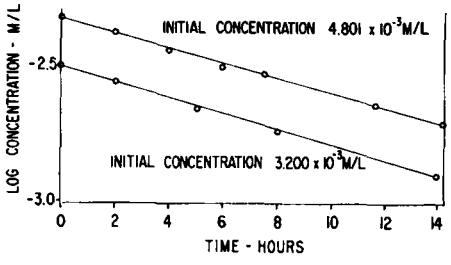


Fig. 2.—Plot showing the effect of initial concentration on the rate of hydrolysis of Compound MA 1050 in acetate buffer pH 5 at 50°.

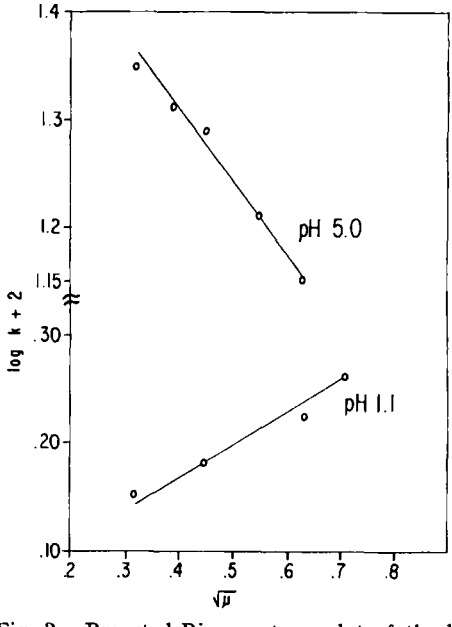


Fig. 3.—Bronsted-Bjerrum-type plot of the logarithm of the observed rate constants against $\sqrt{\mu}$ for the hydrolysis of compound MA1050 at pH 1.1 and 5 at 60°.

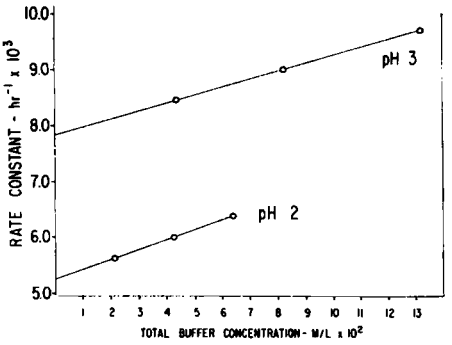


Fig. 4.—Effect of buffer concentration on the hydrolysis of Compound MA1050 in phosphate buffers of pH 2 and 3 at 60° at constant ionic strength.

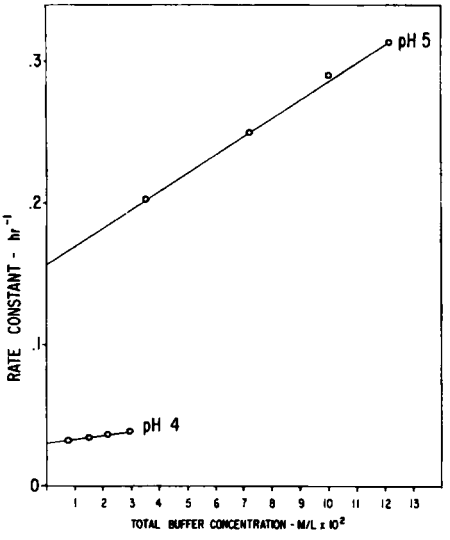


Fig. 5.—Effect of buffer concentration on the hydrolysis of compound MA1050 in acetate buffers of pH 4 and 5 at 60° at constant ionic strength.

RESULTS AND DISCUSSION

Figure 1 is a typical plot of the logarithm of the concentration remaining in solution against time for the hydrolysis of compound MA1050 and demonstrates that the hydrolysis is pseudo first order with respect to compound MA1050 and is a function of the pH of the medium. The first-order rate constants for the reaction at 60° are given in Table I.

Figure 2 demonstrates that the rate is directly dependent on the concentration of MA1050.

Effect of Ionic Strength.—No salt was noticed at pH 2. At pH 3, 4, and 5, an increase in the ionic strength resulted in a decrease in rate. At pH 1.1, however, increasing the ionic strength had the reverse effect of accelerating the rate. The data were tested using the Bronsted-Bjerrum equation (Eq. 1) for bimolecular reactions.

$$\log k = \log k_0 + 2QZ_AZ_B\mu^{1/2} \quad \text{(Eq. 1)}$$

where k is the observed rate constant, k_0 is the rate constant at zero ionic strength, Q is a function of such properties of the solution as dielectric constant, temperature, etc., and thermodynamic constants; Z_A and Z_B are the charges on the reacting species, and μ is the ionic strength of the medium.

Figure 3 is a plot of the logarithm of the observed rate constants against the square root of the ionic strengths for the hydrolysis at pH 1.1 and 5 at 60°. The positive slope for the hydrolysis at pH 1.1 indicates a reaction between ions of like charges. Conversely, the negative slope for the hydrolysis at pH 5 indicates a reaction between ions of unlike charges. At pH 2, where there is no salt effect, the value of Z_AZ_B is zero, which would be consistent for a reaction between uncharged species or between an ion and a neutral molecule. The values for Z_AZ_B were determined from the slopes of the lines in Fig. 3, and from the calculated values for Q at 60°. These were 0.28 and −0.58 at pH 1.1 and 5, respectively. The finding of nonintegral values for Z_AZ_B could be due to high ionic strength, the occurrence of more than one reaction at the point of determination, or to a significant contribution by the secondary salt effect.

Effect of Buffers as General Acid or General Base Catalysts.—Under the experimental conditions used in this study, compound MA1050 was subject to specific acid and general base catalysis. The observed rate constant is a summation of the catalytic effect of all the species involved. In accordance with the treatment of Frost and Pearson (7) it can be represented as

$$k_{obs} = k_1(H^+) + k_2(OH^-) + \Sigma_i k_i(HA_i) + \Sigma k_i(A^-_i) + \Sigma k_i(B_i) + \Sigma k_i(B_iH^+) \quad \text{(Eq. 2)}$$

where HA is a general acid and A^- its conjugate base, and B is a general base and BH^+ its conjugate acid. Figures 4 and 5 are plots of the observed rate constants against the total buffer concentrations at pH 2, 3, 4, and 5 at 60°, the ionic strength of all solutions being the same. No attempt was made to calculate separately the specific contribution of the individual general acids or general bases to the total buffer catalysis. The increasingly positive slopes of the lines with increasing pH indicate that the catalytic effect of the various species was limited to their basic forms.

pH-Rate Profile.—According to Eq. 2, the plots of the observed rate constants against buffer concentrations extrapolated to zero buffer concentration give the rate constant uninfluenced by the buffer species. These values were obtained from Figs. 4 and 5 and plotted as a function of pH in Fig. 6 to show the pH dependency of the rate and a maximum stability at pH 2.

A number of chemical reactions can lead to the same observed experimental dependence. Representing the basic form of compound MA1050 as A and a general base as B, the probable reactions are

$$1,\ A^{++} + H^+ \xrightarrow{k_{H^+}}$$

$$2,\ A^{++} + H_2O \xrightarrow{k_{H_2O}}$$

$$3,\ A^{++} + B \xrightarrow{k_B}$$

$$4,\ A^+ + H^+ \xrightarrow{k'_{H^+}}$$

$$5,\ A^+ + H_2O \xrightarrow{k'_{H_2O}}$$

$$6,\ A^+ + B \xrightarrow{k'_B}$$

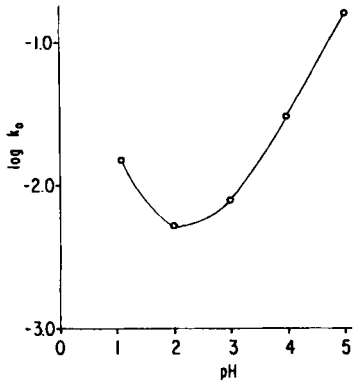


Fig. 6.—Effect of pH on the hydrolysis of compound MA1050 in buffered aqueous solutions at 60° and at constant ionic strength.

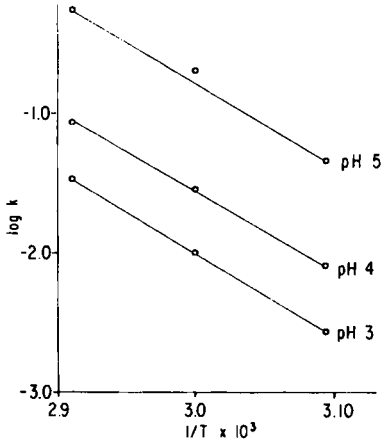
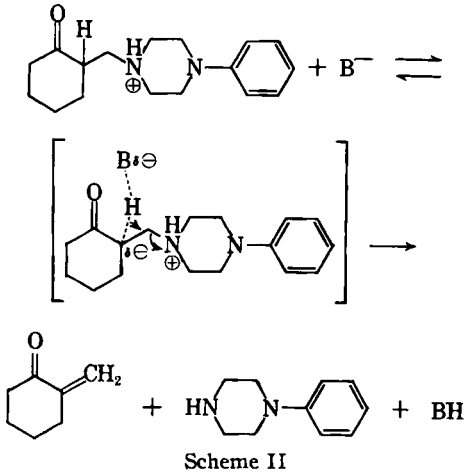


Fig. 7.—Arrhenius-type plot of the logarithm of the observed rate constants against the reciprocal of absolute temperature for the hydrolysis at pH 3, 4, and 5.

Reaction *3* can be ruled out because one of the nitrogens in compound MA1050 is weakly basic and under conditions where it is protonated the hydroxyl ion concentration will be negligible. The predominant reactions appear to be *1*, occurring below pH 2, and *6*, occurring at higher pH. Theoretically, the slopes of the plot in Fig. 6 should be an indication of the order of the reaction with respect to the hydrogen and hydroxyl ion concentrations. The numerical value of the slope varies from 0 to 0.72. These fractional values are an indication of the complexity of the reaction in the pH region under study.

Temperature Effect on the Rate.—The effect of temperature on the rate constants is demonstrated by the Arrhenius plot (Fig. 7). Data obtained at pH 3, 4, and 5 at 50°, 60°, and 70° were used in this plot. The energy of activation, uncorrected for the heat of ionization of water, was calculated from the slopes of the lines and presented in Table II. The average value for the energy of activation was 27.3 Kcal./mole. The frequency factor, A, and the entropy of activation, ΔS^{+}, were calculated from this value using standard equations. These values are given in Table II. The frequency factor was in the range of 10^{12} to 10^{13}, which seems high for a bimolecular reaction. Of interest was the entropy of activation change from a negative value at pH 3 to a positive value at pH 5, for which no rationale could be developed.

Mechanism of Hydrolysis.—The isolation and characterization of the degradation products of compound MA1050 as the hydrated, rearranged dimer of 2-methylenecyclohexanone and *N*-phenylpiperazine indicate that the primary path of breakdown is the hydrolytic attack at the methylene-nitrogen bond. The hydrolysis is first order with respect to the concentration of compound MA1050, but of a complex order with respect to hydrogen and hydroxyl ion concentrations making if difficult to write a rate equation. There is a positive salt effect at pH 1.1, no salt effect at pH 2, and a negative salt effect at higher pH, which suggests that more than one mechanism is involved. Although this study was not designed to investigate the mechanism of hydrolysis of compound MA1050, two pathways can be postulated, which appear to be in agreement with the kinetic data. In solutions of pH higher than 2, a pathway consistent with the negative salt effect and the marked catalytic effect of the acetate ion at pH 5 would be as shown in Scheme II.

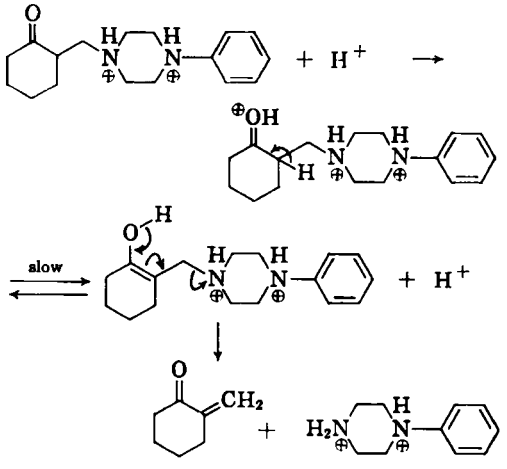


Scheme II

In solutions of pH lower than 2, the pathway might be as shown in Scheme III.

TABLE II.—VALUES FOR THE ENERGY OF ACTIVATION, FREQUENCY FACTOR, AND THE ENTROPY OF ACTIVATION CALCULATED FROM THE SLOPES OF THE ARRHENIUS PLOT

	pH 3			pH 4			pH 5		
	50°	60°	70°	50°	60°	70°	50°	60°	70°
Frequency factor, A, sec.$^{-1}$	2.1×10^{12}	1.9×10^{12}	6.0×10^{11}	6.5×10^{12}	7.1×10^{12}	6.0×10^{12}	3.7×10^{13}	1.9×10^{13}	3.9×10^{13}
Entropy of activation, cal./mole deg.	−2.28	−2.57	−4.93	−0.08	0.04	0.35	3.36	2.00	3.34
Energy of activation, Ea, Kcal./mole	27.71			26.85			27.31		



Scheme III

The 2-methylenecyclohexanone shown in the postulated reactions appears to dimerize and rearrange according to the pathway suggested by Warnhoff and Johnson (5).

Pharmaceutical Consideration.—The immediate practical application of this study was in the elimination or control of factors affecting the rate of breakdown of a parenteral preparation for clinical pharmacological work. The pH of an aqueous solution of compound MA1050 is 5.0. The kinetic data indicated that a solution at this pH could not be sterilized by autoclaving, nor could a sterile solution be prepared aseptically and stored even at refrigeration temperature for reasonable lengths of time without appreciable breakdown. Formulation considerations were therefore directed to the prepasation of a sterile lyophilized powder.

SUMMARY

A Mannich base, 2-(4-phenyl-1-piperazinylmethyl) cyclohexanone, was susceptible to hydrolytic attack at its methylene-nitrogen linkage. The breakdown products were isolated and characterized as *N*-phenylpiperazine and the hydrated dimer of 2-methylenecyclohexanone.

A kinetic study of the hydrolysis reaction in buffer solutions of pH 1.1 to 5.5 and factors influencing it are reported. The hydrolysis was pseudo first order in nature and was specific acid and general base catalyzed. The pH profile showed the compound to have maximum stability in solution at pH 2. At pH values other than 2, ionic concentration of the medium affected the rate. The energy of activation for the hydrolysis in buffered aqueous solutions, uncorrected for the heat of ionization of water, was 27.3 Kcal./mole. In view of the uncertainty of the hydrogen or hydroxyl ion dependency, no rate equation is given. However, two probable pathways for the hydrolysis are suggested.

Knowledge from the evaluation of stability factors influencing compound MA1050 was applied to pharmaceutical considerations with the development of a lyophilized product as the most stable dosage form.

REFERENCES

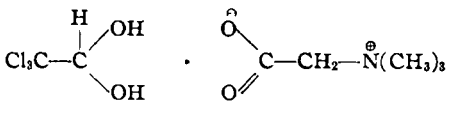
(1) Mannich, C., and Heilner, C., *Ber.*, **55B**, 356(1922).
(2) Blicke, F. F., and Burckhalter, J. H., *J. Am. Chem. Soc.*, **64**, 451(1942).
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Drug Standards

Qualitative and Quantitative Tests for Chloral Betaine

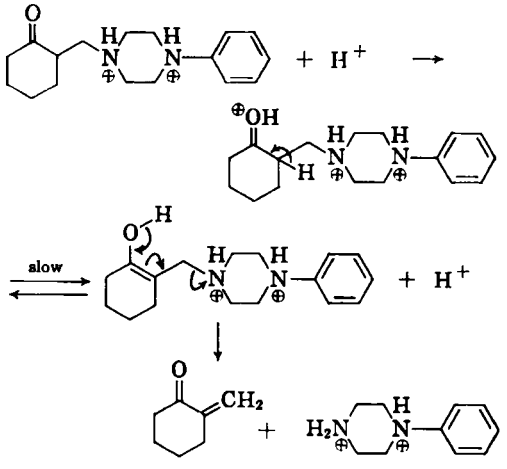
Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

CHLORAL HYDRATE-betaine adduct; $C_7H_{14}Cl_3NO_4$; mol. wt. 282.55. The structural formula of chloral betaine may be represented as



Physical Properties.—Chloral betaine occurs as a white, crystalline powder having a faint aromatic,

Received March 13, 1964, from the Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, D. C.
Accepted for publication April 6, 1964.
Mead Johnson and Co., Evansville, Ind., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.



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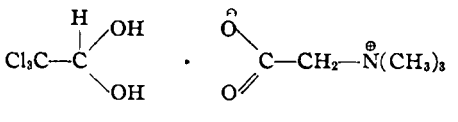
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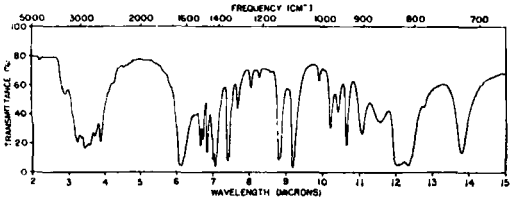


Fig. 1.— Infrared spectrum of chloral betaine in potassium bromide disk (0.5%). Perkin-Elmer model 21 spectrophotometer; sodium chloride prism.

penetrating, slightly acrid odor characteristic of chloral hydrate and a slightly bitter taste, m.p. 122–124.5°, U.S.P. XVI Class Ia. It is very soluble in water, freely soluble in alcohol, and very slightly soluble in chloroform.

Identity Tests.—Warm chloral betaine with a few drops of aniline and of sodium hydroxide T.S.: the mixture has the intensely disagreeable odor of phenyl isocyanide. (*Caution:* poisonous.)

The infrared spectrum of a 0.5% dispersion of chloral betaine in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 1.

Purity Tests.—Char about 1 Gm. of chloral betaine, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.2%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of chloral betaine in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U.S.P. XVI heavy metals test, method I: the heavy metals limit for chloral betaine is 20 p.p.m.

Determine the water content of chloral betaine by the titrimetric (Karl Fischer) method: chloral betaine contains not less than 5.85% and not more than 7.35% water.

Assay.—*Betaine.*—Transfer about 200 mg. of chloral betaine, accurately weighed, to a 200-ml. tall-form beaker and dissolve in 50 ml. of glacial acetic acid. Add 5 ml. of acetic anhydride, 1.5 to 2 ml. of a 1 in 5000 solution of *p*-naphtholbenzein in glacial acetic acid, and titrate with 0.1 *N* acetous perchloric acid. Perform a blank titration and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 11.72 mg. of $C_5H_{11}NO_2$. The amount of betaine found is not less than 40% and not more than 44%.

Chloral Hydrate.—Transfer about 500 mg. of chloral betaine, accurately weighed, to a 125-ml. conical flask and dissolve in 25 ml. of water. Add 25.0 ml. of 0.1 *N* sodium hydroxide, swirl to mix, and allow the mixture to stand for 2 minutes. Add phenolphthalein T.S. and titrate the residual alkali at once with 0.1 *N* hydrochloric acid. Each milliliter of 0.1 *N* sodium hydroxide is equivalent to 16.54 mg. of $C_2H_3Cl_3O_2$. The amount of chloral hydrate found is not less than 56% and not more than 59.5%.

DOSAGE FORMS OF CHLORAL BETAINE

Chloral Betaine Tablets

Identity Tests.—Remove the film coating from one chloral betaine tablet by cutting with a sharp blade or by shaking with chloroform for about 1 hour and triturate the tablet to a fine powder. The infrared spectrum of a 0.5% dispersion of the powdered tablet in potassium bromide, in a disk of about 0.82 mm. thickness, corresponds to that of chloral betaine (Fig. 1).

Assay.—Transfer 10 whole tablets to a 200-ml. volumetric flask, add 160 ml. of water, and shake mechanically for 45 minutes or until tablet disintegration is complete. Add water to volume and mix. Filter, rejecting the first portion of filtrate. Pipet a portion of the filtrate, equivalent to about 250 mg. of chloral hydrate, into a 125-ml. conical flask. Add 20.0 ml. of 0.1 *N* sodium hydroxide and allow the mixture to stand for 2 minutes. Add phenolphthalein T.S. and titrate the residual alkali at once with 0.1 *N* hydrochloric acid. Each milliliter of 0.1 *N* sodium hydroxide is equivalent to 16.54 mg. of $C_2H_3Cl_3O_2$. The amount of chloral hydrate found is not less than 95% and not more than 110% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Chloral betaine,[1] a chemical complex of chloral hydrate and betaine, is an oral sedative and hypnotic compound.

Quantitative Methods.—The quantitative methods provided are simple to perform and possess sufficient accuracy and precision for their intended purposes. Titrimetric determination of the water content gave an average value of 6.34 ± 0.10%.[2] Nonaqueous titration of this compound with acetous perchloric acid gave an average value equivalent to 41.2 ± 0.1% betaine. Acidimetric determination of the chloral hydrate content gave an average value of 58.8 ± 0.1%. Analysis of commercial tablets gave an average value of 102.3 ± 0.03% based on the chloral hydrate content.

[1] Marketed as Beta-Chlor by Mead Johnson and Co., Evansville, Ind.

[2] Maximum deviation from the mean value.

New Air Suspension Apparatus for Coating Discrete Solids

By H. C. CALDWELL and EARL ROSEN

A patent for the apparatus and method for coating discrete solids by means of air suspension and a modification of the basic Wurster design has recently been issued. Experience with this new apparatus and its differences from previously reported air suspension coating equipment are discussed.

SINCE WURSTER'S original patents on means for applying coating to tablets or the like (1, 2), several reports have appeared (3–12) on the use of an air suspension coating (ASC) apparatus patterned after his inventions. The Wurster apparatus comprises in combination a vertical coating chamber, a screen means extending transversely across the chamber at its lower end, and an air source and conduct means for directing an air stream through the chamber in a vertical direction in order to support the materials to be suspended in the lower part of the coating chamber. An expanded part at the top of the coating chamber is provided to reduce the air velocity and thereby prevent pneumatic transport of the particles being coated. Figure 1 is a schematic diagram of such an apparatus.

The process consists simply of supporting particles in the vertical column with an upwardly moving air stream, during which time a coating solution is atomized onto the suspended particles. The velocity of the air stream is adjusted so that the suspended particles are maintained in a region of the column relatively confined.

An experimental study by Wilhelm and Valentine (13) has disclosed the sequence of the relationships between the various phenomena that comprise the entire fluidized state spectrum. The Wilhelm and Valentine apparatus, the elements of which are shown in Fig. 2, was essentially a vertical glass tube of 4-in. diameter, fitted on its side with ports for addition of solids and admission of air. The tube was unobstructed over its entire length. For its operation spheres of 0.02 to 0.03 in. in diameter were introduced while air entered simultaneously. The number of particles and air rates could be varied over wide ranges, and it was possible to observe the behavior of the particles directly.

The results of these experiments indicated that with the proper adjustment of solids and air flows virtually all phenomena of the fluidized state, extending from the dense-phase bed all the way through the dispersed phase into pneumatic transport, could be produced. The diagrams of Fig. 3, suggested by Wilhelm and Valentine, will

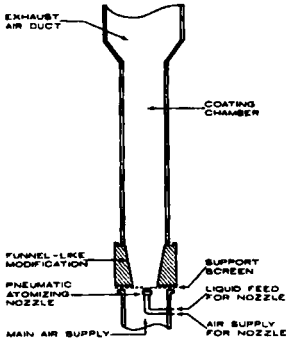


Fig. 1.—Schematic diagram of Wurster air-suspension coater as reported by Singiser and Lowenthal (5).

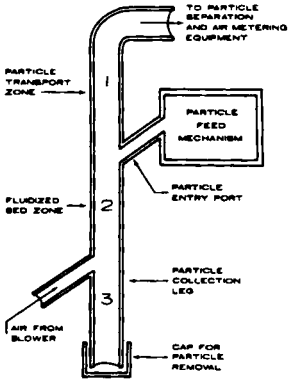


Fig. 2.—Elements of Wilhelm and Valentine apparatus (13).

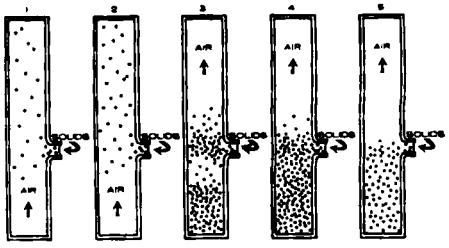


Fig. 3.—Pictorial representation of transition from concurrent to countercurrent flow of particles in rising gas stream in Wilhelm and Valentine apparatus (13).

Received April 1, 1964, from Smith Kline and French Laboratories, Philadelphia, Pa.
Accepted for publication June 30, 1964.

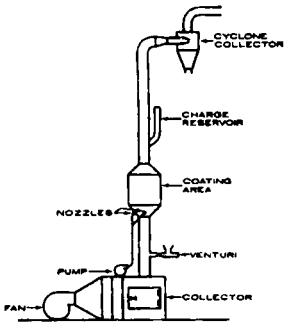


Fig. 4.—Schematic diagram of the Mesnard-Rosen-Scott air-suspension apparatus (14).

be helpful in establishing the relationship among solids, air rate, and resulting bed density. The drawings in Fig. 3 represent the upper two sections of the apparatus. Given a fixed-solids feed rate, the air rate is gradually reduced from its original high value in *1*. Owing to this high air velocity in *1*, the particles will be blown out of the apparatus. As the air rate is reduced as shown in *2*, the particle population increases as the void space decreases slightly. However, the pellets still move concurrently upward with the air. For another small reduction in air rate, *3*, solids will tend to crowd together, and violent slugging will occur with the solids moving downward. The void space decreases sharply. A condition of minimum voidage is observed near *4*, and the solids now have a well-defined upper boundary. With a further reduction, the solids in the bed decrease to reach a limiting value.

In our laboratories air suspension coating apparatuses of the Wurster design operated under conditions described by Fig. 3, *3*, *4*, and *5*. It was possible to operate under these conditions of relatively dense bed as long as the coating materials applied were nontacky and not of great quantity—about 10% of the starting charge. However, under conditions of general operation the regions *3*, *4*, and *5* were relatively unstable and sufficiently sensitive to air flow rate to make frequent adjustments necessary during application of significant (over 10% of starting charge weight) quantities of coating.

To overcome this problem and provide maximum flexibility of the apparatus a series of experiments was undertaken to modify the design. Since the area of highly dispersed phase was far less sensitive to variations in air flow rate, special attention was given to this spectrum of the fluidized state.

EXPERIMENTAL

Although the area of pneumatic transport provided a starting point for experimentation, it was not a satisfactory condition in a vertical system since there was not sufficient time for particle build-up during passage of a particle through the spray pattern of a single nozzle. However, when a horizontal or air slide arrangement with a plurality of spraying nozzles was used, encouraging results were obtained.

After considerable experimentation with vertical apparatuses, a device schematically described in Fig. 4 was evolved (14).

This apparatus differs from the Wurster design primarily in that materials to be coated are truly air suspended and do not need support for screens or other air-regulating devices (such as vanes) in a highly dispersed state approaching that of pneumatic transport, in an expanded chamber situated at the top of a vertical column providing a laminar cushion of air. Also the materials to be applied are delivered out of the main suspending air stream in a direction opposite or countercurrent to the direction of the movement of the particles to be coated. This construction makes possible the application of significant quantities of coating materials and those of a tacky nature without regulating the rate of air flow to keep the solids in suspension as their weight increases incident to the application of the coating material. Furthermore, this construction makes possible the use of a single apparatus to coat charges of particles of widely varying magnitude in the same apparatus.

The expanded chamber used for coating is constructed to provide a dense zone of particles falling countercurrent to the spray pattern in the immediate area of the nozzle. This permits the most economical utilization of the coating material since there is a minimum mean free path for coating material to be entrained out of the chamber without contacting a particle to be coated. Second, the truncated cone construction permits pellets or tablets to roll down the sides, thereby achieving some of the smoothing effect of rolling before falling into the central air stream, a zone of maximum sheer and disengagement. The highly dispersed state reached in the upper chamber insures complete drying of the separated particles before they again fall into the coating zone.

Equipment.—(*a*) A Zenith laboratory metering unit type QE equipped with a Graham variability speed transmission model 45 R2.8 with infinite variable speed motor from 0 to 200 r.p.m. and 1/4 hp. master electric explosion-proof motor were used to supply fluids to atomizing nozzles. Pumps with 0.58 ml./rev. (size 1), 1.168 ml./rev. (size 2), and 2.920 ml./rev. (size 5) were used in these studies. Curves for each of these pumps were drawn to indicate the relationship of transmission setting to grams of coating pumped per minute. (*b*) New York blower No. 14 General Industrial fan arrangement No. 1, which has a capacity of 1400 C.F.M. at 12-in. static pressure at 3710 r.p.m., was driven by a 5 hp., 440 v., 1750 r.p.m., three-phase motor (Crocker Wheeler Electric Manufacturing Co.). Air flow rate was set by a variable-speed drive on the fan or by a restriction of the air at the fan intake port. (*c*) Internal and external mixing spraying systems pneumatic atomizing nozzles were used for most of these studies. Internal mixing nozzle setup No. 26 and external mixing nozzle setup No. 4 were satisfactory for most spraying needs. (*d*) A cyclone powder collector was fabricated to our specifications by the E. D. Menold Co., Philadelphia, Pa. The cyclone was designed to recover 95% of all powders. (*e*) A venturi tube was fabricated of glass in our laboratories. Powder was introduced into this venturi with a model F-00 Syntron equipped with a controller. Special tubular and flat pan troughs were used to convey the powder.

Since it was desirable to spray coatings that melt

TABLE I.—APPLICATION OF POWDERS TO PELLETS

No.	Pellet Charge, Kg.	Powder Charge, Kg.	Coating Time, Min.	% Powder Charge Applied
1	10.0	4.90[a]	5	48
2	10.0	5[a]	6	63
3	10.0	5[a]	6	57
4	10.0	5[a]	6	62
5	10.0	5[a]	7	66
6	10.0	5[b]	7	64
7	10.0	5[b]	7	60
8	17.9	7.3[c]	9	82
9	30.0	4.3[d]	10	77
10	25.0	4.3[d]	11	49

[a] Starch:sugar (1:1). [b] Calcium sulfate, dihydrate. [c] Powdered sugar. [d] Dextro-amphetamine sulfate coating powder containing 82.5% active drug.

at about room temperature, the liquid reservoir, fluid lines, and nozzles were heated (electrically or with steam) to avoid congealing in the system. Also, since heated air was needed for some experiments, a series of steam-heated pipes was installed in the air ducts between the fan and the coating zone. The temperature was regulated to ±1° using a series of steam reducer valves.

Charge Magnitude and Operation.—The following procedure is employed during a typical coating operation. The blower is turned on and the particles to be coated are permitted to fall from a reservoir into the coating chamber. The coatings are then applied *via* the nozzle assembly and/or venturi. When all of the coating agents have been applied, the blower is turned off, and the particles are permitted to fall into an appropriate container.

Using the apparatus described in Fig. 4, it is possible to suspend a minimum charge of 1 Kg. and a maximum charge of 40 Kg. of sugar pellets ranging in size from 40–16 mesh with negligible loss through entrainment. The optimal charge range for this equipment is 10–25 Kg.

Powder Coating onto Sugar Pellets.—Conventional pan-coating procedures for application of powder onto sugar pellets are lengthy since several coats are required (15). Thus, it was of interest to determine if coating time could be reduced in the ASC device. A variety of powders were applied onto 20–25-mesh sugar pellets with an adhesive consisting of 10% acidified gelatin in a hydroalcoholic solvent. The adhesive solution was sprayed through the nozzle assembly while the powder was applied simultaneously through the venturi tube. Results of 10 representative experiments are reported in Table I.

The cyclone collector was used to collect coating powder that did not adhere to the pellets on the first pass. Although the collected powder was suitable for subsequent recycling, the figures for per cent powder applied refer to the quantity that adhered on the first pass since the process was not set up for recycling of powder.

Lipid Coating of Pellets.—The classical method of preparing sustained-release pellets involves spraying of a wax and/or a fat on medicated pellets in a coating pan as described by the MacDonnell (15) and Blythe (16) patents. This pan-coating operation requires a number of separate applications to permit solvent evaporation between coats. Therefore, pan coating of sustained-release pellets is long and relatively expensive due to the costs of labor and solvent involved.

In contrast, a batch of sustained-release pellets can be prepared by the ASC procedure in a fraction of the time required for pan coating. Furthermore, the use of the ASC apparatus permits the application of waxes and fats as melts rather than as solutions in organic solvents. For example, the authors have successfully sprayed melted polyethylene, microcrystalline wax, glyceryl monostearate, glyceryl distearate, beeswax, and 12-hydroxystearyl alcohol, etc.

Data on nine batches of sustained-release pellets using 12-hydroxystearyl alcohol and glyceryl di- and monostearate–white wax as the coating agents are reported in Table II. The criteria for comparing the various sustained-release groups are the *in vitro* release profiles obtained by the method of Souder and Ellenbogen (17). The pellets coated in this case were from the same batch of *d*-amphetamine sulfate pellets. In all cases the lipid was sprayed as a melt.

The amount of coating on the pellets in Table II definitely influences the *in vitro* release of the drug; as more coating is applied, the release is depressed.

TABLE II.—APPLICATION OF SUSTAINED-RELEASE COATING TO DRUG PELLETS

No.	Starting Charge, Kg.	Spraying Time, Min.	Actual % Coating on Final Product	% *d*-Amphetamine Sulfate Released at Hr. Indicated 0.5	2	4.5	7
1	12	16.0	9.0[a]	45	75	87	93
2	12	22.5	12.5[a]	20	50	67	77
3	12	28.8	16.0[a]	7	23	44	59
4	12	35.0	19.4[a]	3	11	25	38
5	19.2	13.4	14.2[b]	44	67	96	99
6	21	18	18.8[b]	29	51	79	96
7	21	46	19.3[b]	19	43	65	93
8	20	36	25.6[b]	16	32	77	98
9	21	29.5	27.4[b]	10	16	51	92

[a] 12-Hydroxystearyl alcohol. [b] Glyceryl monostearate, 36%; glyceryl distearate, 54%; white wax, 10%.

TABLE III.—STUDY OF COATING CONDITIONS

No.	Starting Charge, Kg.	Spraying Time, Min.	Gm. Molten Coating[a] Sprayed Per Min.	Actual % Coating on Final Product	Air Temp., °C.	% *d*-Amphetamine Sulfate Released at Hr. Indicated 0.5	2	4.5	7
1	10	11.7	257	21.4	40	17	28	69	94
2	10	11.7	257	21.7	30	31	44	87	99
3	20	23.4	257	21.6	30	34	45	93	99
4	10	23.4	128	21.3	45	3	25	47	81

[a] Glyceryl monostearate, 36%; glyceryl distearate, 54%; white wax, 10%.

To determine the best coating conditions, some of the variables involved in the ASC of sustained-release pellets were investigated. Table III shows the results of one study which consisted of four experiments. Each experiment was run under different conditions to determine factors critical in influencing the *in vitro* release profile of the final product.

Table III shows the effect of these different conditions. Perhaps the most important discovery is that desired quantities of coating agent can be applied to pellets with a high degree of reproducibility. Compare the amount of coating on batch 1 to the other three batches. Also, a comparison of the *in vitro* disintegration profiles for batches 2 and 3 reveals that sustained-release pellets prepared at the same air temperature are indeed comparable. Note that the weight of the starting charge and coating time for batch 3 is twice that of batch 2. Thus, it appears that the weight of the starting charge is critical.

Studies with a Laboratory Model Air Suspension Apparatus.—The above studies suggested that a minimum charge of 10 Kg. was required to obtain optimal coating results. Thus, it was desirable to make a scaled-down model with a charge capacity of 1-5 Kg. for laboratory use to study the effects of a host of possible variables encountered in air coating. While the major emphasis was on studying the variables encountered in the preparation of medicated and sustained-release pellets, it was also desirable to determine if the apparatus had utility in other coating operations.

Lipid Coating of Pellets.—To exploit the potential advantages of the ASC procedure for the routine manufacture of lipid-coated pellets, it was necessary to study the effects of the possible variables encountered in their preparation.

Results of one series of experiments in which molten 12-hydroxystearyl alcohol was sprayed onto dextro-amphetamine sulfate pellets are reported in Table IV.

Perhaps the most important part of this study was to determine if results obtained with the laboratory model could be obtained routinely with the larger device. A comparison of the *in vitro* release profiles of batch 1 with 2 and 3, and batch 4 with 5 and 6 reveals that pellets coated in either the small or large ASC device under similar conditions possess similar release profiles. Therefore, no scale-up problems are anticipated.

Another important consideration is batch-to-batch reproducibility. Note that batches 5–9, coated under similar conditions, were coated with nearly identical quantities of 12-hydroxystearyl alcohol and had similar release profiles. Of course, this illustrates that a high degree of batch-to-batch reproducibility can be expected.

Among the variables studied, the most critical was suspending air temperature. A comparison of the *in vitro* disintegration profiles for batches 9 and 10 demonstrates this. It is thought that the warm air allows the coating to be applied more efficiently.

Other variables studied that were not critical under our conditions are rate of application and use of different nozzles. For example, when the *in vitro* disintegration profiles for batch 6 are compared to batch 9, it is evident that an increase in the rate of application from 39 to 65 Gm./minute has little effect. Also a comparison of the profiles of batches 7 and 8 to batches 5, 6, and 9 reveals that changing nozzles has a minor effect.

Enteric Pellets.—The usual methods for preparing enteric-coated pharmaceutical products are laborious. Therefore, when a development project required enteric-coated pellets, it was decided to determine if the laboratory model air suspension apparatus could be used. Since supplies of the chemical in question were in short supply, a model run was conducted with dextro-amphetamine sulfate pellets. In this study an enteric coating solution consisting of ethyl acetate (38%), ethyl lactate (11%), alcohol (38%), cellulose acetate phthalate (8.5%), and glyceryl monostearate (4.5%) was sprayed in 67 minutes with a Spraying Systems pneumatic atomizing nozzle

Table IV.—Study of Coating Variables

Batch	Starting Charge, Kg.	Spraying Time, Min.	Gm. Molten Coating[a] Sprayed Per Min.	Actual % Coating on Final Product	Suspending Air Temp., °C.	Nozzle[b]	% *d*-Amphetamine Sulfate Released at Hr. Indicated 0.5	2	4.5	7
1	12	16.0	86	9.0	40	*2*	45	75	87	93
2	3	9.0	39	9.6	40	*2*	51	80	93	100
3	3	9.0	39	10.2	40	*2*	44	77	88	100
4	12	22.5	86	12.5	40	*2*	20	50	67	77
5	3	9.9	53	14.1	40	*2*	17	45	61	80
6	3	8.0	65	14.3	40	*2*	19	47	71	82
7	3	13.4	39	14.1	40	*1*	18	52	70	80
8	3	12.5	39	14.2	40	*1*	20	52	69	82
9	3	13.4	39	14.0	40	*2*	17	45	65	78
10	3	13.4	39	14.0	R.T.	*1*	33	58	78	87

[a] 12-Hydroxystearyl alcohol. [b] Spraying systems pneumatic atomizing nozzles: *1*, external mixing nozzle set-up No. 3; *2*, external mixing nozzle set-up No. 4.

Table V.—Enteric Capsules Prepared in ASC Device

Batch	Charge, Kg.	Capsule Size	Capsules, No.	Actual % Coating on Final Product	Spraying Time, Min.	No. of Capsules Remaining After 4 Hr. in U.S.P. GF (with Disks)
1	0.7	2	1660	5.4	10	9 of 11
2	0.7	2	1660	9.5	6	18 of 18
3	1.0	1	2200	7.6	5	18 of 18
4	1.0	1	2200	8.8	5	18 of 18
5	1.0	4	4800	7.9	7	18 of 18

TABLE VI.—ENTERIC TABLETS PREPARED IN ASC DEVICE

Batch No.	Charge, Kg.	Tablets, No.	% Enteric Coating, Wt. Gain	Coating Time, Min.	No. of Tablets Remaining After 4 Hr. in U.S.P. GF (with Disks)
1	0.6[a]	1320	14.6	40.5	18 of 18
2	0.6[a]	1320	7.1	7	18 of 18
3	1.5[a]	3110	6.2	15	18 of 18
4	1.5[b]	6500	1.9	13	18 of 18

[a] Ecotrin tablets coated with acacia and coating powder (lot 2516). [b] Placebo cores.

TABLE VII.—DISINTEGRATION OF ASC ENTERIC TABLETS

Ref.	Disintegration, Min. in U.S.P. GF at 37°C. with Disks		Disintegration, 1 Hr. in U.S.P. GF with Disks, then U.S.P. IF with Disks, Min.		Hardness (Units), Electric Strong-Cobb	
	Range	Mean	Range	Mean	Range	Mean
Ecotrin Cores Lot 2516	0.5–2	1.3	...	...	8–12	10
Placebo Cores Batch No.	5–15	10	...	...	12–20	16
1	...	...	10–15	9	...	...
2	...	...	6–9	8	...	...
3	...	...	5–10	7	...	...
4	...	...	18–29	24	...	...

set up No. 5 onto 2 Kg. of pellets which was suspended in air at 45°. Duplicate release values after 1.5-hour exposure to U.S.P. gastric fluid showed that only 1 and 3% of the drug was released. In contrast, the pellets completely disintegrated in less than 30 minutes of exposure to U.S.P. intestinal fluid.

This experiment shows that the air suspension apparatus can be used to prepare enteric-coated pellets. It is likely that the spraying time could be shortened considerably with further work.

Enteric Capsules.—Although enteric capsules are not used extensively commercially, they are often used for clinical studies. These capsules are usually prepared in pans by applying numerous coats of an enteric coating solution. Since this coating process usually requires 1 day for each batch of enteric capsules, it was of interest to determine if the process could be speeded up in the ASC apparatus.

To prevent the capsules from opening in the air stream, they were sealed in a coating pan with a solution containing ethyl acetate (43%), ethyl lactate (14%), alcohol (41%), and cellulose acetate phthalate (2%). The enteric solution reported by Blythe *et al.* (18) was diluted with an equal volume of acetone: alcohol (46% acetone: 54% alcohol) and sprayed with a Spraying Systems pneumatic atomizing nozzle set up No. 5. The usual coating time was 6 minutes.

The data reported in Table V indicate that enteric capsules of various sizes can be prepared in the ASC device. This procedure requires only a fraction of the time required for pan coating. Thus the ASC device offers an excellent method for the preparation of enteric-coated capsules. It should be of particular value for the preparation of clinical supplies.

Enteric Tablets.—Singiser and Lowenthal (5) reported that the air suspension coating technique could be used to prepare enteric ammonium chloride tablets in 30 to 60 minutes. The authors have used the apparatus for this purpose also and wish to report on this phase of our ASC studies. Aspirin tablets with sealing and rounding coats as described by Blythe *et al.* (18) and uncoated compressed placebo tablets were used for these studies. The enteric solution and nozzle are the same as used for the enteric capsules. Results of four experiments are summarized in Table VI.

In Table VII original tablet disintegration and hardness data are presented which indicate that the disintegration times of the enteric tablets in intestinal fluid were within normal limits. Therefore, it appears that the ASC device also can be used for the preparation of enteric tablets.

SUMMARY

A versatile new air suspension coating apparatus has been described. This apparatus can be used in a number of pharmaceutical coating processes. For example, it has been used successfully to apply powder and lipid materials to pellets. Furthermore, it also has been used to prepare enteric-coated pellets, tablets, and capsules.

The most critical coating variable encountered in the preparation of lipid-coated pellets was the temperature of the suspending air.

A high degree of batch-to-batch reproducibility was obtained when similar conditions were used to prepare lipid-coated pellets. Reproducible results were obtained with both the large and the laboratory models. Thus, no scale-up problems were encountered.

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Kinetic Analysis of Blood Levels and Urinary Excretion in the Absorptive Phase after Single Doses of Drug

By JOHN G. WAGNER and EINO NELSON

The use of per cent absorbed *versus* time plots to elucidate kinetic models for drug absorption is described. The following kinetic models, which are illustrated with experimental data, are considered: absorption by a zero-order process, by two zero-order processes operating in parallel, by a first-order process, by two parallel first-order processes, and by simultaneous zero-order and first-order processes.

THE MATHEMATICAL description of blood, serum, or plasma drug concentration and time data and data on amounts of drug excreted in various times is receiving increased attention by academic, clinical, and industrial researchers. The industrial phamacist, in particular, is faced with the task of devising methods which hopefully will allow him to predict from *in vitro* measurements the characteristic of a product with respect to the rate and extent it releases the active ingredient contained for absorption. This may require him to make measurements *in vivo* that allow him to describe absorption rate as a function of time and interpret these in a manner consistent with the physiological reality of the *in vivo* system studied. Although the intact animal may seem to be a system too complex for any interpretation of experimental data except gross ones, this is not necessarily the case. Once data are described in mathematical terms within the experimental error of the measurements, they become subject to interpretation which must be consistent with the physiological reality of the system and the physical-chemical, physical, and pharmaceutical characteristics of the product administered.

Several parameters have been used to estimate rate of drug absorption when a drug is administered in pharmaceutical dosage forms. Rate of absorption has been assessed by time of appearance of measurable blood level, time of appearance of maximum blood, serum, or plasma levels, amount of drug or drug metabolite excreted in the urine in a given time or time of appearance of maximum urinary excretion rate. All of these parameters give only qualitative indication of rate of drug absorption because they are only indirectly related to rate. Quantitative methods for estimating rate of absorption have been developed by Dominguez (1) and Nelson (2). The method of Dominguez is applicable to blood, serum, or plasma level *versus* time data and the method of Nelson to urinary excretion rate *versus* time data. Among other quantities, the method of Dominguez requires knowledge of the apparent volume of distribution; the method of Nelson requires knowledge of the fraction of a dose excreted as unchanged drug, the fraction of the dose absorbed, and the derivative of excretion rate *versus* time data. These parameters may be difficult to obtain with accuracy.

Recently (3), modifications of the methods of Dominguez (1) and of Nelson (2) have been described which greatly simplify calculation of drug absorption rate because values of per cent of total absorption are obtained. Use of these equations does not require a knowledge of a drug's volume of distribution, what fraction of a dose is absorbed, or the fraction of the dose absorbed that is excreted as unchanged drug.

It is the purpose of this paper to illustrate the applicability of these expressions in elucidating kinetic models that describe the various processes by which drugs may be absorbed. While it may not be possible always to determine the kinetics of the absorption process, sufficient data are available to illustrate the usefulness of the methods. Equation 1 is for blood, serum, or plasma level *versus* time data, and Eq. 2 is for urinary excretion data. The derivation of these expressions is given in the *Appendix*. In Eq. 1, A_T is the cumulative amount of drug absorbed from time zero to time T in any convenient units, A_∞ is the amount

$$\% \text{ absorbed} = \frac{A_T}{A_\infty} \times 100 = \frac{C_T + K\int_{t=\infty}^{t=T} C\,dt}{K\int_{t=0}^{T=\infty} C\,dt} \times 100 \quad \text{(Eq. 1)}$$

$$\% \text{ absorbed} = \frac{A_T}{A_\infty} \times 100 = \frac{(1/K)\cdot(dX_u/dt) + X_u}{(X_u)_\infty} \times 100 \quad \text{(Eq. 2)}$$

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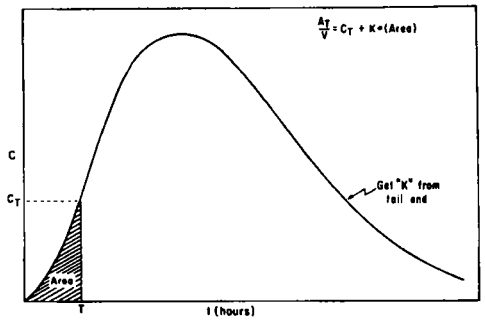


Fig. 1.—Illustrates graphically the operations necessary to obtain A_T/V (*i.e.*, the cumulative amount absorbed per unit volume of the apparent volume of distribution between time zero and time T) from blood level data.

eventually absorbed in the same units, K is the over-all rate constant for elimination from blood, etc., in reciprocal time, and C_T is blood, serum, or plasma concentration at time T. In Eq. 2, A_T, A_∞, and K have the same meanings as in Eq. 1; dX_u/dt is urinary excretion rate at time T, and X_u is the cumulative amount excreted in the same time. The method for using Eqs. 1 and 2 and the assumptions on which they are based have been described previously (3). However, their use is best illustrated by simulated blood level *versus* time curves and cumulative amount excreted *versus* time curves.

Figure 1 shows a hypothetical blood level *versus* time curve with various markings which will be used to illustrate the application of Eq. 1. The vertical line delineates an area under a blood level *versus* time curve from $t = 0$ to $t = T$ on the abscissa of the graph. These areas may be estimated by planimetry or by use of the trapezoidal rule. It is only necessary to multiply each of these cumulative areas by the value of K, as determined by well-known procedures (4), and add to each the value of blood concentration at corresponding times to obtain the cumulative amount absorbed per unit volume of the apparent volume of distribution. This is continued until a maximum or asymptotic value is obtained which is divided into the value obtained at earlier times to obtain per cent absorbed *versus* time data.

Figure 2 is a plot of cumulative amount of drug excreted *versus* time. Excretion rate values from curve in Fig. 2 are selected at various times (obtained from the slope of the curve as shown on the figure) and divided by K and added to the amount excreted in the same time as determined from the cumulative excretion curve. A maximum or asymptote will be obtained; when this quantity is obtained, division of it into the values of the quantities obtained at earlier times will give the per cent absorbed at various times in accord with Eq. 2.

Cases of Recycling via the Enterohepatic Cycle.—It is known that some drugs may be absorbed *via* the gastrointestinal tract, pass *via* the portal vein to the liver, and then be partially excreted *via* the bile back into the intestinal contents. Part or all of this drug may then be reabsorbed; some of it may be excreted in the feces.

In such cases the serum (or plasma) level–time plot may have a major maximum and a minor maximum or a major maximum and a shoulder rather than the usual exponential fall-off, beginning shortly following a single maximum in the plot. When the per cent absorbed time plot is derived from such a set of data, the cumulative per cent absorbed values may rise progressively, then decrease for a time, then increase again. At first glance this may appear to be inconsistent. However, we define rate of absorption as the rate of appearance of unchanged drug in the volume of distribution. This rate may be negative if the rate of efflux of drug from the blood back into the tract is greater than the rate of influx of drug from the tract to the blood. The latter can occur at certain times when recycling *via* the enterohepatic cycle takes place to a marked degree. In these cases it may be best to define the slope of the cumulative per cent absorbed–time plot calculated by the method of Wagner and Nelson (3) as the net flux across the gastrointestinal barrier, which is the result of influx from the tract to the blood and efflux from the blood and/or bile back into the tract. This takes care of the apparent inconsistency that the cumulative per cent absorbed can be less than it has been at earlier times, then gradually increase again.

Kinetic Models.—The kinetics of absorption, distribution, metabolism, and excretion of drugs can be adequately described by multicompartment models if unchanged drug is measured in the blood or in the urine, and measurements are made during the absorption phase. Differential equations and their solutions which depend upon the assumption that amount of drug, and not concentration of drug, is the

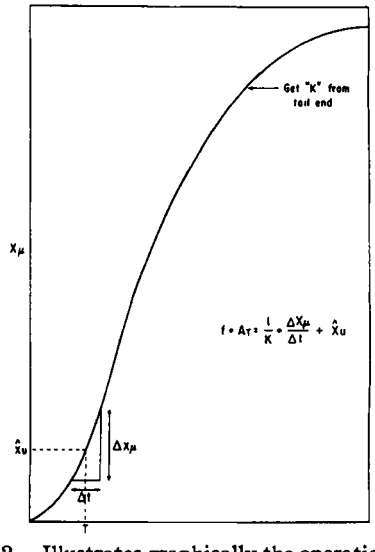


Fig. 2.—Illustrates graphically the operations necessary to obtain A_T/V (*i.e.*, the cumulative amount absorbed per unit volume of the apparent volume of distribution between time zero and time T) from a plot of cumulative amount of unchanged drug excreted in the urine against time.

driving force also are applicable to these kinetics. This is true since the assumption also is applicable to these kinetics. The assumption and definitions used herein include the following concepts: (*a*) only the volume of one compartment—namely, the apparent volume of distribution—is involved; (*b*) the rate of absorption is defined as the rate of appearance of ***unchanged*** drug in the apparent volume of distribution in amount/unit time, or per cent of total drug absorbed/unit time; hence, although the driving force may be the concentration in the intestinal contents, the volume of the intestinal content does not enter into the calculations and may be ignored; (*c*) the amount of unchanged drug excreted in the urine in a given time is the quantity measured and used in the kinetic calculations; hence, the volume of the urine is of importance only in the estimation of the amount of drug in the urine.

The apparent volume of distribution, V, is simply the number by which to multiply the plasma, serum, or whole blood concentration, so that there is material balance according to Eq. 3.

$$C_T V = A_T - E_T - M_T \qquad \text{(Eq. 3)}$$

where C_T is the plasma, serum, or whole blood concentration of unchanged drug at time T, A_T is the cumulative amount of unchanged drug absorbed to time T, E_T is the cumulative amount of unchanged drug which has been eliminated in the urine to time T, and M_T is the cumulative amount of drug metabolized to time T. No physical significance should be assigned to V since part of the drug in the volume, V, may be bound to protein or stored temporarily in one particular organ. Also, it is obvious that depending upon whether plasma, serum, or whole blood concentration is measured, the value of V will change even though everything else is held constant.

As previously explained, the method of calculation using blood (serum or plasma) unchanged drug concentration data involves estimation of A_T/V at various times after administration of the dose. The maximum or asymptotic value of A_T/V, $(A_T/V)_{max.}$ is FD/V, where F is the fraction of the dose absorbed and D is the dose. Hence, FD is the amount of drug absorbed and FD/V is the amount of drug absorbed per unit of apparent volume of distribution in concentration units. By the method of calculation one never knows the absolute values of F or V but the value of FD/V is obtained. This value is comparable to, but not the same as, the C_0 obtained by extrapolating a semilogarithmic plot of blood level against time. The C_0 obtained by such a method is an approximation (and frequently a poor one) for the desired value FD/V. Due to the relationship

$$FD = VK \int_{t=0}^{t=\infty} C dt \qquad \text{(Eq. 4)}$$

where K is the first-order rate constant for loss of drug from the volume of distribution, and the integral is the area under the blood level curve from time zero to time infinity, we have

$$\frac{FD}{V} = K \int_{t=0}^{t=\infty} C dt \qquad \text{(Eq. 5)}$$

Equation 5[1] provides a method of checking the value of FD/V obtained from the A_T/V *versus* time plot if sufficient blood samples are taken. Once the exponential fall-off of C with t is established, the area for the tail end of the C *versus* t plot can be estimated by

$$C = C_0 \exp[-Kt] \qquad \text{(Eq. 6)}$$

which describes this part of the blood concentration *versus* time curve. In Eq. 6, C is blood, etc.; concentration at time t and C_0 is defined below. If t' is the time when the exponential fall-off of blood concentration begins, and $C_{t'}$ is the blood concentration at this time, then the area under the blood concentration *versus* time curve from t' to t_∞ is given by

$$\int_{t=t'}^{t=\infty} C dt = \int_{t=t'}^{t=\infty} C_{t'} \exp[-K(t-t')] dt = \int_{t=\infty}^{t=t'} C_0 \exp(-Kt) dt = \frac{C_0}{K} \exp(-Kt') \qquad \text{(Eq. 7)}$$

where $\ln C_0 = \ln C_{t'} + Kt'$. This area must be added to the area under the C, t plot from $t = 0$ to t' to yield the area $\int_{t=0}^{t=\infty} C dt$.

Hence

$$\frac{FD}{V} = K \int_{t=0}^{t=\infty} C dt = K \left[\int_{t=0}^{t=t'} C dt + \int_{t=t'}^{t=\infty} C dt \right] \qquad \text{(Eq. 5a)}$$

If sufficient blood samples are taken during the interval $t = 0$ to $t = t'$, then the middle integral of Eq. 5a may be estimated with good accuracy using the trapezoidal rule. Also, as a consequence of the foregoing, the following theoretical relationship holds:

$$\frac{FD}{V} = \left(\frac{A_T}{V}\right)_{max.} = K \int_{t=0}^{t=\infty} C dt = C_{t'} + K \int_{t=0}^{t=t'} C dt \qquad \text{(Eq. 8)}$$

Variability of Blood Level and Urinary Excretion Data.—True variability of blood level and urinary excretion data may be attributed to the following factors: (*a*) assay error, (*b*) biological variation (*i.e.*, variation in V, f, F, and K) not only from subject to subject but also for a given subject from drug to drug or with a given drug, and (*c*) dosage form effects (*i.e.*, variation in F and dA/dt) not only from subject to subject tested with the same formulation of the same drug but also for a given subject from day to day with the same formulation.

Apparent variability of blood level and urinary excretion data may be attributed to (*a*) using a time interval too long for excretion rate measurement, (*b*) using the wrong mathematical method with a particular set of data (the mathematical method may be wrong since the method is based on certain assumptions which are invalid for the particular set of data being evaluated), (*c*) using the wrong criterion to estimate variability.

When comparing urinary excretion data with blood level data, blood (serum or plasma) levels must be measured at the midpoints of the urinary collection intervals to expect the proper equation to apply.

[1] Or its equivalent, Eq. 5a.

The correlation of excretion rate with blood levels must be done for each subject independently, since the theoretical relationship (4) indicates the slope of the straight line relating these variables will be KfV, and this will vary from subject to subject. Furthermore, this equation is true only where free unchanged drug is measured in the blood and the urine. Hence, if excretion rate does not correlate linearly with blood level when one measures, for example, a radioactive tracer such as tritium, etc., one should not blame the mathematical method. For example, the conclusion that, "The urinary excretion of both drugs varied widely among individuals, indicating this parameter is limited in value as a reflection of oral absorption," which has been made (5) was not justified on the basis of the evidence the authors presented. The authors measured tritium radioactivity in blood and urine; hence, one would expect that there may not be a quantitative relationship between urinary excretion rate and plasma level. Their first excretion interval of 0 to 4 hours was too large and probably covered all the absorption phase or even more, especially for phenylephrine hydrochloride. They could have compared the serum levels at 2 hours, with excretion in the 0 to 4-hour interval (since only in this instance were the serum levels at the midpoints of the excretion intervals); yet they failed to do so. They did not calculate per cent standard deviations—the most valid way to compare variability when the averages and dimensions of that which one is comparing are different. In fact, the per cent standard deviations calculated from their data (5) in Tables II and III were 49.7% for the plasma levels at 10 hours and 47.1% for the urinary excretion in the 8- to 12-hour interval for phenylephrine given as the hydrochloride, and 25.9% and 28.6%, respectively, for phenylephrine given as the tannate. In each pair, these are of the same order of magnitude. Another factor not recognized was that drug metabolites are usually eliminated more rapidly than they are formed. Hence, in the nonspecific assay used, no correlation between drug blood level and urinary excretion rate should be expected.

Correlation of excretion rate and serum level of tetracycline in three normal humans has been reported recently (6). The experimental design and mathematical methods need not be repeated here; the reader is referred to that paper for the details. In this particular set of experiments, it was necessary to carry out three assays on each serum sample to obtain about the same accuracy and precision as one obtains with a single urine assay.

Rate of urinary excretion of unchanged drug is given by (4)

$$\frac{dX_u}{dt} = KfVC \qquad \text{(Eq. 9)}$$

where dX_u/dt is urinary excretion rate, and f is the fraction of a dose eventually excreted unchanged in the urine, and the other terms have their usual meaning. Integration of Eq. 9 between the limits $t=0$ and $t=\infty$ and transposition of the f term gives

$$\frac{(X_u)_\infty}{f} = VK\int_{t=0}^{t=\infty} Cdt \qquad \text{(Eq. 10)}$$

Hence, another possible test of the variability of urinary excretion and blood levels is to compare the per cent standard deviation of $(X_u)_\infty$ and of $K\int_{t=0}^{t=\infty} Cdt$ obtained with a panel of known subjects in whom these quantities could be estimated at the same time. Experiments of this type were recently carried out at the organization with which one of the present authors (7) is affiliated using commercial capsules of tetracycline hydrochloride and a panel of eight male adults. The per cent standard deviation was 23.0% for $(X_u)_\infty$ based on 96-hour urinary excretion, 36.9% for the asymptotic value of $C_T + K\int_{t=0}^{t=T} Cdt$ (which is theoretically equivalent to $K\left(\int_{t=0}^{t=\infty} Cdt\right)$ in one experiment, 36.0% for $(X_u)_\infty$ based on 96-hour urinary excretion, and 24.3% for the asymptotic value of $C_T + K\int_{t=0}^{t=T} Cdt$ obtained in the second experiment. From these data one could *not* conclude that urinary excretion data were less reliable indices of physiological availability than blood level data.

Physiological Availability.—Oser and his associates (8) introduced the concept of physiological availability. They defined physiological availability as

$$\%\ \text{availability} = \frac{\%\ \text{of dose excreted in given time after test dose}}{\%\ \text{of dose excreted in same time after aqueous solution of drug is taken}} \times 100 \qquad \text{(Eq. 11)}$$

It is important to collect urine for a period long enough that essentially all drug excreted is collected. Otherwise, the estimated availability will be erroneous.

A more generalized equation for calculating physiological availability is

$$\%\ \text{availability} = \frac{\text{amount (or \%) absorbed after test dose}}{\text{amount (or \%) absorbed following the same dose in most readily available form}} \times 100 \qquad \text{(Eq. 12)}$$

Wagner *et al.* (9) showed that physiological availability could be estimated from blood level data as well as from urinary excretion data. Although the example utilized Eq. 8, the calculation was unnecessarily complicated by estimation of V in the individual subject by extrapolation of a plot of the logarithm of C against t.

If a crossover study is carried out in a panel of humans in which one or more test formulations of a given drug is compared with a readily available form of the drug, then the methods for estimating physiological availability are

Method 1:

$$\% \text{ availability} = \frac{\text{Average asymptotic value of } C_T + K\int_{t=0}^{t=T} Cdt \text{ for test preparation}}{\text{Average asymptotic value of } C_T + K\int_{t=0}^{t=T} Cdt \text{ for the readily available preparation}} \times 100 \quad \text{(Eq. 13)}$$

Method 2:

$$\% \text{ availability} = \frac{\text{Asymptotic value of } C_T + K\int_{t=0}^{t=T} Cdt \text{ calculated from average blood levels for test preparation}}{\text{Asymptotic value of } C_T + K\int_{t=0}^{t=T} Cdt \text{ calculated from average blood levels for readily available preparation}} \times 100 \quad \text{(Eq. 14)}$$

Method 3:

$$\% \text{ availability} = \frac{\text{Asymptotic value of } \frac{1}{K}\frac{dX_u}{dt} + X_u \text{ for test preparation}}{\text{Average asymptotic value of } \frac{1}{K}\frac{dX_u}{dt} + X_u \text{ for readily available preparation}} \times 100 \quad \text{(Eq. 15)}$$

Method 4:

$$\% \text{ availability} = \frac{\text{Asymptotic value of } \frac{1}{K}\frac{dX_u}{dt} + X_u \text{ calculated from average amounts excreted for test preparation}}{\text{Asymptotic value of } \frac{1}{K}\frac{dX_u}{dt} + X_u \text{ calculated from average amounts excreted for readily available preparation}} \times 100 \quad \text{(Eq. 16)}$$

Method 5:

$$\% \text{ availability} = \frac{\text{Average } (X_u)_\infty \text{ for test preparation}}{\text{Average } (X_u)_\infty \text{ for readily available preparation}} \times 100 \quad \text{(Eq. 17)}$$

Method 6:

$$\% \text{ availability} = \frac{\text{Average } \int_{t=0}^{t=\infty} Cdt \text{ for test preparation}}{\text{Average } \int_{t=0}^{t=\infty} Cdt \text{ for readily available preparation}} \times 100 \quad \text{(Eq. 18)}$$

Method 7:

$$\% \text{ availability} = \frac{\int_{t=0}^{t=\infty} Cdt \text{ calculated from average blood levels of test preparation}}{\int_{t=0}^{t=\infty} Cdt \text{ calculated from average blood levels of readily available preparation}} \times 100 \quad \text{(Eq. 19)}$$

Method 5, which is equivalent to the method of Oser and associates (8), requires collection of urine for approximately seven to ten biological half-lives of the particular drug involved. Urine must be collected for this interval of time to ensure collection of about 99% of the unchanged excreted drug. *Methods 6* and *7* require blood samples to be collected at intervals frequent enough and for a time long enough that there is obtained a good estimate of the area under the blood level–time curve from zero time to the time that the blood level is no longer measurable. *Methods 3* and *4* require that urine be collected only during the absorption phase, provided K is known. *Methods 1* and *2* require that blood samples be collected only during the absorption phase, provided K is known.

Usually, but not always, the asymptotic value of $C_T + K\int_{t=0}^{t=T} Cdt$ will be reached at, or shortly after, the peak of the *C versus t* plot. A pilot experiment will usually determine whether this is true for the particular formulation under study. If a pilot experiment is conducted first, one cannot only estimate K for the particular drug but also establish a reasonable blood and urine sampling scheme which is applicable to the particular drug and formulations. The most important blood and urine samples are those between the time of administration and the time when the maximum blood level is reached and those taken in the terminal part of the blood level–time plot or cumulative urinary excretion–time plot. The absorption phase is covered by the first group of samples and the value of K obtained from the latter group of samples. Hence, often a reduction or elimination of several blood or urine samples can be made between the time of the peak blood level and the time the blood or urine levels become essentially zero. Unfortunately, most blood level data reported in the literature were obtained with such sampling schedules that most of the results fall in the latter range.

If unchanged drug is measured in the blood and urine, then all seven methods above should give, within experimental error, the same estimate of physiological availability. Large deviations between the percentage obtained by different methods of calculations, say about 20%, should lead to the suspicion that one or more of the assumptions involved in making one or more of the estimates is invalid for the particular set of data.

Biological Examples.[2]—The first example in this

[2] Averaged data are used in fitting to various equations discussed. This may lead to errors in interpretation of the kinetic model which actually holds (11). This consideration is only important when wide variation in rate constants occurs in test subjects (12). Extremely large variations did not occur in the cases illustrated. However, the definitive test of the correctness of a given kinetic model lies in the demonstration that the individual rather than the average data obey the model.

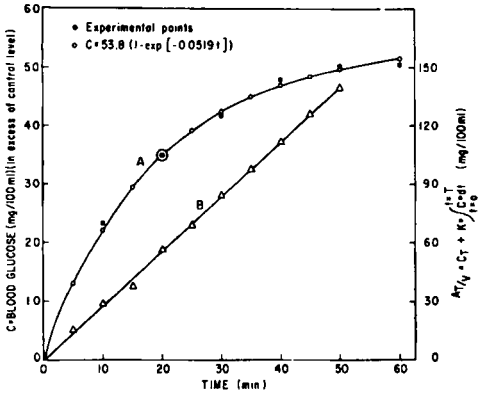


Fig. 3.—Curve A is a plot of blood glucose level against time taken from Jokipii and Turpeinen (10). The solid line is the one described by the equation A_T/V, and the solid dots are the experimentally observed values. Line B is a plot of A_T/V against time calculated according to the method described in the text.

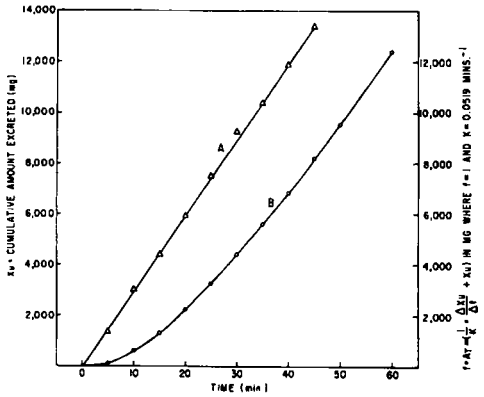


Fig. 4.—Agreement between theoretically predicted infusion rate of glucose calculated with the appropriate equation (right ordinate) and that known to be experimentally correct. Curve A is the least squares line, and the triangles are calculated values. Curve B represents hypothetical cumulative urinary excretion of glucose.

section will consider the case where the drug enters the fluids of distribution at a constant rate (zero-order process) and is eliminated from these fluids at a rate directly proportional to the existing concentration at a given time (first-order process). This sequence of processes is shown as *Model I* in the *Appendix*, and the expression which describes blood concentration as a function of time is Eq. 5A of the appendix.

The data used are the results of the experiments of Jokipii and Turpeinen (10). Glucose was infused as 5 to 6% isotonic solutions into the cubital vein at constant rates. The mean rate of infusion in 24 normal subjects was 297 mg./minute. The mean preinjection blood sugar was 84.9 mg./100 ml. This is the zero level at time zero shown on Fig. 3. The mean blood sugar level at 60 minutes is shown also in Fig. 3. The solid dots are the arithmetic values. The open circles are those obtained by the best fit—namely, $C = 53.8\,[1-\exp(-0.0519t)]$.

The triangles in Fig. 3 are values of $C_T + K\cdot$(area) calculated from these data. The slope of this line through these points is 2.794 ml./minute 100 ml. The line is straight, a reflection that the glucose was infused at a constant rate.

Since it is known that the actual infusion rate was 297 mg./minute, an estimate can be made of the apparent volume of distribution, V, from this rate and the slope of the A_T/V time plot. The calculated V is 10,600 ml. Exactly the same answer was obtained by Jokipii and Turpeinen (10) by assuming that the *Model 1* applied to these data.

Assume all the glucose in the blood was excreted unchanged in the urine, and urine was assayed for glucose. Figure 4 shows a hypothetical cumulative excretion curve for this case.

It should be noted that the cumulative excretion curve, X_u *versus* t, is curved throughout the 60-minute infusion period. It appears linear from 40 to 60 minutes, but the rate calculated in this interval is 274 mg./hour, which is 7.7% lower than the actual rate of 297 mg./minute.

By the method previously outlined, values of $1/K\cdot(\Delta X_u/\Delta t) + X_u$ (see Eq. 42A of the *Appendix*) were calculated and are shown as the triangles here. The slope of the line drawn through these points is 297 mg./minute, which is exactly the known infusion rate. These results are supporting evidence that the simple graphical method is highly accurate. The apparent linearity of the hypothetical cumulative excretion plot in the 40 to 60-minute interval is shown to be only apparent in Fig. 4 when the excretion rate is plotted against time (*cf.* Fig. 5). A cumulative excretion plot is very "insensitive" compared with the derivative plot.

If a loading dose, exactly equal to k_0/K, is injected rapidly intravenously and at the same time the constant rate infusion is started, then the blood level will remain constant,[3] and a plot of cumulative amount of unchanged drug in the urine against time will be linear as long as the constant infusion rate is maintained.

It may be that absorption proceeds into the fluids of distribution by two parallel zero-order processes (*Model 2* of the *Appendix*). Data are available with which this can be shown. Tanaka *et al.* (13) reported blood levels of sulfanilamide in five dogs administered in two different formulations of sulfanilamide orally. Sample I was a micropellet formulation containing 33.2% of sulfanilamide in gelled gelatin. Sample II was a similar micropellet formulation which had been treated with 10% formalin-isopropanol at 2 to 5° for 24 hours, then dried; it contained 19.0% sulfanilamide. Both preparations were administered orally to the dogs at a dose of 250 mg. of sulfanilamide per kilogram of body weight. The results obtained with the second preparation were interpreted in terms of the model just discussed after computing per cent absorbed *versus* time plots. The results are summarized in Fig. 6. As may be seen, two zero-order processes were in operation during absorption. The values of the constants and material balance are summarized in Table I.

In a study in which 200-mg. doses of lincomycin were injected intramuscularly, data (7) were obtained which could be interpreted as consistent with *Model 3* of the *Appendix*. Nearly 70% of the dose was absorbed rapidly, comparable to a rapid intra-

[3] Actually, in the usual medical experiment the loading dose is less than or greater than k_0/K, and the constant rate infusion is continued until the measured blood level becomes apparently constant.

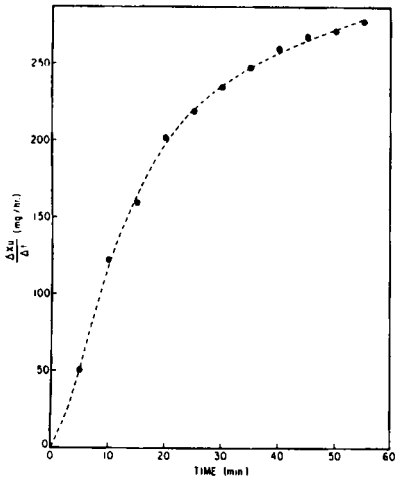


Fig. 5.—Hypothetical urinary excretion rate of glucose *versus* time. (See text regarding this curve.)

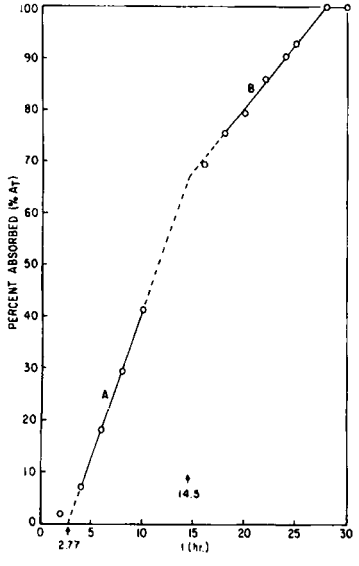


Fig. 6.—Per cent absorbed *versus* time plot calculated from blood level *versus* time data obtained following the administration of sulfanilamide in sustained-release form (see text for reference). Two zero-order processes were apparently in operation. The equation describing segment A is $\% A_T = 5.67t - 15.7$, and the equation for segment B is $\% A_T = 2.48t + 30.5$. The arrow on the figure indicates the time in hours when the first process ceased.

venous injection, and about 30% by a first-order process (see Fig. 7). Hence, data were fitted to the sum of Eqs. 7A and 20A of the *Appendix*. The correlation between experimentally observed and theoretically predicted values of serum level *versus* time by the resulting equation is shown in Table II. It will be seen from data in this table that the correlation was excellent.

It may also be the case that drug is absorbed by two first-order processes operating in parallel, as in the scheme shown as *Model 4* in the *Appendix*.

Data in the literature (14) on blood levels of sulfaethylthiadiazole as a function of time after the administration of a sustained-release suspension of this substance have been interpreted in terms of this model.

Per cent absorbed *versus* time values were calculated using Eq. 1. When plotted (Fig. 8), it appeared that the line connecting the points might be one delineating a cumulative first-order process.

TABLE I.—SUMMARY OF DATA FOR A CASE OF ABSORPTION AND ELIMINATION CONFORMING TO *Model 2*

Clock Time, Hr.	Time Interval, Hr.	% of Dose Absorbed/ Hr.[a,b]	Total % of Dose Absorbed
2.8–14.5	11.7	3.19	37.3
2.8–28	25.2	2.48	62.5
		Total	99.8

[a] The value shown for the first interval is that calculated by subtracting the value for the second interval from the slope of the first linear segment of the two shown in Fig. 6. The slope of the latter mentioned line was 5.67% per hour. [b] The true dimensions of a zero-order rate constant are amount/unit time. Therefore, because of the method of calculation, *viz.*, per cent absorbed *versus* time, the values in this column should be thought of as multiplying the amount eventually absorbed.

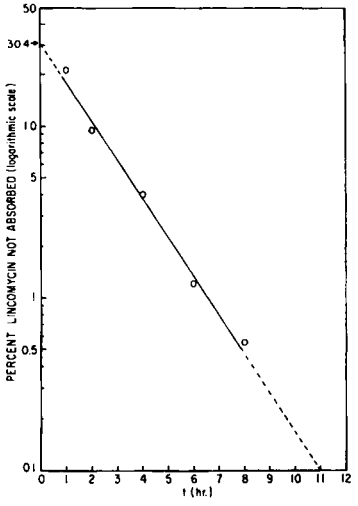


Fig. 7.—Apparent first-order absorption of part of the dose of lincomycin (hydrochloride) following intramuscular injection. The arrow on the ordinate indicates the percentage absorbed by this process. The initial part of the dose was absorbed at a rate comparable to that observed following intravenous injection. The line shown on the graph would be described by $100 - A_T/A_\infty = 30.4 \exp(-0.515t)$ on Cartesian coordinate paper. The open circles are values calculated from serum level data using Eq. 1 and after subtraction of these from 100.

TABLE II.—COMPARISON BETWEEN OBSERVED AND THEORETICALLY PREDICTED LINCOMYCIN SERUM LEVELS AT VARIOUS TIMES[a]

Time, Hr.	Observed Serum Levels, mcg./ml.	Theoretically Predicted Serum Levels, mcg./ml.
1	3.8	3.5
2	3.8	3.8
4	3.0	2.8
6	2.3	2.3
8	1.7	1.7
12	0.9	0.9

[a] Following a 200-mg. intramuscular dose.

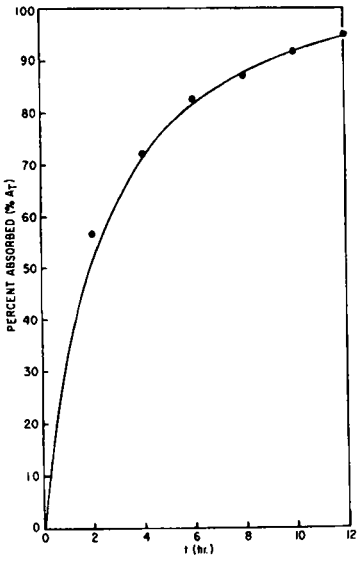


Fig. 8.—Per cent absorbed *versus* time plot calculated from blood level *versus* time data obtained following oral administration of sulfaethylthiadiazole in sustained-release form (14). The solid circles are values directly calculated from average serum levels. The line is based on the analysis shown in Fig. 9.

Therefore, per cent drug unabsorbed was plotted *versus* time on semilogarithmic paper. (The procedure was the same as in the preceding case.) This plot is shown in Fig. 9. If the differences between line B and extrapolated line C are plotted as a function time, another straight line, A, results. This could be interpreted as an indication that absorption occurred by two parallel apparent first-order processes.[4]

The appropriate equation to estimate the blood levels of sulfaethylthiadiazole is Eq. 28A in the *Appendix*. For this particular example, the value FD/V = 20 mg.% was obtained by extrapolating a plot of A_T/V *versus* time (analogous to extrapolating Fig. 8 to 100%). The other constants were obtained as follows: f_i = 0.334 from the intercept of line A and f_s = 0.666 from the intercept of line C of Fig. 9; k_a = 1.074 hr.$^{-1}$ from the slope of line A and k_a' = 0.219 hr.$^{-1}$ from the slope of line C of Fig. 9; K = 0.0928 hr.$^{-1}$ was obtained from data in the same paper (14) obtained following administration of crystalline (noncoated) sulfaethylthiadiazole. Figure 10 compares the estimated blood levels (dotted line) with the observed blood levels (solid dots). The estimated blood levels agree to within 0.3 to 5% (average 2.1%) of the observed blood levels. Even better agreement of estimated and observed blood levels could have been obtained with a computer using the blood level and time values themselves rather than the logarithms of the blood level values.

Hypothetical Example.—A hypothetical example of *Model 5* of the *Appendix* illustrates the accuracy of the method when absorption occurs by simultaneous zero-order and first-order processes. The parameters chosen (Eq. 32A) were as follows: FD = 100%, f_s = 0.70, k_0 = 7%/hour (hence, 10 hours were required to release all the "drug" from compartment A at a constant rate), f_i = 0.30, k_a = 0.693 hour^{-1}, and K = 0.1386 hour^{-1} corresponding to a biological half-life of 5 hours. Figure 11 is a plot of the per cent of drug in compartment C

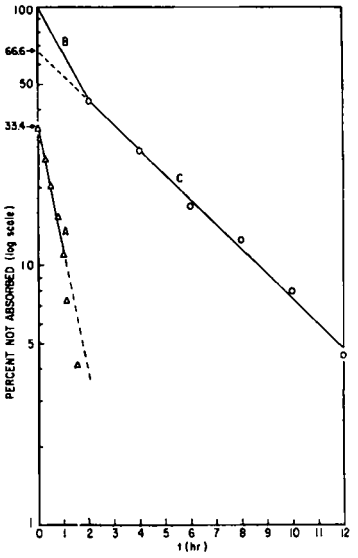


Fig. 9.—A plot of per cent sulfaethylthiadiazole unabsorbed (logarithmic scale) *versus* time showing two components in the absorption phase. The open circles delineate a line described by 100 − A_T = 66.6 exp (−0.219t) on linear coordinate paper. The points for the line delineated by triangles were obtained by subtracting values given by the extrapolated portion of the other line and those given by the line connecting the last open circle and the 100% value. The equation of this line on linear coordinate paper is described by 100 − A_T = 33.4 exp (−1.074 t). The arrows on the ordinate indicate the percentages not absorbed by the two parallel first-order process. (Additional explanation is given in the text.)

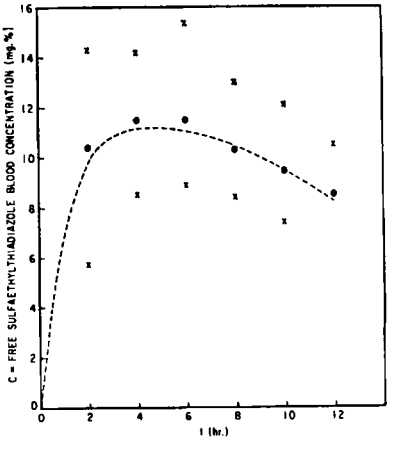


Fig. 10.—Agreement between theoretically predicted sulfaethylthiadiazole blood levels and time (line) and experimentally observed levels (solid circles). The X's represent range of individual subject experimental values observed. Mean values were used to obtain the plotted solid circles and the theoretical line.

[4] It also could be the case that two consecutive processes were involved. However, this was shown not to be the case by further mathematical analysis.

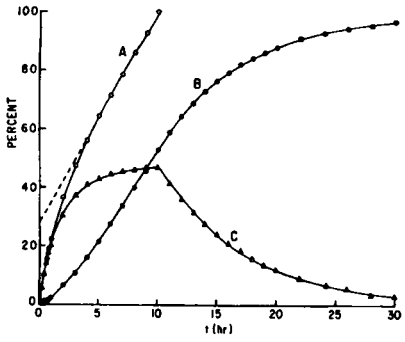


Fig. 11.—Hypothetical percentages of drug in various compartments of distribution for a specific case when drug is absorbed by parallel zero and first-order processes. This is *Model 5* of the *Appendix*. (See text under *Hypothetical Example* for explanation of curves.)

(analogous to the blood level) and the cumulative amount of drug in compartment D (analogous to unchanged drug excreted in the urine plus metabolites). The amounts remaining in compartments A and B at times t—namely, X_a and X_b, respectively—were directly calculated and used to produce the third line, $\%\ A_T = 100-(X_a + X_b)$, shown in Fig. 11. From 5 to 10 hours the latter line is essentially linear. However, it is not exactly linear, since at 5 hours 6.25% of the original 30% of the drug in compartment A is still there and being released at a first-order rate. However, this is a relatively small amount compared with the 35% being released at a rate of 7%/hour which is in compartment B at the same time. By assuming linearity of the $\%\ A_T$, t plot in the 5- to 10-hour interval, some error is introduced. The magnitude of this error is shown in Table III in the first row. The second row in the table gives the constants back-calculated from the compartment C data using Eq. 1; the third row in Table III gives the constants back-calculated from the compartment D data using Eq. 2. It may be seen that the new methods introduce little or no more error than is caused by the assumption that the $\%\ A_T$, t plot is linear in the 5- to 10-hour period. These results were achieved simply with a pencil and paper using the trapezoidal rule to estimate the areas and reading the X_d values directly off the original graph. Both analog and digital computers yielded the same results.

This model illustrates another important point. Even when 70% of the dose was released at a constant rate, the blood level (amount in compartment C) never became flat or constant, and the cumulative excretion curve (amount in compartment D) never became linear. Even if the proper loading dose—namely, $k_0/K = 7\%/0.1386 = 50.5\%$—had been placed in compartment B initially and the remaining 49.5% put in compartment A and released over 10 hours at a constant rate of 4.95%/hour, the resulting compartment C plot would be nearly, but not exactly, parallel to the time axis for only a short interval of time.

Equations to Estimate Blood Levels.—The equation to provide estimated blood levels at any time t after administration of a single dose of a drug may also be derived by using only the numerator of Eq. 1 and by using Eq. 8. In this case, the total initial amount in compartments A and B of the models in the *Appendix* becomes FD/V and is in the same concentration units as the measured blood levels. In this case, the per cent absorbed *versus* time plot is replaced by an A_T/V *versus* time plot, where A_T/V is in the units of the measured blood levels. This procedure results in considerable time saving since the conversion to percentages is eliminated.

APPENDIX

This appendix considers various models to describe drug absorption, metabolism, and excretion with which new data and data in the literature may be interpreted (in most cases). Also contained is the derivation of the mathematical expressions which describe blood, serum, or plasma level and in some cases, urinary excretion of drug in the models presented.

Model 1.—The first model to be considered is

$$A \xrightarrow{k_0} C \xrightarrow{K} D$$

In *Model 1*, A represents drug at an absorption site or in an infusion apparatus being transferred to a compartment C (drug in blood and the other fluids of distribution in apparent equilibrium) by a process whose rate is constant (zero-order process) and from which it is removed from the body by metabolism

TABLE III.—CONSTANTS OF *Model 5* BACK-CALCULATED BY VARIOUS METHODS AND THE PERCENTAGE ERROR OF THE CALCULATED VALUES FROM THE ACTUAL VALUES USED TO GENERATE THE MODEL CURVES

	Constant Involved							
	100 f_b, %		100 f_s, %		k_1, Hr.$^{-1}$[a]		k_0, %/Hr.	
Method of Calculation	Calcd. Value	% Error	Calcd. Value	% Error	Calcd. Value	% Error	Calcd. Value	% Error
Graphically from actual X_a and X_b values from 5 to 10 hr.	28	−6.67	72	+2.86	0.717[a]	+3.46	7.18	+ 2.6
From X_c, t values using $X_c + K\int_{t=0}^{t=T} X_c dt$	28.1	−6.33	71.9	+2.71	Not estimated		7.17	+ 2.4
From X_d, t values using $\frac{1}{K}\frac{\Delta X_d}{\Delta t} + X_d$	28.2	−6.00	71.8	+2.57	Not estimated		7.19	+ 2.7
From actual X_d, t values from 5 to 10 hr., *assuming* excretion rate is constant	These values indeterminate by this method						6.27	−10

[a] $k_1 = \frac{-2.303}{t}\log_{10}\left[\frac{100 - \%\ A_T - (100\ f_s - k_0 t)}{100\ f_b}\right]$. Values of k_1 were calculated for t = 0.2, 0.4, 0.6, 0.8; 1, 2, 3, and 4 hr. then averaged.

and/or excretion of unchanged drug at a rate directly proportional to the concentration present in compartment C (first-order process) to compartment(s) D. The quantities k_0 and K are the zero and first-order rate constants, respectively, with dimensions of amount per unit time and reciprocal time, respectively. The rate of change of concentration C in compartment C with time in *Model 1* will be equal to

$$\frac{dC}{dt} = \frac{k_0}{V} - KC \qquad \text{(Eq. 1A)}$$

where V is the apparent volume in which drug becomes distributed (*i.e.*, the volume of compartment C). Rearranging Eq. 1A and integrating gives

$$-\frac{1}{K} \ln \left(\frac{k_0}{V} - KC\right) = t + \text{constant} \quad \text{(Eq. 2A)}$$

If at zero time no drug is absorbed, the value of the constant is

$$-\frac{1}{K} \ln (k_0/V) = \text{constant} \quad \text{(Eq. 3A)}$$

Inserting Eq. 3A in Eq. 2A and rearranging gives

$$\ln \frac{k_0/V - KC}{k_0/V} = -Kt \qquad \text{(Eq. 4A)}$$

If Eq. 4A is raised to power of the natural logarithm and rearranged, the following expression results which gives blood, serum, or plasma concentration as a function of time:

$$C = \frac{k_0}{KV} [1 - \exp(-Kt)] \quad \text{(Eq. 5A)}$$

If the constant of integration in Eq. 2A is evaluated under the condition that at zero time $C = C^0$, then the expression becomes after rearranging and raising it to powers of the natural logarithm

$$C = \frac{k_0}{KV} + \left(\frac{k_0}{KV} - C^0\right) \exp(-Kt) \quad \text{(Eq. 6A)}$$

If C^0 is selected to equal k_0/KV, then Eq. 6A reduces to

$$C = \frac{k_0}{KV} \qquad \text{(Eq. 7A)}$$

and blood, etc., concentration would be constant for as long as drug is absorbed or introduced at a constant rate. This is the case when an appropriately calculated initial loading dose is given by rapid intravenous injection, and drug infusion starts immediately at a constant rate by the intravenous route.

If accumulation of unchanged drug is followed in compartment D, assuming that some part of the dose is excreted unchanged in the urine, an expression can be developed to describe this accumulation. Urinary excretion rate will be given by (4)

$$\frac{dX_u}{dt} = k_1 VC \qquad \text{(Eq. 8A)}$$

where k_1 is the first-order urinary excretion rate constant. Substituting for C by its value as given in Eq. 5A yields

$$\frac{dX_u}{dt} = \frac{k_1 k_0}{K} [1 - \exp(-Kt)] \quad \text{(Eq. 9A)}$$

Integrating Eq. 9A gives

$$X_u = \frac{k_1 k_0 t}{K} + \frac{k_1 k_0 \exp(-Kt)}{K^2} + \text{constant} \quad \text{(Eq. 10A)}$$

If at zero time no drug has been excreted, the constant of integration has the following value:

$$\text{constant} = -\frac{k_1 k_0}{K^2} \qquad \text{(Eq. 11A)}$$

Inserting this expression in Eq. 10A and rearranging gives

$$X_u = \frac{k_1 k_0 t}{K} - \frac{k_1 k_0}{K^2} [1 - \exp(-Kt)] \quad \text{(Eq. 12A)}$$

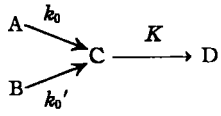
as the expression desired. After a sufficiently long time (seven to ten half-lives), the term containing the exponential in Eq. 12A becomes negligible and the equation reduces to

$$X_u = \frac{k_1 k_0 t}{K} - \frac{k_1 k_0}{K^2} \qquad \text{(Eq. 13A)}$$

and a plot of cumulative amount of unchanged drug excreted would become linear.

In Eq. 13A the ratio, k_1/K, is the fraction of a dose, f, eventually excreted unchanged.

Model 2.—Another model in which the drug absorption part would conceivably be found in practice is



The constants k_0 and K have the same meaning as previously. The constant, k_0', is a zero-order rate constant for an absorption or infusion process which operates simultaneously with the other zero-order process. In *Model 2* rate of change of blood, etc. (compartment C) concentration with time is given by

$$\frac{dC}{dt} = \frac{k_0 + k_0'}{KV} - KC \qquad \text{(Eq. 14A)}$$

By the procedure identical to that used to obtain Eq. 5A, blood concentration as a function of time in *Model 2* will be given by

$$C = \frac{k_0 + k_0'}{KV} [1 - \exp(-Kt)] \quad \text{(Eq. 15A)}$$

Model 3.—It could be the case that absorption proceeds by a single first-order process and the situation may be depicted as

$$FD \text{ at } t = 0\} \quad A \xrightarrow{k_a} C \xrightarrow{K} D$$

In *Model 3*, k_a is the first-order rate constant for the absorption process. The rate of change of blood, etc., concentration is given by

$$\frac{dC}{dt} = \frac{k_a X_a}{V} - KC \qquad \text{(Eq. 16A)}$$

In Eq. 16A, X_a is the amount of the drug at the absorption site at any time, t, and the other terms have the same meaning as previously described.

If the drug disappears by a first-order absorption process, then X_a will be given by

$$X_a = FD \exp(-k_a t) \quad \text{(Eq. 17A)}$$

where D is the dose and F the fraction of it eventually absorbed by the first-order process. Inserting the value of X_a as given above in Eq. 16A and integrating the resulting expression gives

$$C = (\text{constant}) \exp(-Kt) + \frac{k_a FD}{V(K - k_a)} [\exp(-K_a t) \quad \text{(Eq. 18A)}$$

If at zero time no drug is in the blood, the constant of integration has the following value

$$\text{constant} = -\frac{k_a FD}{V(K - k_a)} \quad \text{(Eq. 19A)}$$

Inserting the value of this constant in Eq. 18A and rearranging the resulting expression gives

$$C = \frac{F \cdot D}{V} \frac{k_a}{K - k_a} [\exp(-k_a t) - \exp(-Kt)] \quad \text{(Eq. 20A)}$$

Using Eq. 8A, urinary excretion rate of unchanged drug (if any) will be described by

$$\frac{dX_u}{dt} = \frac{k_1 k_a FD}{K - k_a} [\exp(-k_a t) - \exp(-Kt)] \quad \text{(Eq. 21A)}$$

Equation 21A may be integrated to obtain

$$X_u = \frac{k_1 k_a FD}{K - k_a} \left[\frac{\exp(-Kt)}{K} - \frac{\exp(-k_a t)}{k_a}\right] + \text{constant} \quad \text{(Eq. 22A)}$$

If at zero time no drug has been excreted, the constant of integration has the following value:

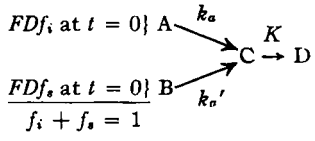
$$\text{constant} = -\frac{k_1 FD}{K - k_a} \left(\frac{k_a - K}{K}\right) \quad \text{(Eq. 23A)}$$

If Eq. 23A is substituted in Eq. 22A and resulting expression rearranged, the following equation may be obtained:

$$X_u = \frac{k_1 FD}{(K - k_a)} [1 - \exp(-k_a t)] - \frac{k_1 k_a FD}{(K - k_a)} [1 - \exp(-Kt)] \quad \text{(Eq. 24A)}$$

Equation 24A describes cumulative amount of unchanged drug excreted, and again the ratio, k_1/K, is equal to the fraction of a dose excreted in this form.

Model 4.—Absorption may also precede by two parallel first-order processes as shown:



In *Model 4* f_i and f_s are, respectively, the fractions of the dose absorbed by the indicated processes. In this model, drug blood, etc., level will be described by the following expression, where A and B are the two sources of drug, and k_a and k_a' are the first-order rate constants for the indicated processes.

$$\frac{dC}{dt} = \frac{FD}{V}(k_a X_a + k_a' X_b) - KC \quad \text{(Eq. 25A)}$$

Equation 25A may, after substituting for X_a and X_b as given in Eq. 17A, be rearranged and integrated to give

$$C = (\text{constant}) \exp(-Kt) + \frac{k_a FDf_i}{V(K - k_a)} \exp(-k_a t) + \frac{k_a' FDf_s}{V(K - k_a')} \exp(-k_a' t) \quad \text{(Eq. 26A)}$$

If no drug is in circulation at zero time, then the constant of integration has the value

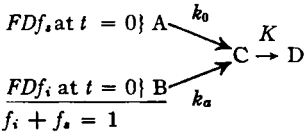
$$\text{constant} = -\frac{k_a FDf_i}{V(K - k_a)} - \frac{k_a FDf_s}{V(K - k_a')} \quad \text{(Eq. 27A)}$$

Inserting Eq. 27A in Eq. 26A gives, on rearrangement

$$C = \frac{FD}{V}\left\{\left[\frac{f_i k_a}{(K - k_a)}\right][\exp(-k_a t) - \exp(-Kt)] + \left[\frac{f_s k_a'}{(K - k_a')}\right][\exp(-k_a' t) - \exp(-Kt)]\right\} \quad \text{(Eq. 28A)}$$

to describe blood (compartment C) level as a function of time.

Model 5.—In the preceding model two first-order absorption processes were assumed to be in operation simultaneously. It may also be that both a zero and first-order process operate simultaneously as shown in the following model:



Rate of change of blood (compartment C) concentration with time in the *Model 5* above is given by

$$\frac{dC}{dt} = \frac{k_0}{V} + \frac{k_a X_a}{V} - KC \quad \text{(Eq. 29A)}$$

After substituting for X_a in Eq. 29A, as given by Eq. 17A, rearranging, and integrating, the following expression is obtained:

$$C = (\text{constant}) \exp(-Kt) + \frac{k_0}{KV} + \frac{k_a FDf_i}{V(K - k_a)} \exp(-k_a t) \quad \text{(Eq. 30A)}$$

If at zero time no drug is present in the blood, the constant of integration in Eq. 30A has the value

$$\text{constant} = -\left[\frac{k_0}{KV} + \frac{k_a FDf_i}{V(K - k_a)}\right] \quad \text{(Eq. 31A)}$$

Substituting for the constant in Eq. 30A by its value as given by Eq. 31A gives

$$C = \left\{\left(\frac{FD}{V}\right)\left(\frac{f_i k_a}{K - k_a}\right)[\exp(-k_a t) - \exp(-Kt)]\right\} + \left\{\frac{k_0}{VK}[1 - \exp(-Kt)]\right\} \quad \text{(Eq. 32A)}$$

as the expression which describes blood level as a function of time. This equation is valid during the time zero-order absorption is occurring.

The derivation of the expressions given in the text to calculate per cent of the absorbed dose of a drug as a function of time begins with a material balance accounting for the dose absorbed at all times. This material balance is

$$FD = X_{a+b} + X_c + X_d \quad \text{(Eq. 33A)}$$

where X_{a+b} is the amount at absorption site at any time, X_c is the amount in the body at the same time, and X_d is the amount eliminated from the body also in the same time. Another material balance is

$$A_T = X_c + X_d \quad \text{(Eq. 34A)}$$

Hence,

$$\frac{dA}{dt} = \frac{dX_c}{dt} + \frac{dX_d}{dt} \quad \text{(Eq. 35A)}$$

Furthermore, since $X_c = VC$ and $dX_c/dt = V \cdot dC/dt$ and since $dX_d/dt = KVC$ (loss from V is assumed to be first order), Eq. 35A may be written

$$\frac{dA}{dt} = V\frac{dC}{dt} + KVC \quad \text{(Eq. 36A)}$$

Integration of Eq. 36A between the limits $t=0$ and $t=T$ yields

$$A_T = V\left(C_T + K\int_{t=0}^{t=T} Cdt\right) \quad \text{(Eq. 37A)}$$

Integration of Eq. 36A between the limits $t=0$ and $t=\infty$ yields

$$A_\infty = V \cdot K \cdot \int_{t=0}^{t=\infty} Cdt \quad \text{(Eq. 38A)}$$

By definition, we have

$$\%\ \text{absorbed} = \frac{A_T}{A_\infty} \times 100 \quad \text{(Eq. 39A)}$$

Hence,

$$\%\ \text{absorbed} = \frac{C_T + K\int_{t=0}^{t=T} Cdt}{K\int_{t=0}^{t=\infty} Cdt} \times 100 \quad \text{(Eq. 40A)}$$

The denominator of Eq. 40A is the maximum or asymptotic value of the numerator.

If urinary excretion of unchanged drug is followed, Eq. 40A may be modified. Urinary excretion rate of unchanged drug is given by (4):

$$\frac{dX_u}{dt} = fVKC \quad \text{(Eq. 41A)}$$

Solving Eq. 41A for C, substituting into Eq. 40A, and simplifying yields

$$\%\ \text{absorbed} = \frac{(1/K)\cdot[(dX_u/dt) + X_u]}{(X_u)_\infty} \times 100 \quad \text{(Eq. 42A)}$$

GLOSSARY

dA/dt, instantaneous absorption rate (units of amounts per unit time).
A_T, amount absorbed from time zero to time T (weight units).
A_∞, amount eventually absorbed, $A_\infty = FD$ (weight units).
C, the blood (serum or plasma) concentration (concentration units) assumed to be representative of compartment C in the models.
C_T, the blood (serum or plasma) concentration at time T (concentration units).
V, the apparent volume of distribution (volume units). See *Introduction* for definition.
K, the first-order rate constant for loss of drug from V units (units of reciprocal time).
D, the dose of drug administered (weight units).
F, the fraction of the drug absorbed (*i.e.*, the fraction of the dose which reaches V). This is a dimensionless number and $0 \leq F \leq 1$.
f, the fraction of the drug reaching the circulation (*i.e.*, V) which is excreted unchanged in the urine. This is a dimensionless number and $0 \leq f \leq 1$.
X_c, amount of unchanged drug retained in V at a given time (weight units).
X_d, amount of drug eliminated from V by all processes in a given time (weight units).
X_u, amount of unchanged drug eliminated in the urine to a given time (weight units) and $X_u = f \cdot X_d$.
dX_u/dt, the instantaneous rate of urinary excretion of unchanged drug at time t. This is the first derivative of a plot of cumulative amount of unchanged drug excreted in the urine against time (units of weight per unit time).
d^2X_u/dt^2, the rate of change of urinary excretion rate. This is the second derivative of a plot of cumulative amount of unchanged drug excreted in the urine against time.
$(X_u)_\infty$, the amount of unchanged drug excreted in the urine in infinite time (weight units).
% A_T, the amount of drug absorbed to time T expressed as a percentage of the total amount absorbed [*i.e.*, % $A_T = (A_T/A_\infty) \times 100$].
$\int_{t=0}^{t=T} Cdt$, the area under a plot of blood (serum or plasma) concentration against time between time zero and time T (units of concentration × time).
$\int_{t=0}^{t=\infty} Cdt$, the area under a plot of blood (serum or plasma) concentration against time between time zero and infinite time (units of concentration × time).

Interpretation of the Models

A and/or B, drug in the dosage form and/or gastrointestinal tract or site of injection (*i.e.*, drug in A and/or B is unabsorbed).
C, drug in V of which blood (serum or plasma) is a part. In the models C is the concentration of compartment C, whose volume is V. Hence, $C = X_c/V$.
D, drug in the urine and/or metabolites.
k_0, a zero-order rate constant (amount/unit time).
k_1 and k_2, first-order rate constants (units of reciprocal time).
X_a, X_b, X_c, and X_d, amounts of drug in the compartments A, B, C, and D at time t, respectively.
fi, that fraction of the drug absorbed which is absorbed at rapid rates from compartment A.
fs, that fraction of the drug absorbed which is absorbed slowly from compartment B.
k_a and k_a', first-order absorption rate constants (units of reciprocal time).

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Polarographic Analysis of Some Sulfonic Acids

By EDWARD E. SMISSMAN and CHARLES F. BARFKNECHT

Sulfonic acids gave fine polarographic waves. Alkyl sulfonic acids have a half-wave potential of −1.6 v., while the half-wave potential of aromatic sulfonic acids can vary with substituents.

During the investigation of the antimicrobial factor in the giant milkweed bug, *N*-methyltaurine (I) was isolated and identified (1). The authors concluded that since the spot test for sulfonic acids involves the reduction to sulfite (2), the polarographic reduction should also occur.

$$CH_3NCH_2CH_2SO_3H$$
$$H$$

I

Using a rapid-dropping mercury electrode, *N*-methyltaurine gave a fine wave and a half-wave potential of −1.6 v. Camphor-10-sulfonic acid and methane sulfonic acid also gave the same half-wave potential. These values neglect internal resistance within the cell.

Polarographic reductions of sulfonic acids have not been reported previously, although the reduction of sulfonic esters, amides, and acid chlorides have been observed (3). Zuman reported a correlation of half-wave potentials and Hammett sigma values for a large number of aromatic sulfonic acid derivatives. Some correlation appears to exist for aromatic sulfonic acids (Table I). Benzene sulfonic acid and *p*-toluenesulfonic acids exhibit half-wave potentials at −1.6 v., *p*-acetamidobenzene sulfonic acid, somewhat more negative, and *p*-nitrobenzenesulfonic acid exhibited a half-wave potential at −1.5 v. Irregular behavior was observed in polarograms of *ortho*-, *meta*-, and *para*-aminobenzenesulfonic acids. These three compounds all exhibited half-wave potentials of −1.6 v. Zuman (3) had reported that groups capable of mesomeric effects (*e.g.*, *p*-hydroxy, *p*-amino) generally did not fit his plots of half-wave potentials *versus* Hammett sigma values. He did not attempt to explain this observation.

Table I.— Half-wave Potentials of Sulfonic Acids

Compd.	Half-wave Potential, v.
N-Methyltaurine	−1.6
Camphor-10-sulfonic acid	−1.6
Benzenesulfonic acid	−1.6
p-Toluenesulfonic acid	−1.6
p-Nitrobenzenesulfonic acid	−1.5
p-Acetamidobenzenesulfonic acid	> −1.6
p-Aminobenzenesulfonic acid	−1.6
m-Aminobenzenesulfonic acid	−1.6
o-Aminobenzenesulfonic acid	−1.6

If the amino group existed as the ammonium cation in the aromatic sulfonic acids, the half-wave potential should have been less negative, since the cation would withdraw electrons from the ring and in turn make the sulfur more positive. If the amino group were present in the free form, the *para* and the *ortho* isomers could have participated in resonance stabilization involving the sulfur which should have made the half-wave potential more negative. Since neither of these possibilities appeared to occur, it might be attractive to postulate the removal of the amino group prior to reduction of the sulfonic acid to account for the identical half-wave potentials.

EXPERIMENTAL

To 1 ml. of 10^{-3} *M* sulfonic acid solution was added 9 ml. of 10^{-1} *M* tetramethylammonium iodide solution. In those cases where 10^{-3} *M* solutions could not be formed, a saturated solution was employed.

The solution was deoxygenated using nitrogen gas and the polarograms run within 30 minutes of mixing. A Metrohm polarograph equipped with a rapid-dropping mercury electrode was used *versus* an Ag, AgCl, anode.

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Modified Method for Studying Anorexigenic Agents in Dogs

By H. J. ADAMS and L. C. GREENE

Mongrel dogs, selected to accept a food offering every 10 minutes for 8 hours and to respond reproducibly to oral test doses of dextroamphetamine sulfate, have been employed in a test procedure designed to evaluate anorexigenic agents quantitatively. Linear dose-response and dose-duration curves have been obtained, demonstrating the validity of the procedure and affording the possibility of quantitative comparisons of potency and duration of anorectic activity of various drugs.

SEVERAL METHODS of testing anorexigenic agents in dogs have been described in the literature. Various end points, such as reduced consumption of food (1), complete refusal of food (2), and weight loss (3) have been employed to ascertain anorectic activity of test compounds. No attempts were made to obtain dose-response curves or time-action curves in order to compare potency and duration of action of different compounds. These methods are therefore qualitative or at best semiquantitative. Wendel *et al.* (4) described a quantitative test procedure for determining time-action curves of anorexigenic agents in sustained-release forms. A modification of their method is reported in this paper.

The objectives of this study were twofold: first, to establish a test in which *reproducible responses* could be obtained in unoperated animals under normal physiological conditions; and second, to establish a test that would be pharmacologically valid as shown by (*a*) a linear relationship between the log of the dose of a drug and the biological response elicited, (*b*) a linear relationship between the log of the dose and the duration of action of the dose, and (*c*) observing the desired biological activity at dosage levels far below those eliciting toxic side effects.

METHODS

Animal Selection.—Healthy mongrel dogs of either sex, weighing between 10 and 20 Kg., were tested and selected by the following criteria.

Each animal, after 18 hours without food, accepted 1/24 lb. of canned dog food every 10 minutes for 8 hours, then consumed an additional 1 lb. of food (feeding control). Each animal showed some degree of anorexia at a dose of 0.75 mg./Kg. of dextroamphetamine sulfate. No animal was so sensitive that it refused all six food offerings during any hour beyond the fifth hour at a dose of 0.4 mg./Kg. of dextroamphetamine sulfate.

Test Procedure.—A working colony of 10 animals selected from 17 dogs was maintained throughout the study. Frequent feeding controls and drug controls were conducted on the colony to insure reproducibility of the responses. During a feeding control each animal was offered 1/24 lb. of dog food every 10 minutes for 8 hours. An animal that did not accept every food offering was replaced. For the drug controls, the animals received orally one of the selected doses of dextroamphetamine sulfate (0.4, 0.6, or 0.75 mg./Kg.) and were then offered food every 10 minutes for the subsequent 8 hours. The anorectic response of the whole colony and the responses of the individual animals were checked; any animal failing to conform fully was replaced. In addition, animals that showed marked and persistent changes in their patterns of refusal after receiving one of the standard doses of dextroamphetamine sulfate were replaced. Only by taking such precautions can valid and reproducible results be obtained.

The drug, in capsules, was included in the first food offering. If any food offering was not consumed, it was removed from the cage and reoffered. In this way no animal was presented with more than one food offering at a time. To facilitate the evaluation of the data, the following calculation was employed:

$$\% \text{ Anorexia for each hr. interval} = \frac{\text{Total No. of refusals of colony during this hr.}}{\text{Total No. of offerings to colony}} \times 100$$

The colony was used not more than 1 day a week.

TABLE I.—TYPICAL ANORECTIC RESPONSES OF THREE ANIMALS TO 0.6 mg./Kg. OF DEXTROAMPHETAMINE SULFATE[a]

Animal No.	Date	Intervals Post Drug, Hr. 1	2	3	4	5	6	7	8
2	11/15/61	0	5	6	6	6	6	6	5
	11/29/61	2	6	6	6	6	6	6	4
	1/3/62	1	6	6	6	6	6	5	0
7	11/15/61	0	3	6	6	6	0	0	0
	11/29/61	0	4	6	6	1	0	0	0
	1/3/62	0	5	6	6	3	0	0	0
29	11/15/61	0	3	6	6	6	6	6	5
	11/29/61	2	6	6	6	6	6	6	5
	1/3/62	0	5	6	6	6	6	6	5

[a] Numbers in the table show the number of refusals in any hourly interval.

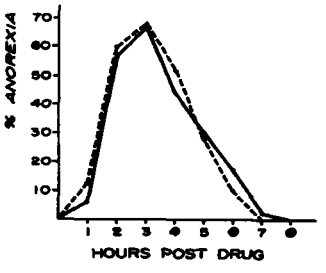


Fig. 1.—Time-action curves of anorectic activity of dextroamphetamine sulfate (0.4 mg./Kg.) administered on two occasions about 1 year apart. Data for each curve obtained on 10 animals. Calculation of per cent anorexia is explained in text. Key: ——— May 31, 1961; - - - - May 16, 1962.

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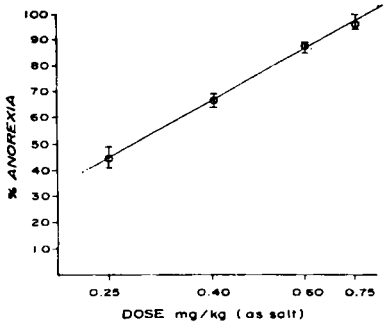


Fig. 2.—Dose-response curve for graded doses of dextroamphetamine sulfate administered orally to dogs. Peak activity in all cases occurred at 3 hours post drug. Confidence limits (95%) of the line are shown.

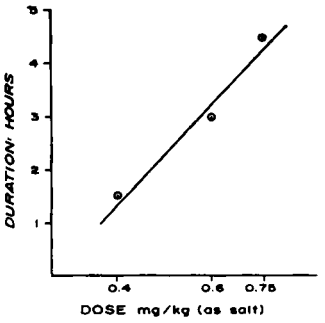


Fig. 3.—Dose-duration curve for graded doses of dextroamphetamine sulfate administered orally to dogs. The duration in hours required to descend from the peak to 50% anorexia has been plotted against the log of the dose.

TABLE II.—SUMMARY OF DATA OBTAINED IN THIS STUDY

Drug	Dose, mg./Kg. as Salt	Dogs per Trial, No.	Trials, No.	Max. Responses, % Anorexia	Duration of Action, Hr.[a]	Relative Areas under Time-Action Curves
Dextroamphetamine sulfate	0.25	10	2	45	...	1.0
	0.40	10	3	67	1.5	2.1
	0.60	10	3	88	3.0	3.3
	0.75	10	2	96	4.5	>4.0
l-Amphetamine sulfate	2.50	10	2	86	2.8	3.0

[a] For the purposes of this study duration of action was defined as the time required for anorectic activity to descend from the maximum to 50% anorexia.

RESULTS AND DISCUSSION

Since the aims of this study were to develop a test procedure that would give reproducible results and be pharmacologically valid, the results will be discussed accordingly.

Reproducibility. — *Individual Animals.* — There was considerable individual variation in the response to the anorectic agents. This, however, appeared to be of minor importance as long as responses of the single animal and the colony as a whole were consistent. Nevertheless, the pattern of refusals was usually remarkably constant as shown in Table I for three typical dogs on 3 different days.

Colony as a Whole.—The reproducibility of the colony as a whole tested on two occasions about 1 year apart is summarized in Fig. 1.

Validity.—The linearity of the log dose-response relationship obtained with four doses of dextroamphetamine sulfate clearly demonstrates that the colony functioned as a pharmacologically valid test object (Fig. 2). Since the processes (redistribution, metabolism, excretion) responsible for decreasing drug concentration at the site of action are usually first-order processes, the decrease in the effective concentration of a drug is exponential. Therefore, the time required for the peak response to descend to some selected level should be linearly related to the log of the dose. Thus, the relationship between log dose and duration of action has been examined with our test procedure and was found to be linear (Fig. 3). Some of the pertinent data are summarized in Table II. A plot of relative areas under the time-action curves (total effectiveness) against log dose will also yield a linear relationship, which constitutes additional proof for the validity of the test procedure. Recently, Friedman (5) stated that the potency of the anorexigenic effect of *l*-amphetamine sulfate had not been evaluated. In our test procedure it required almost exactly four times as much of the *levo* form (2.5 mg./Kg.) to produce the same maximum anorexigenic activity as 0.6 mg./Kg. of the *dextro* form, and the total effectiveness (area under the time-action curve) was also nearly equal.

Tolerance to the anorexigenic activity of dextroamphetamine sulfate was not seen; undesirable side effects, such as excitation and salivation, occurred only rarely at the highest dose used (0.75 mg./Kg.).

SUMMARY

A test procedure in dogs for the quantitative evaluation of anorexigenic agents has been described. Strict adherence to the criteria established yields reproducible responses and linear log dose-response relationships, indicating that the procedure and the test object are pharmacologically valid. Anorectic activity can be demonstrated at doses well below those producing marked signs of CNS stimulation. Time-action curves of different agents and of several doses of the same agent can be obtained. Therefore, the test procedure appears to be extremely useful for comparing the relative potencies and durations of action of anorexigenic agents.

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Essential Oils and their Constituents XXIII. Chemotaxonomy of the Genus *Mentha*

By K. L. HANDA*, D. M. SMITH, I. C. NIGAM, and LEO LEVI

Essential oils derived from *Mentha aquatica* L.; *M. citrata* Ehrh. (bergamot mint); *M. pulegium* L. (European and North African pennyroyal); *Hedeoma pulegioides* (L.) Pers. (American pennyroyal); *M. rotundifolia* and *M. sylvestris* L. were examined by gas-liquid partition chromatography using a polar (SAIB) and nonpolar (Ucon) substrate, respectively. Quantitative data illustrating the extent of compositional differences between these products are presented and compared with similar criteria of authenticity for *M. arvensis* (mint); *M. piperita* (peppermint); *M. cardiaca, M. spicata,* and *M. viridis* (spearmints). The occurrence of isopiperitenone as a major constituent of *M. rotundifolia* derived from a French mutant strain and the detection of several terpenoids occurring as trace constituents in these species are reported for the first time. Correlation of the chemical data illustrates their value in plant classification.

THE CLASSIFICATION of aromatic plants considered to belong to the genus *Mentha*—an important source of basic raw materials for the food, drug, and cosmetic industries—has always proved a difficult task to the botanist, geneticist, and physiologist engaged in natural products research. As yet, the many species of this genus cannot be unambiguously identified by a single technique. It is the purpose of this paper to demonstrate the value of using chemical analysis of the essential oils they yield for the recognition of botanical origins.

EXPERIMENTAL

Apparatus and analytical methods were described in previous publications (1–4).

Essential Oils.—The *Mentha aquatica, M. citrata, M. sylvestris,* and *Hedeoma pulegioides* used were obtained through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich. Plants used as the source of these oils were grown under the direction of Dr. M. J. Murray—herbarium specimens available at the Cornell University Herbarium and the Missouri Botanical Gardens, St. Louis, Mo.; *M. rotundifolia* was obtained through the courtesy of Dr. Sumio Shimizu, Laboratory of Agricultural Chemistry, Shinshu University, Ina-Nagano Ken, Japan. Plants used as the source of these oils were grown at the Okayama Agricultural Experiment Station and Laboratory of Plant Breeding under the direction of Drs. Sumio Shimizu and Nagamori Ikeda. *Mentha pulegium*—authenticated commercial products were obtained through the courtesy of Dr. E. Guenther, Fritzsche Brothers, Inc., New York, N. Y., Lautier Fils, New York, N. Y., and Destilaciones Bordas Chinchuretta, S. A., Seville, Spain.

RESULTS AND DISCUSSION

Essential oils are typical end-products of plant metabolism and as such their composition reflects all biogenetic and biochemical processes associated with and characteristic of plant life. Thus, essential oils analysis can assume a key role in plant authentication.

Table I lists the various constituents occurring in the products examined. Relative retention times and quantitative data for the components are also given. They show that distinct qualitative and quantitative differences as well as marked compositional similarities exist between essential oils of different botanical provenance. The value of specific ratios of biochemically related constituents as characteristic criteria of authenticity for several essential oils of the genus *Mentha* has already been demonstrated. Limonene-cineole ratios, for example, were shown to distinguish readily *M. arvensis* from *M. piperita* (major constituent menthol) and provide reliable parameters of geographical provenance as well. They permitted, likewise, unambiguous distinctions between *M. cardiaca, M. spicata,* and *M. viridis* (major constituent carvone) of different geographical origins (1, 2). The present study shows the specificity of this ratio for *M. aquatica* (0.29); *M. sylvestris* (0.48); *M. citrata* (5.5); and *M. rotundifolia,* French mutant strain (4.8), Italian strain (9.7). These oils can, moreover, be readily distinguished from one another by analysis for their major constituents, *e.g., M. aquatica,* menthofuran (51.3%); *M. citrata,* linalyl acetate (45.6%); *M. pulegium,* pulegone (77.6 ± 6.3%); *M. rotundifolia,* French strain, neo-iso-isopulegol (52.3%), Italian strain, piperitenone oxide (87.3%); *M. sylvestris,* piperitone oxide (66.3%).

The occurrence of isopiperitenone in *M. rotundifolia* (French strain) was established following isolation and purification of the compound by preparative gas chromatography. Hydrogenation of the isolate with Adams catalyst in methanol showed the presence of two double bonds in the molecule and yielded a product consisting of isomenthone (71%), menthone (17%), and isomeric menthols (12%). Ultraviolet examination ($\lambda_{max.}^{EtOH}$ 233.5 mμ) suggested the presence of an α,β-unsaturated keto group. Infrared analysis of the compound (see Fig. 1) confirmed this deduction (1670 cm.$^{-1}$ absorption) and showed, moreover, the presence of a terminal methylene group in a side chain (3080, 1787, 1640, and 888 cm.$^{-1}$), as well as a trisubstituted double bond conjugated with a keto group (3035, 1617, and 787 cm.$^{-1}$).

One of the oils examined, *Hedeoma pulegioides* (L.) Pers. (American pennyroyal), generally considered belonging to a different genus of the Labiatae family, contained all constituents present in oil of *M. pulegium* L. (European pennyroyal). Yet characteristic quantitative differences were detected, *e.g.,* limonene-cineole ratio for *H. pulegioides* 6.1;

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TABLE I.—ESSENTIAL OILS OF THE GENUS *Mentha*

No.	Constituent	Retention time (relative to menthol) UCON	SAIB	Essential Oil Composition % — *M. aquatica* (5, 6, 11)	*M. citrata*[b] (5, 6, 11)	*H. pulegioides* (6, 11)	*M. pulegium* (5, 6, 11)	*M. rotundifolia* French Strain (7, 8, 11)	*M. rotundifolia* Italian Strain (7, 8, 11)	*M. sylvestris* (5, 6, 11)	*M. arvensis* (1, 3, 11)	*M. piperita* (1, 3, 9, 11)	*M. cardiaca* (2, 9, 11)	*M. spicata* (2, 9, 11)	*M. viridis* (2, 11)
1	α-Pinene	0.18	0.16	0.9[a]	0.6[a]	0.4	0.7 to 1.2	0.9[a]	0.7[a]	1.3[a]	0.2 to 0.5	0.2 to 1.0	0.5 to 1.0	0.5 to 0.9	0.7 to 0.8
2	β-Pinene	0.23	0.22	2.3[a]	0.9[a]	0.2	0.7 to 0.8	2.1[a]	1.7[a]	4.5[a]	0.9 to 1.3	0.7 to 1.9	1.3 to 2.6	1.8 to 4.4	4.4 to 4.5
3	Limonene	0.27	0.27	6.4[a]	1.1[a]	0.6	0.1 to 0.6	2.9[a]	2.9[a]	4.9	4.3 to 6.0	2.7 to 6.8	17.7 to 29.7	13.3 to 2[illegible].5	1[illegible].9 to 16.2
4	Cineole	0.30	0.30	22.4	0.2[a]	0.1[a]	0.2 to 0.8[a]	0.6[a]	0.3[a]	10.2	0.4 to 0.8	5.2 to 13.5	1.8 to 2.5	2.9 to 4.4	6.0 to 6.8
5	3-Octanol	0.36	0.38	1.9[a]	...	0.6[a]	1.0 to 1.3[a]	0.9[a]	0.7[a]	0.2[a]	0.2 to 0.8	...	1.6 to 2.6	0.7 t 1.4	0.6 to 0.7
6	3-Octyl acetate	0.36	0.46	0.3[a]	...				0.5[a]	...	...	...	0.3	0.2 to 0.5	0.3
7	Sabinene hydrate	0.49	0.94	...	...	...	...	...	...	...	...	0.3 to 0.9	0.1 to 0.4	0.8 to 1.5	...
8	Sabinene acetate	0.49	0.94	...	...	...	...	...	...	...	...				...
9	Linalool	0.62	0.67	...	32.4	...	...	...	...	0.2[a]	0.0 to 0.8	...	0.0 to 0.4	0.0 to 0.4	0.1 to 0.2
10	Menthofuran	0.65	0.68	51.3	0.1	<0.1	0.2 to 0.3	<0.1	<0.1	<0.1	<0.1	0.7 to 9.2	<0.1	<0.1	<0.1
11	Menthone	0.67	0.75	...	...	1.4	0.4 to 1.7	...	0.2	...	9.9 to 17.1	8.9 to 31.6	0.9 to 2.2	0.1 to 0.5	0.2
12	Isomenthone	0.70	0.78	...	...	0.8	0.0 to 1.2	...	...	...	2.7 to 4.2	2.2 to 5.9	0. to 0.3	0.0 to 0.1	0.0
13	Linalyl acetate	0.81	0.94	...	45.0	0.6	1.7 to 4.1	...	...	0.1[a]	...	...	...	...	...
14	Neomenthol	0.86	0.94	...	...	...	...	...	...	...	1.6 to 2.2	2.8 to 5.3	...	...	...
15	Carvomenthone	0.88	1.03	...	...	...	...	...	...	...	...	...	0.1 to 0.6	0.7 to 1.3	0.2
16	Menthyl acetate	0.94	1.06	6.2[a]	...	...	...	...	...	...	4.7 to 6.9	3.8 to 11.6	...	...	...
17	Neo-iso-isopulegol	0.98	1.00	...	...	...	...	52.3	0.2[a]	...	...	...	...	...	...
18	Dihydrocarvone	1.00	1.11	...	...	...	...	...	...	...	...	...	2.5 to 6.9	3.4 to 5.5	3.5 to 4.6
19	Menthol	1.00	1.00	2.6	...	0.1	0.2 to 0.7	...	...	...	61.5 to 68.6	33.2 to 48.7	...	...	...
20	Pulegone	1.16	1.24	1.8	8.1	82.3	71.3 to 83.9	...	...	...	0.2 to 1.1	0.5 to 4.6	0.0 to 0.9	0.1 to 0.9	1.7 to 1.9
21	Neo-iso-isopulegyl acetate	1.30	1.38	...	...	...	...	7.2	0.1	...	...	...	...	...	...
22	Dihydrocarveyl acetate	1.32	1.59	...	...	...	...	...	...	...	...	...	0.4 to 1.3	0.5 to 4.1	4.4 to 7.7
23	Isopiperitenone	1.44	1.39	...	...	...	...	13.4[a]	...	...	...	...	...	...	...
24	Dihydrocarveol	1.45	1.38	...	...	...	...	...	...	...	...	...	0.0 to 0.1	0.5 to 0.8	4.8
25	Carvone	1.45	1.59	0.3	...	...	...	...	...	...	...	...	53.9 to 68.2	59.1 to 68.1	58.2 to 62.1
26	*trans*-Carveyl acetate	1.45	1.68	...	...	...	...	...	...	...	...	...	0.7 to 0.9	0.8 to 1.0	0.9
27	Piperitone	1.48	1.67	0.8	4.2	0.9	0.3 to 1.7	7.3	0.1[a]	4.5	1.8 to 2.7	0.5 to 4.1	...	...	...
28	Piperitone oxide	1.63	1.70	...	3.0	...	...	...	...	66.3	0.3	<0.1	...	...	...
29	*cis*-Carveyl acetate	1.66	1.92	...	...	...	...	...	...	...	...	...	0.0 to 1.6	0.5 to 2.6	0.9 to 1.7
30	*trans*-Carveol	1.79	1.59	...	...	...	...	...	...	...	...	...	...	0.2	0.5
31	*cis*-Carveol	1.84	1.77	...	...	...	...	...	...	...	...	...	0.0 to 0.9	0.0 to 1.0	0.2
32	Piperitenone	2.33	2.65	...	...	4.7	1.1 to 12.6	8.6	2.4	2.5	...	...	...	...	...
33	Piperitenone oxide	3.30	3.42	...	...	...	...	...	87.3	2.7	...	...	...	...	...
34	Unaccounted	...	...	2.6	3.8	7.2	5.6	3.7	2.8	2.5	0.0 to 0.9	0.6 to 1.8	0.0 to 0.4	0.0 to 0.8	0.0 to 0.7

[a] Presence of this constituent reported for first time. [b] Presence of 0.6% citral in this oil established by the BAC-method of analysis (10).

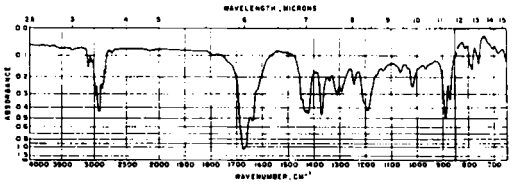


Fig. 1.—Infrared spectrum of isopiperitenone. 4000–850 cm.$^{-1}$ CCl_4 solution; 850–650 cm.$^{-1}$ contact film.

for *M. pulegium* 0.12 to 0.30. The somewhat higher linalyl acetate content of the European oil may likewise be a valuable indicator of botanical identity.

None of the essential oils was fractionated or pretreated prior to analysis. All samples were injected directly as the crude natural products obtained by conventional steam distillation. Coupled gas liquid–thin layer chromatography was used successfully for the detection of trace constituents. Similar analyses of fractions isolated by rectification, column chromatography, and/or other separation techniques would undoubtedly establish the presence in these oils of many more terpene compounds still unaccounted for as shown in Table I.

The experimental data presented summarize results of a series of compositional studies on essential oils of the genus *Mentha*. They serve to illustrate further that distinct biochemical relationships exist between constituents synthesized by its different species and that such data, obtained exclusively by physiochemical analysis, provide important criteria for species characterization and classification *via* qualitative and quantitative chemotaxonomy.

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Quantitative Evaluation of Infiltration Anesthetics in Albino Mice

By W. R. JONES, T. L. KERLEY, and L. C. WEAVER

Infiltration anesthetics were quantitatively evaluated using a test procedure based on the vocalization of mice in response to electrical stimulation.

MOST LABORATORIES today prefer to use the mouse as a laboratory animal for preliminary toxicity work and primary pharmacodynamic evaluation of chemical compounds. The use of this species for the evaluation of local anesthetic activity is advantageous because of economy and the opportunity to compare more tests in the same species. Weidmann and Petersen (1) first used the mouse to study surface anesthesia, and a slight modification of this method has been used in our laboratories (2). In addition, mice have been used for the evaluation of anesthetics injected directly into the tissues (3, 4). A method using mice for the quantitative assessment of infiltration anesthetics is described in this communication.

EXPERIMENTAL

Male Swiss-Webster albino mice were used in the experiments. A constant volume (0.03 ml.) of drug solution was injected beneath the skin on the plantar surface of one hind foot, and an equal volume of 0.9% sodium chloride solution was injected similarly into the opposite foot. Ten minutes after injection, the control foot of each animal was stimulated rapidly and repeatedly until the animal vocalized, then continued to vocalize in response to 10 successive stimulations. Immediately afterward, the treated foot was stimulated five times, and any animal that failed to vocalize one or more times was classified as anesthetic. An electrical current (100 v. d.c.), delivered by a Grass model S-4 stimulator, was used as the stimulus. Because of tissue damage resulting from the intense stimuli, each animal was used only once. To facilitate conduction, each foot was moistened with 10% sodium chloride solution just prior to contact with the stimulating electrodes. Cocaine hydrochloride, procaine hydrochloride, dibucaine hydrochloride, and dyclonine hydrochloride were administered to groups of 10 animals at a minimum of three different concentrations to establish dose response curves. The anesthetic dose for 50% of mice (AD_{50}) was calculated for each drug and relative potency determined with respect to cocaine hydrochloride (5, 6).

RESULTS

The AD_{50} for each of the drugs was determined on four separate occasions, and the results obtained are presented in Table I. Experiments *A*, *B*, and *C* were each completed during a single day for all of the local anesthetics. For the preliminary experiment, the data (except for cocaine) were obtained over a period of 2 days. The results obtained show that the AD_{50} values were consistent for procaine and dyclonine. There was one AD_{50} value that was slightly low for dibucaine and one high for cocaine.

The potency of these agents relative to cocaine

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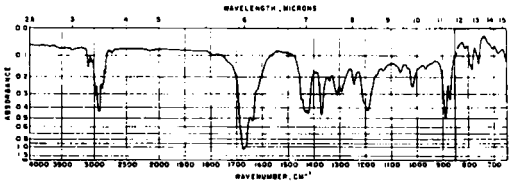


Fig. 1.—Infrared spectrum of isopiperitenone. 4000–850 cm.$^{-1}$ CCl_4 solution; 850–650 cm.$^{-1}$ contact film.

for *M. pulegium* 0.12 to 0.30. The somewhat higher linalyl acetate content of the European oil may likewise be a valuable indicator of botanical identity.

None of the essential oils was fractionated or pretreated prior to analysis. All samples were injected directly as the crude natural products obtained by conventional steam distillation. Coupled gas liquid–thin layer chromatography was used successfully for the detection of trace constituents. Similar analyses of fractions isolated by rectification, column chromatography, and/or other separation techniques would undoubtedly establish the presence in these oils of many more terpene compounds still unaccounted for as shown in Table I.

The experimental data presented summarize results of a series of compositional studies on essential oils of the genus *Mentha*. They serve to illustrate further that distinct biochemical relationships exist between constituents synthesized by its different species and that such data, obtained exclusively by physiochemical analysis, provide important criteria for species characterization and classification *via* qualitative and quantitative chemotaxonomy.

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Quantitative Evaluation of Infiltration Anesthetics in Albino Mice

By W. R. JONES, T. L. KERLEY, and L. C. WEAVER

Infiltration anesthetics were quantitatively evaluated using a test procedure based on the vocalization of mice in response to electrical stimulation.

MOST LABORATORIES today prefer to use the mouse as a laboratory animal for preliminary toxicity work and primary pharmacodynamic evaluation of chemical compounds. The use of this species for the evaluation of local anesthetic activity is advantageous because of economy and the opportunity to compare more tests in the same species. Weidmann and Petersen (1) first used the mouse to study surface anesthesia, and a slight modification of this method has been used in our laboratories (2). In addition, mice have been used for the evaluation of anesthetics injected directly into the tissues (3, 4). A method using mice for the quantitative assessment of infiltration anesthetics is described in this communication.

EXPERIMENTAL

Male Swiss-Webster albino mice were used in the experiments. A constant volume (0.03 ml.) of drug solution was injected beneath the skin on the plantar surface of one hind foot, and an equal volume of 0.9% sodium chloride solution was injected similarly into the opposite foot. Ten minutes after injection, the control foot of each animal was stimulated rapidly and repeatedly until the animal vocalized, then continued to vocalize in response to 10 successive stimulations. Immediately afterward, the treated foot was stimulated five times, and any animal that failed to vocalize one or more times was classified as anesthetic. An electrical current (100 v. d.c.), delivered by a Grass model S-4 stimulator, was used as the stimulus. Because of tissue damage resulting from the intense stimuli, each animal was used only once. To facilitate conduction, each foot was moistened with 10% sodium chloride solution just prior to contact with the stimulating electrodes. Cocaine hydrochloride, procaine hydrochloride, dibucaine hydrochloride, and dyclonine hydrochloride were administered to groups of 10 animals at a minimum of three different concentrations to establish dose response curves. The anesthetic dose for 50% of mice (AD_{50}) was calculated for each drug and relative potency determined with respect to cocaine hydrochloride (5, 6).

RESULTS

The AD_{50} for each of the drugs was determined on four separate occasions, and the results obtained are presented in Table I. Experiments *A*, *B*, and *C* were each completed during a single day for all of the local anesthetics. For the preliminary experiment, the data (except for cocaine) were obtained over a period of 2 days. The results obtained show that the AD_{50} values were consistent for procaine and dyclonine. There was one AD_{50} value that was slightly low for dibucaine and one high for cocaine.

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TABLE I.—REPRODUCIBILITY OF AD_{50} DETERMINATIONS[a]

	Preliminary Expt.	Expt. *A*	Expt. *B*	Expt. *C*
Cocaine HCl	1.38 (0.67–2.10)	2.22 (1.44–2.99)	3.53 (2.42–4.65)	1.24 (0.90–1.59)
Procaine HCl	19.95 (11.22–28.68)	27.11 (24.02–30.20)	23.36 (16.45–30.07)	19.01 (10.69–27.51)
Dibucaine HCl	0.90 (0.57–1.24)	2.12 (1.45–2.79)	3.10 (2.40–3.90)	2.85 (1.73–3.97)
Dyclonine HCl	4.50 (3.06–5.36)	2.56 (1.48–3.65)	4.52 (2.96–6.09)	2.51 (1.37–3.65)

[a] Dose expressed as milligrams per milliliter. Figures in parentheses are 95% confidence limits.

TABLE II.—REPRODUCIBILITY OF RELATIVE POTENCY DETERMINATIONS[a]

	Expt. *A*	Expt. *B*	Expt. *C*
Procaine HCl	Slopes Not parallel	0.15 (0.10–0.22)	0.06 (0.04–0.10)
Dibucaine HCl	0.95 (0.61–1.50)	1.35 (0.50–3.63)	0.41 (0.17–0.64)
Dyclonine HCl	0.94 (0.55–1.62)	0.72 (0.46–1.14)	0.49 (0.30–0.81)

[a] Cocaine hydrochloride used as standard for comparisons. Figures in parentheses are 95% confidence limits.

TABLE III.—LOCAL ANESTHETIC ACTIVITY BASED ON THE COMBINATION OF RESULTS OF REPEATED ASSAYS

Drug	Mice, No.	AD_{50}, mg./ml.	Activity Ratios (Cocaine = 1)
Cocaine HCl	126	2.03 (0.52–7.91)	1
Procaine HCl	88	26.10 (20.82–32.73)	0.11 (0.02–0.25)
Dibucaine HCl	108	2.45 (1.25–4.79)	0.67 (0.35–1.27)
Dyclonine HCl	87	3.28 (1.98–5.44)	0.69 (0.52–0.91)

TABLE IV.—LOCAL ANESTHETIC ACTIVITY TESTED 15 MINUTES AFTER SUBCUTANEOUS INJECTION OF THE DRUGS TO TAILS OF MICE (4)[a]

Drug	Mice, No.	AD_{50}, mg./ml.	Activity Ratios (Cocaine = 1)
Cocaine HCl	160	0.66 (0.53–0.81)	1
Procaine HCl	120	4.25 (3.40–5.31)	0.15 (0.10–0.20)
Dibucaine HCl	140	0.42 (0.31–0.55)	1.57 (1.20–2.29)

[a] Figures in parentheses are 95% confidence limits. Adrenaline hydrochloride (10 mcg./ml.) was added to all solutions.

is shown in Table II. In experiments *A*, *B*, and *C* the results were reproducible. The data for the preliminary experiment were not included because the drugs were not compared simultaneously. In one case (procaine), the slopes of the lines were not parallel, and a statement of relative potency could not be made. Despite considerable variation in relative potencies, there was an overlap of confidence limits for each anesthetic agent.

DISCUSSION

While it is desirable to obtain absolute reproducibility of AD_{50} and relative potency values, it must be recognized that the standard error of a test can only reflect causes of variation that influence the results of the initial test. There may be other factors that are constant at one time or in one laboratory, but that vary from time to time or from laboratory to laboratory (7). For example, in a collaborative study of lethality data obtained in several laboratories, Swope (8) found that not only was there a significant difference in results between laboratories but also between tests within a single laboratory.

To obtain more reliable estimates of the AD_{50} values and relative potency determinations, the results of the separate tests were combined as described by Miller *et al.* (6) and Stewart and Young (9). The results are presented in Table III. The 95% confidence limits for the relative potency determinations for cocaine hydrochloride, procaine hydrochloride, and dibucaine hydrochloride overlap with those reported by Bianchi (4), who compared the effectiveness of these compounds in producing anesthesia in the mouse's tail (Table IV). This illustrates that relative potency determinations from different laboratories are frequently equivalent, even though dissimilar investigative methods are used. Obviously, this degree of agreement between the AD_{50} values is not to be expected.

Since the precision of assays is generally compared by means of the lambda index, an approximate value for lambda was obtained by averaging the reciprocals of the slopes obtained in the separate AD_{50} determinations in these tests and the antilogs of the slope factors reported by Bianchi (4). The results obtained were 0.35 and 0.41, respectively. Both of these values compare favorably with the 0.38 lambda index reported by Mongar (10) for the intradermal wheal method with guinea pigs.

The results obtained by the method described in this paper exhibited considerable variability. Nevertheless, the accuracy of this test appears comparable to that enjoyed by similar biological tests.

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Tricin from Alfalfa—Isolation and Physiological Activity

By E. M. BICKOFF, A. L. LIVINGSTON, and A. N. BOOTH

Tricin was isolated from the press juice of fresh alfalfa (*Medicago sativa*) and identified by comparing its physical and chemical properties to authentic material. Tricin caused smooth muscle relaxation when in contact with isolated guinea pig intestinal strips but did not produce bloat in sheep. It exhibited only slight activity as an antioxidant, measured by the protection of epinephrine from oxidation. The activity of tricin as an estrogen was slight.

THE FLAVONE, tricin, recently reported in the seed of *Phoelipaea ramosa* (1), was originally isolated from wheat (2) and subsequently reported in alfalfa (3). The latter report stated that it was isolated by ether extraction of clarified juice and purified by high-vacuum sublimation but gave no further details. In this laboratory, extraction of dehydrated alfalfa with 95% alcohol, using the procedure of Anderson (2), gave a low yield of tricin. Furthermore, a crude product, including much extraneous material, was extracted with ether from alfalfa juice by the procedure of Ferguson *et al.* (3). The isolation procedure finally developed was a modification of these two procedures.

Ferguson's observation that tricin relaxed and partially paralyzed excised rabbit intestine led him to postulate that the substance might cause bloat (4–6). However, he was not able to obtain sufficient tricin to test this theory.

The subject of estrogen-like substances in forages has been of continuing interest since 1948, when Curnow *et al.* (7) prepared estrogenic extracts from subterranean clover and showed their relation to reproductive problems of sheep grazing this forage. To date, five estrogen-like substances have been isolated from alfalfa. These include the coumarin-like compound, coumestrol (8), and the four isoflavones, genistein, daidzein, formononetin, and biochanin A (9). No estrogenic flavones have been isolated yet from forage, although Wenzel and Rosenberg (10) in a study on the estrogenic activity of flavones reported that 4′-6-dihydroxyflavone was active.

EXPERIMENTAL

Preparation of Tricin from Alfalfa.—Fresh alfalfa was passed through a double-roller cane crusher and the juice collected. The bagasse was extracted twice with fresh portions of water and the juice expressed. The combined juices were heated to 60° for 5 minutes, then rapidly cooled with ice. Following removal of the green sediment by centrifugation, the clear brown juice was concentrated about tenfold under vacuum. Centrifugation was followed by extraction of the juice in a separator with an equal volume of ether. Small quantities of alcohol were added to the mixture to prevent the formation of emulsions. Greenish-yellow crystals formed as the ether layer was slowly evaporated on a steam bath. Before permitting the solution to go down to dryness, the crystals were collected on a filter and recrystallized from ethanol and dimethylformamide.

A total of about 4000 lb. of fresh alfalfa was processed to collect about 20 Gm. of tricin. Following this procedure, in numerous individual isolations from 100- to 200-lb. portions of fresh alfalfa, the quantity isolated varied greatly, ranging from almost none to about 0.02% of the dry weight of the alfalfa.

Identification of Tricin.—The analytical sample was recrystallized from ethanol and dimethylformamide, m.p. 291–294° dec.; no depression in melting point with an authentic sample.

Anal.—Calcd. for $C_{17}H_{14}O_7$: C, 61.8; H, 4.24. Found: C, 61.3; H, 4.57.

5,7,4′-Triacetoxy-3′,5′-dimethoxyflavone.—One-hundred milligrams of tricin was acetylated in the usual way with acetic anhydride and fused sodium acetate. Recrystallization from chloroform gave 95 mg. of colorless needles of the triacetate, m.p. 251–254°; no depression in melting point with an authentic sample.

Anal.—Calcd. for $C_{23}H_{20}O_{10}$: C, 60.5; H, 4.38; OCH_3, 13.56; CH_3CO, 28.2. Found: C, 60.5; H, 4.43; OCH_3, 13.3; CH_3CO, 27.5.

Identical X-ray diffraction patterns, infrared, and ultraviolet spectra were obtained with the isolated tricin and an authentic sample as well as with the acetate derivative and an authentic sample of the acetate.

Pharmacological Studies with Tricin

Effect on Guinea Pig Intestine.—The direct action of tricin on smooth muscle strips was compared to the action of the flavonoid, quercetin. Two milligrams of tricin in the 50-ml. tissue bath was required to produce a relaxation equal in magnitude to that produced by 0.5 mg. of quercetin. Wilson and DeEds (11) have reported the correlation between the antioxidant properties of various flavonoids and their ability to prolong the epinephrine relaxation time of excised guinea pig colon segments. Using their technique, Fig. 1 shows that the epinephrine relaxation time was only slightly prolonged by tricin, in contrast to the striking effect of an equal weight of quercetin.

Effect on Bloat in Sheep.—The procedure for the administration of test materials to sheep has been previously described (12). Ten grams of tricin was administered in this same manner but failed to reduce ruminal motility or cause bloat in the sheep.

A water suspension of 25 Gm. of quercetin administered to five intact sheep also failed to produce

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Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

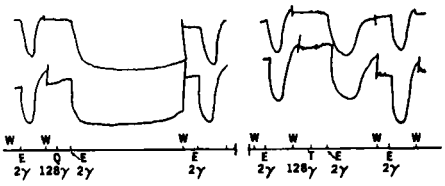


Fig. 1.—Prolongation of epinephrine (E) relaxation time of guinea pig colon segments in the presence of quercetin (Q) and tricin (T). W = wash out and replacement of bath fluid (50 ml.) with fresh Tyrode solution.

bloat symptoms. On the basis of muscle relaxing power, the quercetin administered was equivalent to 100 Gm. of tricin (12).

In another study (13) each of three cows were given 2 Gm. of quercetin on 1 day, followed by 10 Gm. each on 2 successive days. The cows showed no indication of bloat. If we assume a tricin content of 0.02% in alfalfa, it would be impossible for cows to consume enough alfalfa to ingest 10 Gm. of tricin in 1 day.

Mouse Uterine Weight Bioassay Procedure.—In this previously described report (14), weanling mice (five per cage) were fed diets containing the test materials. At the end of 4 days, the mice were sacrificed, and the uteri were excised and weighed. The results are presented in Table I. For comparative purposes, coumestrol was included in the study. The data indicate that tricin was weakly estrogenic (p = <0.01).

Metabolic Fate Studies.—That quercetin is absorbed from the intestinal tract is shown by the appearance of three split products in the urine when the compound is fed to rats by mouth (15). However, when tricin was fed under similar conditions, no split products like syringic acid were detected. Thus, it is doubtful that tricin is absorbed from the intestinal tract.

TABLE I.—EFFECT OF TRICIN, COUMESTROL, AND DIETHYLSTILBESTROL ON THE IMMATURE MOUSE UTERUS

Group[a]	Compd.	Quantity Fed per Mouse, mg.	Mean Uterine Wt., mg.
1	Control	0	9.6 ± 0.25
2	Tricin	20	11.2 ± 0.57
3	Tricin	40	11.4 ± 0.75[b]
4	Tricin	40	12.4 ± 1.02[b]
5	Coumestrol	0.6	32.4 ± 2.63
6	Diethylstilbestrol	0.0001	29.2 ± 0.40

[a] Five weanling female mice per group. Supplements incorporated into control diet and fed 4 days (10 Gm. diet/mouse). [b] p = <0.01.

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Effects of Gibberellic Acid on the Fixed Oils of Four Plants

By C. DWAYNE OGZEWALLA*

The influence of gibberellic acid on the quality of fixed oils from four plants has been studied. Sesame, castor bean, sunflower, and flax plants were treated with aqueous solutions of gibberellic acid when the plants reached blooming size and at 2-week intervals until harvest. The iodine values and the quantity of unsaponifiable matter varied between samples and between oils from different species of plants. Significant variations from normal were produced in castor and sesame oils. The differences consisted of higher saponification values in both oils and a lower acid value in the latter. A higher saponification value indicating shorter chain fatty acids was the only characteristic common to all oils from treated plants. Some of the changes obtained were from plants that had insignificant or no visible morphological differences.

THE GIBBERELLINS have been known in the United States and Europe for only about 12 years, and they have been available for extensive experimental use for less than a decade (1). The number of research papers involving the gibberellins runs into the thousands; there have been a number of reviews which give detailed information regarding such aspects as the physiology, chemistry, and recommended economic applications (1–5).

Sciuchetti (6), in a recent review, has included the influence of gibberellic acid (GA) on volatile oil, glycoside, and alkaloid producing plants. Only a few of the long list of references deal with changes in the active components, and no references are listed relative to changes in fixed oils. The percentages of certain terpenes in volatile oils have been changed by the use of GA. A slight increase

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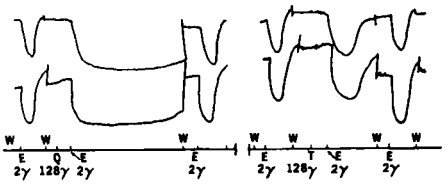


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* Fellow of the American Foundation for Pharmaceutical Education, 1957–1959. S. B. Penick Fellow, 1958–1959. Present address: University of Cincinnati, Cincinnati, Ohio.
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TABLE I.—CHARACTERISTICS OF FIXED OILS FROM CONTROL AND GA-TREATED PLANTS

Test	Treatment	Sesame Oil	Castor Oil	Sunflower Oil	Linseed Oil
Iodine value	Control	105	90	96	162
	GA	101	88	97	179
	Change	—	—	+	+
Unsaponifiable matter	Control	0.2	0.1	0.3	0.5
	GA	0.6	0.6	1.4	0.3
	Change	+	+	+	—
Saponification value	Control	191	183	199	188
	GA	197	187	200	191
	Change	+[a]	+[a]	+	+
Acid value	Control	35	0.5	10	1.7
	GA	15	0.3	7	1.3
	Change	−[a]	—	—	—
Ester	Control	156	182	189	186
	GA	182	187	193	190
	Change	+	+	+	+

[a] Statistically significant at 0.05 level.

of menthol in peppermint oil was detected (7); published results of gas chromatograms indicated changes in volatile constituents of this same oil, although the peaks were not identified or their percentages calculated (8). The altered morphological changed in belladonna and stramonium which have been observed have been accompanied by changes in the total alkaloid content and in the distribution of alkaloids through plant parts (9). In these plants both the biosynthesis and transportation mechanisms are undoubtedly involved.

The objectives of this experiment were (*a*) to ascertain whether GA would bring about changes in the quality of fixed oils and to determine the direction of those changes and (*b*) to determine if chemical changes are invariably accompanied by visible morphological deviations. Alterations in quantity of oil produced were recorded but were not intended as a part of the study.

EXPERIMENTAL

Flax (*Linum usitatissimum* L. var. redwood, family *Linaceae*) was chosen as one of the study plants because it reportedly does not respond to low concentrations of exogenous GA (10). Three other oil bearing plants that grow under cultivation in Western Oklahoma were used. These are sesame (*Sesamum indicum* L. var. *blanco*, family *Pedaliaceae*), sunflower (*Helianthus annus* L., family *Compositae*), and castor bean (*Ricinus communis* L., family *Euphorbiaceae*). Seeds were planted in a small irrigated garden in 1961 and 1962 at the appropriate time of spring. The soil of the drug plant garden was not homogeneous, and other environmental factors were not uniform. A randomized block design was chosen using two adjoining castor or sunflower plants as a block—one treated and one control. Both sesame and flax plantings were divided into four approximately equal sized blocks; half of each was treated with GA.

When the plants reached blooming size and the first flowers appeared, treatment was initiated using 10 p.p.m. aqueous solution of GA.[1] It was impossible to prevent a spray from blowing from one treatment to another because of persistent winds. This was reduced to an insignificant level by the use of a laundry sprinkler purchased in a dry goods store. Sunflower, sesame, and flax plants were sprinkled until the solution dripped from the leaves. The leaves of castor plants shed the droplets of solution so readily that another method of treatment was chosen. GA was injected into the pith cavity of the stems with a hypodermic needle and syringe. Approximately 0.5 mg. of GA per plant was used on the sunflower and castor plants and smaller amounts on the others. At the time of treatment the large leaves of the sunflower and sesame plants had reached their maximum number, and few leaves increased in size thereafter. Records were kept of the height of the plants. The number, width, and length of leaves were measured and the shape noted.

The fruit of sunflower and the seed of the other plants were harvested, threshed, and cleaned by hand. A quantity of sesame seed and sunflower fruit was lost due to natural causes. However, each block of the experiment was harvested at the most opportune time for the particular plant. Seed from each castor plant and from each block of flax and sesame were collected, extracted, and assayed separately. The fruit from the sunflower treatments was pooled because of heavy losses of seed due to insect and storm damage to the flowerheads.

Oil was obtained from the ground seed or fruit by the use of a Soxhlet extractor using benzene as the solvent. The benzene was evaporated at room temperature. U.S.P. XVI (11) procedures were employed to determine iodine value (Hanus method), saponification value, unsaponifiable matter, and free fatty acids. The acid value was calculated from the free fatty acid test and the ester value was obtained by taking the difference between the saponification value and the acid value.

Analysis of variance tests were run using the randomized block design. Notation was made when the resultant *F* test indicated that the results were significant at the 0.05 level. The results are indicated in Table I.

RESULTS

Morphological changes resulting from GA application were observed in castor plants and consisted of internode elongation and a reduction in the number of capsules and seeds. The height of the treated plants was 8% greater than the controls; there was a 6% reduction in the number of seeds ob-

[1] Gibberellic acid was supplied as the potassium salt through the courtesy of Merck and Co., Inc., Rahway, N. J.

tained. A reduction of oil per plant was proportional to the reduction in number of seeds. Significant changes were noted in no other plants. A smaller quantity of fruit from sunflower and of seed from sesame was collected; but due to natural losses of the plant parts, no percentage reduction was calculated. An increase of 5% in flax seed was obtained. Further studies will need to be made to verify this finding and to determine if the number of fruits per plant or the number of seeds in each fruit are increased.

Because the oil of sunflower seed was pooled, no statistically significant differences in the quality of the oils were possible. The iodine value and the saponification value of the sunflower oil were somewhat different from those reported in the literature (12). It is known, however, that the composition of sunflower oil varies according to the locality in which it is grown (13). No significant differences were noted in the linseed oil samples. Significant differences in sesame oil consisted of higher saponification values and lower acid values in oils from plants treated with GA. A higher saponification value was the only significant deviation obtained from castor oils.

Saponification values were consistently higher in the oils from treated plants. The average acid value was consistently lower; however, there was considerable variation between individual castor oil samples and the confidence limits overlapped. The iodine values and the percentages of unsaponifiable matter obtained varied between species of oils and between samples.

DISCUSSION

The reduction in yield in several species of oil bearing plants is not surprising, as a smaller percentage of active constituents has been observed by other workers (4). One author (8) was lead to conclude "that gibberellic acid is of doubtful value in drug plant cultivation." While more studies will need to be made to justify such a broad statement, it does illustrate that hoped-for increases have seldom been obtained. An investigation of proper timing, methods of application, and dosage will need to be conducted.

Morphological changes were either insignificant or absent in some of the treated plants; yet there were changes in the quality of the oils. With more refined methods, slight changes may be detected in the plants used. The choice of economic crops for investigation with GA need not be limited to those that show a striking growth response.

Oils produced from plants treated with GA differed from the controls in their chemical characteristics. The increased saponification values indicated that the oils from treated plants are composed of shorter chain fatty acids. This characteristic was constant in all the species and samples of oils tested. It is more likely to be a general physiological effect of GA than any other. Acid values tended to be lower, an indication that more of the fatty acids were esterified. The resultant higher ester values would indicate that a greater percentage of glyceryl esters occurred in the oils from plants treated with GA. Other characteristics varied between species. The photosynthetic products which build the fixed oils are produced in the leaves some distance from the place where they are stored. A physiologically abnormal catabolism or transportation or both might have been involved in the plants used.

SUMMARY

Slight changes in the chemical constants of oils from GA-treated plants were observed. Significant changes were seen in sesame and castor oils.

Increased saponification values from all treated plants indicate that shorter chain fatty acids were present in the oils. Acid values tended to be lower, an indication of less free fatty acids. Iodine values and unsaponifiable matter varied among species.

Changes were observed in the quality of oils obtained from plants that had insignificant or no visible morphological differences.

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2. Equation 23 contains the term $2K^2/K^3$. This should be replaced by $2k^2/K^3$.
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Significance of Phosphorus–Nitrogen Ratio in U.S.P. Thyroid

By ALBERT D. WILLIAMS, LESTER MEISTER, MARJORIE FAIRCLOTH, and WARNER H. FLORSHEIM

Biologically defective U S.P. thyroid tablets which have appeared recently on the American market have been prepared from triple strength U.S.P. desiccated thyroid powder supplied by a European distributor. This material, with an unnaturally high iodotyrosine and low iodothyronine content, also has a phosphorus–nitrogen ratio greater than that found in genuine U.S.P. thyroid. Chemical evidence suggests that suboptimally iodinated casein was added to thyroid powder to increase the organic iodine content of the blend. Artificial mixtures have been prepared which closely resemble the physical, chemical, and biological characteristics of known defective U.S.P. thyroid.

A CHEMICAL METHOD for the detection of biologically defective but pharmacopeial thyroid preparations recently has been described (1). Since the organic iodine content in these samples met U.S.P. requirements, even though the thyronine iodine content was reduced, the balance of the organic iodine in the defective preparations was thought to consist of nonhormonal iodinated organic compounds. Twenty-two of 24 biologically defective thyroid preparations studied have been traced to a single European distributor of triple strength U.S.P. desiccated thyroid powder.

The possibility existed that an unnatural iodinated protein had been added to genuine desiccated U.S.P. thyroid, resulting in a mixture with an organic iodine content of approximately 0.6%; the product had characteristics capable, on dilution, of satisfying all current U.S.P. requirements. Several iodinated proteins which could have been used for this purpose were iodinated casein, iodinated peanut protein, or artificially iodinated thyroid powder.

More than 30 years ago, Gutman *et al.* (2) suggested the feasibility of adulterating desiccated thyroid with iodinated casein and pointed out that such adulteration would be undetectable by existing pharmacopeial methods. Although it is possible to identify such substandard thyroid preparations by biological and chemical means (1), the adulterant has not yet been directly identified. Evidence suggesting the presence of iodinated casein in defective U.S.P. thyroid is reported here based on established knowledge that casein is inexpensive and easily iodinated and may be the only known phosphoprotein having such characteristics.

PROCEDURE

Iodine Assay.—Methods for the determination of total iodine in thyroid, iodoproteins, and iodinated compounds have been described (1).

Phosphorus Assay.—Powdered samples of thyroid or iodoprotein were extracted twice with 10% trichloroacetic acid (TCA) to remove inorganic phosphorus. The residue was extracted with dimethoxymethane. The phosphorus content of the lipid-free, TCA-insoluble residue was determined by King's modification (3) of the Fiske and Subbarow method (4).

Nitrogen Assay.—Nitrogen analysis of the samples was done by a micro-Kjeldahl digestion, followed by steam distillation of the ammonia into boric acid for titrimetric measurement (5).

Column Chromatography.—Dowex-1 anion-exchange resin columns and eluting solutions were prepared as described by Galton and Pitt-Rivers (6). Following alkaline hydrolysis of 20–60-mg. samples of iodinated protein, desiccated thyroid or pure iodinated compounds (1), the filtered hydrolysates were neutralized and diluted to known volumes. Suitable portions were washed into Dowex-1 columns previously equilibrated with pH 5.6 acetate buffer. The columns were developed with aqueous acetic acid of progressively decreasing pH, and the pH 1.8 and pH 1.4 effluents were collected separately. These effluents were analyzed for total iodine. The fraction of the total iodine of the original sample which appeared in the pH 1.8 and in the pH 1.4 effluent then was calculated.

Preparation of Poorly Iodinated Casein.—Casein was iodinated by a modification of a procedure of Reineke and Turner (7). To a solution of 1 Gm. $NaHCO_3$ in 700 ml. of water at 70°, 20 Gm. of casein was added slowly with constant stirring. After the casein had dissolved, 0.5 Gm. of finely ground iodine was added slowly over a period of 4 hours with constant stirring, and the mixture was stirred at 70° for 20 hours. After dialyzing against flowing tap water overnight, the iodinated casein was precipitated by slow addition, with constant stirring, of 3 *N* HCl to pH 3.9. The precipitate was recovered by filtration and washed with acidified (pH 4.0) water. This iodocasein (sample 115) was dried at 60°, ground to pass through an 80-mesh screen, and had an iodine content of approximately 1%. Another iodocasein (sample 119) was prepared as described above, except that iodination and incubation temperatures were 40°.

RESULTS

Column Chromatography.—Recovery of the following pure compounds from Dowex-1 anion-exchange columns was evaluated: sodium L-thyroxine (T-4), sodium L-3,3′,5-triiodothyronine (T-3), L-3,5-diiodothyronine (T-2), L-3,5-diiodotyrosine (DIT), and L-3-monoiodotyrosine (MIT).[1] Alkaline hydrolysates of duplicate samples of each pure compound were neutralized and filtered. Portions of the diluted filtrates were chromato-

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[1] These compounds were purchased from California Corporation for Biochemical Research, Los Angeles, Calif.

TABLE I.—DOWEX-1 COLUMN RECOVERY OF HYDROLYZED PURE COMPOUNDS

Compd.	% of Total Iodine in Compd. Iodine in pH 1.8 Effluent	Iodine in pH 1.4 Effluent
Sodium L-thyroxine	1.2	92.7
Sodium L-3,3′,5-triiodothyronine	2.0	75.2
L-3,5-Diiodothyronine	0.6	100.0
L-3,5-Diiodotyrosine	95.4	2.3
L-3-Monoiodotyrosine	94.7	1.2

graphed on Dowex-1 columns, collecting the pH 1.8 and the pH 1.4 effluents separately.

The values for iodine recovered in the pH 1.8 and in the pH 1.4 effluents (Table I), expressed as per cent of total iodine in each compound, are the averages of duplicate experiments, which agreed closely. These results show a 95% recovery of MIT and DIT iodine in the pH 1.8 effluent with only 1–2% appearing in the pH 1.4 effluent. In contrast, 93% of the T-4, 75% of the T-3, and the bulk of the T-2 iodine were recovered in the pH 1.4 effluent with only 1–2% appearing in the pH 1.8 effluent. These results are comparable to those reported by Galton and Pitt-Rivers (6), who separated I^{131}-labeled iodinated tyrosines and thyronines by Dowex-1 anion-exchange column chromatography.

In the results reported here, the iodine in the pH 1.8 effluent from the Dowex-1 columns is considered to represent DIT + MIT (iodotyrosine) iodine, while the iodine in the pH 1.4 effluent represents T-4 + T-3 (iodothyronine) iodine. No attempt was made to separate MIT from DIT or T-4 from T-3.

The mean values for DIT + MIT and T-4 + T-3 iodine of several different iodocaseins, an iodoardein sample,[2] and a number of effective and defective U.S.P. thyroid samples appear in Table II. The organic iodine content of each preparation was determined after two extractions with TCA to remove inorganic iodine known to be present in some of the synthetic iodoproteins. TCA-soluble iodine was not found in the U.S.P. thyroid samples or in iodocasein samples 86, 115, and 119. However, TCA-soluble iodine constituted 10–30% of the total in iodoardein and in iodocasein samples 70 and 46.

The iodothyronine iodine contents of samples 86 and 70, unlike those of iodotyrosine iodine, were similar (Table II). Both were samples of iodinated casein[3]; sample 86 was supplied as pharmaceutical grade, *i.e.*, free of inorganic iodine. Sample 70 contained appreciable inorganic iodine, and the possibility that the alkaline hydrolysis procedure resulted in some iodination of tyrosine residues was not investigated.

The mean iodothyronine iodine content of the effective thyroid samples is significantly higher than that of the defective samples ($p < 0.001$). Conversely, the mean iodotyrosine iodine content of the defective samples is higher ($p < 0.001$) than that of the effective samples (Table II).

[2] Iodinated peanut protein. Marketed as Ardein by Imperial Chemical Industries, Ltd., England. Kindly supplied by Professor A. S. Parkes, Cambridge, England.

[3] Marketed as Protamone by Agri-Tech, Inc., Kansas City, Mo.

Phosphorus Content of Iodoproteins.—The content of phosphorus and nitrogen in thyroglobulin, iodocasein, iodoardein, and effective and defective U.S.P. thyroid samples was measured and the mean values, expressed as milligrams of phosphorus per gram of nitrogen, are given in Table III. The phosphorus–nitrogen (P/N) ratio in defective U.S.P. thyroid is significantly higher ($p < 0.001$) than in effective thyroid. Iodinated casein has the highest P/N ratio.

Studies with Artificial Mixtures.—Sufficient suboptimally iodinated casein (approximately 1% organic iodine) was blended with effective U.S.P. thyroid powder to produce mixtures with a total organic iodine content of approximately 0.6%. The components in Table IV used for the preparation of two artificial mixtures containing no detectable inorganic iodine are listed together with the total iodine content of each. The iodotyrosine and iodothyronine iodine content and the P/N ratios of each mixture in Table IV can be compared to corresponding values in Tables II and III for effective and defective U.S.P. thyroid samples. The biological activity of one of the artificial mixtures reported in Table IV was estimated to be less than 60% of that of a standard thyroid preparation; the other artificial mixture was inert.

DISCUSSION

In this extension of an earlier report of chemically detectable differences between biologically effective and defective U.S.P. thyroid (1), a high iodotyrosine and a low iodothyronine iodine content of defective U.S.P. thyroid has been demonstrated (Table II). Estimates of the MIT + DIT and T-4 + T-3 iodine content of effective desiccated thyroid are similar to those reported for hydrolysates of fresh thyroid glands from several species (8, 9). A low iodothyronine and a high iodotyrosine content in suboptimally iodinated casein having a total organic iodine content of about 1% has been observed by Roche and Michel (10).

The mean P/N ratio of the iodocasein samples (Table III) agrees well with published data for casein, a phosphoprotein (11). In accordance with others (12), thyroglobulin has a low P/N ratio. The results (Table III), an indication of a P/N ratio significantly higher in defective than in effective U.S.P. thyroid, seem notably relevant since they strongly suggest the presence of iodinated phosphoprotein in defective thyroid. The increased P/N ratio in the defective preparations is explained most readily by assuming that an iodocasein was blended with desiccated thyroid powder. This would raise the organic iodine content of the mixture to triple strength U.S.P. but with a concomitant elevation of phosphorus.

Determination of the P/N ratio in thyroid offers a secondary procedure for detection of nongenuine thyroid. Although the P/N ratio of commercial thyroid preparations seems at present a reliable indicator of adulteration by iodinated casein, it does not necessarily predict the hormonal content of these preparations. The hormonal content of iodocasein is chiefly related to its organic iodine content, for which there is an optimal level approximating 6% (7, 10). Optimally iodinated casein

TABLE II.—FRACTIONATION OF HYDROLYSATES OF IODINATED PROTEINS AND DESICCATED THYROID ON DOWEX-1 COLUMNS

Sample	Organic Iodine, %	% of Organic Iodine: DIT + MIT Iodine (pH 1.8 Effluent)	% of Organic Iodine: T-4 + T-3 Iodine (pH 1.4 Effluent)
Iodocasein (No. 86)[a]	5.92	29.9	19.7
Iodocasein (No. 70)[a]	5.10	48.5	20.9
Iodocasein (No. 46)[b]	6.15	54.9	14.1
Iodocasein (No. 115)	0.96	80.4	11.3
Iodocasein (No. 119)	0.89	86.2	4.5
Iodoardein	0.70	24.5	8.4
Effective U.S.P. thyroid (11 samples):		44.4 ± 1.2[c] (38.7–47.6)	14.4 ± 0.7[c] (11.6–19.6)
Defective U.S.P. thyroid (11 samples):		61.5 ± 1.7 (53.1–67.5)	7.7 ± 0.4 (6.7–10.3)

[a] Kindly supplied as Protamone by Agri-Tech, Inc., Kansas City, Mo. [b] Produced by Agri-Tech, Inc., by a process since modified. [c] Mean, standard error, and range of values.

TABLE III.—PHOSPHORUS CONTENT OF IODINATED PROTEINS AND DESICCATED THYROID

Sample	Samples, No.	mg. P per Gm. N: Mean	S.E.	Range
Effective U.S.P. thyroid	9	14.0	0.71	11.1–17.6
Thyroglobulin	3	4.4[a]	0.57	3.7– 5.6
Defective U.S.P. thyroid	10	30.2[a]	3.17	18.8–52.0
Iodocasein	5	56.8[a]	4.10	46.6–67.7
Iodoardein	1	4.6		

[a] Significantly different from effective U.S.P. thyroid ($p < 0.001$).

may have a thyroxine content of 1% in addition to small but biologically significant amounts of triiodothyronine (13)—although compared to desiccated thyroid, the hormone in iodocasein is biologically less available perorally in the human (14) and in the rat (15).

Present analytical procedures (1, 16) overestimate the iodothyronine content in iodocasein. Therefore, it is theoretically possible to adulterate U.S.P. thyroid with an optimally iodinated casein yielding a product with substandard clinical thyroactivity yet having an acceptable P/N ratio and an apparently satisfactory iodothyronine content. However, commercial adulteration of thyroid with optimally iodinated casein probably would be precluded due to the effort and expense involved.

The mixtures of suboptimally iodinated caseins with U.S.P. thyroid closely mimic triple strength U.S.P. thyroid powder physically but are low in biological activity. Although the total organic iodine content is similar in these mixtures (Table IV), the iodothyronine content varies by a factor of 2, being dependent on the iodocasein moiety (Tables II and IV).

Since Ardein may be commercially available and has been studied in its iodinated forms for thyroactivity (17), the possibility existed that iodoardein had been used to adulterate desiccated thyroid. However, the low P/N ratio and the relatively low iodotyrosine content of the single iodoardein sample available for study (Tables II and III), in contrast to high corresponding values in defective U.S.P. thyroid, make this possibility most unlikely.

It seemed possible that desiccated thyroid powder could have been artificially iodinated to increase the organic iodine content of the material. Such treatment would not change the P/N ratio of the product; in view of the high P/N ratios of the defective thyroid samples, it is improbable that thyroid powder was iodinated directly.

SUMMARY AND CONCLUSIONS

Appropriate amounts of two noncommercial iodinated casein preparations have been mixed with effective U.S.P. thyroid to obtained two products, each having a total iodine content equivalent to triple strength U.S.P. thyroid and containing no inorganic iodine. These mixtures have biological activities varying from inert to 60% of a standard commercial thyroid preparation and have a P/N ratio higher than genuine U.S.P. thyroid. Chemical characteristics (P/N ratios and iodothyronine and iodotyrosine content) of the biologically less active artificial mixture resemble corresponding values for a group of biologically defective commercial thyroid preparations. Clinically defective commercial U.S.P. thyroid preparations differ strikingly from effective samples in relative iodothyronine and iodotyrosine content and in P/N ratios.

The evidence presented supports the hypothesis that suboptimally iodinated casein, free of inorganic iodine, has been added to desiccated thyroid, raising the total iodine content of the mixture to a level equivalent to triple strength U.S.P. thyroid. Such material, satisfying all current

TABLE IV.—CHARACTERISTICS OF ARTIFICIAL THYROID MIXTURES

Mixture No.	Components	Organic Iodine[a] %	% of Organic Iodine: DIT + MIT Iodine	% of Organic Iodine: T-4 + T-3 Iodine	Mg. P / Gm. N	Bioassay[b]
1	Iodocasein[c] Desiccated thyroid[d]	0.620	42.1	13.1	45.8	<60%
2	Iodocasein[e] Desiccated thyroid[d]	0.665	51.2	6.3	46.7	Inert

[a] The artificial mixtures contained no detectable inorganic iodine. [b] The authors thank the Wilson Laboratories, Chicago, Ill., for bioassay of these samples by a rat goiter-prevention method. [c] Sample 115, Table II (iodinated at 70°). [d] Effective U.S.P. desiccated thyroid powder (0.20% iodine). [e] Sample 119, Table II (iodinated at 40°).

U.S.P. chemical requirements for desiccated thyroid —although biologically substandard—may thus have been used for the manufacture of defective U.S.P. thyroid tablets. It has not been possible to ascertain with certainity the primary source of the nongenuine thyroid material.

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A colorimetric method was employed in the estimation of the normal iron content of mice. An estimate of the total amount of iron present per mouse was 1.457 ± 0.152 mg. This represented an average of 52 ± 6 mcg. per Gm. body weight of mouse with approximately 11.5 per cent of the total iron per mouse present in the gastrointestinal tract and 88.5 per cent in the remainder of the carcass.

AN ESTIMATION of normal iron content of mice, the total amount contained therein compared with the amount contained in the separated entire gastrointestinal tract, is indicated in studies of iron absorption following oral administration.

There are numerous methods available for estimating iron content; *o*-phenanthroline, α,α'-dipyridyl, and benzidine methods (1–13). A sodium sulfocyanate or potassium thiocyanate method was described by Kennedy (14), improved and simplified by Farrar (15), Andes and Northup (16), and Wong (17), resulting in the method for the determination of iron in blood and hemoglobin published by the Fisher Scientific Company (18).

Employing a modification of the Fisher method, it was the purpose of this investigation to determine an estimate of the normal amount of iron present in a whole mouse carcass and that present in the separated gastrointestinal tract. This latter method of separation of carcass and gastrointestinal tract is essentially the method described by Cori *et al.* (19–21).

EXPERIMENTAL

The method employed was a colorimetric analysis with the Fisher model AC electrophotometer. The procedure as published (18) was followed except for the elimination of the tungstate solution. Since the tungstate solution was only involved in precipitating protein, it was unnecessary in the procedures employed in this investigation.

The iron, after being liberated and oxidized with sulfuric acid and potassium persulfate, was treated with potassium thiocyanate. The electrophotometer scale reading of the resulting colored solution was determined, and the iron concentration was calculated from this using a calibration curve.

Following the determination and calculation of the standard iron concentration curve, as described in the Fisher method, iron determinations were done on whole mice fasted at least 20 hours but not longer than 24 hours. Six B4BC male mice, 5 weighing 27 Gm. and one weighing 26 Gm., were sacrificed and digested in a Kjeldahl flask using concentrated sulfuric and nitric acids. The complete digestion resulted in a clear solution of sulfuric acid with or without a small amount of white precipitate (calcium salts) in the bottom of the flask.

The sulfuric acid solution was cooled and slowly diluted to 100 ml. with distilled water and was used as the sample solution for iron determination using the modified Fisher method.

At all times, precautions were taken to use glass distilled water and carefully cleaned glassware.

A separate determination on the gastrointestinal tract and on the remainder of the mouse carcass was done on three 32.5-Gm. B4BC male mice employing the same procedure.

In experiments done to observe the method and obtain more preliminary control figures for the amount of iron present, mice in groups of 6 were fasted, sacrificed, separated as to carcass and entire gastrointestinal tract, digested, and an aliquot amount obtained for sample determination. In a number of animals equaling that of the controls, the same procedure was employed, except that 1 hour prior to sacrificing the animals received a total oral dose of 1, 2, or 4 mg. of ferrous sulfate in solution, the concentration of which was varied to provide near equal volumes orally administered.

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EXPERIMENTAL

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TABLE I.—IRON DETERMINATIONS ON A SERIES OF INDIVIDUAL MICE

Mouse No. and Wt. in Gm.	% Absorption	Sample Size, ml.	Total Iron Present, mg.	mcg. Iron per Gm. wt. of Mouse (Av. of Samples)
1, 26	8.0	1	1.5	
	30.0	4	1.425	56
2, 27	9.5	1	1.75	
	34.5	4	1.64	63
3, 27	9.0	1	1.65	
	32.0	4	1.53	59
4, 27	8.0	1	1.5	
	30.5	4	1.45	55
5, 27	34.5	5	1.32	
	35.0	5	1.34	
	34.5	5	1.32	
	34.5	5	1.32	50
6, 27	33.5	5	1.28	
	33.5	5	1.28	
	33.0	5	1.26	
	33.0	5	1.26	47

TABLE II.—SEPARATE IRON DETERMINATIONS OF INDIVIDUAL MOUSE CARCASS AND GUT

Mouse, No.	Sample Size, ml.	% Absorption	Total Iron Present, mg.	mcg. Iron per Gm. wt. of Whole Mouse
1, Body	5	33.0	1.26	
1, Gut	10	10.0	0.185	44
2, Body	5	38.0	1.44	
2, Gut	10	5.0	0.09	47
3, Body	5	37.0	1.40	
3, Gut	10	12.0	0.22	50

trol group and the other an iron-treated group, receiving a total oral dose of 1 mg. ferrous sulfate, the feces were digested and the iron content determined along with the respective determinations on the carcasses and gastrointestinal tracts.

RESULT AND DISCUSSION

The values read from the electrophotometer for each initial whole mouse tested were based upon the values obtained from the standard iron concentration curve. The values tabulated in Table I by observation show the variability in the method to be relatively small, largely attributed to differences in the mice. The different and replicate sample sizes were employed simply to check the method of procedure without applying any statistical analyses on this number of animals employed.

The following averages were calculated from the data in Table I: average iron present per mouse, 1.470 mg.; average weight per mouse, 26.83 Gm.; average mcg. iron per Gm. mouse, 56 mcg.

The values obtained from the separate gastrointestinal and carcass determinations are recorded in Table II. The sample size, absorption reading, and the amount of iron present are given. The amount or size of the sample, the 1/2 ml. ratio of the sample sizes for the mouse and gut samples, respectively, was arbitrarily chosen. The two separate determinations would be necessary as controls for use in absorption studies where the gut and rest of the body could be treated separately in the attempt to determine whether alteration in absorption had taken place. Knowing the amount of iron administered, the amount in the gut, and the amount in the rest of the body, it would be possible to ascertain whether absorption had been changed by any experimental procedure employed.

The average iron per mouse from the data in Table II was 1.532 mg. (1.470 mg., Table I); the average mcg. iron per Gm. mouse was 47 (56 mcg., Table I).

From these data, an average percentage of approximately 11% of the total iron present was found in the gastrointestinal tract, leaving approximately 89% in the remainder of the carcass.

In the expanded experiments on mice in groups of six, the averages of iron present per mouse carcass and gut, separate and combined, are noted in Table III. Although there is some individual overlapping, the average figures observed indicate a reliable positive trend toward the differences between the control and iron treated groups. However, the finding of less iron in the animals treated with the larger doses of iron is apparently due to the colorimetric method employed, the test solution being too concentrated over extending Lambert-Beer's law. The amount of iron apparently unaccounted for here was linearly related to the amount of iron that had been added, again indicating the overextension of the Lambert-Beer's law. In the groups of mice treated orally with the 2- and 4-mg. iron doses, the animals apparently had an inherent low iron content prior to treatment, thus showing a low carcass iron content. However, when compared on a microgram iron content per gram weight basis the figure shows the expected increase, although no

TABLE III.—AVERAGES FROM MICE IN GROUPS OF SIX OF IRON PRESENT PER MOUSE CARCASS AND GUT, SEPARATE AND COMBINED[a]

Group	Av. Iron Content in Carcass, mg.	Av. Iron Content in Gut, mcg.	Av. Iron Content per Mouse, mg.	Av. Iron Content per Gm. Mouse, mcg.
Controls	1.45	210	1.660	47
	1.20	192	1.392	55
	1.12	110	1.230	56
Combined Av.	1.26	171	1.427	53
With total oral dose of 1 mg. ferrous sulfate	1.61	342	1.952	61
	1.43	410	1.840	59
	1.26	380	1.635	68
Combined Av.	1.43	377	1.809	63
With total oral dose of 2 mg. ferrous sulfate	1.265	425	1.690	68
With total oral dose of 4 mg. ferrous sulfate	1.380	530	1.910	73

[a] Single figures indicate averages of mice in groups of six with combined averages of these where more than one group was tested.

apparent increase in carcass iron (iron absorption) was observed to occur in the limited (1 hour) time period prior to sacrificing and determination in any of the iron treated groups.

From these data on the control groups, an average percentage of approximately 12% of the total iron present was found in the gastrointestinal tract, leaving approximately 88% in the remainder of the carcass. The average iron content per mouse was 1.427 mg.; the average iron content per gram mouse was 53 mcg.

From the determination of the iron content in the feces from two groups of mice, the average iron content of the feces per mouse in the control group was 27 mcg. and in the iron treated group 13 mcg. This would appear to indicate that estimates of fecal iron could be ignored in selected studies.

SUMMARY AND CONCLUSIONS

As modified in this laboratory, the Fisher method was observed to be relatively accurate within concentration limits and comparatively easy to accomplish. The digestion of the animal was the most tedious part of the technique.

An average estimate of the total amount of iron present per mouse was 1.457 ± 0.152 mg. This represented an average of 52 ± 6 mcg. per Gm. body weight of mouse with approximately 11.5% of the total iron per mouse present in the gastrointestinal tract (168 ± 54 mcg.) and 88.5% in the remainder of the carcass (1.31 ± 0.14 mg.).

In the animals treated orally with 1, 2, and 4 mg. of iron 1 hour prior to sacrificing and determination, the average percentages of iron present in the gastrointestinal tracts were 21, 25, and 28, with 79, 75, and 72% in the remainder of the carcasses, respectively. Low inherent carcass iron content must be considered even when comparisons are made on a microgram iron content per gram weight basis as no apparent increase in carcass iron (iron absorption) was observed to occur in the limited (1 hour) time period prior to sacrificing and determining in any of the iron treated groups.

Employing this method, the quantity of iron apparently unaccounted for in the animals treated orally with iron was in direct linear relationship to the amount of iron administered or added and due to the inherent properties of the colorimetric method itself; therefore, the quantities of iron determined are relative and indicative of the true quantities of iron present.

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3-Bromopyruvic Acid, a Highly Selective Antimicrobial Agent Against Certain Yeasts

By WALTER A. ZYGMUNT and TELLIS A. MARTIN

3-Bromopyruvic acid has been shown to be a highly effective growth inhibitor of certain yeasts. Wide differences were noted in the inhibitory activity of the compound among different species and genera of yeast.

THE USE of halogens as antimicrobial agents is well documented, and certain halogenated metabolites possess potent antimetabolite activity. Among such compounds are fluoroacetic acid (1), halogenated phenylalanines (2), 5-halogenated pyrimidines (3), and many others.

Busch *et al.* (4) have reported that β-halogenated pyruvic acids have pharmacological activity as cholinesterase inhibitors. However, there appears to be only limited information in the literature on the activity of such compounds as possible antimicrobial agents. 3-Bromopyruvic acid was selected as a representative compound. This report summarizes our findings on its inhibitory activity against a variety of microorganisms.

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EXPERIMENTAL

Since significant growth inhibition was noted only with certain yeasts and yeast-like fungi, description of the testing techniques employed will be restricted to this group of microorganisms. Yeast nitrogen base medium (Difco), supplemented with 1.5% dextrose and an initial medium pH of 4.5, were used. Solutions of 3-bromopyruvic acid and—where applicable—of reversal agents were adjusted to pH 4.5, sterilized by Seitz filtration, and portions added to previously autoclaved flasks containing sterile distilled water and basal medium. Only freshly prepared solutions of test compound and reversal agents were used. The final volume was 10 ml. per 50-ml. conical flask. In the preparation of inocula, all cells were carefully washed and standardized to a constant absorbance reading. All flasks were agitated on a rotary shaker during incubation for 20 hours at 28°. Growth was measured turbidimetrically as absorbance using a Coleman Junior spectrophotometer at a wavelength of 620 mμ.

All yeast cultures tested, except for *Candida albicans* (ATCC 10231), were kindly supplied by Dr. L. J. Wickerham, Northern Utilization Research

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3-Bromopyruvic Acid, a Highly Selective Antimicrobial Agent Against Certain Yeasts

By WALTER A. ZYGMUNT and TELLIS A. MARTIN

3-Bromopyruvic acid has been shown to be a highly effective growth inhibitor of certain yeasts. Wide differences were noted in the inhibitory activity of the compound among different species and genera of yeast.

THE USE of halogens as antimicrobial agents is well documented, and certain halogenated metabolites possess potent antimetabolite activity. Among such compounds are fluoroacetic acid (1), halogenated phenylalanines (2), 5-halogenated pyrimidines (3), and many others.

Busch *et al.* (4) have reported that β-halogenated pyruvic acids have pharmacological activity as cholinesterase inhibitors. However, there appears to be only limited information in the literature on the activity of such compounds as possible antimicrobial agents. 3-Bromopyruvic acid was selected as a representative compound. This report summarizes our findings on its inhibitory activity against a variety of microorganisms.

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EXPERIMENTAL

Since significant growth inhibition was noted only with certain yeasts and yeast-like fungi, description of the testing techniques employed will be restricted to this group of microorganisms. Yeast nitrogen base medium (Difco), supplemented with 1.5% dextrose and an initial medium pH of 4.5, were used. Solutions of 3-bromopyruvic acid and—where applicable—of reversal agents were adjusted to pH 4.5, sterilized by Seitz filtration, and portions added to previously autoclaved flasks containing sterile distilled water and basal medium. Only freshly prepared solutions of test compound and reversal agents were used. The final volume was 10 ml. per 50-ml. conical flask. In the preparation of inocula, all cells were carefully washed and standardized to a constant absorbance reading. All flasks were agitated on a rotary shaker during incubation for 20 hours at 28°. Growth was measured turbidimetrically as absorbance using a Coleman Junior spectrophotometer at a wavelength of 620 mμ.

All yeast cultures tested, except for *Candida albicans* (ATCC 10231), were kindly supplied by Dr. L. J. Wickerham, Northern Utilization Research

TABLE I.—INHIBITION IN PER CENT OF YEAST AND YEAST-LIKE FUNGAL GROWTH BY 3-BROMOPYRUVIC ACID

Culture	3-Bromopyruvic Acid, mcg./ml. 1.0	2.5	5.0	10	20	40
C. lipolytica	80	98	100	100	100	100
C. brumpti	63	94	97	100	98	97
C. zeylanoides	44	64	91	100	100	100
C. flareri	0	11	47	88	100	100
C. guilliermondii	12	21	33	72	91	95
C. utilis	0	20	24	42	61	81
C. albicans (477)	6	36	44	62	82	89
C. albicans (10231)	3	12	18	45	68	94
C. monosa	0	5	21	62	97	100
C. krusei	0	0	0	27	94	100
C. pulcherrima	0	0	0	0	0	2
C. reukaufii	0	0	0	0	0	0
C. tropicalis	0	0	0	0	0	0
H. anomala	17	46	85	97	98	98
G. candidum	0	0	0	7	23	78
S. cerevisiae	0	0	0	0	7	23

Laboratories, U. S. Department of Agriculture, Peoria, Ill. These cultures included *C. albicans* (NRRL Y-477), *C. guilliermondii* (NRRL Y-488), *C. flareri* (NRRL Y-245), *C. krusei* (NRRL Y-301), *C. monosa* (NRRL Y-1079), *C. pulcherrima* (NRRL Y-775), *C. reukaufii* (NRRL Y-1348), *C. utilis* (NRRL Y-900), *C. zeylanoides* (NRRL Y-106), *C. tropicalis* (NRRL Y-1410), *C. brumpti* (NRRL Y-311), *C. lipolytica* (NRRL Y-1094), *Geotrichum candidum* (NRRL Y-552), *Hansenula anomala* (NRRL Y-1737), and *Saccharomyces cerevisiae* (NRRL Y-9763).

3-Bromopyruvic acid was synthesized according to the method of Dickens and Williamson (5). Identity of the compound was established by bromine analysis (Calcd. for $C_3H_3BrO_3$: Br, 47.86. Found: Br, 47.57), infrared spectrum, and condensation with thiourea to form a known derivative (2-imino-4-thiazoline-4-carboxylic acid) (6).

RESULTS AND DISCUSSION

Data in Table I show the potent antimicrobial activity of 3-bromopyruvic acid against certain yeasts and yeast-like fungi. The results also clearly indicate wide differences in sensitivity to the inhibitory activity of the compound within a single yeast genus. Among 13 cultures of *Candida* representing 12 different species, a minimum of 50% growth inhibition occurs at less than 1 mcg./ml. of 3-bromopyruvic acid with two cultures, one culture at 2.5 mcg./ml., four cultures at 10 mcg./ml., and three cultures at 20 mcg./ml. Three cultures show no growth inhibition at 40 mcg./ml. of test compound.

In addition, single species of three other yeast genera also vary widely in susceptibility to the compound. *H. anomala* is highly sensitive to growth inhibition by 3-bromopyruvic acid; whereas *G. candidum* is moderately sensitive, and *S. cerevisiae* is only slightly susceptible. Potassium bromide at concentrations up to 100 mcg./ml. shows no growth inhibition.

Growth of three different species of bacteria in chemically defined media, *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium phlei*, is not effectively antagonized by 3-bromopyruvic acid. In all cases, the minimal inhibitory concentrations are greater than 100 mcg./ml.

The fungistatic nature of the growth inhibition is suggested by the increased concentrations of 3-bromopyruvic acid required for complete growth inhibition of *C. lipolytica* with increased incubation periods. Where at 24 hours growth is inhibited by 2.5 mcg./ml., these concentrations increase to 5, 10, and greater than 80 mcg./ml. at 48, 72, and 96 hours, respectively. Thus, the fungicidal concentration of the compound is greater than 80 mcg./ml.

Of the seven organic acids tested (Table II), acetate and pyruvate are the most effective reversal agents for 3-bromopyruvic acid growth inhibition in *C. lipolytica*. This growth inhibition is not easily reversed, however, in that a ratio of 2000 to 1 of pyruvic acid to test compound (based on carbon concentrations of both compounds) is needed to cause a 90% reversal of the growth inhibition at 1 mcg./ml. of test compound. Therefore, these data suggest an interference in the metabolism of pyruvic acid by 3-bromopyruvic acid in those yeasts which are highly susceptible to growth inhibition by this compound. Alternately, those species of yeast which are relatively insensitive to the inhibitory activity of this compound may possess such permeability characteristics that the test compound cannot readily permeate the cells.

TABLE II.—PER CENT REVERSAL OF 3-BROMOPYRUVIC ACID GROWTH INHIBITION IN *Candida lipolytica* BY VARIOUS ORGANIC ACIDS[a]

Organic Acids, mcg./ml. as C	Pyruvate I	Pyruvate II	Acetate I	Acetate II	α-Ketoglutarate I	α-Ketoglutarate II	Succinate I	Succinate II	Fumarate I	Fumarate II	L-Malate I	L-Malate II	Citrate I	Citrate II
0.4	8	0	21	0	0	0	0	0	5	0	18	0	0	0
4	44	0	34	0	22	0	1	0	0	0	1	0	12	0
40	50	0	42	0	18	0	0	0	25	0	0	0	0	0
400	92	28	79	13	33	0	19	0	44	0	7	0	14	0
4000	100	86	[b]		52	12	86	3	[b]		75	2	38	4

[a] 3-Bromopyruvic acid was added at concentrations of 1.0 mcg./ml.(I) and 10 mcg./ml.(II). [b] Growth inhibition in the absence of test compound.

Previously, Kalnitsky and Barron (1) showed that fluoroacetate, and much less effectively bromoacetate, inhibited oxidation of acetate and pyruvate in *S. cerevisiae*. Interestingly, inhibition of pyruvate oxidation by fluoroacetate was much less marked in several species of bacteria.

In summary, wide differences in the susceptibility to the growth inhibitory activity of 3-bromopyruvic acid exist among different genera of yeast and also between different species of yeast within the same genus. Data suggest that the observed growth inhibition may be due to an interference in pyruvate metabolism.

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Synthesis of 5-[(2-Fluoroethyl)(2-chloroethyl)amino]-6-methyluracil (Fluorodopan)

By HENRYK DUBICKI, FLORIAN ZIELINSKI, and FRED W. STARKS

Fluorodopan has been synthesized and its antitumor activity compared to 5-[2-(bis-chloroethyl)amino]-6-methyluracil (dopan).

LARIONOV (1) describes the excellent activity of fluorodopan as a chemotherapeutic agent for cancer. However, the author indicates no preparative method. This paper reports the synthesis of this compound as performed in our laboratory.

Fluorodopan has shown activity[1] against the Walker 256 (subcutaneous) tumor system and against the Walker 256 (intramuscular) tumor system. In the former system, the therapeutic index was equal to 4, compared to 13 for dopan (2); in the latter system, fluorodopan has shown complete tumor inhibition in three cases. However, the doses required to produce this effect also reflected considerable toxicity to the host in both weight loss and death. Minimal activity was displayed by fluorodopan against the Dunning leukemia system. This was the same response for dopan. No reproducible activity was seen when fluorodopan was tested against the sarcoma 180, lymphoid leukemia 1210, and 9KB cell culture cytotoxicity test systems.

EXPERIMENTAL

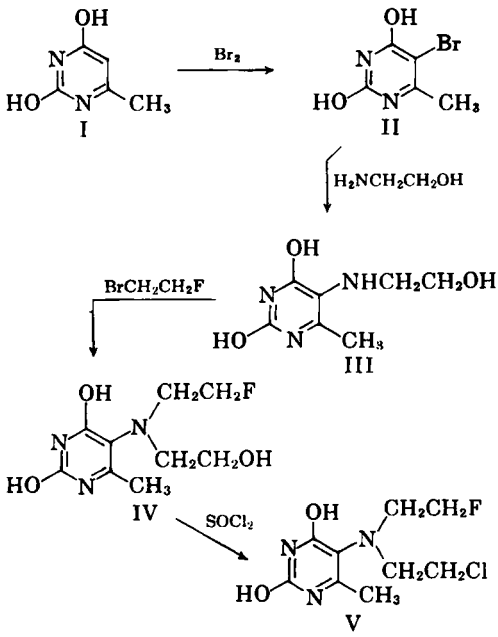
5-Bromo-6-methyluracil (II).—To 150 Gm. (1.19 moles) of 6-methyluracil (I) contained in a 2-L. round-bottomed flask was added 240 Gm. (1.50 moles) of liquid bromine. The mass was stirred manually at room temperature to cause intimate contact of bromine with the solid uracil. When the product had changed to a homogeneous red color, the mass was powdered. The flask was evacuated on a flash evaporator at 90° to remove excess bromine and hydrogen bromide. A heating period of 2–3 hours was required to cause the reaction material to become a constant yellow-white. The product was washed in 4 L. of boiling water for 20 minutes, cooled to 20°, and recovered by filtration. The yield, after recrystallizing from water, was 209.5 Gm. (86%), m.p. 230–237° [lit. (3) m.p. 230°].

Anal.—Calcd. for $C_5H_5BrN_2O_2$: C, 29.3; H, 2.5; Br, 39.0; N, 13.7. Found: C, 29.4; H, 2.5; Br, 39.1; N, 13.6.

5-Hydroxyethylamino-6-methyluracil (III).[2]—A 1-L. steel autoclave was charged with 240 Gm. (1.17 moles) of II, 276 Gm. (4.53 moles) of 2-aminoethanol, and 120 ml. of water. The mixture was agitated manually at room temperature until a homogeneous paste formed. The autoclave was sealed and heated at 160° for 5 hours, then cooled to room temperature in a water bath. A clear brown oil was obtained, which was dissolved in 2 L. of methanol and cooled to −20°, causing the crystalline product to precipitate. The product was decolorized with Norit and recrystallized from methanol to yield 85.7 Gm. (39.6%), m.p. 183–184°.

Anal.—Calcd. for $C_7H_{11}N_3O_3$: C, 45.4; H, 6.0; N, 22.7. Found: C, 45.3; H, 6.0; N, 22.7.

5 - [(2 - Fluoroethyl)(2 - hydroxyethyl)amino]-6-methyluracil (IV).—A 2-L. round-bottomed flask, fitted with a water-cooled condenser, was charged with 350 ml. of dimethylformamide and 131 Gm. (0.708 mole) of III. The mixture was stirred magnetically and heated to 90°. Bromoethylfluoride (90 Gm., 0.708 mole) and cyclohexene oxide (64.4 Gm., 0.708 mole) were added to the reaction mixture in equal portions during eight 3-hour intervals. After the reaction mixture had been heated a total of 27 hours, the dimethylformamide was removed *in vacuo* to leave a dark-red oil. The oil was dissolved in a minimum amount of methanol (about 100 ml.) and added to 2.0 L. of ethyl acetate, causing the precipitation of a red-black oil fraction which was discarded. The supernatant ethylacetate solution was evaporated *in vacuo* to a light-colored oil which was triturated with boiling ethyl acetate (10 × 200 ml.). The combined ethyl acetate solutions were concentrated *in vacuo* to about 500 ml., heated to boiling, and decanted from a yellow oil which had been deposited. The hot ethyl acetate was seeded with authentic product[3] IV, cooled at a rate of 20°/hour, stored



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The authors are grateful to Dr. Howard J. Schaeffer for stimulating discussions during the course of this work.

[1] These data were obtained from the Screening Section, Drug Evaluation Branch, Cancer Chemotherapy National Service Center, National Cancer Institute.

[2] The method is similar to that employed for the production of 5-(2-hydroxyethylamino) uracil (4).

[3] Seed crystals were obtained by a chromatographic separation of the reaction residue on activated alumina. When eluted with ethyl acetate, the desired fraction produced a white fluorescence (U.V.) on filter paper. When the color turned blue, collection of the fraction was stopped. Concentration of the white fluorescing fraction *in vacuo* produced a crystalline product (IV).

at room temperature overnight, then at −10° for 24 hours; yield, 53.1 Gm. (32.6%), m.p. 116–119°. Purity of this material was sufficient for the succeeding reaction to form fluorodopan. Product IV may be purified by suspending it in boiling ethyl acetate (10 Gm., 250 ml.), followed by the dropwise addition of methanol until complete dissolution occurs. The melting point of pure IV was 123–123.5°.

Anal.—Calcd. for $C_9H_{14}FN_3O_3$: C, 46.7; H, 6.1; F, 8.2; N, 18.2. Found: C, 46.9; H, 6.0; F, 8.1; N, 18.0.

5 - [(2 - Fluoroethyl)(2 - chloroethyl)amino]-5-methyluracil (V).—To a stirred solution of 800 ml. of 1,2-dimethoxyethane, 21 ml. of absolute ethanol, and 0.5 ml. of water, was added 28.6 ml. (0.39 mole) of thionyl chloride. After 5 minutes, the solution was cooled to 0°, and 80 Gm. (0.35 mole) of IV was added, then 89.6 ml. (1.23 moles) of thionyl chloride (5). The suspension was stirred at 0–5° for 6 hours, then permitted to warm to room temperature. The suspension was stirred an additional 18 hours, then filtered. The product filter cake was washed by resuspending in benzene (5 × 500 ml.) and recovered by filtration; yield, 89 Gm. (83.2%), m.p. 130–140°. The product hydrochloride was recrystallized from ethanol by the addition of ethyl ether, m.p. 159–160°. Since purity of the recrystallized product was poor, the free base was liberated by neutralizing a water suspension of the salt with saturated aqueous sodium bicarbonate; yield from 89 Gm., 67 Gm. (86.2%). The free base was purified by crystallization from ethyl acetate, m.p. 166–167°.

Anal.—Calcd. for $C_9H_{13}ClFN_3O_2$: C, 43.3; H, 5.3; Cl, 14.2; F, 7.6; N, 16.8. Found: C, 43.3; H, 5.3; Cl, 14.3; F, 7.7; N, 17.1.

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By S. S. MISHRA and J. P. TEWARI

A phytochemical investigation was conducted on *Piper chaba* Hunter. β-Sitosterol, piperine, and piplartine have been isolated in pure form from the stems of this plant and identified.

PIPER CHABA Hunter (*Piperaceae*), known as chaba, is a glabrous, fleshy plant. It is cultivated in various parts of India and Malaya. The assumed medicinal effects of this plant are briefly described by Kirtikar and Basu (1), Chopra (2), and Nadkarni (3). The phytochemical study was initiated primarily because preliminary screening tests indicated that *P. chaba* possessed hypotensive and smooth muscle relaxant activity as shown in acute experiments (4). This observation and a survey of the available literature, which revealed no apparent report on the exact chemistry of this plant, prompted this investigation.

β-Sitosterol, piperine, and a new alkaloid—piplartine (m.p. 124°)—have been isolated from the petroleum ether extract of the stems of *P. chaba* and identified. This finding in the *Piper* family is not unusual (5). Piplartine has also been isolated from the stems of *P. longum* Linn., by Atal and Banga (6); its structure has recently been elucidated (7) as piperidine amide of 3,4,5-trimethoxycinnamic acid. Piperlongumine, having the melting point of piplartine and identical to it in many respects, has also been isolated from the roots of the same species by Chatterjee and Dutta (8). They proposed its structure as piperidone amide of 3,4,5-trimethoxycinnamic acid and confirmed it by synthesis, disproving the structure claimed by Atal and Banga.

EXPERIMENTAL

Plant Material.—The chemical studies were conducted on an authentic sample of stems of *P. chaba* Hunter, free of adulteration and foreign materials, obtained from M/S Jai Bharat Traders, Lucknow, India. The stems were comminuted to a No. 40 mesh powder.

Extraction Studies.—Suitable accurately weighed samples of powdered material were extracted successively with a series of solvents in a Soxhlet continuous extraction apparatus by the Rosenthaler method (9). The percentage of extractives were: petroleum ether (b.p. range 40–60°), 0.8%; solvent ether, 0.5%; chloroform, 0.5%; ethanol, 3.2%; 70% ethanol, 3.8%. Ash value, determined by the B.P. (1963), was 4.5% of the dry weight of the crude drug.

Two kilograms of the powdered drug was extracted with light petroleum, b.p. 40–60°, in a Soxhlet apparatus until an aliquot of the colorless percolate left no residue when evaporated to dryness. On keeping the extract overnight, a white deposit was obtained and separated by filtration. On further concentration and standing overnight, a further greenish-yellow deposit, which was again separated by filtration, was obtained. On removal of the solvent, the filtrate yielded a fatty residue.

Isolation and Purification of Piplartine.—The white deposit obtained above was crystallized from methanol. Silky white needles of piplartine, m.p. 124°, were obtained on three crystallizations from methanol in poor yield. It was insoluble in water and in dilute acids and alkalies but dissolved in concentrated acid and alkalies. With concentrated sulfuric acid, it turned yellow. It was soluble in chloroform and sparingly soluble in ethanol, methanol, and benzene. It did not reduce Fehling's solution even after hydrolysis. Piplartine decolorized an aqueous permanganate and bromine in glacial acetic acid. It gave positive test for nitrogen and for alkaloid with various alkaloidal reagents.

Anal.—Calcd. for $C_{17}H_{19}NO_6$: C, 64.35; H, 5.99; N, 4.41. Found: C, 64.6; H, 5.82; N, 4.40.

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The mixed melting point remained unaltered with an authentic sample of piplartine.

Isolation of Piperine.—The greenish-yellow deposit was dissolved in methanol and filtered hot. On cooling, more piplartine was obtained as fine needles, which were separated. The mother liquor was chromatographed over Brockmann alumina using benzene and benzene–chloroform mixture (1:3). Elution with benzene gave a steroid, while elution with benzene and chloroform mixture yielded piperine. After several crystallizations from benzene, light yellow crystals of piperine, m.p. 128–129°, were obtained. The test with concentrated sulfuric acid and gallic acid for methylene dioxy group was positive. A mixed melting point with an authentic specimen of piperine was not depressed.

Isolation of β-Sitosterol from Fatty Residue.—The fat was hydrolyzed with 0.5 *N* alcoholic potassium hydroxide, and the mixture of phytosterols was extracted with ether (10). The sterol mixture obtained after removal of solvent was chromatographed over alumina. The column was eluted with benzene and benzene–chloroform mixture (1:3). The benzene eluent was too small for further study. The residue obtained from benzene–chloroform mixture gave a positive Liebermann-Burchard test. On several crystallizations from methanol, the residue gave colorless needles, m.p. 136°, $(\alpha)_D^{25}$ −36.5° ($CHCl_3$). The sterol was freely soluble in benzene, chloroform, and petroleum ether and sparingly soluble in cold methanol and ethanol.

Anal.—Calcd. for $C_{29}H_{50}O$: C, 84.05: H, 12.09. Found: C, 83.84; H, 12.13.

The sterol acetate, benzoate, and digitonide were prepared in the usual manner; m.p. 127, 144, and 221° dec., respectively. β-Sitosterol and its acetyl and benzoyl derivatives did not show a change in melting point when admixed with the respective authentic specimen.

Thus, from a comparison of the data with those for known sitosterol, this sterol was identified as β-sitosterol. The characteristics of the sterol are in conformity with the earlier observation of the authors (11) on the sterol from *Ipomoea degitata* Linn. (*Convolvulaceae*).

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Thimerosal as a Preservative in Biological Preparations. III. Factors Affecting the Concentration of Thimerosal in Aqueous Solutions and in Vaccines Stored in Rubber-Capped Bottles

By J. BIRNER and J. ROS. GARNET

Inactivation of the antiseptic properties of thimerosal contained in vaccines stored in bottles which have been sealed with rubber closures is governed by the type of rubber used for sealing the containers, the temperature and duration of storage, and the ratio of volume of liquid to surface area of the rubber to which the thimerosal is exposed.

THE POLAROGRAPHIC method of estimating thimerosal[1] in dilute solutions has been applied to its determination in samples of pertussis vaccine and triple antigen[2] during storage of the preparations in contact with rubber closures of differing compositions for varied periods of time and temperature. The purpose of this investigation was to assess the suitability of thimerosal as a preservative in these vaccines and to provide a basis for their storage under optimum conditions.

There is no question that the preservative effect of thimerosal in the low concentration employed in biological preparations is appreciably diminished when those preparations are stored in containers sealed with rubber closures (1).

Considerations of safety in the use of vaccines dispensed in multidose containers which have been sealed with rubber caps make it important to know at what stage in the shelf life of the preparation the concentration of the preservative may be expected to fall below what can be regarded as an effective level.

As shown in a previous paper (2) the determination of thimerosal with the polarograph can be carried out rapidly and with no less accuracy than is possible with the customarily used biological method.

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The valued and helpful criticism of Dr. S. J. Leach, Principal Research Officer, Division of Protein Chemistry, C.S.I.R.O., Melbourne, is gratefully acknowledged. The authors also thank the Director, Commonwealth Serum Laboratories, for permission to publish this paper.

[1] Thimerosal, sodium ethylmercurithiosalicylate, is official in the "British Pharmacopoeia" as Thiomersal. It is trademarked as Merthiolate.

[2] Triple antigen is an alternate name for diphtheria, tetanus, and pertussis vaccine B.P.

The mixed melting point remained unaltered with an authentic sample of piplartine.

Isolation of Piperine.—The greenish-yellow deposit was dissolved in methanol and filtered hot. On cooling, more piplartine was obtained as fine needles, which were separated. The mother liquor was chromatographed over Brockmann alumina using benzene and benzene–chloroform mixture (1:3). Elution with benzene gave a steroid, while elution with benzene and chloroform mixture yielded piperine. After several crystallizations from benzene, light yellow crystals of piperine, m.p. 128–129°, were obtained. The test with concentrated sulfuric acid and gallic acid for methylene dioxy group was positive. A mixed melting point with an authentic specimen of piperine was not depressed.

Isolation of β-Sitosterol from Fatty Residue.—The fat was hydrolyzed with 0.5 *N* alcoholic potassium hydroxide, and the mixture of phytosterols was extracted with ether (10). The sterol mixture obtained after removal of solvent was chromatographed over alumina. The column was eluted with benzene and benzene–chloroform mixture (1:3). The benzene eluent was too small for further study. The residue obtained from benzene–chloroform mixture gave a positive Liebermann-Burchard test. On several crystallizations from methanol, the residue gave colorless needles, m.p. 136°, $(\alpha)_D^{25}$ −36.5° ($CHCl_3$). The sterol was freely soluble in benzene, chloroform, and petroleum ether and sparingly soluble in cold methanol and ethanol.

Anal.—Calcd. for $C_{29}H_{50}O$: C, 84.05: H, 12.09. Found: C, 83.84; H, 12.13.

The sterol acetate, benzoate, and digitonide were prepared in the usual manner; m.p. 127, 144, and 221° dec., respectively. β-Sitosterol and its acetyl and benzoyl derivatives did not show a change in melting point when admixed with the respective authentic specimen.

Thus, from a comparison of the data with those for known sitosterol, this sterol was identified as β-sitosterol. The characteristics of the sterol are in conformity with the earlier observation of the authors (11) on the sterol from *Ipomoea degitata* Linn. (*Convolvulaceae*).

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THE POLAROGRAPHIC method of estimating thimerosal[1] in dilute solutions has been applied to its determination in samples of pertussis vaccine and triple antigen[2] during storage of the preparations in contact with rubber closures of differing compositions for varied periods of time and temperature. The purpose of this investigation was to assess the suitability of thimerosal as a preservative in these vaccines and to provide a basis for their storage under optimum conditions.

There is no question that the preservative effect of thimerosal in the low concentration employed in biological preparations is appreciably diminished when those preparations are stored in containers sealed with rubber closures (1).

Considerations of safety in the use of vaccines dispensed in multidose containers which have been sealed with rubber caps make it important to know at what stage in the shelf life of the preparation the concentration of the preservative may be expected to fall below what can be regarded as an effective level.

As shown in a previous paper (2) the determination of thimerosal with the polarograph can be carried out rapidly and with no less accuracy than is possible with the customarily used biological method.

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[2] Triple antigen is an alternate name for diphtheria, tetanus, and pertussis vaccine B.P.

TABLE I.—EFFECT OF STORAGE TEMPERATURE, CONTAINER, AND SEAL ON THE THIMEROSAL AND ZINC CONTENT OF 1 ml. OF TRIPLE ANTIGEN STORED FOR 22 WEEKS[a]

Seal	Type of Container											
	Ampul						Bottle					
	6° C.		25° C.		37° C.		6° C.		25° C.		37° C.	
	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./mg.	Zinc, mcg./ml.
Hermetic	58	Nil	40	Nil	32	Nil	Nil	8	Nil	10	Nil	11
White rubber cap	...	...	...	...	...	...	Trace	Nil	Below limit	Nil	Below limit	Nil
Gray rubber cap	...	...	...	...	...	...	Trace	Nil	Below limit	Nil	Below limit	Nil
Lacquered red rubber cap	...	...	...	...	...	...						

[a] Initial concentration of thimerosal, 100 mcg./ml.

With this instrument, the authors have been able to confirm the results of biological tests which show that zinc ions contributed by the rubber are not an important factor in the loss of antiseptic activity.

The present investigation was not designed to identify the substance responsible for the degradation of thimerosal, but to determine the conditions under which it occurs. There is reason to believe that sulfur, commonly incorporated into rubber mixes, reacts with thimerosal.

An experiment was carried out in which an aqueous solution containing 100 mcg. of thimerosal per milliliter was incubated at 37° for 72 hours with 1000 mcg./ml. of precipitated sulfur.

At the end of storage, polarographic assay indicated that only 42% of the thimerosal was present; this finding was confirmed by biological assay.

Rubber, of all compositions examined so far, is invariably blackened with mercury sulfide when left in contact with thimerosal solution.

The mercury-sulfur reaction, being irreversible, would account for the loss of activity of the antiseptic and explain why the loss is governed by the several factors noted in this paper.

EXPERIMENTAL

A series of three experiments was planned. In *Experiment A*, at the beginning and end of storage for several weeks in contact with rubber closures of differing compositions, the polarographic method was applied to the determination of the thimerosal content of samples of pertussis vaccine. The amount of antiseptic initially added was deliberately varied to give a series of concentrations of 80, 40, and 20 p.p.m.

The preparations containing the two higher concentrations of thimerosal gave characteristic polarographic waves; but those of the sample with an initial concentration of 20 p.p.m. showed only a wave characteristic of zinc ions.

Experiment B was carried out with samples of thimerosal and triple antigen, a biological preparation containing the antiseptic in an initial concentration of 0.01%.

The samples in 1-ml. quantities were stored for approximately 22 weeks in hermetically sealed ampuls and in bottles sealed with a selection of rubber closures. They were inverted at weekly intervals during storage in order to mix the contents and bring them into contact with the rubber. Samples of the triple antigen were held in three groups—at 6, 25, and 37° C. The results of the polarographic analysis of the contents are recorded in Table I.

No values for zero time assays are given, since the purpose of the experiments was to demonstrate differences which occurred between samples stored in all-glass and rubber-capped containers.

Experiment C was set up with triple antigen to determine to what extent the inactivation of thimerosal was affected by the ratio of volume of vaccine to surface area of rubber exposed to it.

The antiseptic was used in the same initial concentration of 0.01%. The vaccine was used in 5- and 10-ml. quantities distributed in hermetically sealed glass ampuls and in glass bottles closed with lacquered red rubber and gray rubber caps, both natural rubbers.

Thimerosal estimations were carried out periodically during storage at 6° for 18 months.

TABLE II.—EFFECT OF SAMPLE VOLUME, CONTAINER, AND SEAL ON THE STABILITY OF 100 mcg./ml. THIMEROSAL IN TRIPLE ANTIGEN STORED FOR 18 MONTHS AT 6° C.

		Type of Container					
		Ampul			Bottle		
Vol. of Stored Sample, ml.	Seal	Commencement Thimerosal, mcg./ml.	6 Mo. Thimerosal, mcg./ml.	18 Mo. Thimerosal, mcg./ml.	Commencement Thimerosal, mcg./ml.	6 Mo. Thimerosal, mcg./ml.	18 Mo. Thimerosal, mcg./ml.
5	Hermetic	...	...	83	...	...	...
	Gray rubber cap	...	...	...	89	85	71
	Lacquered red rubber cap	...	...	...	89	80	62
10	Hermetic	89	85	83	...	...	...
	Gray rubber cap	...	...	...	89	85	72
	Lacquered red rubber cap	...	...	...	89	85	72

Throughout the time of storage the containers were inverted at weekly intervals in order to bring the vaccine into direct contact with the rubber of the cap. The period of actual contact was 9 months in the aggregate—one-half of the full period of storage. The results of the test are recorded in Table II.

RESULTS AND DISCUSSION

Experiment A with pertussis vaccine indicated clearly that thimerosal will be an unsuitable preservative if the vaccine is dispensed in small volume quantities, such as 1-ml. containers sealed with rubber closures.

At the end of 22 weeks of storage at room temperature (region of 25°), the polarographic wave characteristic of the thimerosal ion was altogether absent from samples which had contained the preservative in the proportion of 20 p.p.m.

Although the pH of pertussis vaccine is within the range 7.2 to 7.4 the characteristic zinc wave was observed when white rubber stoppers were used as closures.

Table I summarizes the results of *Experiment B*. *Below limit* in Table I means that the concentration of thimerosal registered by the polarograph was less than 20 p.p.m.; no quantitative measure is possible below about 12.5 p.p.m., a value which is indicated by *trace*.

It will be noted that, irrespective of the temperature of storage, triple antigen in 1-ml. quantities retains a negligible proportion of the amount of thimerosal originally added after contact for a period totaling about 3 months with rubber of any of the three types used.

Apart from the fact that the white, zinc oxide-filled rubber yielded a measurable amount of zinc into the vaccine during storage, the interesting observation appears that even after prolonged storage under relatively adverse conditions more than 30% of the added thimerosal remained in the vaccine contained in hermetically sealed glass ampuls; of course, no zinc was detected.

No values for zero time assays are given because the purpose of the experiments was to demonstrate differences which occur between samples stored in rubber-capped and all-glass containers.

Experiment C is summarized in Table II. It was set up to determine to what extent the inactivation of thimerosal was affected by the ratio of volume of vaccine to surface area of rubber exposed to it.

The results, as tabulated, again demonstrate that in the absence of contact between liquid and rubber (as in the hermetically sealed glass containers) the diminution in the quantity of thimerosal is relatively small; but when rubber closures are used, a progressive loss occurs—a loss greater in the 5 ml. than in the 10-ml. volumes. However, even with the lower ratio of volume of vaccine to surface area of vaccine exposed to it, the maximum loss did not exceed 30%.

SUMMARY AND CONCLUSIONS

The inactivation of thimerosal contained in vaccines stored in bottles sealed with rubber closures is governed by the type of rubber used for sealing the containers, the temperature and duration of storage, and the ratio of volume of liquid to area of rubber surface to which the thimerosal is exposed.

Under the conditions of the experiment it is considered that the residual thimerosal in 5- and 10-ml. rubber-sealed multidose containers is adequate for the purpose it is intended to serve—that of a bacteriostat.

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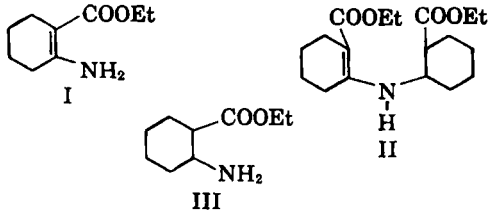
Rhodium-Catalyzed Hydrogenation of Ethyl Anthranilate

By KENNETH J. LISKA

Room temperature hydrogenation of ethyl anthranilate employing 5 per cent rhodium on alumina at both low and high pressures yields the 3,4,5,6-tetrahydro derivative. At elevated temperatures, ethyl *cis*-hexahydroanthranilate is formed in good yield. An unsaturated dicyclohexylamine diester appears to form in a side reaction in the room temperature hydrogenations.

In synthetic approaches to potential psychotomimetics, it was necessary to investigate the hydrogenation of ethyl anthranilate (ethyl *o*-aminobenzoate). The desired hexahydro derivative has been obtained in useful amounts by reduction with sodium in boiling isoamyl alcohol (1). Houben and Pfau obtained the hexahydro compound by platinum-catalyzed reduction, but they did not give yields or physical data (2).

Five per cent rhodium on alumina at low pressures and room temperatures catalyzed the hydrogenation of ethyl anthranilate to ethyl 3,4,5,6-tetrahydroanthranilate (I) but not to the hexahydro compound.



The tetrahydro derivative was identified by elemental analyses and by comparison with an authentic sample prepared by Dieckmann's ammonolysis method (3). With the same catalyst, room temperature hydrogenations at pressures up to 1000 p.s.i. again yielded the tetrahydro compound I, an indication of the stability of this bond toward hydrogenation. However, when the reaction temperature was raised to 85° and at 500 p.s.i., hydrogenation was complete to ethyl *cis*-hexahydroanthranilate (III) in approximately 70% yield. The conversion of ethyl anthranilate to the tetrahydro compound and finally to the hexahydro can be followed very well by U.V. spectroscopy. To prove the *cis* nature of the hexahydro product, it was hydrolyzed to the known *cis*-hexahydroanthranilic acid (4).

Both the low-pressure and the high-pressure room temperature rhodium-catalyzed hydrogenations always yielded a higher boiling liquid fraction in weight amounting to about one-half that of compound I. Rast molecular weight determinations gave values in the 300 to 400 range. Though insoluble in water, the liquid was soluble in hydrochloric acid; elemental analyses indicated only one nitrogen atom per molecule. The compound was an absorber in the U.V. (log E = 4.21 at 303 mμ) as strong as compound I (log E = 4.17 at 286 mμ). This by-product was identified tentatively as ethyl 2-(2-ethoxycarbonylcyclohexyl)aminocyclohexene-1-carboxylate (II). It could form *via* intermolecular primary amine addition to an olefin, similar to the well-known addition of amines to acrylate, followed by hydrogenolysis of one nitrogen atom. Almost exactly the same type of by-product was reported by Huenig and Kahanek (4) in their platinum oxide-catalyzed hydrogenation of ethyl 3,4,5,6-tetrahydroanthranilate, except that their dicyclohexylamine diester had no olefinic bond in either ring. That compound II was not identical to their saturated diester was indicated by the strong U.V. absorption of II, a conjugated olefinic ester, plus elemental analyses and I.R. spectral studies. The I.R. spectrum of compound II showed bands attributable to a saturated ester (1720 cm.$^{-1}$), an α,β-unsaturated chelated ester (1635 cm.$^{-1}$), an olefin conjugated to an ester (1595 cm.$^{-1}$), and the N—H stretching vibration of a secondary amine (3250 cm.$^{-1}$). By comparison, the I.R. spectrum of compound I showed no band attributable to a saturated ester, and the spectrum of compound III showed no band attributable to an α,β-unsaturated ester.

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EXPERIMENTAL[1]

Ethyl 3,4,5,6-Tetrahydroanthranilate (I).—A solution of 16.5 Gm. (0.1 mole) of ethyl anthranilate in 100 ml. of absolute ethanol was shaken at room temperature with 10 Gm. of 5% rhodium-on-alumina (K&K Laboratories, Inc.) in a Parr hydrogenator at an initial pressure of 61.1 p.s.i. for a total of 16 hours over a 2-day period. The total pressure drop was equal to 16.9 p.s.i., but most of the hydrogen was absorbed in the first 7 hours. Removal of catalyst and solvent, followed by distillation, gave 7.0 Gm. of product (41%), b.p. 159–164° (29 mm.), which when cooled gave a colorless solid, m.p. 71–73°. An authentic sample of this compound, prepared according to Dieckmann (3), melted at 73–75°; mixed m.p. 75–78°.

Anal.—Calcd. for $C_9H_{15}NO_2$: C, 63.88; H, 8.94; N, 8.28. Found: C, 64.04; H, 9.02; N, 8.19.

Compound I is a strong absorber in the U.V.: log E = 4.17 at 286 mμ. Its HCl salt could not be prepared in the solid state. Carbonyl stretching frequency of this α,β-unsaturated chelated ester: 1665 cm.$^{-1}$.

Ethyl 2-(2-Ethoxycarbonylcyclohexyl)aminocyclohexene-1-carboxylate (II).—The high-boiling fraction remaining after removal of the tetrahydro derivative was further distilled, yielding 4.8 Gm. of viscous yellow oil, b.p. 185–192° (1.6 mm.), N^{23} = 1.5208.

Anal.—Calcd. for $C_{18}H_{29}NO_4$: C, 66.84; H, 9.04; N, 4.33. Found: C, 66.75; H, 8.89; N, 3.53.[2]

Compound II is a strong absorber in the U.V.: log E = 4.21 at 303 mμ. The HCl salt of this

[1] Melting points are uncorrected. Analyses performed by Galbraith Laboratories and Schwarzkopf Laboratories.

[2] Einhorn and Bull state that ethyl hexahydroanthranilate loses nitrogen (as ammonia) when distilled at atmospheric pressure. [*Ann.*, 295, 204(1897).] Two other analyses from different runs fell in the range 4.7 to 5.1.

aminoester could not be prepared in the solid state. Infrared spectral bands in cm.$^{-1}$: 3250(w), 1720(s), 1635(s), 1595(s), 1445(s), 1360(m), 1233(s), 1165(s), 1083(m), 1060(m), and 1036(m).

Ethyl cis-Hexahydroanthranilate (III).—A mixture of 10 Gm. of ethyl anthranilate in 150 ml. of absolute ethanol and 8 Gm. of 5% rhodium on alumina was stirred in a Parr pressure reaction apparatus at 500 p.s.i. and at temperatures up to 85° for a total of 10 hours over a 2-day period. Removal of catalyst and solvent gave an oil which distilled at 115–119° (20 mm.). Huenig and Kahanek report 103–104° (11 mm.) (4). The yield of hexahydro ester was 7.3 Gm. (70.1%). For analysis, the previously unreported HCl salt of III was prepared in the usual manner with dry ether and HCl gas and melted at 131–133° after two crystallizations from acetone/alcohol.

Anal.—Calcd. for $C_9H_{18}ClNO_2$: C, 52.05; H, 8.67; N, 6.74. Found: C, 51.82; H, 8.71; N, 6.62.

The free acid was prepared by refluxing III in plain water, followed by evaporation. The *cis*-hexahydroanthranilic acid so obtained was recrystallized from alcohol/water, m.p. 233–234°. Huenig and Kahanek (4) report 235° for the *cis* acid; the *trans* acid, also prepared by plain water hydrolysis (1), melts at 274°.

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Antistaphylococcal Activity of Seeds of *Psoralea corylifolia*

By K. N. GAIND, R. N. DAR, and R. N. KAUL

The petroleum ether extract of the seeds of *Psoralea corylifolia* Linn. inhibits the growth of *Staphylococcus aureus*, in the concentration of 10 mcg./ml. From the extract a pure compound, $C_{12}H_{16}O$, b.p. 155°/0.3 mm., has been isolated by chromatography and subsequent fractional distillation. It inhibits the growth of *S. aureus* in the concentration of 0.5 mcg./ml.

PSORALEA CORVLIFOLIA Linn. (*Leguminosae*) is a common herbaceous weed, grows throughout the plains of India, and is commonly known as babchi. The seeds of this plant have long been used in the indigenous system of medicine for leprosy, inflammatory diseases of the skin, leucoderma, psoriasis, as an anthelmintic, a diuretic and a diphoretic in febrile conditions (1). In southern India, the drug is widely used as a stomachic, deobstruent, and in various cutaneous diseases (2). The seeds have an estrogenic effect on certain laboratory animals (3). Jois and Manjunath (4) and Seshadri and Venkattarao (5) isolated o fixed and a volatile oil, psoralen and iso-psoralen. Chakravarti *et al.* (6) isolated a new crystalline compound, psoralidin, from the pericarp. Gupta *et al.* (7) recently reported the antistaphylococcal activity of the petroleum ether extract of the seeds in the concentration of 2–4 mcg./ml. Jois *et al.* (8) obtained from a petroleum ether extract of the seeds a fixed oil, psoralen, and iso-psoralen. In this investigation, these components did not show activity against *Staphylococcus aureus*. Volatile oil from the seeds has not revealed appreciable activity (7). A systematic investigation of the seeds was undertaken and an attempt made to isolate and characterize the antistaphylococcal principle.

MATERIAL AND METHODS

Test Organism.—*S. aureus* (Oxford culture) was employed for testing the activity of the various fractions. The organism was maintained on nutrient agar slants from which regular subculturing was done in nutrient broth. A 24-hour suspension of this organism in broth was used for all subsequent investigations.

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Minimum Inhibitory Concentration.—The antistaphylococcal activity of the various fractions (Table I) was tested by serial dilution method. A solution (10 mg./ml.) of the test compound was prepared in alcohol (90%). One-half milliliter of the dilution was then transferred to 4.5 ml. of sterile nutrient broth (pH 7.2–7.4) from which 0.5 ml. of the dilution was further transferred to 4.5 ml. of the broth, etc., until a varied range of concentrations was obtained in the broth. The total volume was kept at 5 ml. Various dilutions of the test compound ranging from 0.1 to 100 mcg./ml. were obtained. The solution of the test compound

TABLE I.—ANTISTAPHYLOCOCCAL ACTIVITY OF VARIOUS CHROMATOGRAPHIC FRACTIONS OF PETROLEUM ETHER EXTRACT OF *P. corylifolia* SEEDS

Eluents	Physical Appearance	Antistaphylococcal Activity[a]
Petroleum ether (1–18)	Dark brown liquid	−
10% Benzene in petroleum ether (19–29)	Light green liquid	−
50% Benzene in petroleum ether (30–40)	Viscous green liquid	+
Benzene (41–53)	Viscous green liquid	+
10% Ether in benzene (54–57)	Viscous brown liquid	−
50% Ether in benzene (58–67)	Viscous brown liquid	−
Ether (68–75)	Viscous brown liquid	−
10% Alcohol in ether (76–79)	Yellow solid mass	−
50% Alcohol in ether (80–83)	Yellow solid mass	−
Alcohol (84–90)	Yellow solid mass	−

[a] −, No inhibition; +, inhibition.

aminoester could not be prepared in the solid state. Infrared spectral bands in cm.$^{-1}$: 3250(w), 1720(s), 1635(s), 1595(s), 1445(s), 1360(m), 1233(s), 1165(s), 1083(m), 1060(m), and 1036(m).

Ethyl cis-Hexahydroanthranilate (III).—A mixture of 10 Gm. of ethyl anthranilate in 150 ml. of absolute ethanol and 8 Gm. of 5% rhodium on alumina was stirred in a Parr pressure reaction apparatus at 500 p.s.i. and at temperatures up to 85° for a total of 10 hours over a 2-day period. Removal of catalyst and solvent gave an oil which distilled at 115–119° (20 mm.). Huenig and Kahanek report 103–104° (11 mm.) (4). The yield of hexahydro ester was 7.3 Gm. (70.1%). For analysis, the previously unreported HCl salt of III was prepared in the usual manner with dry ether and HCl gas and melted at 131–133° after two crystallizations from acetone/alcohol.

Anal.—Calcd. for $C_9H_{18}ClNO_2$: C, 52.05; H, 8.67; N, 6.74. Found: C, 51.82; H, 8.71; N, 6.62.

The free acid was prepared by refluxing III in plain water, followed by evaporation. The *cis*-hexahydroanthranilic acid so obtained was recrystallized from alcohol/water, m.p. 233–234°. Huenig and Kahanek (4) report 235° for the *cis* acid; the *trans* acid, also prepared by plain water hydrolysis (1), melts at 274°.

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Antistaphylococcal Activity of Seeds of *Psoralea corylifolia*

By K. N. GAIND, R. N. DAR, and R. N. KAUL

The petroleum ether extract of the seeds of *Psoralea corylifolia* Linn. inhibits the growth of *Staphylococcus aureus*, in the concentration of 10 mcg./ml. From the extract a pure compound, $C_{12}H_{16}O$, b.p. 155°/0.3 mm., has been isolated by chromatography and subsequent fractional distillation. It inhibits the growth of *S. aureus* in the concentration of 0.5 mcg./ml.

PSORALEA CORVLIFOLIA Linn. (*Leguminosae*) is a common herbaceous weed, grows throughout the plains of India, and is commonly known as babchi. The seeds of this plant have long been used in the indigenous system of medicine for leprosy, inflammatory diseases of the skin, leucoderma, psoriasis, as an anthelmintic, a diuretic and a diphoretic in febrile conditions (1). In southern India, the drug is widely used as a stomachic, deobstruent, and in various cutaneous diseases (2). The seeds have an estrogenic effect on certain laboratory animals (3). Jois and Manjunath (4) and Seshadri and Venkattarao (5) isolated o fixed and a volatile oil, psoralen and iso-psoralen. Chakravarti *et al.* (6) isolated a new crystalline compound, psoralidin, from the pericarp. Gupta *et al.* (7) recently reported the antistaphylococcal activity of the petroleum ether extract of the seeds in the concentration of 2–4 mcg./ml. Jois *et al.* (8) obtained from a petroleum ether extract of the seeds a fixed oil, psoralen, and iso-psoralen. In this investigation, these components did not show activity against *Staphylococcus aureus*. Volatile oil from the seeds has not revealed appreciable activity (7). A systematic investigation of the seeds was undertaken and an attempt made to isolate and characterize the antistaphylococcal principle.

MATERIAL AND METHODS

Test Organism.—*S. aureus* (Oxford culture) was employed for testing the activity of the various fractions. The organism was maintained on nutrient agar slants from which regular subculturing was done in nutrient broth. A 24-hour suspension of this organism in broth was used for all subsequent investigations.

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Minimum Inhibitory Concentration.—The antistaphylococcal activity of the various fractions (Table I) was tested by serial dilution method. A solution (10 mg./ml.) of the test compound was prepared in alcohol (90%). One-half milliliter of the dilution was then transferred to 4.5 ml. of sterile nutrient broth (pH 7.2–7.4) from which 0.5 ml. of the dilution was further transferred to 4.5 ml. of the broth, etc., until a varied range of concentrations was obtained in the broth. The total volume was kept at 5 ml. Various dilutions of the test compound ranging from 0.1 to 100 mcg./ml. were obtained. The solution of the test compound

TABLE I.—ANTISTAPHYLOCOCCAL ACTIVITY OF VARIOUS CHROMATOGRAPHIC FRACTIONS OF PETROLEUM ETHER EXTRACT OF *P. corylifolia* SEEDS

Eluents	Physical Appearance	Antistaphylococcal Activity[a]
Petroleum ether (1–18)	Dark brown liquid	−
10% Benzene in petroleum ether (19–29)	Light green liquid	−
50% Benzene in petroleum ether (30–40)	Viscous green liquid	+
Benzene (41–53)	Viscous green liquid	+
10% Ether in benzene (54–57)	Viscous brown liquid	−
50% Ether in benzene (58–67)	Viscous brown liquid	−
Ether (68–75)	Viscous brown liquid	−
10% Alcohol in ether (76–79)	Yellow solid mass	−
50% Alcohol in ether (80–83)	Yellow solid mass	−
Alcohol (84–90)	Yellow solid mass	−

[a] −, No inhibition; +, inhibition.

in alcohol on dilution with broth gave slight turbidity. However, concentrations below 100 mcg./ml. were all clear solutions. The tubes were then inoculated with 0.05 ml. of the suspension of *S. aureus* in broth and incubated at 37°. These were then examined after 24 hours for visible growth.

EXPERIMENTAL

The seeds of the drug were coarsely powdered and subjected to successive extractions with solvents in a Soxhlet apparatus. The per cent residues were: petroleum ether, 8.6; chloroform, 5.2; and alcohol, 2.8.

The petroleum ether extract inhibited the growth of *S. aureus* in the concentration of 10 mcg./ml. Since the chloroform and alcoholic extracts did not show antistaphylococcal activity, these extracts were not taken up for further study.

Isolation of Antistaphylococcal Principle.—Ten grams of petroleum ether extract was chromatographed over 100 Gm. of alumina (Brockmann grade I) using, successively, petroleum ether, benzene, ether, alcohol, and their appropriate mixtures (Table I) as elution solvents. All the solvents were previously dried and purified. Ninety 30-ml. fractions were collected. The solvents were removed *in vacuo*, and the antistaphylococcal activity of the various fractions recorded (Table I). After the elution, the chromatographic column showed the presence of six different bands, which were extruded separately and refluxed with methyl alcohol on a water bath. The residues were tested and did not show antistaphylococcal activity.

The eluates consisting of petroleum ether–benzene mixture (1:1) were pooled and designated as fraction A. After refrigerating for a few days, it deposited needle-shaped crystals which were washed with cold petroleum ether to remove the adhering liquid. A white crystalline compound was obtained which on repeated crystallization from hot dilute alcohol gave a pure compound. It was identified as psoralen and did not show activity against *S. aureus*. The mother liquor was further concentrated, and 1 Gm. of this liquid was again passed through a column containing 5 Gm. of alumina. The column was then eluted with petroleum ether–benzene mixture (1:1). The solvent was removed *in vacuo*, and the residual liquid was distilled under reduced pressure; the fraction B, b.p. 155°/0.3 mm., was collected. It was of a pale, viscous oily nature and inhibited the growth of *S. aureus* in the concentration of 0.5 mcg./ml.

Anal.—Calcd. for $C_{12}H_{16}O$. Found: C, 82.22; H, 8.85, mol. wt. (Rast) 186.

The benzene eluates were also mixed together and did not deposit crystals when cooled. The liquid was distilled under reduced pressure and the fraction, b.p. 155°/0.3 mm., was obtained. The compound on analysis was identical to B ($C_{12}H_{16}O$) and had equal inhibition against *S. aureus*.

The activity of compound B was also tested against *S. aureus* resistant to penicillin, streptomycin, chloramphenicol, and tetracycline, in which it was equally effective.

A survey of the literature has revealed that compound B ($C_{12}H_{16}O$) has not been reported previously in *P. corylifolia*. The preliminary characterization tests have revealed the aldehyde nature of this compound; therefore, the name psoralaldehyde is proposed for it. Detailed work about its characterization and constitution will be communicated later.

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Semiautomatic System for Timing Rotarod Performance

By NATHAN WATZMAN, HERBERT BARRY, III, JOSEPH P. BUCKLEY, and WILLIAM J. KINNARD, JR.

An electronic circuit automatically stops the timer when the animal falls to a platform beneath the rod. This system improves the accuracy and convenience of recording rotarod performance time. The investigator may test several animals simultaneously with only periodic observation.

THE ROTAROD is a device which has been used extensively for the evaluation of drug effects on the central nervous system. Rats and mice perform a task similar to log-rolling; neurological deficit induced by a drug is indicated by the inability of an animal to remain on the rod for a specified period of time (1). Various types of central nervous system depressants (2, 3) and stimulants (4) may be evaluated and compared by the increase or decrease in performance times they produce.

The rod is generally divided into compartments by thin circular disks, larger in diameter than the roller, so that several animals can be tested simultaneously. The experimenter starts a timer as soon as he places each animal on the rod and stops the timer as soon as he sees the animal fall to the platform beneath.

In situations where as many as six animals are being tested at one time, it is necessary for the experimenter to give his undivided attention. Under these circumstances, it is quite possible that, if more than one animal falls in quick succession, the experimenter might not be able to observe each fall

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The authors gratefully acknowledge the technical assistance of Mr. Robert Butchko.

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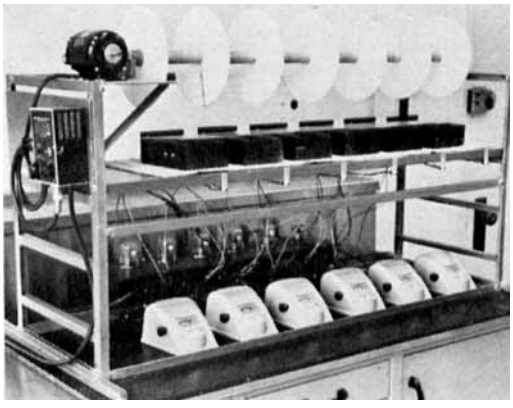


Fig. 1.—Rotarod.

and stop the timers at the exact moment. Since performance time data, under such a system, become dependent on the alertness and reflex abilities of the experimenter, the authors have devised a semiautomatic system for stopping the timers.

APPARATUS

The rotarod is shown in Fig. 1. The supporting structure is composed of 0.75-in. angle iron fastened to a wooden base 24 × 48 × 0.5 in. The wooden catch platform is 43.25 × 11.5 × 0.75 in. and can be adjusted at several heights. A 115-v. d.-c. motor (Bodine Electric Co., NSH-12R; 1/50 H.P.) with speed control (Minarik Electric Co., model No. 5H-14) drives the rod at any selected speed, ranging from 4.1 to 83 r.p.m.

The circuitry for the semiautomatic recording of performance times is shown schematically in Fig. 2. S-1 is a pushbutton switch (S.P.S.T.-N.O. Grayhill 30-1). S-2 is a microswitch (S.P.S.T.-N.C.; Unimax 2 HBW-1) which is mounted on a 6.5 × 4 × 2 in. wooden frame as shown in Fig. 3. The 5 × 7 in. lid, made of Bakelite or plastic, is hinged to the frame and rests lightly on the arm of the microswitch. K-1 represents a 115-v. a.-c. relay (S.P.S.T.-N.O.; Potter and Brumfield KRP5A) which is set in an octal socket housed on a frame (20 × 5 × 3.5 in.) located at the base of the rotarod. The timer is a Phipps and Bird precision Time-It electrical stopclock (78–760) with a manual reset. Each animal compartment has a separate pushbutton switch (S-1), microswitch (S-2), relay coil (K-1), and timer.

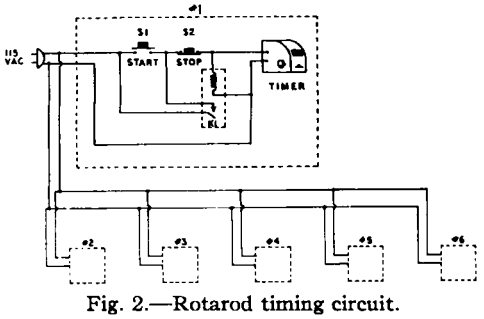


Fig. 2.—Rotarod timing circuit.

OPERATIONAL PROCEDURE

As soon as the experimenter places the animal on the rod, he presses the pushbutton start-switch (S-1) which activates the relay (K-1) and the timer through S-2 (normally closed). The relay contacts, being closed, keep the relay activated and the timer on. When an animal falls from the rod, its weight forces the Bakelite platform downward on the microswitch (S-2), opening it. This de-energizes the relay and stops the timer.

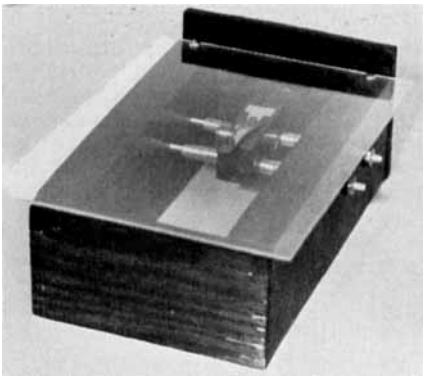


Fig. 3.—Microswitch platform.

CONCLUSIONS

A semiautomatic system for recording rotarod performance times, described in this paper, measures the success or failure of the animal to stay on the rod, independent of the alertness and reflexes of the experimenter. Therefore, the data more accurately reflect the forced coordinated motor ability of the animal. Furthermore, the method does not demand rigid undivided attention on the part of the experimenter; and, indeed, the animals need be observed only periodically. The system is electronically simple and inexpensive to build and repair. It may be expected to function for long periods of time without needing repairs, because the number of operations (on–off conditions) on a typical test day is small compared with the number for which the components are constructed.

We have used this system in our laboratory for several months with completely satisfactory results.

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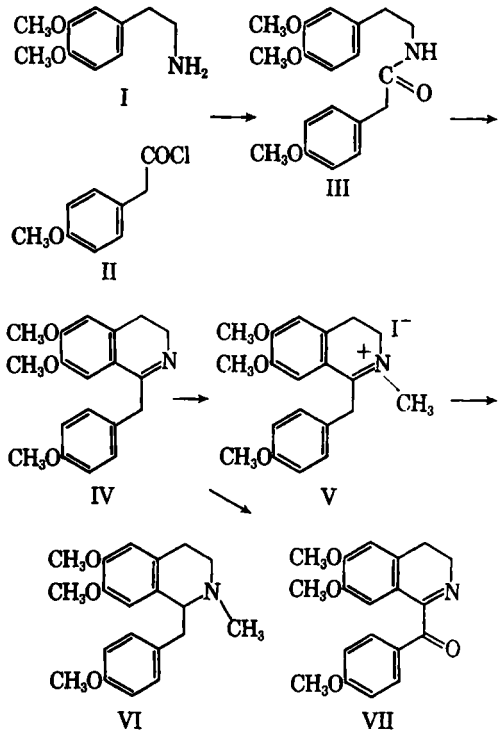
Synthesis of *dl-O*-Methylarmepavine

Sir:

In a continuation of studies on the alkaloids of *Magnolia acuminata* L. (1, 2), what appears to be the first isolation and characterization of *d-O*-methylarmepavine from plant was reported. For purposes of comparison, a reference sample of the alkaloid was required. The *dl* form of the base was synthesized by a method differing from that reported by Marion *et al.* (3) and Tomita and Yamaguchi (4).

In a series of five steps, Marion and co-workers obtained armepavine starting from homoveratrylamine and 4-nitrophenylacetyl chloride. The phenolic base was methylated with diazomethane to yield *dl-O*-methylarmepavine, m.p. 92°. Tomita and Yamaguchi (4) condensed homoveratrylamine with 4 - benzyloxyphenylacetyl chloride to yield armepavine in a series of four steps. Methylation of the phenolic alkaloid with diazomethane yielded its *O*-methyl ether, m.p. 62°. Since the melting point of this synthetic compound and that reported by Marion and co-workers was different, Tomita and Yamaguchi considered erroneous the melting point of 92° for *dl-O*-methylarmepavine.

In our synthesis of *dl-O*-methylarmepavine, homoveratrylamine (I) and 4-methoxyphenylacetyl chloride (II) were condensed to yield the amide (III), m.p. 126–128° [reported m.p. 123.5° (5)]. Cyclization of III under Bishler-Napieralski conditions (4), using phosphorus oxychloride and toluene, yielded from the reaction a white product (IV*a*), m.p. 145–147° (petroleum ether-chloroform). This product was shaken with water, basified with ammonia, and extracted with chloroform in an atmosphere of nitrogen. The chloroform extracts, on evaporation, yielded presumably IV as a syrupy mass. The mass was treated with methyl iodide to obtain the methiodide (V), m.p. 126–128° (petroleum ether-chloroform). The methiodide (V) was reduced with zinc and hydrochloric acid to yield, after basifying with ammonia and extraction with chloroform, a light brown mass. Crystallization of this mass using ether-petroleum ether gave a colorless compound (VI), m.p. 60–61°. The compound was left overnight *in vacuo* (0.500 mm. pressure) and the melting point again recorded. The compound (VI) now melted at 89–90°. This change of ~30° in melting point was again observed when a second crop was obtained and left *in vacuo* as previously.



Reduction of methiodide (V) was also performed with sodium borohydride in methanol (6) to afford a yellowish product. Crystallization of this product with benzene-petroleum ether and seeding with crystals, m.p. 89–90°, from zinc hydrochloric acid reduction yielded VI, m.p. 89–90°.

The synthetic *dl-O*-methylarmepavine, m.p. 89–90°, yielded a crystalline picrate, m.p. 172–173° [reported m.p. 170–172° (7)], and the methiodide, m.p. 138–139° (softens 135°) [reported 135–136° (3)]. The I.R. spectra of this methiodide and methiodide prepared from authentic *l-O*-methylarmepavine were identical. Paper, thin-layer, and gas-liquid chromatographic data and I.R. and U.V. spectra of the crystalline *dl-O*-methylarmepavine, m.p. 89–90°, were identical to authentic *l-O*-methylarmepavine. The NMR spectrum of the synthetic compound, m.p. 89–90°, was the same as that of *d-O*-methylarmepavine isolated from *M. acuminata* leaves (2).

Anal.—Calcd. for $C_{20}H_{25}NO_3$: C, 73.36; H, 7.70 N, 4.28. Found: C, 73.80; H, 7.86; N, 4.62.

In a separate experiment, preparation of Schiff base (IV) was attempted under the following conditions.

The white product (IV*a*) was shaken with water, basified with ammonia, and extracted with benzene. The benzene extracts, on evaporation, yielded a light orange mass. A colorless crystalline compound, m.p. 101–102° [$\lambda_{max.}^{EtOH}$ 226 mμ (ε 23,400), 290 (20,600); on addition of dilute hydrochloric acid, the peaks shifted to 248 (14,720), 308 (18,900), and 373 (10,500)] was isolated from the product. The I.R. spectrum of the compound indicated a carbonyl peak at 1667 cm.$^{-1}$. An A_2B_2 system in the NMR δ = 8.04 and 6.98 p.p.m. also suggested the presence of an anisoyl moiety. The presence of 3,4-dihydroisoquinoline was shown by a four proton complex near δ = 2.8. This compound yielded a semicarbazone, m.p. 200–202°, and the picrate, m.p. 229–230° dec. (darkens 220°). Evidently the reported (8–11) aerial oxidation of 1-benzyl-3,4-dihydroisoquinoline (IV) to 1-benzoyl-3,4-dihydroisoquinoline (VII) had occurred during extraction from alkaline solution with benzene.

Anal.—Calcd. for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 70.58; H, 5.79; N, 4.06. Mol. wt. 325.35. Found: 324 (±4) (vapor pressure osmometer).

Govindachari and Nagarajan (12) reported preparation of VII by oxidizing an alcoholic solution of IV by prolonged exposure to air or with iodine. The melting points reported for the base (VII) and its picrate were 110–112° and 220° dec., respectively.

The observation of the change in the melting point of the *dl-O*-methylarmepavine indicates that the alkaloid exists in polymorphic forms. This alteration in the melting point might explain the discrepancy in the melting point of synthetic *O*-methylarmepavine, m.p. 92°, reported by Marion *et al.* (3) and for *dl* form of the alkaloid, m.p. 62°, synthesized by Tomita and Yamaguchi (4).

Details of the synthesis reported here will be published at a later date.

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Bethesda, Md.

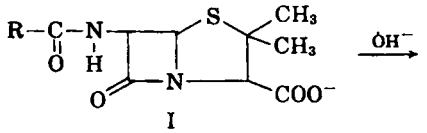
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The authors are indebted to Professor Masao Tomita, Kyoto University, Kyoto, Japan, for the sample of *l-O*-methylarmepavine, and to Mrs. K. S. Warren for spectrometric determinations.

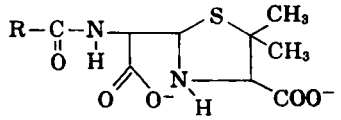
Catalysis of Penicillin Hydrolysis by Catechol and 3,6-Bis-(dimethylaminomethyl)catechol

Sir:

Penicillins (I) are hydrolyzed to penicilloic acids (II) by hydroxyl ion and by the enzyme penicillinase (1). It has now been found that this

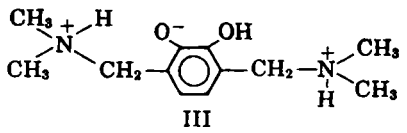


I



II

reaction is also catalyzed by catechol and even more markedly by 3,6-bis(dimethylaminomethyl)catechol (CDM).[1] The rates of penicillin hydrolysis were measured by following the acid produced by the reaction with a recording pH-stat. The rates were pseudo first order at constant pH; Fig. 1 shows the dependence upon pH of the rate of hydrolysis of penicillin G (R = $C_6H_5CH_2$—) in the presence or 1.33×10^{-3} *M* CDM dihydrochloride at 31.5°. This bell-shaped curve with maximum at pH 8.0 is consistent with catalysis by the species III, the monoanion of the dihydrochloride. That III is the monoanion



III

is evidenced by the pKa of 6.35 and 9.65 obtained by potentiometric titration and the bathochromic shift in the ultraviolet absorption maximum noted after neutralization of 1 proton of the dihydrochloride. The latter is typical of phenolic ionization (2). In the case of catechol, the pH dependence of the reaction also showed that the monoanion was the reactive species. Catalytic second-order rate constants for the reaction of the following species with penicillin G at 31.5° were (liter mole^{-1} minute^{-1}): hydroxyl ion, 12.5; monocatecholate ion, 3.8; and species III, 54.0. Based on these constants, the half-life of penicillin G in aqueous solution at pH 8 would be approximately 575 hours[2] with no catalyst added, about 25 hours in the presence of 0.001 *M* catechol, and only 13 minutes in the presence of 0.001 *M* CDM.

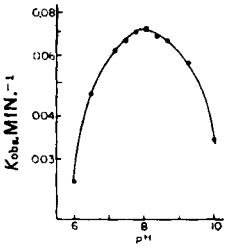


Fig. 1.—Dependence upon pH of observed pseudo first-order rate constants (log scale) for hydrolysis of benzyl penicillin catalyzed by CDM. Concentration of penicillin was 0.002 *M*; CDM was 0.00133 *M*.

In the past, monocatecholate ion has been shown to be an effective catalyst for hydrolysis of phenyl chloroacetate (3) and to react unusually rapidly with isopropyl methylphosphonofluoridate (sarin) (4). In both cases the *ortho* acidic hydroxyl group was necessary for enhanced reactivity. It has also been demonstrated that aminoalkyl groups increase the reactivity of phenol with sarin (5) and the rate of catalysis of *p*-nitrophenyl acetate hydrolysis by imidazole (6). In the latter case, the rate enhancement was attributed to polarization of the ester carbonyl by the charged amine, thus increasing the electrophilicity of the carbonyl carbon.

In the present case, where both an acidic *ortho* hydroxyl group and a charged aminoalkyl group are present, it seems likely that both may be involved in the reaction. Further work is underway to elucidate the mechanism of this catalysis and will be reported at a later date. There is some evidence that the nature of the side chain of the penicillin (R in I) plays a role. It seems probable from past work that CDM will also catalyze the hydrolysis of certain esters; this is also being investigated.

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(2) Edsall, J. T., and Wyman, J., "Biophysical Chemistry," Vol. I, Academic Press Inc., New York, N. Y., 1958, p. 427.
(3) Fuller, E. J., *J. Am. Chem. Soc.*, **85**, 1777(1963).
(4) Epstein, J., Rosenblatt, D. H., and Demek, M., *ibid.*, **78**, 341(1956).
(5) Epstein, J., *et al.*, *ibid.*, **86**, 3075(1964).
(6) Franzen, V., *Angew. Chem.*, **72**, 139(1960).

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[1] This compound was originally synthesized by Dr. J. Epstein and co-workers, U. S. Army Chemical Center, Edgewood, Md., and is highly reactive with phosphonofluoridates. It is expected that their synthesis will be published shortly.

[2] This half-life was computed on the basis of alkaline hydrolysis only. The actual half-life will be somewhat shorter due to a spontaneous hydrolysis which is also known to occur.

Selection of an Optimal Surface for the Preparation of Uniform Detached Films of Adhesive Materials

Sir:

Shellac films are used extensively in the pharmaceutical industry in sugar-coated tablets as waterproof barriers, in enteric medication, and in sustained-release dosage forms. These polymeric films often vary with time because of complex chemical and physical changes, which may be the cause of erratic drug-release patterns observed in several dosage forms.

Studies of the aging of shellac films can be handicapped seriously when they are restricted to applied films, since this procedure can introduce exogenous variables which may lead to erroneous or misleading results. In addition, many properties of the film itself cannot be evaluated after application.

The development of a detached shellac film presents a particularly difficult problem. Shellac displays a high degree of adhesion to surfaces used routinely for film casting. Removal of the cast and dried film from these surfaces can result in destruction of film integrity.

Surfaces such as glass, stainless steel plates, chromium-plated steel plates, celluloid, and formica were evaluated in our laboratories and rejected as inadequate to overcome this problem. Attempts to utilize Gardner's (1) technique of casting on a mercury surface met with only moderate success. Wax-free shellac glazes could be spread on a mercury surface, but a small amount of mercury adhered to the detached dry film. In addition, film thickness was extremely difficult to control. On the other hand, glazes containing natural wax possess a sufficiently high interfacial tension against mercury, so that a negative spreading coefficient results, and they could not be spread uniformly on a mercury surface.

To prepare films of uniform thickness from most types of shellac, a surface upon which the shellac glaze demonstrates a positive spreading coefficient and a low work of adhesion was required. Therefore, the decision was made to investigate silicone-treated paper as a possible film casting surface. These papers are available in a wide range of silicone content and antiadhesive quality. A series of 8 × 11 in. silicone-treated papers was mounted on a level formica-plated platform. Solutions containing 40% (w/w) shellac resin,[1] suitably plasticized with 2 to 5% (w/w) dibutyl phthalate in anhydrous ethyl alcohol, were freshly prepared and cast on the treated papers according to the method described by Kanig and Goodman (2).

The solutions used in this study were prepared from samples of the two basic types of shellac resins: one type is produced by a chemical bleaching process, and the other is purified by mechanical means. Each type was tested with and without the natural wax content. After casting, the films were dried under ambient conditions or in ovens at various temperatures for a period of 24 hours, then manually removed.

Optimum results were obtained utilizing Riegelease MF separator paper No. 53044 for both types of wax-free shellac and Riegelease paper No. J-4043 for both types of wax shellac.[2] The latter paper represents a more highly treated silicone surface. In each case, neither the casting of the glaze nor the removal of the dried film posed difficulty. Test films of 5 and 10 mil wet thickness were readily cast and easily detached. Silicone adherence to the dried films was not evidenced. A high degree of uniformity of the dry film was observed. The dry film thickness, measured by means of an Ames model 2W dial comparator to an accuracy of 10^{-4} in., varied less than ±10%.

Providing that caution is exercised to insure that the solvent system employed has no affinity for the silicone resin impregnated in the paper, the silicone-treated surfaces appear to offer a considerable advantage because, with a minimum of procedural screening, an optimal surface for the casting of free films may be selected quickly. It is suggested that this approach will greatly simplify the study of highly adhesive, tenacious, film-forming polymers.

(1) Gardner, W., and Kappenberg, W., *Ind. Eng. Chem.*, 28, 437(1936).
(2) Kanig, J. L., and Goodman, H., THIS JOURNAL, 51, 77(1962).

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Accepted for publication September 16, 1964.

[1] Obtained through the courtesy of William Zinsser & Co., New York, N. Y.
[2] The authors are indebted to Mr. S. M. Gerbino, Riegel Paper Corp., New York, N. Y., for the generous supply of papers used in this study.

REVIEWS

Ionic Equilibria in Analytical Chemistry. By HENRY FREISER and QUINTUS FERNANDO. John Wiley & Sons Inc., 605 Third Avenue, New York, N. Y., 1963. 15 × 23.5 cm. xiii + 334 pp. Price $4.95.

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Reviewed by Edward F. Salim
American Pharmaceutical Association Foundation
Washington, D. C.

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Structure Elucidation of Natural Products by Mass Spectrometry. Vol. 1: Alkaloids. By HERBERT BUDZIKIEWICZ, CARL DJERASSI, and DUDLEY H. WILLIAMS. Holden-Day, Inc., 728 Montgomery St., San Francisco, Calif., 1964. 233 pp. Price $10.50.

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Minneapolis

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problems are included to give the reader an intuitive understanding of the subject. The book is well written, and the topics discussed are illustrated with clearly presented examples. Problems with answers are included. The first chapter on the logical basis of statistical inference is excellent in its discussion of the design of experiments and the statistical philosophy concerned in this design.

The second, third, and fourth chapters deal with, respectively, quantitative data, enumeration data, and correlation. That the book contains only four chapters should not mislead one into believing that it is incomplete, since this is certainly not the case.

Three nonparametric tests are described and their use illustrated. These are the two-sample rank test, sign test, and the signed-rank test. The author feels that these tests should be more widely used than at present because of their simplicity of application.

This book would be most satisfactory as a textbook in a course on the subject for pharmacy students or as a supplementary text in a course in biopharmaceutics. The book is enthusiastically recommended.

Reviewed by Eino Nelson
School of Pharmacy
State University of New York at Buffalo
Buffalo

Laboratory Guide in Pharmacology. 2nd Ed. By T. S. MIYA, H. G. O. HOLCK, G. K. W. YIM, and T. MYERS. Burgess Publishing Co., 426 South Sixth St., Minneapolis 15, Minn., 1964. 162 pp. 21.5 × 27 cm. Paperbound. Price $3.50.

This laboratory guide, written especially for the pharmacy student, is designed to serve as a companion to lecture and textbook material for a beginning student. The experiments are organized so that the student begins with basic pharmacological principles, including routes of drug administration, factors affecting dosage, and absorption and excretion of drugs by man. Following these are experiments pertaining to the pharmacology of the various bodily systems, toxicology, and gross pharmacological effects of drugs.

Each experiment provides an organized method of recording observations and includes questions to stimulate thinking about the significance of these observations. The clearly presented experiments are intended for student participation and generally do not require complicated or costly equipment.

Absorption and Distribution of Drugs. Based on a symposium held by the Association of Medical Advisers in the Pharmaceutical Industry. Edited by T. B. BINNS. The Williams and Wilkins Company, Baltimore, Md., 1964. pp. xi + 270 Price $7.50.

The 17 papers in *Absorption and Distribution of Drugs* with the 520 references cited are an excellent introduction to the subject matter and also will provide excellent reading for those well acquainted with the field. Four of the papers are concerned with the fundamentals of absorption, two with the fate of drugs, one with the blood-brain barrier, one with the placental barrier, one with protein binding, two with biopharmaceutical aspects of absorption, five with absorption of particular classes of drugs, and one with the significance of serum levels of chemotherapeutic agents. The two papers of Bernard B. Brodie, namely, "Physico-Chemical Factors in Drug Absorption" and "Distribution and Fate of Drugs; Therapeutic Implications," constitute approximately one-third of the book and are very fine reviews. The papers entitled, "Pharmaceutical Manipulation and Therapeutic Efficacy," by K. A. Lees, "The Influence of Particle Size upon the Absorption of Drugs from the Gastrointestinal Tract," by J. A. L. Gorringe and E. M. Sproston, and "Absorption of Steroids with Special Reference to Spironolactone," by G. R. Venning, would be interesting reading not only for the industrial scientists in the pharmaceutical industry but also for the practicing pharmacist.

The only disappointment to the reviewer was the paper entitled, "Kinetics of Drug Absorption: Methods and Interpretations," by R. F. Crampton and D. M. Matthews. There is a vast amount of literature on the kinetic interpretation of drug blood levels and urinary excretion which these authors only touched.

Reviewed by John G. Wagner
The Upjohn Company
Kalamazoo, Michigan

NOTICES

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Review Article

Electron Spin Resonance Spectroscopy

By L. DALLAS TUCK

THE YEARS since World War II have brought a continual and almost geometric increase in the sophistication of physical measurements. While the chemist or pharmaceutical chemist of three decades ago depended for his routine work upon pipets, burets, and balances with the occasional use of the refractometer, pH meter, and the single-beam spectrophotometer, his modern counterpart has at his disposal a profusion of complex electronic instruments. Not only have the old instruments been refined into self-scanning automatic recording systems, but also new atomic and optical principles have been discovered and have led to new types of measurement.

Two new branches of spectroscopy of particular interest in this review are commonly known as nuclear magnetic resonance (NMR) and electron spin resonance (ESR). They are mentioned together because they represent applications of the same principle to two different types of elementary particles. The former, NMR, concerns energy levels due to orientation of the *atomic nucleus* in a magnetic field, while the latter, ESR, has the same relationship with respect to the *electron*. Though there are many differences in the practical details of the instrumentation, there are also many common features, particularly from a viewpoint of the theory. The principal concern of this review is electron spin resonance, but we shall have occasion to compare it with NMR, an instrument probably more familiar to the reader.

The purpose of this article is to set down enough of the physical principles and applications of electron spin resonance to make the contemporary literature readable, with particular emphasis on those applications currently being made in chemistry and biology. For more detailed discussion of the theory or specific applications, a wealth of review and research literature is available (1–12).

There is not a unanimity in the use of the name "electron spin resonance." The name used depends on the particular application and the habits of the research worker (5, 12). From a broad instrumental point of view, "electron spin resonance," "electron paramagnetic resonance," and just plain "paramagnetic resonance" are all synonymous, and only the first mentioned will be used here.

While the spectroscopy of nuclear magnetic resonance requires the presence of nuclei possessing magnetic moments, the mere presence of electrons does not lead to an ESR spectrum. Only an atom or molecule possessing an *unpaired* electron will exhibit ESR absorption. Thus, most chemical compounds are inactive in this respect because their electrons are paired; *i.e.*, every possible electron orbital either is empty or is filled with two electrons of opposite spin.

The following types of substances are susceptible to study in ESR spectroscopy: (*a*) paramagnetic ions of transition group elements, (*b*) free radicals, (*c*) triplet-state molecules, often formed by excitation from the (paired electron) ground state in phosphorescent molecules. A triplet-state molecule has two unpaired electrons; the oxygen molecule is an example, though an atypical one. (*d*) Odd-electron elements and

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molecules in the gaseous state, and (*e*) metals and other solids containing conduction electrons and holes.

This review is devoted to those of the above substances which exist in detectable quantity in liquid solution at ordinary temperatures—principally items *a* and *b*. Item *c* is given only brief mention because of the experimental problems involved in obtaining a spectrum (13).

PHYSICAL PRINCIPLES

It is well known (14)[1] that the electron possesses an axis about which it rotates with an invariant angular momentum, referred to as its spin angular momentum or *spin*. The magnitude of the spin is expressed as $p_s = {}^1/_2(h/2\pi)$, where the *spin quantum number*, ${}^1/_2$, stands in place of an integer in a similar equation for the orbital angular momentum of an atomic electron. Planck's constant, h, has the value 6.62×10^{-27} erg second. The spinning charge comprises an electric current which produces a magnetic dipole moment, μ_s, also an invariant of the electron, whose value is related to the unit of orbital magnetic moment, μ_o, the Bohr magneton, by

$$\mu_s = {}^1/_2\, g\mu_o \qquad \text{(Eq. 1)}$$

In this equation, g is a factor determined by the charge structure of the electron and is called the Lande g factor or the spectroscopic splitting factor; for an electron with no orbital angular momentum, g has the value 2.0023. For the purpose of a simple image not structurally meaningful, it is convenient to imagine the electron consisting of a tiny magnet of pole strength 9.27×10^{-13} electromagnetic units and with north and south poles separated by 1 Å. This is a way of illustrating the *fact* that the spin moment is 9.27×10^{-21} erg/gauss.[2]

Consider a volume of a dilute gas contained within the pole gap of a magnet with parallel pole faces, *i.e.*, within a homogeneous magnetic field. The rule of spatial quantization permits the electron magnets to occur only in two orientations, as illustrated in Fig. 1. This is a consequence of the fact that the spin quantum number S is ${}^1/_2$ and the general rule according to which there are $2S + 1$ permitted orientations. The two permitted states are characterized by a set of quantum numbers representing the orientation of the electron magnet in the magnetic field, $M_\alpha = +{}^1/_2$, $M_\beta = -{}^1/_2$. The only possible transitions are those for which $\Delta M = M_\alpha - M_\beta = 1$ and $\Delta M = M_\beta - M_\alpha = -1$. The orientation, α, which is parallel to the magnetic field, is mechanically unstable because of the repulsion of like poles; while the antiparallel orientation, β, is stable. The work required to rotate the electron magnet from condition β to condition α is proportional to the magnetic moment and the magnetic field strength H (gauss). According to Eq. 1, the energies of the states relative to the condition of zero magnetic field strength can be computed by

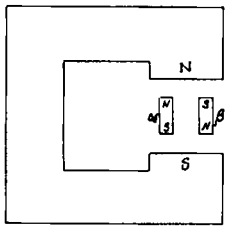


Fig. 1.—Schematic representation of the two permitted electron orientations in a magnetic field. The orientation α is the excited state; β is the ground state.

$$E_\alpha = \mu_s H = {}^1/_2 g\mu_o H \qquad \text{(Eq. 2)}$$

$$E_\beta = -\mu_s H = -{}^1/_2 g\mu_o H$$

The energy of the transition $\Delta M = +1$ is thus

$$\Delta E = E_\alpha - E_\beta = g\mu_o H \qquad \text{(Eq. 3)}$$

Equations 2 and 3 are expressed graphically in Fig. 2, in which the length of the arrow corresponds to ΔE at a given externally applied field.

As applied to electrons in free atoms, one will find in books on spectroscopy (*e.g.*, *Reference 15*) an equation for g which takes into account the orbital angular momentum as well as the spin.[3] Since the orbital motion in molecules is intimately connected with the physical and chemical bonds, it is not possible, except in gaseous systems, to effect reorientation of the orbital moments without absorbing large energies. As expressed in the literature (3), orbital orientation is quenched.[4] The electron spin, however, is not involved in the molecular structure; accordingly, spin orientation readily occurs with energy levels almost identical to those of the free electron. Small variations from the free-spin value due to forces between the orbital and the spin magnetic moments are expressed by adjustment of the value of g as used in Eq. 1. This effect is referred to by the term "spin-orbit coupling."

[1] The concepts fundamental to this discussion are found in numerous books on introductory atomic physics or quantum mechanics. Most of the vocabulary is in a succinct form in *Reference 14*.

[2] In electrodynamics, a distinction is made between the magnetic field strength (gauss) and the magnetic induction (oersted). While the latter is logically appropriate here, common usage dictates the former. Computation results are the same either way since the materials commonly used have unit magnetic permeability.

[3] The equation for g referred to would yield precisely 2 for the free electron and must be corrected for a polarization effect arising in quantum electrodynamics to yield the value 2.0023 referred to above.

[4] An exceptional class of solid compounds occurs in the lanthinide and actinide rare earth salts, in which the unpaired electrons are deep within the atomic kernel (5, 6, 16).

As in other branches of spectroscopy, absorbed electromagnetic energy lifts the energy level of a molecule from a ground state to an excited state. The ground state energy is represented by the point on curve β of Fig. 2 which corresponds to the applied magnetic field strength. Absorption will occur only if a photon of the electromagnetic radiation matches the energy required to lift the system to the value on curve α at the same H value, illustrated by the length of the arrow in Fig. 2. A given value of field strength H requires a definite electromagnetic frequency, ν, which is calculated in cycles per second by combining Einstein's equation, $\Delta E = h\nu$, with Eq. 3 to yield

$$\nu = \frac{g\mu_o H}{h} \qquad \text{(Eq. 4)}$$

It would appear that there is an infinite range of frequencies to choose from, for one need only choose a value of the magnetic field which corresponds to a convenient frequency according to Eq. 4. In practice, the difficulties and cost of generating a steady and homogeneous magnetic field increase in a geometric manner with the field strength. Other desiderata, such as sensitivity and resolvability of the spectrum, are improved by higher frequency and thus higher field strength, so that a compromise is necessary. Though for some purposes frequencies in the 10 to 100 megacycle range may be used, the most common frequency now used is about 9 gigacycles (Gc.) (9000 megacycles) per second, which is in the microwave region of the radiofrequency spectrum. Calculation from Eq. 4 shows that the corresponding magnetic field strength is of the order of 3000 gauss.

INSTRUMENTAL

In gross schematic structure, the ESR spectrometer consists of the magnet, the microwave circuit, and electronic equipment for regulation, detection, and recording. A block diagram of a simplified spectrometer is shown in Fig. 3. The microwave source is a reflex klystron oscillator which sends 9 Gc. per second power by way of a pipe of rectangular cross section called a waveguide into a resonant cavity through a circular iris of about 0.5-cm. diameter. The cavity may be a walled-off section of waveguide, but it is often an especially constructed chamber with built-in field-modulation coils, special fittings for support of the sample, etc. The source frequency must be carefully adjusted to be exactly resonant with the cavity—a condition such that there are an integral number of half-wavelengths in the cavity. A second iris through the far wall of the cavity permits a fraction of the power to escape and be detected by a silicon crystal rectifier, and the magnitude of the exit power is registered on a recording voltmeter. Because of the strict frequency requirement for resonance[5] in the cavity, it is not possible to obtain an ESR spectrum by varying the frequency. According to the conjugate nature of frequency and magnetic field strength (Eq. 4), the same purpose is served by varying the magnetic field at a fixed frequency.

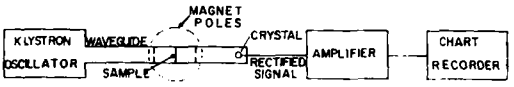


Fig. 3.—Schematic diagram of a simplified ESR spectrometer.

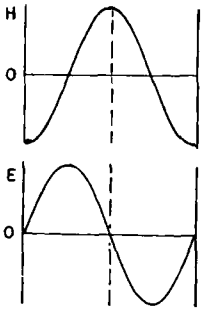


Fig. 4.—Standing - wave amplitudes in a full-wavelength resonant cavity as a function of position along the axis of the cavity. The ordinate of the upper curve is the magnetic field strength; that of the lower curve is the electric field strength.

A practical spectrometer varies in numerous respects from the above, but it has as its main characteristics the following components: an electromagnetic oscillator, a resonant circuit containing the sample within the magnetic field, and provisions for detecting, amplifying, and indicating the power absorbed by the sample.

The position of the sample is a critical factor in the sensitivity of the spectrometer. Ideally, one desires to absorb as much magnetic energy in the sample as possible with no power lost in other ways. Thus, the most favored position for the sample is at the maximum (loop or antinode) of the magnetic standing wave in the cavity. Because the electric and magnetic waves are 90°

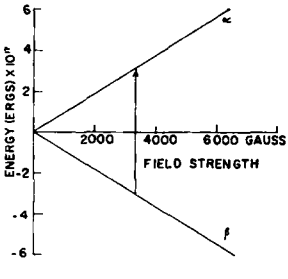


Fig. 2. — Energies of the two states of a free electron as a function of magnetic field strength.

[5] The word resonance is used in this article in two separate senses. In this section, simple instrumental resonance is used as resonance between the source frequency and one of the natural frequencies of the cavity. As used in the name of the phenomenon the word resonance implies the condition that the photons of the source frequency have the exact energy to effect the desired magnetic transition.

out of phase, the antinode of the oscillating magnetic field is at the same position as the node of the electric field, as shown in Fig. 4. As the dielectric loss in matter due to oscillating *electric* dipoles is proportional to the oscillating electric field strength, maximum magnetic absorption and minimum electric absorption occur at the same position.

Of course, the position of the node is merely a mathematical point, so that any actual physical sample would overlap less ideal positions. For many substances, a small amount of overlap is no disadvantage. However, some samples, particularly aqueous solutions, are so lossy that they tend to kill the oscillation in the cavity and must be inserted in capillary tubes or in thin flat quartz cells carefully aligned in the electric nodal plane.

The presentation of the spectrum may take many forms. It could be presented as a graph of the point-by-point d.-c. voltage output of the crystal rectifier as a function of the magnetic field. An automatic device for linear sweeping of the magnetic field together with a recording voltmeter would produce such a graph, as shown in Fig. 5A.

To increase the signal-to-noise ratio, many ESR spectrometers use a device known as a phase-sensitive (lock-in or coherent signal) detector. This device is capable of eliminating extraneous signals and noise voltages by accepting only an alternating voltage of the same frequency and phase as some constant-frequency reference voltage.

An alternating magnetic field is superimposed on the slowly swept magnetic field by means of auxiliary coils parallel to the pole faces of the magnet, referred to as field modulation coils. The frequency of the field modulation is arbitrary, and spectrometers exist in which it is as low as 30 cps. and as high as 1 megacycle. The voltage producing this field is connected to the reference input of the phase-sensitive detector and operates a gate to turn the detector on and off in synchrony with the alternating output voltage from the crystal detector. The output of this device is illustrated in Fig. 5B for the sample whose absorption spectrum is shown in Fig. 5A. As the external magnetic field is slowly swept through the absorption curve of Fig. 5A, the field modulation imposes an alternating field, indicated by the sinusoidal wave form. The crystal receives a d.-c. voltage according to the height of the curve, upon which is superimposed an alternating voltage whose magnitude depends upon the steepness of the curve. The phase-sensitive detector responds only to the alternating voltage, which is

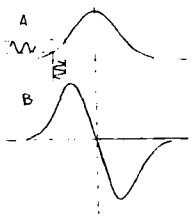


Fig. 5.—Key: A, ESR absorption spectrum of a substance showing no hyperfine structure; B, ESR derivative spectrum resulting from use of field modulation and phase-sensitive detection. The abscissa is magnetic field strength; the ordinate is voltage output at the recording voltmeter. The vertical sine wave represents the field modulation of the magnetic field; the horizontal sine wave is the resulting alternating output voltage.

then rectified. The voltage recorded at each point of the abscissa has a value proportional to the derivative of Fig. 5A at the same H value, as shown in Fig. 5B. This curve is often referred to as a derivative spectrum.[6]

Though the novice may find it difficult to interpret such a curve, with experience it yields more information to the eye than does the original absorption curve, at least in complicated spectra with overlapping of individual lines. This is because subtle changes of the slope are exaggerated in the derivative curve. Some workers have carried this procedure one step farther to the second derivative and claim even better interpretability (17).

A limiting factor in any sensitive instrument is the amount of interference arising from extraneous sources. Electronic instruments utilize grounding, shielding, and regulating techniques to reduce stray pickup; yet every element which emits or carries electric power is an internal source of thermal noise. The klystron oscillator is the most important noise source in a modern ESR spectrometer, and the sensitivity is primarily determined by this tube (18). Since the noise should average out to zero, an average signal taken over a time long enough should give a spectrum of any desired signal-noise ratio, and most electronic instruments are equipped with a low-pass filter of variable time constant for this purpose. To extend the time constant beyond a few seconds, however, has many practical disadvantages and requires an instrument of unattainable stability. An ingenious technique used by Commoner and co-workers (19) takes advantage of the averaging effect by recording the signal from identically prepared samples on the same chart 10 to 15 times. Though in a single sweep it is difficult to distinguish the signal from the noise, the repetition produced a composite

[6] Strictly, the derivative spectrum is found only in the limit as the field modulation approaches zero.

pen track of breadth equal to the noise voltage upon which the signal appears unambiguously.

Electronic devices based on the same principle store the information from successive sweeps, reproducing the average after completion of some hundreds of sweeps (20, 21). Figure 6 compares an ESR spectrum by Klein and Barton (20) of a dilute solution of manganous ion in one sweep with an average of 5000 sweeps over 7 hours.

CHARACTERISTICS OF A SPECTRUM

The absorption spectrum of a free electron appears as a single spike centered at field strength corresponding to that calculated from Eq. 4, with $g = 2.0023$. Spectra of unpaired electrons in atoms and molecules vary from this for several reasons.

Line Shift and g Value.—As discussed in a previous section, interaction with the orbital angular momentum results in a variation of g by spin-orbit coupling. To borrow a term from NMR, this is a kind of chemical shift, as the deviation from the free spin value depends upon magnetic effects in the environment.

Line Broadening.—For precision in the information to be obtained from a spectrum, the line width must be as small as possible. There are several causes of broadening in lines, a knowledge of which is necessary for rational study of ESR spectra.

Some line broadening arises from purely instrumental causes. Lack of homogeneity in the magnetic field, producing a different field strength at different parts of the sample, would produce broadening for obvious reasons. Another common cause of broadening is the use of large values of field modulation alternating field strength. It is clear from inspection of Fig. 5 that if the amplitude of the field modulation is of the order of magnitude of the line width itself, an apparent broadening of the line will occur. Sometimes this may be necessary, however, as increase of the field modulation is one way of increasing the sensitivity; and if the sample is very dilute in paramagnetic solute, a compromise is necessary between detection of the free radical and characterization of its true spectrum.

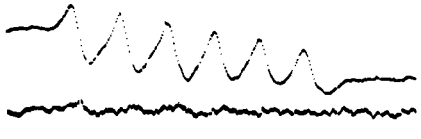


Fig. 6.—ESR spectrum of an aqueous solution containing 8×10^{-11} moles of manganous ion in the spectrometer cell. The lower curve is the direct output of the spectrometer; the upper curve is an electronic average of the same sample as a result of 5,000 traverses.

The presence of neighboring paramagnetic molecules alters the local magnetic field strength. This broadens the spectral line in part because of the statistical deviations of the field owing to momentarily different environments. This effect is called "dipole broadening" or "spin-spin broadening." Dilution of the solution will result in narrower lines by increasing the average distance between molecules.

In complicated organic free radicals at low concentration, the ESR spectrum will often be a single broad line. This broadening could be caused by failure to resolve hyperfine splitting (see *Hyperfine Structure*). If the spectrum consists of a series of narrow lines, large field modulation may wash out this structure by the mechanism discussed above.

The breadth of an ESR spectrum line is actually the result of a complex combination of causes, both intramolecular and environmental. The ultimate line width is related to the lifetime of a magnetic state. Any effect which facilitates energy coupling between the spin center and the environment leads to broadening; any effect that tends to isolate it leads to narrowing. For further information, one should consult the literature on the subject of the spin-relaxation process (3).

Fine Structure.—Students of atomic spectroscopy will recall that fine structure pertains to the separation of electronic energy levels of the same principal quantum number and different angular momentum quantum number (14). Fine structure appears in ESR spectra only in the gaseous and crystalline states. For further discussion, one should refer to the pertinent literature (5, 10–12).

Hyperfine Structure.—An important characteristic of an ESR spectrum arises owing to the presence of nuclei having magnetic moments within the orbital of the unpaired electron. Like that of the electron, the spin of a hydrogen nucleus can be oriented in only two directions. In one such orientation, it adds its field to that of the external field, while in the other it subtracts. Thus, if the electron lies very close to this nucleus, an applied field measurably smaller (or larger) than for a free electron is required to bring the electron to its resonance value. The energy diagram of Fig. 2 may be amended to contain this information. A single proton acting as a magnet will add or subtract a definite amount to the applied field, regardless of the value of the latter. Figure 7 shows two values of H at which resonance occurs at a given frequency—one lower and one higher than that expected in the absence of the proton. Since there will be an

approximately equal number of protons in each of the two states, it will give rise to a doublet symmetrically disposed around the basic line position. This is the simplest case of hyperfine splitting and is analogous to spin-spin splitting in NMR.

It was noted above that two orientations are permitted the hydrogen nucleus. This is a consequence of the nuclear spin quantum number $I = 1/2$, exactly analogous to the rule outlined for the electron with $S = 1/2$. Other nuclei having other values of the nuclear spin quantum number will conform to the rule that the only nuclear orientations permitted are those in which $M_I = \pm I$ and values smaller in magnitude by integers, where M_I is the projection of I along the magnetic field direction. For nitrogen-14, for which $I = 1$, M_I is permitted the values $+1$, 0, -1, leading to a three-peak spectrum. Since one-third of the atoms in a given sample will be in each such state, the three lines will be of equal intensity.

A striking example of such a spectrum is that found for the free radical divalent anion, peroxylamine disulfonate, obtained as the potassium salt, as shown in Fig. 8. The splitting is caused by the nitrogen-14 in the molecule.

Values of the nuclear spin quantum number depend upon both the atomic number and the atomic weight, *i.e.*, both on the number of protons and neutrons. Different isotopes of the same element will thus have different I values. Some values for common nuclides are listed in Table I.

Elements with even mass numbers (total of protons and neutrons) have integral values of I, those with odd mass numbers have half-integral values, and nuclei with even atomic number and even atomic mass number have zero spin. The latter is a result of the pairing of the spins of the respective protons and neutrons.

Table I shows that in an organic compound containing ordinary carbon, hydrogen, and oxygen, only the hydrogen will produce hyperfine splitting. However, splitting due to the small naturally occurring fraction of carbon-13 is sometimes observed (see below).

PRACTICAL AND THEORETICAL APPLICATIONS

Quantitative Analysis.—The use of ESR as a quantitative measure of free radical concentration is based on the fact that the integral of the absorption spectrum is proportional to the number of spin centers within the cavity (3). For spectrometers with first-derivative presentation, this proportionality may be utilized only by performing a double integration on the raw

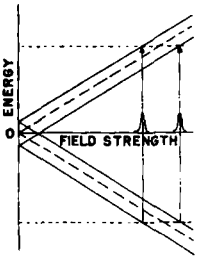


Fig. 7.—Energies of the two magnetic states of an electron as a function of magnetic field strength showing hyperfine splitting by a nucleus with $S = 1/2$. The scale of the splitting is exaggerated.

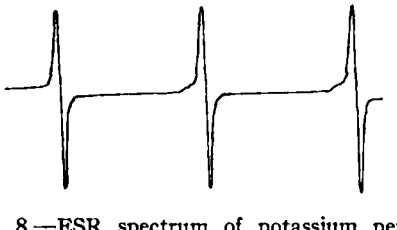


Fig. 8.—ESR spectrum of potassium peroxylamine disulfonate dissolved in an aqueous ammoniacal solution.

spectrum and comparing it with a similar integration for a standard of known titer. Such computations are time consuming and, for reasons listed below, are often of questionable accuracy. Electronic integrators (22) and other computational aids (23) may be used to facilitate the integration, but even these yield only order-of-magnitude results. The inaccuracy of all integration processes as applied to ESR arises from at least two basic causes: (*a*) the instability of the spectrometer, which appears as baseline drift of the integrated spectrum and (*b*) the difficulty of resolving hyperfine structure in a time sufficiently short to obtain an accurate integral. A third cause of inaccuracy occurs, particularly in lossy solutions, if the sample must be removed from the cavity to insert the standard whose dissipative character may be quite different. This difficulty may be avoided by using a double cavity capable of accommodating the two samples and displaying their spectra at the same time (24).

For many purposes only relative concentration of a single substance is required. In this case, the maximum height of the derivative signal serves as a proportional measure of concentration (25).

A standard substance useful and almost indispensable in ESR spectroscopy is 1,1-diphenyl-2-picrylhydrazyl (DPPH) which can be prepared as a polycrystalline solid in the free radical form. In this form, its spectrum is a single peak of about 3 gauss width and a g value of 2.0036 (3). Its line width varies somewhat with crystal size, evidently connected with the surface adsorption of paramagnetic oxygen (26–28). It is stable for months in the solid state but undergoes a few

per cent decay in a year. In dilute solution in benzene and other solvents, it exhibits a spectrum of five lines with line separation of about 10 gauss, a result of hyperfine splitting by the two nearly equivalent nitrogen nuclei.

Other standards of free spin which have been proposed and used include manganous ion (29), ruby crystal (30), and carbonaceous material (31); these are useful for particular applications but have certain practical disadvantages.

Qualitative Analysis and Chemical Structure.—As discussed earlier, there are three important characteristics of an ESR spectrum by which it can be identified—*viz.*, its g value, its line width, and its hyperfine structure. Each of these can play a part in the qualitative identification of a paramagnetic entity and furnish clues to its chemical nature.

As a simple example, manganous ion in an aqueous solution can be readily identified by observation of its six-line spectrum of 100-gauss separation (32) (see Fig. 6). With methods beyond the scope of this discussion, the electronic structure of coordinated complexes and chelates of transition-group ions may be deduced by study of the details of the hyperfine structure (33).

The free radical produced by electric discharge through methane was identified as the methyl radical by its ESR spectrum, consisting of four symmetrical hyperfine lines of intensity ratios 1:3:3:1 (34). Four lines would be expected from a free radical with three equivalent protons from the multiplicity rule, $2I + 1$, since the total nuclear spin of three aligned protons would be $I = {}^3/_2$. The relative intensities of the lines are computed from the statistical weights of the four possible combinations. There is only one permutation for which all of the nuclear magnets point to the north (or the south) pole of the external magnet, which corresponds to $M_I = \pm{}^3/_2$. Reversal of any one of the three protons will yield the result $M_I = \pm{}^1/_2$. Since each permutation is equally probable, the statistical weight of $M_I = {}^1/_2$ is three times that for $M_I = {}^3/_2$. While it is true that most organic free radicals do not have the structural simplicity of the methyl radical, their spectra sometimes do. From the fact that the ESR spectrum of a free radical is a symmetrical triplet of intensity 1:2:1, it is deduced that the unpaired electron is localized on a group containing two equivalent protons (35).

TABLE I.[a]—NUCLEAR SPIN QUANTUM NUMBERS OF SEVERAL COMMON NUCLIDES

Element	At. No.	Mass No.	I
Hydrogen	1	1	${}^1/_2$
Hydrogen-2 (deuterium)	1	2	1
Helium	2	4	0
Carbon-12	6	12	0
Carbon-13	6	13	${}^1/_2$
Carbon-14	6	14	0
Nitrogen	7	14	1
Oxygen-16	8	16	0
Oxygen-17	8	17	${}^5/_2$
Oxygen-18	8	18	0
Fluorine	9	19	${}^1/_2$
Sodium	11	23	${}^3/_2$
Vanadium	23	51	${}^7/_2$
Manganese	25	55	${}^5/_2$

[a] Sullivan, W. H., "Trilinear Chart of Nuclides," Oak Ridge National Laboratory, 1957. See this or other sources for a complete list.

The methods of analysis illustrated above can be extended only in principle to more complex organic and biological free radicals. However, like the infrared spectrum, common features in two spectra are to be associated with similar structural features. Ingram (3) cites at length the work of Gordy *et al.* (36), who showed the similarity of the ESR spectra of free radicals from a number of animal proteins with that of cystine. Schieser (37) has recently pointed out common features in the spectra of free radicals formed in the Marquis test (and other color tests) on various alkaloids. He concludes that the site of the free radical is a phenolic moiety common to all the compounds studied.

Electron Density in π-Radicals.—Free radicals formed by oxidation or reduction of aromatic molecules owe their stability to the participation of the unpaired electron in the aromatic resonance. In the language of the quantum theory, it is in a π-orbital. This is confirmed by the ESR spectra of such radicals, which show hyperfine splitting by all of the ring hydrogens. Moreover, hyperfine splitting is also contributed by the protons of methyl groups attached directly to the ring, which is a direct verification of hyperconjugation (3).

A quantum mechanical analysis of the aromatic ring shows that the hyperfine splitting by a ring proton is directly proportional to the unpaired electron density (the square of the wave function) at the carbon atom to which it is attached (38). Hyperfine splitting calculated from this theory is in good agreement with experimental values (39). Electron densities of both positive and negative radical ions of a number of aromatic hydrocarbons have been computed (40).

Hyperfine splitting in aromatic radicals attributed to the 1% of carbon-13 in natural carbon has been observed in certain spectra with narrow well-resolved lines (41). Figure 9 shows the ESR spectrum of dibenzo-p-dioxin (42). The five principal lines of intensity 1:4:6:4:1 arise from hyperfine splitting by one set of four equiv-

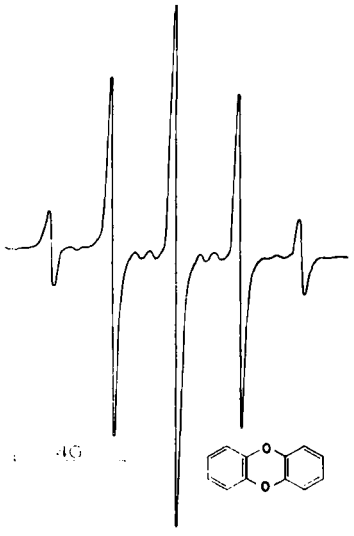


Fig. 9.—ESR spectrum of dibenzo-*p*-dioxin, showing hyperfine splitting by four equivalent protons together with C^{13} splitting by two sets of four equivalent carbon atoms. The C^{13} is that naturally present in 1% abundance.

alent protons. The satellite lines are of the proper intensity to be ascribed to splitting by two sets of four equivalent carbon-13 atoms in their natural abundance. These observations are related to the local electron densities in a way more complicated than the proton splittings (43).

Reaction Kinetics.—Limited as it is to the detection of free radicals and other paramagnetic substances, ESR cannot by itself give direct results of kinetics and mechanisms of reactions. Used with other spectrographic instruments, it is a powerful auxiliary for study of those reactions involving free radicals of reasonably long lifetimes. Its use in this connection may be enhanced by the development of the following accessory techniques: low-temperature measurements, methods for electrolytic generation of free radicals within the instrument, and rapid mixing and flow techniques. All of these have the effect of facilitating the observation of free radicals of short lifetimes.

In direct application to kinetic problems, ESR is used to measure the free radical titer according to the rules outlined in *Quantitative Analysis*. Used in this way, it has had few applications in simple organic systems. This is in part owing to the short lifetimes of most structurally simple free radicals at their usual reacting temperatures.

Of more than casual interest to pharmaceutical chemists, phenothiazine and its derivatives form long-lived free radical intermediates upon oxidation. Borg and Cotzias (44) have carried out studies on chlorpromazine and related substances in the presence of certain inorganic ions by optical spectroscopy, supplemented by ESR data. Tuck and Tozer (45), using ESR supplemented by optical spectrophotometry, studied the decay in acidic solutions of the free radical of phenothiazine and a number of its substituents and have analyzed the mechanism of the decay process. Piette *et al.* (46) have studied the rate of peroxidase catalyzed oxidation of chlorpromazine.

In the field of polymerization, in which the free radicals are stabilized by trapping in solid or viscous media, ESR promises to play an important part in kinetic studies, as suggested by Whiffen (47). In some recent studies it has been used as an auxiliary technique in polymer rate studies (48).

Rates of rapid electron exchange reactions involving a free radical may be estimated by a broadening effect on its spectrum when mixed with the other reactant. Ward and Weissman (49) showed that the complex spectrum of the naphthalene free radical ion lost its resolution as a result of an increase of line width when mixed with unreduced naphthalene. In 0.8 *M* solution in tetrahydrofuran, they estimated the half-life of the individual free radicals to be of the order of a microsecond. This is analogous to the well-known proton exchange effect observed in NMR spectra of substances undergoing keto-enol tautomerism (50).

Biological Applications.—Early in its development, Commoner *et al.* (51) pioneered the application of ESR to biological systems when they packed their rather insensitive instrument full of lyophilized samples of some 22 varieties of vegetable and animal life and proved that they contained paramagnetic matter. As the sensitivity of their instrument was improved, more difficult problems were tackled. The year 1957 brought two exciting developments: Commoner and his staff (19, 52) showed the presence of free radicals in aqueous media containing enzymes, and Calvin and Sogo (53) showed that the free radical concentration in photosynthetic processes responded to the intensity of light. Other workers were close behind (54). Since that time, both of these areas have had continued activity (8, 55, 56), some of which could well have been included in the section on *Reaction Kinetics*.

It must be mentioned here that ESR spectra of enzymic and other biological material (as well as those of polymerical radicals) usually depart from the precise symmetry found in simpler molecular compounds. This is to be associated with two important causes—*viz.*, the simultaneous existence in solution of two or more free radicals

with different g values and the colloidal nature of macromolecular systems, such that the molecular tumbling which averages out spectral anisotropy is inhibited.

Of fundamental importance in biology and nuclear medicine are studies of the effects of ionizing radiation on biochemical substances. The laboratory of Gordy has produced many such studies concerning the effects of X-rays and gamma-rays on native proteins (57) and on other polyamino acids (58). These studies have shown that free radicals are formed as the primary effect of irradiation, and the detailed spectra give direct information about the structure of the free radical and its orientation. Studies of the effect of radiation on biological systems have been carried out (59).

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Inter- and Intrasubject Variations in Drug Absorption Kinetics

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Inter- and intrasubject variations in drug absorption kinetics were studied in 15 subjects who ingested 975 mg. of aspirin in compressed tablets on two separate occasions. The group included unusually rapid and unusually slow eliminators of salicylate. Average as well as individual absorption data could be described as first-order processes preceded by a short induction period. The magnitude of variations in rate constants and induction times between subjects and between tests in any one subject has been determined, and the possible existence of consistently slow and rapid drug absorbers has been examined. *In vitro* dissolution of drug from the dosage form was describable as a first-order process, and a quantitative correlation between *in vitro* dissolution and *in vivo* absorption data has been obtained. Acceptance of a given mathematical model, based on average data, as a description of drug absorption and elimination processes is justified only if the model fits the majority of the individual data. It also must be appreciated that considerable inter- and intrasubject variations in rate constants may exist.

THE RECENT development of convenient mathematical methodology for determining drug absorption rates (1) represents a major advance in biopharmaceutics. Different kinetic models for drug absorption processes, illustrated by several practical examples, have been presented (2). In the present report, studies are described which serve to extend these developments to a consideration of inter- and intrasubject variations in drug absorption kinetics and to the mathematical correlation of *in vitro* and *in vivo* drug-release data.

EXPERIMENTAL

Clinical Study.—Fifteen normal male subjects ingested 975 mg. aspirin[1] in the form of three compressed tablets on two occasions, usually about 1 week apart. The tablets were swallowed whole on an empty stomach with 240 ml. of water. Blood samples were obtained at 15, 30, 60, 90, 120, 180, and 240 minutes after drug ingestion. Serum salicylate levels were determined in duplicate by the colorimetric procedure of Trinder (3). Aspirin tablets used in all phases of the study were from a single lot of a national brand purchased on the open market.

Calculation of Absorption Rates.—The per cent of the dose absorbed at various times was calculated by the procedure of Wagner and Nelson (2)

$$\% \text{ Absorbed} = \frac{A}{A_\infty} \times 100 = \frac{C_T + K \int_{t=0}^{t=T} C\,dt}{K \int_{t=0}^{t=\infty} C\,dt} \times 100 \quad \text{(Eq. 1)}$$

where A is the cumulative amount of drug absorbed at any time T, A_∞ is the total amount of drug eventually absorbed, C_T is the drug concentration in serum at time T, and K is the first-order elimination rate constant for salicylate. Individual values of K were obtained from half-lives which were determined graphically from postabsorptive blood level data that yielded straight lines when the logarithm of serum salicylate concentration was plotted as a function of time, on the basis of the relationship

$$K = \frac{.693}{t_{1/2}} \quad \text{(Eq. 2)}$$

where $t_{1/2}$ is the half-life for elimination, expressed in units of time. Values for the integral in Eq. 1 were obtained by planimetry of respective areas under individual serum salicylate concentration *versus* time curves. In view of the known rapid and complete gastrointestinal absorption of aspirin when it is administered in conventional compressed tablets (4), A_∞ was assumed to equal the administered dose. Considerations concerning the use of the Wagner-Nelson method (1, 2) for the determination of aspirin absorption rates on the basis of salicylate blood levels have been discussed by Levy in a previous publication (5).

Dissolution Rate Determinations.—*In vitro* dissolution rates were determined by the method of Levy and Hayes (6) with the following modifications: the volume of 0.1 *N* HCl was increased to 350 ml., and data for tablets which formed a "mound" at the edge rather than in the center of the bottom of the beaker were excluded. Samples were analyzed as described previously (6).

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[1] Analysis of four individual tablets (no more were available) indicated an average aspirin content of 319 mg. per tablet (range: 315–328 mg.). In view of the small sample, the label claim (325 mg.) was accepted as accurate and was used in subsequent calculations.

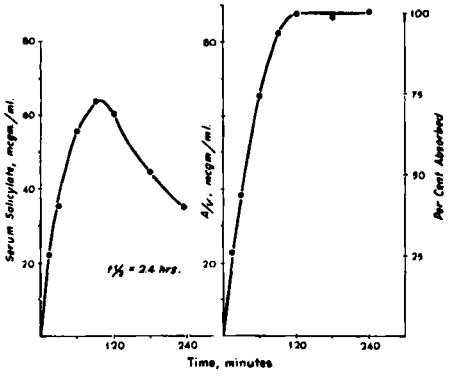


Fig. 1.—Serum salicylate concentration and per cent absorbed as a function of time after ingestion of 975 mg. aspirin by subject H, a rapid eliminator of salicylate.

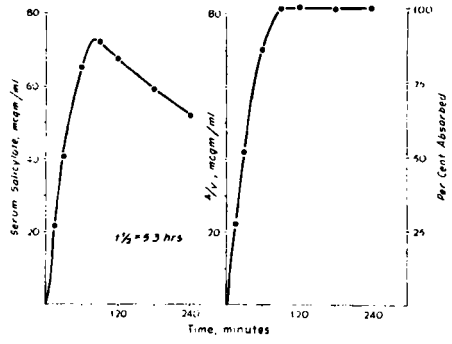


Fig. 2.—Serum salicylate concentration and per cent absorbed as a function of time after ingestion of 975 mg. aspirin by subject P, who eliminates salicylate at an average rate.

RESULTS AND DISCUSSION

Figures 1 to 3 illustrate how absorption rates were determined for each subject. The examples were chosen because they represent subjects who eliminate salicylate at an unusually rapid, average, and slow rate, respectively. Such pronounced inter-subject variations in salicylate elimination kinetics are in accord with the results of previous studies in our laboratories (7, 8). Excellent plateaus were obtained when values of A/V (cumulative amount absorbed/apparent volume of distribution) were plotted as a function of time, an indication of the accuracy of blood level data, the precision of planimetric measurements of areas under the blood level-time curves, and the applicability of apparent K value determinations.[2]

After calculation of individual values for per cent absorbed at various times after drug ingestion, the data were averaged and were plotted on semi-logarithmic paper as per cent unabsorbed *versus* time. Figure 4 represents the average of 30 individual values obtained from 15 subjects in two tests. The data can be described in terms of a first-order process preceded by an apparent induction phase which can be accounted for by a zero time shift. Specifically,

$$A_u = A_o e^{-.038(t-6)}, \quad \text{when } t \geq 6 \text{ minutes} \quad \text{(Eq. 3)}$$

or

$$\log A_u = -0.01(t - 6) + \log A_o, \quad \text{when } t \geq 6 \text{ minutes} \quad \text{(Eq. 4)}$$

where A_u is the amount unabsorbed at time t (expressed in minutes), and A_o is the administered dose. Figure 5 depicts the results obtained when average data from the two tests (15 individual values each) are plotted separately. The results of the two tests are essentially identical.

The impressive reproducibility of absorption kinetics in a group of only 15 subjects might lead one to conclude that the rate of drug absorption in

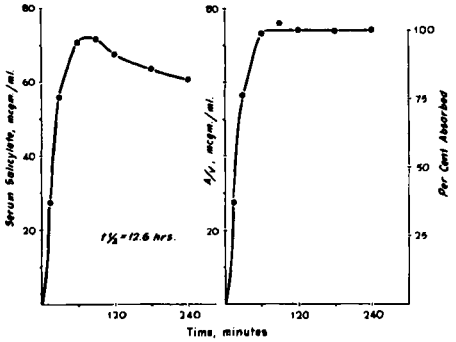


Fig. 3.—Serum salicylate concentration and per cent absorbed as a function of time after ingestion of 975 mg. aspirin by subject J, a slow eliminator of salicylate.

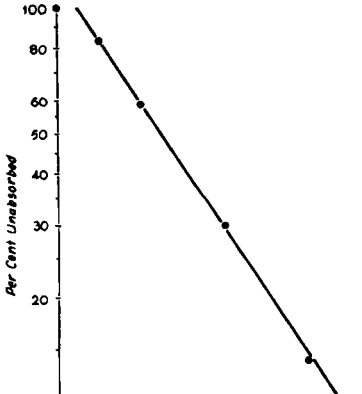


Fig. 4.—Rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Average of 30 determinations in 15 subjects.

[2] It was found recently that elimination of salicylate given in 1-Gm. (aspirin) doses involves initially parallel first-order and zero-order processes (Levy, G., to be published). This cannot be recognized readily from salicylate plasma level *versus* time data, which appear linear rather than curved, when plotted semilogarithmically. Therefore, first-order elimination rate constants (K) referred to in this paper must be considered apparent values. Their use in calculations of salicylate absorption and elimination kinetics does not introduce any significant errors, except in the terminal phase of salicylate elimination.

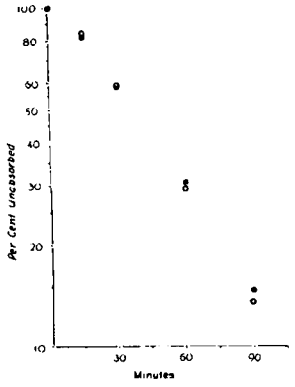


Fig. 5.—Rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Average of 15 determinations in 15 subjects. Key: ●, first test; ○, second test.

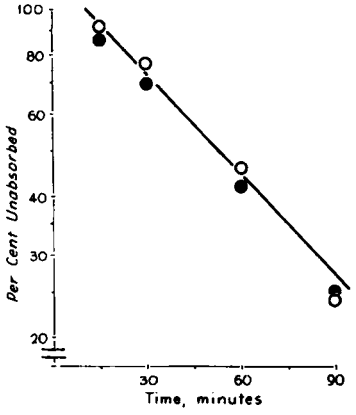


Fig. 6.—Example of consistently slow absorption: rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Results of two separate tests in subject A.

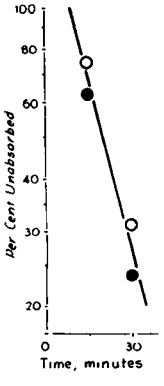


Fig. 7.—Example of consistently rapid absorption: rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Results of two separate tests in subject J.

any one individual is quite consistent in separate experiments. However, such was generally not the case. The group of subjects in the two tests included individuals who behaved as consistently slow absorbers, consistently rapid absorbers, and some who exhibited pronounced differences in absorption kinetics on the two occasions. To illustrate, Fig. 6 shows data representative of an individual who absorbed the drug at a consistently low rate (relative to the average), while Fig. 7 represents data indicative of consistently rapid drug absorption. On the other hand, pronounced differences in absorption kinetics are apparent in the results depicted in Figs. 8–10. These figures were chosen because they illustrate various types of intrasubject variations observed in the present study: (*a*) similar rate constants but different induction times (Fig. 8), (*b*) similar induction times but different rate constants (Fig. 9), and (*c*) different induction times and different rate constants (Fig. 10).

Interestingly, the individual showing rapid absorption (Fig. 7) happens to be an unusually slow drug eliminator (Fig. 3). The unusually rapid drug eliminator (Fig. 1) absorbed aspirin at a slow, though variable rate (Fig. 9). Using data from these two subjects, it is possible to demonstrate the profound intersubject variations in drug absorption and elim-

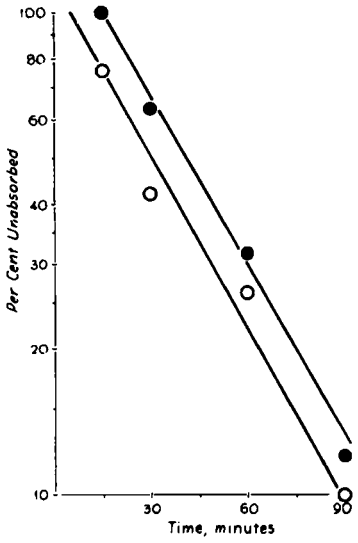


Fig. 8.—Example of variable absorption evidenced by differences in induction times: rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Results of two separate tests in subject L.

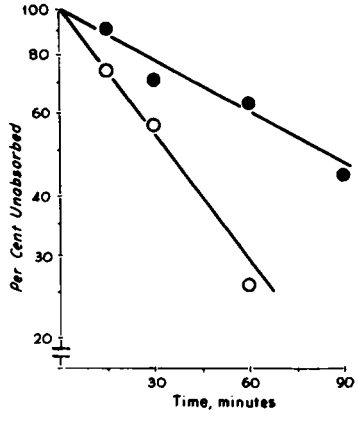


Fig. 9.—Example of variable absorption evidenced by differences in first-order rate constants: rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Results of two separate tests in subject H.

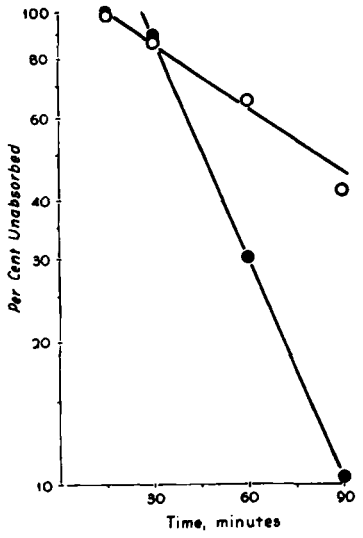


Fig. 10.—Example of variable absorption evidenced by differences in induction times and first-order rate constants: rate of gastrointestinal absorption of aspirin after ingestion of 975 mg. aspirin as three tablets, expressed in terms of per cent unabsorbed as a function of time. Results of two separate tests in subject S.

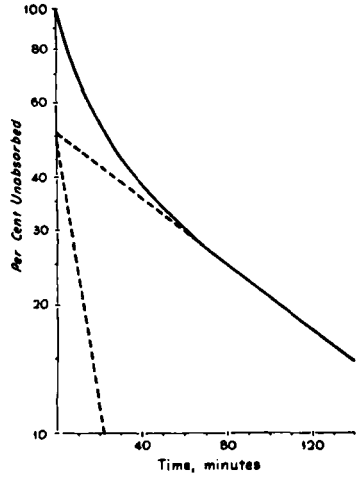


Fig. 11.—Solid line depicts per cent unabsorbed as a function of time for the hypothetical case where average data from two subjects absorbing drug by a first-order process with half-lives of 10 minutes and 80 minutes, respectively, are plotted. Stippled lines indicate rates of component processes if average data are interpreted (mistakenly) to represent two parallel first-order processes.

ination kinetics which may be encountered clinically. Since, in the present study, drug absorption and elimination can be represented as two consecutive first-order processes

$$\frac{dA_b}{dt} = k_a A_u - K A_b \qquad \text{(Eq. 5)}$$

which, upon integration, evaluation of the constant of integration at zero time, and rearrangement yields

$$A_b = \frac{A_o k_a}{K - k_a} (e^{-k_a t} - e^{-Kt}) \qquad \text{(Eq. 6)}$$

where A_b is the amount of drug in the body (exclusive of the gastrointestinal tract) at time t, k_a is the first-order absorption rate constant, and the other symbols have the meanings assigned to them previously. A_b is related to drug concentration in serum (C) in the following manner

$$\frac{A_b}{V} = C \qquad \text{(Eq. 7)}$$

where V is the apparent volume of distribution. In the case of the individual (subject J) who yielded the experimental values shown in Figs. 3 and 7 (rapid absorption and slow elimination),

$$A_b = -1.0\, A_o(e^{-.060(t-9)} - e^{-.00092(t-9)}) \qquad \text{(Eq. 8)}$$

while in the case of subject H, who absorbed the drug slowly and eliminated it rapidly (Fig. 1 and first test in Fig. 9),

$$A_b = -2.3\, A_o(e^{-.0085(t-1)} - e^{-.0048(t-1)}) \qquad \text{(Eq. 9)}$$

The expressions are not applicable when t <9 minutes and <1 minute, respectively.

An even greater difference is obtained in expressions for calculating serum salicylate concentrations (C, in micrograms per milliliter) rather than amount of drug in the body (A_b). Instead of Eq. 8, one obtains

$$C = -.76 \times 10^{-4}\, A_o(e^{-.060(t-9)} - e^{-.00092(t-9)}) \qquad \text{(Eq. 8a)}$$

while

$$C = -2.1 \times 10^{-4}\, A_o(e^{-.0085(t-1)} - e^{-.0048(t-1)}) \qquad \text{(Eq. 9a)}$$

takes the place of Eq. 9. The greater difference is due to the fact that absolute apparent volumes of distribution in the two subjects differ appreciably (subject J: 13,200 ml.; subject H: 11,100 ml.).

It is not too difficult to develop suitable kinetic models to fit most curves representing cumulative amount (or per cent) absorbed *versus* time, since such curves are generally smooth rather than undulating and since they reach a plateau or asymptotic value. It should be recognized, however, that kinetic models derived from average data may be completely meaningless since they may fit only these and none of the individual data. Kinetic models become meaningful only if they can describe the majority, if not all, of the *individual* data. While individual rate constants may vary, all or most of the individual data should be describable, within reasonable limits, by one and the same general type of mathematical expression. Such is the case with the data reported here—the average and the individual data can be described as first-order processes.

From a theoretical point of view, a mathematical expression which describes adequately all individual data need *not* necessarily fit the average data. To illustrate, one may consider the hypothetical case of two subjects, a rapid and a slow absorber.[3] Both subjects absorb a drug in a manner describable as a single first-order process but with half-lives of 10 minutes and 80 minutes, respectively. These are

[3] One may also consider a group of subjects showing bimodality in drug absorption rates, perhaps due to its composition with respect to age, sex, or state of health.

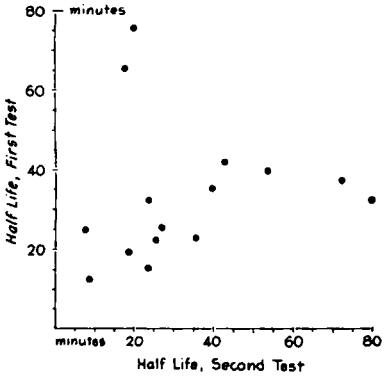


Fig. 12.—Scatter plot of individual half-lives for aspirin absorption (based on absorption rates and neglecting induction times) in two separate tests.

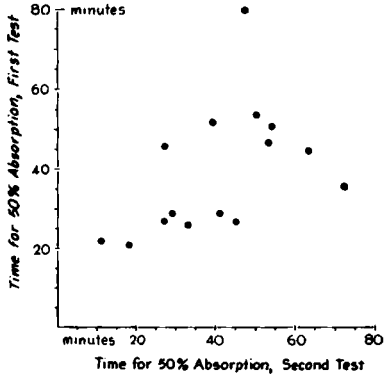


Fig. 13.—Scatter plot of individual times for 50% aspirin absorption (reflecting absorption rates and induction times) in two separate tests.

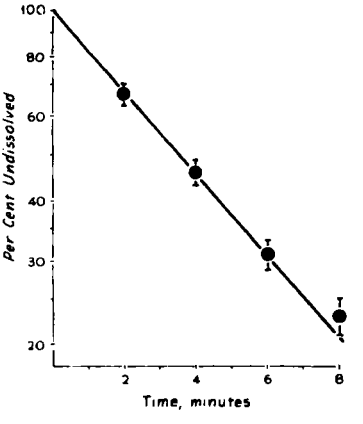


Fig. 14.—*In vitro* dissolution rate of aspirin from compressed tablets (the same lot as the tablets used in the clinical study). Average of six tablets. Vertical bars indicate ± one standard deviation.

realistic values, as evident from data shown in a subsequent figure. A semilogarithmic plot of average per cent unabsorbed as a function of time would yield the concave curve shown in Fig. 11. This curve can be resolved readily to yield two component first-order processes, and thus one may conclude that absorption occurred by two parallel first-order processes. Yet this kinetic model is unrealistic and meaningless because it fits only the average data—examination of individual data would show that absorption is describable as one exponential process.

Van Liew has shown that the sum of several exponential processes with different rate constants may yield a concave curve when plotted on semilogarithmic paper (9). The concavity from a single-mode distribution is determined by the relative magnitude of the standard distribution (SD) and the mean of the distribution. The smaller the SD/mean ratio, the more nearly the composite curve resembles a single exponential process. The straightness of the line in Fig. 4 can be explained in this manner.

The degree of correlation of individual absorption rates during the first test with rates during the second test has been examined in two ways: (*a*) by neglecting the respective induction times and plotting individual half-lives ($t_{1/2}$) for absorption rates in the first test against half-lives in the second test (Fig. 12) and (*b*) by plotting individual times for 50% drug absorption in the first test against those in the second test (Fig. 13). The latter values are a function not only of absorption rate constants but also of the respective induction times. Neither of the data depicted in the two plots yielded statistically significant correlation coefficients. There is an indication that about half of the 15 subjects absorbed drug at individually similar rates in separate tests, while the other half consisted of subjects with pronounced individual variability with respect to the rate of drug absorption. However, this suggestion should be considered as highly speculative, at least until additional data become available.[4]

Extensive studies have shown that the gastrointestinal absorption of aspirin administered in tablet form is rate-limited by the dissolution of drug in gastrointestinal fluids (10). It is reasonable to assume when absorption rate is dissolution rate dependent that the kinetic model which fits the drug absorption process will, in most instances, also apply to the *in vivo* dissolution process. As an extension of this reasoning, the suggestion may be made that a useful *in vitro* dissolution rate test for a given dosage form should yield data which are describable by the same kinetic model as the *in vivo* dissolution process and the *in vivo* absorption process.[5]

Results of dissolution-rate studies of aspirin tablets used in the clinical phase of this investigation are depicted in Fig. 14. Like the *in vivo* absorption process, the *in vitro* dissolution can be described by first-order kinetics, *viz.*,

$$a = a_o e^{-.20t} \qquad \text{(Eq. 10)}$$

or

$$\log a = -.085t + \log a_o \qquad \text{(Eq. 11)}$$

where a_o is the initial amount of drug in the tablet, and a is the amount of drug remaining undissolved at time t (which is expressed in minutes). The Levy-

[4] In some instances, variability in absorption rates can be due to variations in release properties of the dosage form. This was not a factor in the present study.

[5] It is emphasized that this statement applies only to cases where absorption rate is dissolution rate-limited. It must be appreciated also that this reasoning should be interpreted against the background of physiologic realities which may modify the suggested relationship in specific instances.

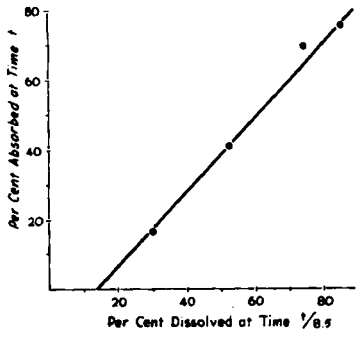


Fig. 15.—Plot of per cent aspirin absorbed at time t after drug administration *vs.* per cent dissolved *in vitro* at time $t/8.5$.

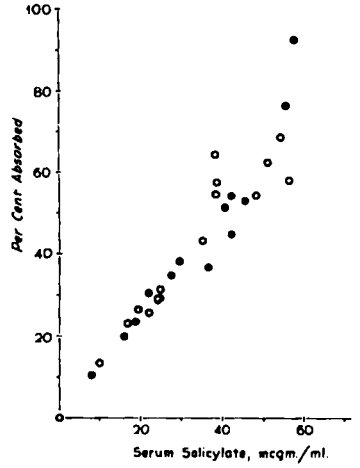


Fig. 16.—Scatter plot of per cent aspirin absorbed *vs.* serum salicylate concentration 30 minutes after ingestion of 975 mg. aspirin. Shown are 30 values from 15 subjects. Key: ●, first test; ○, second test.

Hayes dissolution-rate procedure (6) was modified in this study to obtain greater precision and to reduce saturation effects which became apparent in a previous study (5). Improvement in precision was obtained by excluding data from tablets which formed a "mound" at the edge rather than in the center of the bottom of the beaker,[6] while saturation effects due to concentration build-up in the terminal phase of the dissolution process were reduced by increasing the volume of solvent from 250 ml. to 350 ml.

The first-order rate constant for *in vitro* dissolution was about 8.5 times as great as the first-order rate constant for *in vivo* absorption. This may be a reflection of more intensive agitation of dissolution medium in the *in vitro* test. Using a procedure applied previously by Levy (5), a plot of per cent absorbed *in vivo* at time t *versus* amount dissolved *in vitro* at time $t/8.5$ was constructed and is shown as Fig. 15. The data fit satisfactorily a regression line of slope unity having an intercept at 14% dissolved *in vitro*, and thus can be expressed by

$$\%\text{ of dose absorbed at time } t = \%\text{ of dose dissolved } \textit{in vitro} \text{ at } t/8.5 - 14\% \quad \text{(Eq. 12)}$$

which describes drug absorption up to 86% of the dose. This may be compared with the expression

$$\%\text{ of dose absorbed at time } t = \%\text{ of dose dissolved } \textit{in vitro} \text{ at } t/3 - 10\% \quad \text{(Eq. 13)}$$

which Levy obtained from data on other subjects using different aspirin tablet preparations (5). In each case the data fitted a slope of unity, and in each case similar intercepts on the per cent dissolved axis were obtained. These reflect the induction time in drug absorption evident in Fig. 4. Levy and Jusko (11) have shown in experiments with rats that this induction time is due to initial accumulation of salicylate in the gastrointestinal wall which prevents or reduces markedly the entry of drug into the circulation during the first few minutes after drug administration. The question of why the intensity factor (the divisor of t) should differ almost threefold in the two studies remains. This could, of course, be due to the different subjects and clinical environments involved, but the differences also might be related to the fact that subjects in the present study participated for the first time in a test which involves intensive blood sampling, while subjects in the previous study were indoctrinated and experienced by participation in previous tests (12). Stress and apprehension are known to affect not only gastric motility but also gastric pH (13) and may modify *in vivo* dissolution and, therefore, the rate of absorption.

Finally, it is of interest to evaluate the data in terms of some of their practical implications with respect to comparative studies of absorption rates of drugs (particularly salicylates) administered in different dosage forms. How well do the "raw" blood level values reflect drug absorption rate? Using serum salicylate concentration and per cent absorbed values at 30 minutes (which is the approximate average midpoint for drug absorption in the entire group and shows the greatest spread of individual values), blood level and per cent absorbed values correlated very well (Fig. 16). This only can be the case if the subjects have similar absolute apparent volumes of distribution. Indeed, calculations based on A_∞/V values showed that 13 of the 15 subjects had apparent volumes of distribution which fell within ±10% of the mean value for the group. This fortunate occurrence would not happen if the subjects differ widely in respective body weights[7] or in relative volumes of distribution.[8] For example, the relative volume of distribution of salicylate in arthritics differs from that in normal subjects (14), due to changes in plasma protein composition characteristic of the arthritis syndrome. Consequently, salicylate blood levels from equal doses of drug are lower in arthritics than in normal subjects (14). The same is true in many other clinical situations, like infec-

[6] Data from four out of 10 tablets were excluded in this experiment. Results of all 10 dissolution determinations also fitted first-order kinetics, but standard deviations were considerably larger due to the slower dissolution of tablets which mounded at the edge. Rate constants were 0.20 minute^{-1} and 0.17 minute^{-1} based on the average of six and 10 determinations, respectively.

[7] Evaluation of drug absorption from compressed tablets and many other pharmaceutical dosage forms does not permit individual adjustment of doses to obtain a constant dose on a milligram per kilogram body weight basis.

[8] The average body weight of the 15 subjects was 71 Kg. (SD 8.5 Kg.), and the average relative volume of distribution was 170 ml./Kg. (SD 15.4 ml./Kg.).

TABLE I.—COEFFICIENTS OF VARIATION CALCULATED FROM SERUM SALICYLATE CONCENTRATION AND PER CENT ABSORBED DATA[a]

Time, Min.	First Test S.S.	First Test P.A.	Second Test S.S.	Second Test P.A.	Combined Tests S.S.	Combined Tests P.A.
15	69	66	94	105	83	90
30	53	51	44	52	48	51
60	24	22	28	28	25	25
90	19	13	18	15	18	14

[a] S.S., Coefficients based on serum salicylate concentration; P.A., coefficients based on per cent absorbed.

tion, where plasma albumin concentration is greatly reduced (15). It is also to be expected that correlation of blood level and per cent absorbed data would be poor when drug absorption rate is not considerably higher than the rate of drug elimination (assuming that elimination rate shows the usual intersubject variability).

From a similar point of view, it is of interest to compare the homogeneity of per cent absorbed values to that of blood level data. Considering the possible intersubject variations in volume of distribution and elimination rate, one would expect that actual absorption data would be more homogenous than blood level data (which would reflect also the variations in V and K values). As shown in Table I, coefficients of variation of blood level data and of absorption data obtained in the present study were generally the same, except for the greater homogeneity of absorption data when absorption was near completion (90 minutes). The latter tendency reflects the intersubject differences in elimination rates, which become most apparent in the post-absorption period. The reasons for the lack of greater homogeneity of absorption data (compared to blood level data) in the present study can be attributed to the similarity of volumes of distribution among the test subjects and also the rapid gastrointestinal absorption (compared to elimination) of aspirin. The chance exists also that in this relatively small group of subjects a fortuitous occurrence of opposite effects in any given subject may have dampened the blood level variations. In general, however, and particularly in situations where a greater diversity of V and K values is encountered and where k_a is not much greater than K, the use of actual absorption values should yield more homogenous data than are obtained by use of blood level values. This would permit a more sensitive statistical comparison of drug absorption from different dosage forms and would provide a more direct indication of absorption rates.

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Introduction

By EDWARD G. REMMERS, WILLIAM C. BARRINGER, GEORGE M. SIEGER, and ALBERT P. DOERSCHUK

Metal-acid complexes with a number of members of the tetracycline family have been prepared for chemical and biological evaluation. The studies reported here indicate that these preparations exhibited properties not displayed by the uncomplexed antibiotic. The most interesting properties characteristic of selected complexes in this series include enhanced solubility at pH 4.0–7.0, enhanced alkaline stability, reduced acute toxicity, reduced tissue irritation and rapid tissue diffusion, and enhanced blood levels.

THIS PUBLICATION is the first in a series describing the pharmaceutical properties of metal-acid complexes with members of the tetracycline family. The experimental data reported in this and subsequent publications have been obtained over the last 5 years through the cooperative efforts of a large number of investigators.

Many metal-acid complexes have been prepared having the following general formula: (tetracycline - group antibiotic) - (aluminum)a-(calcium)b-(gluconic acid)c, where a, b, and c vary widely. As the molar ratios of the constituents are varied, the pharmaceutical proper-

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TABLE I.—COEFFICIENTS OF VARIATION CALCULATED FROM SERUM SALICYLATE CONCENTRATION AND PER CENT ABSORBED DATA[a]

Time, Min.	First Test S.S.	First Test P.A.	Second Test S.S.	Second Test P.A.	Combined Tests S.S.	Combined Tests P.A.
15	69	66	94	105	83	90
30	53	51	44	52	48	51
60	24	22	28	28	25	25
90	19	13	18	15	18	14

[a] S.S., Coefficients based on serum salicylate concentration; P.A., coefficients based on per cent absorbed.

tion, where plasma albumin concentration is greatly reduced (15). It is also to be expected that correlation of blood level and per cent absorbed data would be poor when drug absorption rate is not considerably higher than the rate of drug elimination (assuming that elimination rate shows the usual intersubject variability).

From a similar point of view, it is of interest to compare the homogeneity of per cent absorbed values to that of blood level data. Considering the possible intersubject variations in volume of distribution and elimination rate, one would expect that actual absorption data would be more homogenous than blood level data (which would reflect also the variations in V and K values). As shown in Table I, coefficients of variation of blood level data and of absorption data obtained in the present study were generally the same, except for the greater homogeneity of absorption data when absorption was near completion (90 minutes). The latter tendency reflects the intersubject differences in elimination rates, which become most apparent in the post-absorption period. The reasons for the lack of greater homogeneity of absorption data (compared to blood level data) in the present study can be attributed to the similarity of volumes of distribution among the test subjects and also the rapid gastrointestinal absorption (compared to elimination) of aspirin. The chance exists also that in this relatively small group of subjects a fortuitous occurrence of opposite effects in any given subject may have dampened the blood level variations. In general, however, and particularly in situations where a greater diversity of V and K values is encountered and where k_a is not much greater than K, the use of actual absorption values should yield more homogenous data than are obtained by use of blood level values. This would permit a more sensitive statistical comparison of drug absorption from different dosage forms and would provide a more direct indication of absorption rates.

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ties, which are not displayed by salts of these antibiotics, also vary. Much of the experimental effort with these complexes has been devoted to investigation of the effects of different molar ratios on pharmaceutical properties and to determination of the minimum molar ratios of constituents in the complex which still give the desired properties.

EXPERIMENTAL

Preparation of Metal-Acid Complexes.[1]—The aluminum-acid complex was first prepared by adding aluminum isopropoxide to a solution of the acid in water or other suitable solvent to give the desired molar ratios. The resulting mixture was stirred overnight until an essentially clear solution was obtained. The antibiotic was then added as the base or hydrochloride, and stirring was continued until a clear solution was obtained. If a second metal, such as calcium or magnesium, was to be incorporated into the complex, the appropriate amount of the metal oxide was added, and the mixture was stirred until a clear solution was obtained. The pH of the mixture was elevated to 5.0–7.0 with concentrated NaOH if necessary, and the solution was filtered to remove traces of unreacted solids. The complex was recovered by lyophilization or by solvent precipitation in 10–20 vol. of a 2:1 isopropanol–petroleum ether (b.p. 60–68°) mixture. The resulting precipitate was aged for 30 minutes or longer, collected by filtration, washed with isopropanol, and dried *in vacuo* over phosphorus pentoxide at 30–40°.

Solubility Determinations.—The complexes were added to distilled water at a level of about 50 mg./ml. of antibiotic with stirring at room temperature. The pH of the solution was adjusted with either 5 *N* HCl or saturated NaOH. The solution was stirred for approximately 0.5 hour, during which the pH was readjusted to the desired level if necessary. It was then filtered; the filtrate was diluted in water and read in a Cary U.V. spectrophotometer to determine the solubility of the antibiotic.

Blood Level Studies.—Mongrel dogs of each sex, weighing 40 to 60 lb., were divided into groups of three, assigned so that the average body weight of each group was approximately the same. The antibiotic preparations were dissolved in distilled water to give a concentration of 25 to 50 mg. antibiotic/ml. and were injected intramuscularly into the biceps femoris. Blood samples were withdrawn from the cephalic vein at 1, 4, 7, and 24 hours following administration of the antibiotic. The samples were allowed to clot, ringed, and centrifuged. The clear serum was pipeted from the clot and assayed in duplicate microbiologically as tetracycline·HCl, using *B. cereus* as the test organism. The details of this assay procedure have been reported from these laboratories (1). The area under the blood level–time curve from 0 to 24 hours was computed and reported as area under the curve (AUC).

In all blood level experiments, a tetracycline·HCl standard was used in all serum assays. This procedure was followed to reduce all blood level experiments to a common standard and to conform to previous work, as reported by Kunin and Finland (2) and Sweeney *et al.* (3). Thus, all blood levels are reported in terms of tetracycline·HCl equivalents.

LD_{50} Determinations.—The method used for estimating the LD_{50} values has been described by Reed and Muench (4). Swiss albino mice (Taconic Farms) were caged in groups of 10 each; dosage was graduated so that more than 50% of the mice receiving the highest dosage succumbed, and more than 50% survived the lowest dose. The preparation to be tested was dissolved in distilled water at levels such that 0.5-ml. and 0.2-ml. quantities could be administered by intraperitoneal and intravenous routes, respectively. The intravenous dose was injected over an interval of 5–7 seconds. The mice were observed daily for a 6-day test period.

Rabbit Intradermal Irritation Test.—Rabbits weighing 4–6 Kg. were used, usually with three rabbits per group. On the day before the test, the abdomen of each rabbit was clipped with an electric clipper. Immediately before injection, the rabbits were anesthetized by administration of sodium pentobarbital into the marginal ear vein at a dose of 0.2 ml. of a 6% solution per pound of body weight.

Solutions of two chlortetracycline standards (class I "good" and class IV "bad") and two experimental preparations were made at a concentration of 50 mg. antibiotic in 10 ml. of pyrogen-free distilled water. One-milliliter tuberculin syringes were fitted with No. 27 gauge needles with intradermal bevels. One-tenth of a milliliter of each of the four solutions was injected as superficially as possible, just lateral to the mammary glands and away from the movable skin near the front and hind legs. A separate needle and syringe were used for each preparation. The injection sites were examined 16 to 20 hours after injection. The reactions of the test solutions were then compared to the good and bad standards. An average rating was computed from the results obtained with three rabbits. Preparations must have a class I (good irritation) rating before they may be used in parenteral and topical products.

RESULTS AND DISCUSSION

Table I lists some of the antibiotics, metals, and acids used in these complexes. To date, every tetracycline antibiotic tried in these preparations has undergone complex formation. Of the various

TABLE I.—ANTIBIOTICS, METALS, AND ACIDS USED IN METAL-ACID COMPLEXES

Antibiotics	Metals	Acids
Chlortetracycline	Aluminum	Gluconic
6-Demethylchlortetracycline	Calcium	Metaphosphoric
Tetracycline	Magnesium	Pyrophosphoric
Oxytetracycline	Iron	Polyphosphoric
6-Demethyltetracycline	Tungsten	Orthophosphoric
6-Deoxy-6-demethyltetracycline	Uranium	Lactic
6-Deoxytetracycline		Boric
9-Amino-6-demethyl-6-deoxytetracycline		

[1] The procedures employed in preparing these complexes are described more fully by Ritter, I., U. S. pat. 2,736,725 and by Sieger, G. M., and Weidenheimer, J. F., U. S. pat. 3,053,892.

TABLE II.—PHARMACEUTICAL PROPERTIES OF METAL-ACID COMPLEXES

(*a*) Increased solubility in the pH range of 4.0–7.0.
(*b*) Enhanced alkaline stability.
(*c*) Reduced acute toxcity.
(*d*) Reduced tissue irritation and rapid tissue diffusion.
(*e*) Enhanced blood levels.

metals used, aluminum, calcium, magnesium, and iron have produced the most interesting pharmaceutical properties. Unfortunately, complexes containing iron have a relatively dark color due to reaction of iron with a phenolic hydroxyl group of the antibiotic. As a result, a major portion of effort was devoted to complexes with the other metals. Of the various acids employed to date, polyhydroxy acids and phosphorus-containing acids have given the most interesting results. In preparing metal-acid complexes, the two following criteria have been employed in determining if complex formation has occurred: (*a*) changes in the ultraviolet absorption spectrum and (*b*) solubility behavior in the region of pH 4.0–7.0.

If complex formation has occurred, the ultraviolet absorption peak in the 355–370 mμ region, due to the C-7 to C-12 chromophore (5), is shifted to longer wavelengths. The complex will also remain in solution in the region of pH 4.0–7.0, where simple salts of the tetracycline family are hydrolyzed, and the neutral form of the antibiotic precipitates from solution.

The important pharmaceutical properties that have been observed with selected preparations are shown in Table II. Each property will be discussed in detail.

Increased Solubility.—Compared to the uncomplexed antibiotic, metal-acid complexes generally are more soluble in the pH range of 4.0 to 7.0, in which many pharmaceutical preparations are formulated. Figure 1 compares the pH solubility curve for 6-demethylchlortetracycline (DMCTC) to the corresponding curves for two DMCTC-metal-acid complexes. Over the pH range of 3.0 to 7.0, the uncomplexed antibiotic is soluble to the extent of 3 to 7 mg./ml. In contrast, the DMCTC complexes exhibit comparatively high solubility over this pH range. The solubility of DMCTC-aluminum-gluconate (molar ratio 1:4:6.6) exceeds 50 mg./ml. of antibiotic over the pH range of 2.0 to 10.0. The pH solubility curve for DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:2:12) indicates the effect on solubility of incorporation of

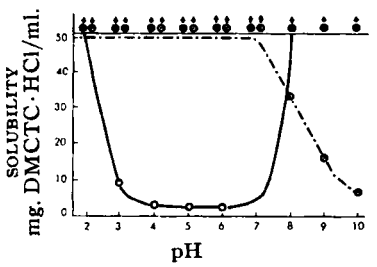


Fig. 1.—pH solubility curves for DMCTC and two DMCTC-metal-acid complexes. Key: O, DMCTC; ●, DMCTC-Al-gluconate (1:4:6.6); ⊙, DMCTC-Al-Ca-gluconate (1:4:2:12).

calcium into the complex. When DMCTC-aluminum-gluconate complexes are prepared with graduated amounts of calcium in the complex, the solubility on the alkaline side of neutrality is depressed.

Enhanced Alkaline Stability.—The enhanced alkaline stability of aluminum-gluconate complexes with graduated amounts of calcium has been reported by Remmers *et al.* (6), where the incorporation of 1.0 or more molar parts of calcium into the complex markedly increased the alkaline stability of most, but not all, of the tetracycline antibiotics. Of those tested to date, only chlortetracycline is unusual since studies with this antibiotic have repeatedly failed to demonstrate greatly enhanced alkaline stability.

Acute Toxicity.[2]—Prior to extensive biological evaluation of these complexes, representative preparations were submitted for acute toxicity studies in mice. Table III presents the LD_{50} determinations for DMCTC·HCl and three selected DMCTC-metal-acid complexes in mice following intraperi-

TABLE III.—LD_{50} VALUES FOR SELECTED METAL-ACID COMPLEXES IN MICE[a]

Derivative	Administration Rt. i.p.	i.v.
DMCTC·HCl	273	89
DMCTC-aluminum-gluconate (molar ratio 1:4:6.6)	495	698
DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12)	420	306
DMCTC-aluminum-pyrophosphate (molar ratio 1:1:1)	1,810	260

[a] Milligrams DMCTC·HCl per kilogram body weight.

TABLE IV.—RESULTS FROM RABBIT INTRADERMAL IRRITATION TEST

Prepn.	Rating
Bad chlortetracycline standard	Class IV
DMCTC neutral (starting material for complex)	Class II
DMCTC-aluminum-calcium-gluconate (1:4:5:12)	<Class I
Good chlortetracycline standard	Class I

toneal and intravenous administration. The LD_{50} values for the complexes are approximately 1.5 to 8.0 times higher than the values obtained with the uncomplexed antibiotic when compared on an antibiotic-content basis.

The DMCTC-aluminum-pyrophosphate complex (molar ratio 1:1:1) resulted in a comparatively high LD_{50} value after intraperitoneal administration and a relatively low LD_{50} value after intravenous administration. This phenomenon is probably the result of serum binding of calcium; complexes whose calcium binding has not been satisfied produce blood pressure depression when administered intravenously By incorporating calcium into the complex or by administering the complex in a solution containing available calcium ions, the blood pressure depression can be essentially eliminated.

Changes in Tissue Irritation and Diffusion.—An important characteristic of these complexes is their ability to minimize or to eliminate the irritating

[2] Acute toxicity studies and blood level experiments were performed under the direction of Dr. J. J. Corbett and Mr. N. Anagnostakos, Biological Assay Development Laboratory.

TABLE V.—MOLECULAR WEIGHT DETERMINATIONS FOR SELECTED METAL-ACID COMPLEXES

Derivative	Mol. Wt.	Degree of Homogeneity
DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12)	990	Homogeneous
DMCTC-aluminum-pyrophosphate (molar ratio 1:1:1)	1,350	Homogeneous
DMCTC-aluminum-metaphosphate (molar ratio 1:4:12)	2,000 [Higher molecular wt. fraction]	Two fractions

properties of selected batches of antibiotic in the rabbit intradermal irritation test.[3] Table IV presents the results obtained with a metal-acid complex; approximately 20 hours following intradermal administration, the complex has diffused away from the injection site and has produced no signs of erythema. Metal-acid complexes consistently diffuse so rapidly from the injection site that in many cases it is difficult to identify the site 16 to 20 hours later. When DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12) is prepared from DMCTC having a class II rating, the resulting complex has an irritation rating less than class I.

Molecular weight determinations were made on selected metal-acid complexes in the ultracentrifuge.[4] Table V indicates that molecular weights ranged from 990 to 2,000. In most cases the complexes behaved as a homogeneous entity in the ultracentrifuge. The DMCTC - aluminum - metaphosphate complex (molar ratio 1:4:12) was the only preparation displaying heterogeneity. The molecular weight of the lighter fraction was too low to be estimated accurately. Disk diffusion studies performed on several microbiological media indicated that DMCTC-metal-acid complexes diffused as rapidly as uncomplexed DMCTC·HCl.[5]

Blood Levels.—A third important property observed with selected metal-acid complexes is the enhanced blood levels obtained compared to levels resulting from salts of the corresponding uncomplexed antibiotic. Figure 2 shows blood levels obtained with DMCTC-aluminum-magnesium-gluconate (molar ratio 1:4:5:12) and DMCTC·HCl when administered intramuscularly to dogs at a level of 1.5 mg. antibiotic per pound of body weight. Blood levels of the group of three dogs receiving the DMCTC·HCl resulted in an AUC of 20.3 mcg./hours/ml. When the dogs received the antibiotic as the DMCTC-aluminum-magnesium-gluconate complex, an AUC of 99.6 mcg./hours/ml. was obtained or nearly a fivefold enhancement. After administration at a level of 1.5 mg. per pound of body weight, irritation evidenced by limping was noted in both groups of dogs in this experiment.

Figure 3 presents the results of a blood level study in which the DMCTC complex was administered at a reduced dosage. The control group of three dogs received DMCTC·HCl at a level of 2.5 mg. per pound of body weight. A second group received DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12) at a level of 0.8 mg. DMCTC·HCl per pound of body weight. After a dosage reduction, in terms of antibiotic content that was approximately threefold, the DMCTC complex resulted in blood levels nearly twice as high in terms of AUC as those obtained with DMCTC·HCl. The level of 2.5 mg. DMCTC·HCl per pound of body weight produced considerable irritation in the dogs, while little or no irritation occurred in dogs receiving the DMCTC complex. Thus, it is possible to reduce the dosage of the antibiotic when administered as the complex to a level where little or no irritation is produced, but where acceptable blood levels are maintained.

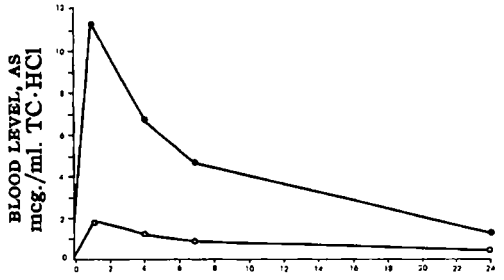


Fig. 2.—Average blood levels of three dogs per group following intramuscular administration of DMCTC·HCl and DMCTC-metal-acid-complex at a dose of 1.5 mg. DMCTC·HCl/lb. body weight. Key: O, DMCTC·HCl, AUC = 20.3 mcg./hours/ml. (formulation pH 4.0); ●, DMCTC-Al-Mg-gluconate (1:4:5:12), AUC = 99.6 mcg./hours/ml. (formulation pH 4.5).

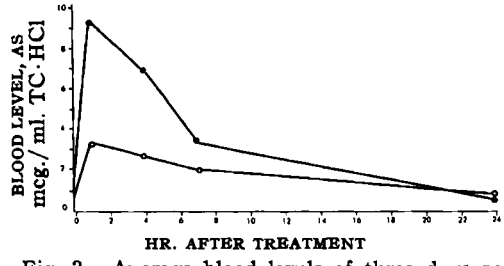


Fig. 3.—Average blood levels of three dogs per group following intramuscular administration of DMCTC·HCl and DMCTC-metal-acid complex. Key: O, DMCTC·HCl at 2.5 mg./lb., AUC = 45.5 mcg./hours/ml. (formulation pH 4.0); ●, DMCTC-Al-Ca-gluconate (1:4:5:12) at 0.8 mg. DMCTC·HCl/lb., AUC = 80.1 mcg./hours/ml. (formulation pH 4.5).

[3] Rabbit intradermal irritation studies were performed under the direction of Mr. G. R. Personeus and Mr. A. Tonelli, Quality Control Section.

[4] Molecular weight determinations were made under the direction of Mr. R. L. Davies, Biochemical Research Section.

[5] Disk diffusion studies were performed under the direction of Mr. A. C. Dornbush, Biochemical Research Section.

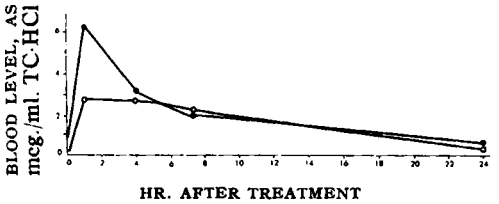


Fig. 4.—Average blood levels of three dogs per group following intramuscular administration of OTC and DMCTC-Al-Ca-gluconate. Key: O, OTC at 2.5 mg. OTC neutral/lb., AUC = 38.6 mcg./hours/ml. (formulation pH 8.5). ●, DMCTC-Al-Ca-gluconate (1:4:5:12) at 0.3 mg. DMCTC·HCl/lb., AUC = 45.3 mcg./hours/ml. (formulation pH 4.5).

Figure 4 shows the blood levels obtained with DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12) and a commercially available preconstituted oxytetracycline intramuscular formulation in 75% propylene glycol (Pfizer) for human administration. The DMCTC complex was administered intramuscularly to dogs at 0.3 mg. DMCTC·HCl per pound of body weight and the oxytetracycline (OTC) at a level of 2.5 mg. OTC base per pound of body weight. On an antibiotic-weight basis, approximately one-eighth as much antibiotic was administered as the complex. However, the blood levels produced nearly equal AUC's in terms of tetracycline·HCl equivalents (Fig. 4). Some of the blood level enhancement observed in this experiment results from the fact that DMCTC is more active microbiologically on a weight basis than OTC when assayed against a tetracycline·HCl standard. To date, many blood-level studies have been made in dogs, rabbits, and rats with complexes containing various tetracycline antibiotics orally, intramuscularly, and intravenously. The degree of blood level enhancement, which has been demonstrated repeatedly, is dependent on the route of administration and the constituents in the complex.

The mechanism by which blood-level enhancement occurs is not completely understood. One hypothesis is that blood-level enhancement results from limited tissue penetration of the antibiotic complex from the blood stream.

Blood Level Experiments in Humans.—Katz (7) and Katz and Fedorko (8) reported the results of a clinical trial in humans using DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12). They found that intramuscular administration of this complex in single doses equivalent to 25 and 50 mg. of DMCTC·HCl produced significantly higher serum levels than would have been expected from considerably larger doses of tetracycline. These doses were in a range normally expected to be therapeutic. However, several patients with tetracycline-antibiotic-susceptible infections did not respond satisfactorily at a dose of 50 mg. per day. The response was improved by increasing the dose to 100 mg. per day.

SUMMARY

Chemical and biological evaluation of metal-acid complexes with members of the tetracycline family indicate that these preparations exhibited properties that were not displayed by the uncomplexed antibiotic. The most interesting properties which are characteristic of selected complexes include enhanced solubility and alkaline stability, reduced acute toxicity and tissue irritation, and enhanced blood levels.

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ERRATUM

In the paper titled "Preparation of a Phase Diagram for Coacervation" (1), Eq. 6 on page 519 should read:

$$n = \frac{(\Delta \text{ sp. gr.})(D) - (\Delta \text{ R. I.})(\Delta \text{ sp. gr. } + B)}{(AD - BC) - (\Delta \text{ sp. gr.})(C - D)} \qquad \text{(Eq. 6)}$$

(1) Phares, R. E., Jr., and Sperandio, G. J., This Journal, **53**, 518(1964).

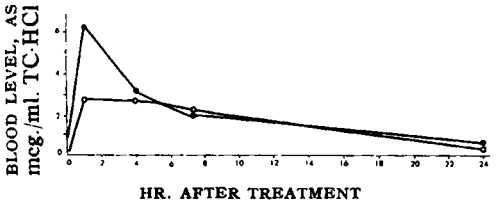


Fig. 4.—Average blood levels of three dogs per group following intramuscular administration of OTC and DMCTC-Al-Ca-gluconate. Key: O, OTC at 2.5 mg. OTC neutral/lb., AUC = 38.6 mcg./hours/ml. (formulation pH 8.5). ●, DMCTC-Al-Ca-gluconate (1:4:5:12) at 0.3 mg. DMCTC·HCl/lb., AUC = 45.3 mcg./hours/ml. (formulation pH 4.5).

Figure 4 shows the blood levels obtained with DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12) and a commercially available preconstituted oxytetracycline intramuscular formulation in 75% propylene glycol (Pfizer) for human administration. The DMCTC complex was administered intramuscularly to dogs at 0.3 mg. DMCTC·HCl per pound of body weight and the oxytetracycline (OTC) at a level of 2.5 mg. OTC base per pound of body weight. On an antibiotic-weight basis, approximately one-eighth as much antibiotic was administered as the complex. However, the blood levels produced nearly equal AUC's in terms of tetracycline·HCl equivalents (Fig. 4). Some of the blood level enhancement observed in this experiment results from the fact that DMCTC is more active microbiologically on a weight basis than OTC when assayed against a tetracycline·HCl standard. To date, many blood-level studies have been made in dogs, rabbits, and rats with complexes containing various tetracycline antibiotics orally, intramuscularly, and intravenously. The degree of blood level enhancement, which has been demonstrated repeatedly, is dependent on the route of administration and the constituents in the complex.

The mechanism by which blood-level enhancement occurs is not completely understood. One hypothesis is that blood-level enhancement results from limited tissue penetration of the antibiotic complex from the blood stream.

Blood Level Experiments in Humans.—Katz (7) and Katz and Fedorko (8) reported the results of a clinical trial in humans using DMCTC-aluminum-calcium-gluconate (molar ratio 1:4:5:12). They found that intramuscular administration of this complex in single doses equivalent to 25 and 50 mg. of DMCTC·HCl produced significantly higher serum levels than would have been expected from considerably larger doses of tetracycline. These doses were in a range normally expected to be therapeutic. However, several patients with tetracycline-antibiotic-susceptible infections did not respond satisfactorily at a dose of 50 mg. per day. The response was improved by increasing the dose to 100 mg. per day.

SUMMARY

Chemical and biological evaluation of metal-acid complexes with members of the tetracycline family indicate that these preparations exhibited properties that were not displayed by the uncomplexed antibiotic. The most interesting properties which are characteristic of selected complexes include enhanced solubility and alkaline stability, reduced acute toxicity and tissue irritation, and enhanced blood levels.

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ERRATUM

In the paper titled "Preparation of a Phase Diagram for Coacervation" (1), Eq. 6 on page 519 should read:

$$n = \frac{(\Delta \text{ sp. gr.})(D) - (\Delta \text{ R. I.})(\Delta \text{ sp. gr. } + B)}{(AD - BC) - (\Delta \text{ sp. gr.})(C - D)} \qquad \text{(Eq. 6)}$$

(1) Phares, R. E., Jr., and Sperandio, G. J., This Journal, **53**, 518(1964).

Analogs of Tetrahydrofolic Acid XV

Mode of Binding of 2,4-Diaminopyrimidines to Folic Reductase and Thymidylate Synthetase

By B. R. BAKER and BENG-THONG HO

A series of compounds related to 5-(3-anilinopropyl)-2,4-diamino 6-methyl-4-pyrimidine (XIII) was synthesized and evaluated as inhibitors of dihydrofolic reductase and thymidylate synthetase. Strong evidence was obtained that both amino groups were binding groups to both enzymes. Inhibition by 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (I*b*) was lost with both enzymes when the 2-amino group was replaced by 2-dimethylamino (XXII). When the 4-amino group of XIII was replaced by a 4-dimethylamino, a 26-fold decrease in binding to dihydrofolic reductase was observed. In contrast, this structural change caused little change in the extent of inhibition of thymidylate synthetase. The 2-amino group of I*b* probably binds to both enzymes (E) through an N—H ← E hydrogen bond. The 4-hydroxyl group of I*b* probably binds to dihydrofolic reductase by an O—H ← E hydrogen bond of the less favored 4-hydroxy tautomer but to thymidylate synthetase by a C=O → H—E hydrogen bond of the more stable 4-oxo tautomer. Since 2,4-diaminopteridines bind to folic reductase 10 to fiftyfold stronger than similarly substituted 2,4-diamino-5,6-dialkylpyrimidines, it is probable that N_5 or N_8 of the pteridine is an additional binding group. In contrast, 2-amino-4-hydroxy-5,6-dialkylpyrimidines bind to folic reductase about fivefold better than the corresponding 2-amino-4-hydroxypteridines, an indication that neither N_5 nor N_8 of the latter is a binding group. It is proposed that the imidazole ring of a histidine is present in the active site of folic reductase in order to transfer a proton to one side of a pyrazine double bond when hydride ion is transferred from TPNH to the other side of the double bond. It is also proposed that this imidazole ring forms a complex (*a*) with N_5 or N_8 of 2,4-diaminopteridines, (*b*) with a 6-phenyl or 6-benzyl group attached to a 2-amino-4-pyrimidinol, or (*c*) with a 5-phenyl group attached to a 2,4-diaminopyrimidine.

THE HIGH biological potency of 4-amino-4-deoxyfolic acid (aminopterin) (XXVIII) and the use of this compound as an antileukemic agent have been known for about 15 years (1). The lethal effect of aminopterin (XXVIII) on mammalian cells is most probably due to its strong inhibition of the important enzyme, folic reductase, which reduces folic acid (XXIX) to its cofactor form, tetrahydrofolic acid (1). Some years later, Werkheiser (2) showed that aminopterin (XXVIII) was bound to folic reductase 10,000 times stronger than the substrate, folic acid (XXIX), a remarkable increase in binding for such a simple structural change.

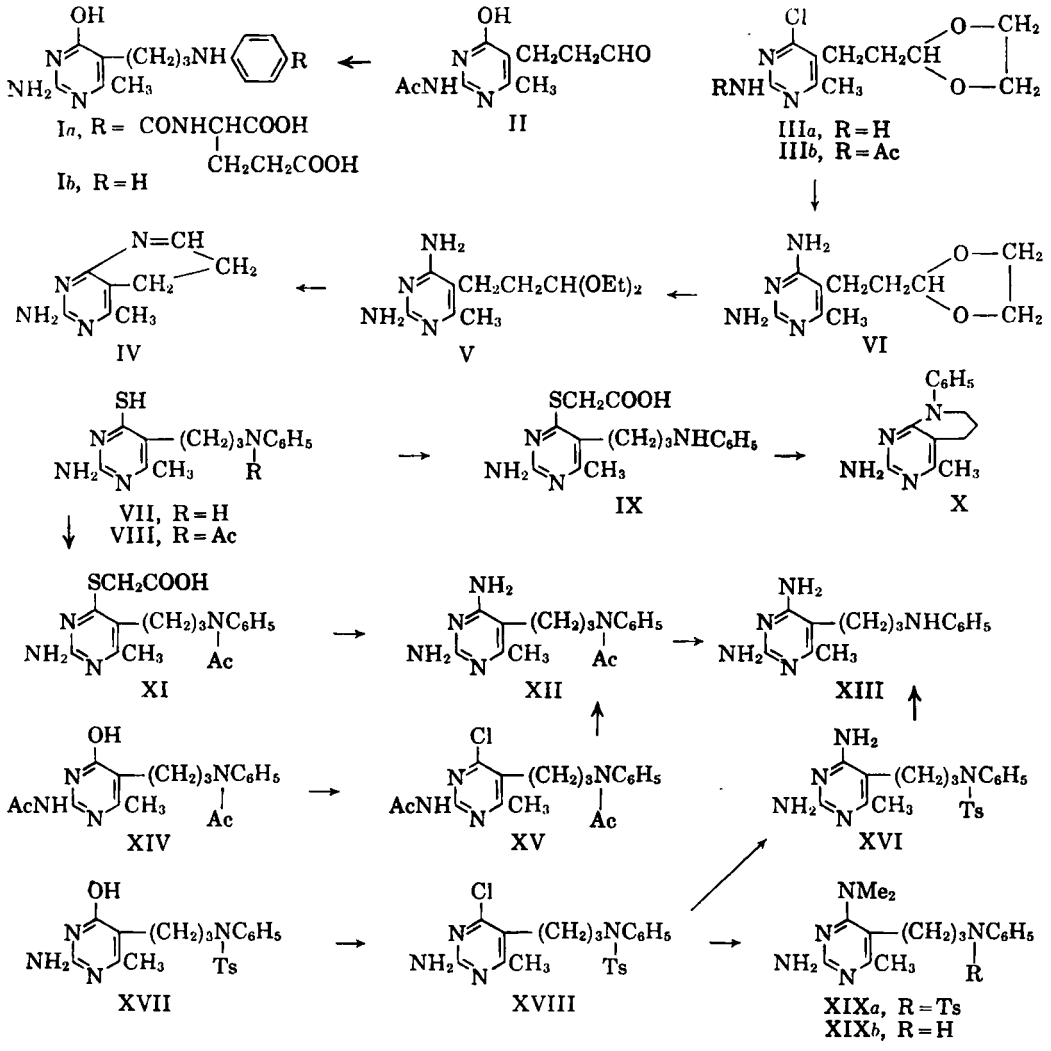
On the basis that the 2,4-diaminopteridine moiety of aminopterin was a much stronger base than the 2-amino-4-hydroxypteridine moiety of folic acid, Baker (3) proposed that the greater extent of protonation at the 2-amino group of aminopterin could lead to increased binding to an anionic site on the enzyme, and such an ionic bond could account for 6–7 Kcal. increase in free energy of binding. Zakrzewski (4) showed by thermodynamic studies that folate most probably had to be converted by the enzyme from its normal 3-H-4-oxo tautomer to the 4-hydroxy tautomer before hydrogen binding of this group to the enzyme could take place. The 4-amino group of aminopterin already exists mainly as the 4-amino tautomer and could therefore readily form two hydrogen bonds with the enzyme through the 4-NH and 3-N groups. Since hydrogen bonds have 2–5 Kcal. (5) of bonding energy, two additional hydrogen bonds in the 4-amino structure would give 4–10 Kcal. of free energy of binding, possibly within the range of the observed 6–7 Kcal. increase in free energy of binding with aminopterin. Furthermore, Zakrzewski (6) observed that there was no correlation between inhibition of folic reductase by a series of 2,4-diaminoheterocycles and their basicity, which made Baker's earlier hypothesis (3) seemingly untenable; however, these 2,4-diamino compounds (6) had other structural changes which might have influenced binding in a way to negate correlation with basicity. Therefore, the authors felt that a further study of binding to folic reductase with a more closely related series of compounds might shed additional light on the

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Previous paper: Baker, B. R., Santi, D. V., and Shapiro, H. S., THIS JOURNAL, 53, 1317(1964).



remarkably strong binding of aminopterin (XXVIII).

A previous paper (7) from this laboratory showed that the tetrahydrofolate analog (I*a*) with $K_i = 2 \times 10^{-6}$ was bound to folic reductase stronger than the substrate, folic acid, with $K_m = 10 \times 10^{-6}$. Furthermore, the simplified structure I*b* without the carboxy-L-glutamate moiety was still a reasonably good inhibitor of folic reductase with $K_i = 63 \times 10^{-6}$ (8). A further study (9) showed that the 2-amino group of I*b* contributed more to binding to (dihydro)folic reductase than the 4-hydroxyl group did, but binding at the 4-position was increased with a 4-mercapto group. The synthesis and mode of binding of some pyrimidyl analogs, where the substituents at the 2- and 4-positions of I*b* have been varied with amino, dimethylamino, and hydrogen substituents, will now be reported. The effects of these groupings on binding of I*b* to thymidylate synthetase are also reported.

EXPERIMENTAL

Methods

Analogs of type I were originally synthesized by reductive condensation of the 5-pyrimidylpropionaldehyde (II) with the appropriate arylamine (7, 8). Therefore, the initial efforts toward synthesis of the 2,4-diamino analog (XIII) were aimed at synthesis of the key intermediate, 2,4-diacetamido-6-methyl-5-pyrimidylpropionaldehyde. Reaction of the 2-amino-4-chloropyrimidine bearing a dioxolane blocked aldehyde group (III*a*) (9) with methanolic ammonia for 24 hours in a sealed vessel at 150° gave the hydrochloride salt of VI in good yield. In the synthesis of II and related compounds it was necessary to exchange the dioxolane blocking group for the diethyl acetal blocking group (7, 10) before the aldehyde function could be regenerated; therefore, the 2,4-diaminopyrimidyl dioxolane (VI) was reacted with alcoholic sulfuric acid. Instead of the expected diethylacetal (V), an air-unstable product was obtained that showed a bathochromic shift in the ultraviolet from that expected for V. It appeared that cyclization to IV had taken place, a compound that would be expected to be unstable to air and would absorb at longer wavelength than V.

The availability of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinethiol (VII) (10) suggested that attempts be made to replace the mercapto group by amino. Since the carboxymethylthio group is a better leaving group than the methylthio group for nucleophilic displacement on a pyrimidine ring (11), VII was reacted with chloroacetic acid in water. Less than 2% of the desired 4-carboxymethylthiopyrimidine (IX) was formed; the major product was the cyclized piperidino [2,3-*d*] pyrimidine (X) formed by anchimeric displacement of the carboxymethylthio group of IX by the neighboring anilino group. The cyclic compound (X) had an ultraviolet spectrum almost identical to that of 2-amino-4-(*N*-methylanilino)-5,6-trimethylenepyrimidine (12), as would be expected. Thus, it was apparent that even if the yield of IX could be increased by conducting the reaction in acid solution to protonate the anilino group, it would not be possible to displace the carboxymethylthio group of IX with ammonia since the anchimeric cyclization to X would be more rapid.

To avoid cyclization of the anilino group, the *N*-acetylanilino pyrimidinethiol (VIII) (13) was reacted with chloroacetic acid in boiling methanol; the rate of reaction was followed by the shift of 350 mμ peak of VIII to 310 mμ. The reaction required 20 hours for completion. Reaction was faster in water, being complete in less than 2 hours. The yield of XI was better (91%) in the aqueous medium than in methanol (45%). Unfortunately, the carboxymethylthio group of XI was not a leaving group sufficiently strong to be displaced by ammonia below the decomposition temperature of XI. At 140° for 24 hours in concentrated ammonia water, conditions strong enough to convert a 2-amino-4-chloropyrimidine (III) to the diamine (VI), XI was recovered unchanged. At higher temperature (190°) conversion to XII apparently occurred along with some decomposition, as shown by the characteristic shift in ultraviolet spectrum in such a conversion (12). Although this route was probably successful, the length of the synthesis (15 steps) was considered impractical, and a shorter synthesis was sought.

Acetylation of I*b* with acetic anhydride in pyridine at about 90° gave an 80% yield of crystalline diacetyl derivative (XIV). Reaction of XIV with phosphorus oxychloride in benzene at 85° seemed rapid (10), but the resultant chloropyrimidine (XV) was difficult to purify and may have been contaminated with unchanged XIV. Treatment of crude XV with ammonia in methanol at 140° gave XII as a glass that had the proper ultraviolet spectrum; if the anilino *N*-acetyl group had not remained intact, then the product could have been expected to be piperidino-pyrimidine, X, which has a quite different ultraviolet spectrum. Hydrolysis of the *N*-acetyl group with boiling 6 *N* hydrochloric acid gave a mixture of the desired diaminopyrimidine (XIII) and the starting 2-amino-4-pyrimidinol (I*b*); these two compounds were separated without much difficulty by taking advantage of the stronger basicity of XIII; but the over-all yield was low. Whether the I*b* arose from contamination of the chloropyrimidine (XV) with unchanged XIV or whether the 2,4-diaminopyrimidine (XIII) was partially hydrolyzed to I*b* during the acid treatment (14) was not resolved.

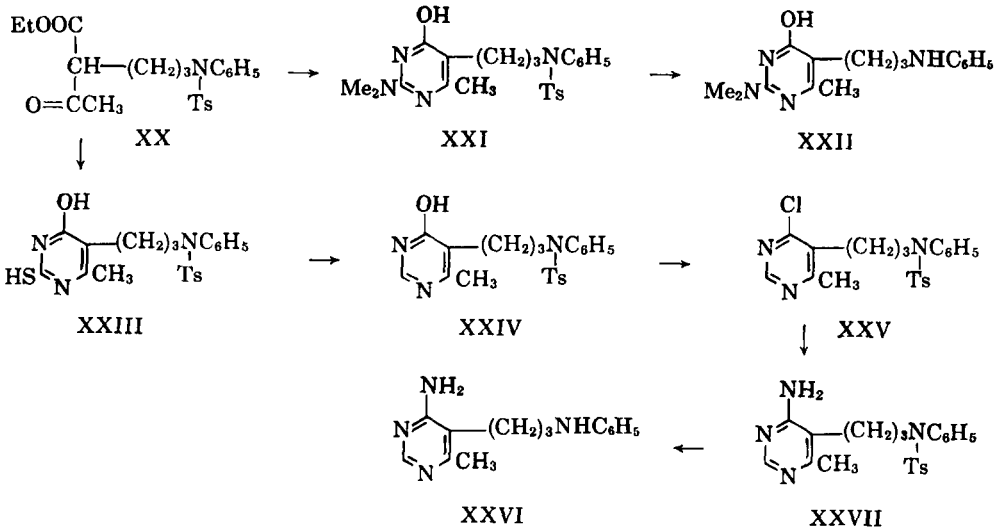
This route to the diaminopyrimidine (XIII) still involved 11 steps *via* 2-acetamido-4-hydroxy-6-methyl-5-pyrimidylpropionaldehyde (II) and I*b*, and the over-all yield was quite low. However, the high potency of XIII as an inhibitor of dihydrofolic reductase and the concomitant emergence of a facile three-step synthesis (15) of the *N*-tosyl derivative (XVII) of I*b* suggested that the conversion of XVII to the diaminopyrimidine (XIII) be investigated.

Reaction of pure XVII with boiling phosphorus oxychloride required 45 minutes for completion, and the crystalline chloropyrimidine (XVIII) was obtained in 80% yield. Treatment of XVIII with methanolic ammonia at 150° for 24 hours afforded the pure crystalline, blocked diaminopyrimidine (XVI) in 64% yield as its hydrochloride. The fusion of ammonium acetate with the chloropyrimidine (XVIII) at 165°—as described for the conversion of certain 2-methylthiopyrimidines to 2-aminopyrimidines (16)—mainly gave reversion to the pyrimidinol (XVII) in 71% yield, and only a 6% yield of the diaminopyrimidine (XVI) was obtained. The tosyl group of XVI was smoothly removed by reductive cleavage with sodium in liquid ammonia (15) to give the desired diaminopyrimidine (XIII) in 85% yield. Thus, this last synthesis of XIII required only six steps, each of which proceeded in 70–90% yield. The *N*-tosyl group of XVI could also be cleaved with 30% hydrogen bromide in acetic acid in the presence of phenol (15, 17) to give 82% of XIII.

Reaction of the chloropyrimidine (XVIII) with ethanolic dimethylamine at 140° for 24 hours—the conditions used for conversion of XVIII to XVI—led to a dark brown syrup which had the ultraviolet spectra expected for a 2-amino-4-dimethylaminopyrimidine (12); however, it could not be crystallized. The severity of the conditions was gradually reduced to the minimum, which surprisingly was only 1 hour at 120°. White crystalline XIX*a* was then readily isolated in 40% yield with the unexpectedly low melting point of 74°. Cleavage of the *N*-tosyl group of XIX*a* with hydrogen bromide in acetic acid containing phenol (15, 17) gave the dimethylamino pyrimidine (XIX*b*) in satisfactory yield.

When the keto ester (XX) (15) was reacted with *N,N*-dimethylguanidine, the crystalline 2-dimethylaminopyrimidine (XXI) was obtained in only fair yield. Cleavage of the *N*-tosyl group with sodium and liquid ammonia afforded the desired 2-dimethylamino - 5 - (anilinopropyl) - 6 - methyl - 4 - pyrimidinol (XXII) in 81% yield.

The final compound needed for enzymic comparison was the 4-aminopyrimidine (XXVI). The synthesis of XXVI started with condensation of the keto ester (XX) (15) with thiourea in methanolic sodium methoxide; the crystalline 2-mercaptopyrimidine (XXIII) was readily isolated in 71%. Desulfurization of XXIII with sponge nickel in boiling 2-methoxyethanol afforded 58% of the 4-pyrimidinol (XXIV). Treatment of XXIV with phosphorus oxychloride in boiling benzene gave a crystalline precipitate that was the phosphate salt of the chloropyrimidine (XXV). Conversion of the phosphate salt to the free base with aqueous sodium bicarbonate afforded an 84% over-all yield of crystalline XXV from XXIV. The chloro group of XXV was displaced with ammonia in methanol at 140° during 5 hours, giving a quantitative yield of the



blocked 4-aminopyrimidine (XXVII). Detosylation with sodium in liquid ammonia proceeded smoothly to afford the desired crystalline 4-aminopyrimidine (XXVI) in 76% yield.

Synthesis

Melting points were taken on a Fisher-Johns apparatus, and those below 230° are corrected. Infrared spectra were determined with a Perkin-Elmer 137 B spectrophotometer in KBr disk unless otherwise indicated; ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer.

2,4 - Diamino - 5 - [2 - (1,3 - dioxolan - 2 - yl)-ethyl]-6-methyl-4-pyrimidine (VI) Hydrochloride.—A mixture of 500 mg. of pure III*b* (10) and 10 ml. of ethanol saturated with ammonia at 0° was heated in a steel bomb at 150–155° for 24 hours. Evaporation to dryness *in vacuo* left 506 mg. of crude crystalline product, m.p. 207–210°. Recrystallization from methanol-acetone gave 234 mg. (51%) of white crystals, m.p. 214–215°; $\nu_{max.}$ 3300, 3150 (NH); 1660 (C=NH$^+$) 1625, 1520 (NH, pyrimidine); 1100, 1040, 1015 cm.$^{-1}$ (ether C—O—C); $\lambda_{max.}^{pH\ 1,7}$ 277 mμ (ε 7800); $\lambda_{max.}^{pH\ 13}$ 287 mμ (ε 8100).

Anal.—Calcd. for $C_{10}H_{17}ClN_4O_2$: C, 46.1; H, 6.57; N, 21.5. Found: C, 45.7; H, 6.40; N, 21.2

Similarly, III*a* (9) gave 65% of recrystallized product and was considered the method of choice because of the ease of obtaining pure III*a* and the large losses involved in obtaining pure III*b* (10).

2 - Amino - 4 - methyl - 8 - phenylpiperido[2,3-d]-pyrimidine (X).—A mixture of 100 mg. (0.364 mmole) of VII and 34 mg. (0.364 mmole) of chloroacetic acid in 2 ml. of water was refluxed with stirring for 45 minutes. After being cooled, the mixture was filtered, and the insoluble crystals were collected on a filter; yield, 2 mg. (1.6%). Its ultraviolet spectra indicated its structure was IX, since it was close to that described for 2-amino-4-methylthio-5,6-trimethylenepyrimidine (12); $\lambda_{max.}^{pH\ 1}$ 240, 274, 318, and $\lambda_{max.}^{pH\ 13}$ 310 mμ.

The filtrate was adjusted to pH 12 with 10% sodium hydroxide. The crystalline X was collected on a filter and washed with water; yield, 25 mg. (29%), m.p. 141°; $\nu_{max.}$ 3320, 3200 (NH); 1600, 1500 (NH, pyrimidine, C=C); 760, 695 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}^{pH\ 1,7}$ 290 (ε 13,100), inflection centering at 245 mμ (ε 9200); $\lambda_{max.}^{pH\ 13}$ 305 (ε 12,500), inflection centering at 252 mμ (ε 8200).

Anal.—Calcd. for $C_{14}H_{16}N_4$: C, 70.0; H, 6.71; N, 23.3. Found: C, 69.7; H, 6.70; N, 23.6.

2 - Amino - 4 - carboxymethylthio - 5 - (N - acetyl-3-anilinopropyl)-6-methylpyrimidine (XI).—A mixture of 100 mg. (0.32 mmole) of VIII (13), 4 ml. of water, and 41 mg. (0.43 mmole) of chloroacetic acid was refluxed for 2 hours, then chilled and neutralized to pH 5. The product was collected on a filter and washed with water; yield, 107 mg. (91%), m.p. 218–221°. Recrystallization from ethanol raised the melting point to 225–226°; $\nu_{max.}$ 3400, 3000, 2960, 2750, 2350, 1950–1860 (NH, NH$^+$); 1700 (C=NH$^+$); 1600 cm.$^{-1}$ (COO$^-$, pyrimidine), indicating a zwitterion structure; $\lambda_{max.}^{pH\ 1}$ 271 (ε 10,500), shoulder at 232 mμ (ε 20,600); $\lambda_{max.}^{pH\ 7,13}$ 233 (ε 20,000), 306 mμ (ε 9900).

Anal.—Calcd. for $C_{18}H_{22}N_4O_3S$: C, 57.7; H, 5.92; N, 15.0. Found: C, 57.7; H, 5.89; N, 14.8.

In boiling methanol, 22 hours were required for completion of the reaction, and XI was obtained in 45% yield. In boiling 0.1 *N* hydrochloric acid, reaction required 3 hours; XI was obtained in 68% yield.

2 - Acetamido - 5 - (N - acetyl - 3 - anilinopropyl)-6-methyl-4-pyrimidinol (XIV).—A solution of 520 mg. (2.01 mmoles) of I*b* (8) in 4 ml. of reagent pyridine and 2 ml. of acetic anhydride was heated in a bath at 90–92° for 1 hour, then spin-evaporated *in vacuo*. The gummy residue was spin-evaporated *in vacuo* with toluene (3 × 5 ml.). Trituration of the residue with ethyl acetate gave 543 mg. (80%) of product, m.p. 127–130°. Recrystallization from ethyl acetate afforded white crystals, m.p. 126–127°; $\nu_{max.}$ 3200, 2950 (NH, OH); 1650, 1600, 1570, 1530 (C=O, NH, pyrimidine, C=C); 697 cm.$^{-1}$ (phenyl CH); $\lambda_{max.}^{EtOH}$ 242 (ε 12,900), 294 mμ (ε 9100); $\lambda_{max.}^{pH\ 13}$ 278 (ε 8100), inflection centering at 247 mμ (ε 10,700).

Anal.—Calcd. for $C_{18}H_{22}N_4O_3$: C, 63.1; H, 6.48; N, 16.4. Found: C, 63.0; H, 6.49; N, 16.2.

2 - Amino - 4 - chloro - 6 - methyl - 5 - (N-tosyl-3-anilinopropyl)pyrimidine (XVIII).—A mixture of 1.075 Gm. (2.6 mmoles) of dry, recrystallized XVII (15) (m.p. 232–233°) and 5 ml. of phos-

phorus oxychloride was heated under a condenser protected from moisture in an oil bath preheated to 110°. After 45 minutes, the solution was cooled and poured into 50 Gm. of crushed ice and 15 ml. of petroleum ether with vigorous mechanical stirring. The gummy precipitate gradually solidified. The solid was ground with water, then filtered; this process was repeated until an aqueous washing was no longer acidic. After being dried over potassium hydroxide for 18 hours, the crude product weighed 1.166 Gm. (104%), m.p. 158–162°. Recrystallization from ethanol-water gave 0.892 Gm. (80%) of white crystals, m.p. 161–164°, that were suitable for further transformations. Several more recrystallizations from ethanol-water gave the analytical sample, m.p. 166–168°; $\nu_{max.}$ 3450, 3300, 3170 (NH); 1625, 1575, 1520 (NH, pyrimidine, C=C); 1340, 1150 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1}$ 232, 316 mμ; $\lambda_{max.}^{pH\ 7,13}$ 236, 305 mμ.

Anal.—Calcd. for $C_{21}H_{22}ClN_4O_2S$: C, 58.5; H, 5.38; N, 13.0. Found: C, 58.3; H, 5.55; N, 12.8.

2,4 - Diamino - 6 - methyl - 5 - (N - tosyl - 3 - anilinopropyl)pyrimidine (XVI) Hydrochloride.—A mixture of 240 mg. (0.557 mmole) of XVIII and 15 ml. of methanol saturated with ammonia at 0° was heated in a steel bomb in a bath at 150° for 24 hours. The solution was clarified by filtration, then spin-evaporated *in vacuo*. The residue was spin-evaporated *in vacuo* with methanol (3 × 5 ml.), leaving 260 mg. (104%) of crude product, m.p. 230–239° dec. Recrystallization from ethanol-petroleum ether (b.p. 60–110°) gave 160 mg. (64%) of white crystals, m.p. 254–257° dec.; $\nu_{max.}$ 3350, 3100 (NH); 1660 (C=NH$^+$); 1630, 1580 (C=C, C=N); 1350 1160 cm.$^{-1}$ (−SO_2N−).

Anal.—Calcd. for $C_{21}H_{26}ClN_5O_2S$: C, 56.3; H, 5.85; N, 15.6. Found: C, 56.5; H, 5.86; N, 15.4.

5 - (3 - Anilinopropyl) - 2,4 - diamino - 6 - methylpyrimidine (XIII).—*Preparation A.*—To a magnetically stirred suspension of 500 mg. (1.12 mmoles) of XVI hydrochloride in 60 ml. of liquid ammonia was added sodium in small pieces until a permanent blue color was obtained that did not bleach in 15 minutes; a total of 167 mg. of sodium was required. After the cautious addition of 0.55 Gm. of ammonium chloride, the ammonia was allowed to evaporate, and the last traces were removed *in vacuo*. The residue was dissolved in 10 ml. of water plus 1.5 ml. of 6 *N* hydrochloric acid. The filtered solution was adjusted to pH 12 with 30% aqueous sodium hydroxide with cooling. The product was collected on a filter and washed with water; yield, 245 mg. (85%), m.p. 180–182°. Recrystallization from ethanol-water gave the analytical sample, m.p. 183–184°; $\nu_{max.}$ 3500, 3350, 3200 (NH); 1700, 1625, 1600, 1575 (NH, pyrimidine, C=C); 750, 690 (phenyl CH); no −SO_2N− near 1350 or 1160 cm.$^{-1}$; $\lambda_{max.}^{pH\ 1}$ 277 mμ (ε 7200); $\lambda_{max.}^{pH\ 7}$ 236 (ε 20,600), 282 mμ (ε 8200); $\lambda_{max.}^{pH\ 13}$ 237 (ε 20,500), 288 mμ (ε 10,100).

Anal.—Calcd. for $C_{14}H_{19}N_5$: C, 65.3; H, 7.44; N, 27.2. Found: C, 65.1; H, 7.51; N, 27.1.

Preparation B.—A suspension of 245 mg. (0.547 mmole) of XVI hydrochloride in 3.75 Gm. of 30% hydrogen bromide in acetic acid and 103 mg. of phenol (17) was stirred for 18 hours protected from moisture, then diluted with 15 ml. of anhydrous ether. The white hydrobromide salt was collected on a filter and washed with ether; yield, 247 mg., m.p. 253–255°. The hydrobromide salt was dissolved in 10 ml. of water; the solution, clarified by filtration, was basified to pH 12 with 30% aqueous sodium hydroxide. The product was collected on a filter and washed with water; yield, 115 mg. (82%), m.p. 183–184°, that was identical to *Preparation A*.

If the hydrogen bromide–acetic acid to XVI ratio was decreased threefold, most of the XVI was recovered unchanged due to its insolubility.

Preparation C.—A magnetically stirred mixture of 400 mg. (1.17 mmoles) of XIV, 4 ml. of benzene, and 0.4 ml. of phosphorus oxychloride was heated under a condenser in an oil bath preheated and maintained at 85°. Solution was completed in 1 minute, and the reaction was stopped after 3 minutes when the solution started to turn turbid; the mixture was immediately poured into a stirred solution of 2.6 Gm. of anhydrous sodium acetate in 10 ml. of water. After being stirred 10 minutes, the layers were separated, and the aqueous layer was extracted with dichloromethane (5 × 5 ml.). The combined organic solutions, dried with magnesium sulfate, were spin-evaporated *in vacuo*. Trituration of the residue with a mixture of ethyl acetate and ether gave 220 mg. (52%) of crude XV, m.p. 134–137°, which was difficult to purify further.

A mixture of 0.85 Gm. (2.36 mmoles) of XV and 8.5 ml. of ethanol saturated with ammonia at 0° was heated in a steel bomb at 140–145° for 24 hours. Spin-evaporation *in vacuo* left 0.87 Gm. of crude XII hydrochloride as a glassy solid; $\lambda_{max.}^{pH\ 1}$ 276 mμ, $\lambda_{max.}^{pH\ 7}$ 278 mμ; $\lambda_{max.}^{pH\ 13}$ 287 mμ; these spectra were in agreement with a 2,4-diamino-5,6-dialkylpyrimidine (12).

A solution of 700 mg. crude XII hydrochloride in 2 ml. of 6 *N* hydrochloric acid was refluxed for 2 hours, then spin-evaporated *in vacuo*. The residue was a mixture of *Ib* and XIII which were readily separated by fractional neutralization of an acid solution; *Ib* separated at pH 5, a mixture of the two at pH 7.5, and pure XIII at pH 13. Refractionation of the pH 7.5 precipitate gave additional XIII; yield, 26 mg. (9%), m.p. 182–184°, that was identical to *Preparation A*.

The yield of *Ib* was over 50%; since the ultraviolet spectrum of crude XII did not indicate that this much of the 2-amino-4-hydroxypyrimidine derivative was present, it would appear that *Ib* arose by acid cleavage of XIII (14).

Preparation D.—A solution of 19 mg. of XI in 4 ml. of concentrated ammonia water was heated in a steel bomb at 185–195° for 20 hours. The filtered solution was evaporated *in vacuo*, leaving 10 mg. of a gummy yellow solid whose ultraviolet spectra were in agreement with structure XII or XIII; $\lambda_{max.}^{pH\ 1}$ 275 mμ, $\lambda_{max.}^{pH\ 13}$ 288 mμ.

2 - Amino - 4 - dimethylamino - 6 - methyl - 5 - (N-tosyl-3-anilinopropyl)pyrimidine (XIXa).—A mixture of 280 mg. (0.65 mmole) of XVIII and 15 ml. of 20% ethanolic dimethylamine was heated in a steel bomb at 120° for 1 hour. The solution was spin-evaporated *in vacuo*. Crystallization of the residue from aqueous ethanol gave 115 mg. (40%) of white crystals, m.p. 70–74°, with softening at 65°. Recrystallization from the same solvent gave white crystals of unchanged melting point; $\nu_{max.}$ 3450, 3350, 3200 (NH); 1620, 1600, 1560–1540 (NH, pyrimidine, C=C); 1340, 1160 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1,7}$ 295 mμ with an inflection at 235 mμ; $\lambda_{max.}^{pH\ 13}$ 305 mμ.

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE AND THYMIDYLATE SYNTHETASE BY 2-R_1-4-R_2-5-(3-ANILINOPROPYL)-6-METHYLPYRIMIDINES

Compd.	R_1	R_2	Dihydrofolic Reductase[a,b] mM Concn. Inhibitor	% Inhibition	Inhibitor : Substrate[d]	Thymidylate Synthetase[b,c] mM Concn. Inhibitor	% Inhibition	Inhibitor : Substrate[e]	Synthetase : Reductase[f]
I*b*[g]	NH_2	OH	0.60[h]	43	130	0.62[i]	50	50	0.39
XIII[g]	NH_2	NH_2	0.0022	50	0.37	0.80	50	63	170
A[g]	NH_2	H	0.95	50[j]	80	0.70[i]	35	100	1.2
XXVII	H	NH_2	0.14	50	23	1.0	26	210	9.2
B[g]	H	OH	1.5	0	>1000	2.0[i]	26	420	<0.4
XXII	Me_2N	OH	6[h]	0	>4000	1.5[k]	0	>480	
XIX*b*	NH_2	NMe_2	0.058	50	9.7	1.3	50	100	10

[a] Dihydrofolic reductase from pigeon liver was prepared and assayed with 6 μm. dihydrofolate and 12 μm. TPNH in 0.05 *M* tris buffer at pH 7.4 as previously described (9). [b] The technical assistance of Mrs. Rita Zielinsky with these assays is acknowledged. [c] Thymidylate synthetase from *E. coli* B. was prepared and assayed with 80 μm. 2′-deoxyuridylate and 25.7 μm. *dl*-tetrahydrofolate, magnesium chloride, and formaldehyde in 0.05 *M* tris buffer at pH 7.4 as previously described (9). [d] Ratio of concentrations of inhibitor to dihydrofolate giving 50% inhibition. [e] Ratio of concentrations of inhibitor to the active 1-isomer of 5, 10-methylene-tetrahydrofolate giving 50% inhibition. [f] Ratio of inhibitor:substrate for 50% inhibition of thymidylate synthetase to inhibitor:substrate for 50% inhibition of dehydrofolic reductase. [g] Enzyme data from *Reference 9*. [h] Cell also contained 10% *N,N*-dimethylformamide. [i] Cell also contained 3% *N,N*-dimethylformamide. [j] In the presence of 12 μm. dihydrofolate. [k] Cell also contained 5% 2-methoxyethanol.

Anal.—Calcd. for $C_{23}H_{29}N_5O_2S$: C, 62.8; H, 6.65; N, 15.9. Found: C, 62.7; H, 6.59; N, 15.8.

2 - Amino - 5 - (3 - anilinopropyl) - 4 - dimethylamino-6-methylpyrimidine (XIXb) Dihydrochloride.—To 1.4 Gm. of 30% hydrogen bromide in acetic acid and 107 mg. of phenol (17) was added 250 mg. of XIX*a*. After being stirred for 16 hours, the mixture was processed as described for *XIII, Preparation B*. When the aqueous solution was basified to pH 12, a gum separated. The mixture was extracted with chloroform (5 × 4 ml.). The combined extracts were dried with magnesium sulfate and spin-evaporated *in vacuo*, leaving 145 mg. of crude XIX*b* as a gum. The gum was dissolved in 1 ml. of ethanol and the solution treated with 1 ml. of 12 *N* hydrochloric acid. Spin evaporation *in vacuo* left 150 mg. (74%) of crude XIX*b* dihydrochloride, m.p. 144–149°. Two recrystallizations from absolute ethanol–petroleum ether afforded 106 mg. (52%) of white crystals, m.p. 172–176°, which was unchanged after one more recrystallization. The analytical sample had $\nu_{max.}$ 3450, 3340, 3200 (NH); 2960–2900, 2740–2660 (NH^+); 1650 (C=NH^+); 1625, 1550 (C=C, C=N); 760, 690 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}^{pH\ 1}$ 291 mμ (ε 9800); $\lambda_{max.}^{pH\ 7}$ 242 (ε 19,700), 292 mμ (ε 11,100); $\lambda_{max.}^{pH\ 13}$ 302 (ε 8700), 240 mμ (shoulder, ε 18,300).

Anal.—Calcd. for $C_{16}H_{25}Cl_2N_5$: C, 53.6; H, 7.03 N, 19.5. Found: C, 53.9; H, 7.16; N, 19.3.

2 - Dimethylamino - 6 - methyl - 5 - (N - tosyl-3-anilinopropyl)-4-pyrimidinol (XXI).—A mixture of 2.75 Gm. of crude XX (73% pure by ultraviolet analysis) (15), 0.90 Gm. (3.29 mmoles) of 1,1-dimethylguanidine sulfate, 0.36 Gm. (6.59 mmoles) of sodium methoxide, and 15 ml. of methanol was refluxed with magnetic stirring for 20 hours protected from moisture. The mixture was spin-evaporated *in vacuo* and the residue triturated with 30 ml. of water; an oil separated that soon solidified. The crude product was collected on a filter and washed with water, then ethanol; yield, 1.36 Gm. (65%), m.p. 197–201°. Recrystallization from absolute ethanol gave 0.705 Gm. (34%) of pure product, m.p. 243–245°, that was unchanged on further recrystallization. The compound had $\nu_{max.}$ 1625, 1600, 1525 (pyrimidine, C=C); 1340, 1160 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1}$ 234, 265 mμ; $\lambda_{max.}^{pH\ 7}$ 230, plateau at 290–306 mμ; $\lambda_{max.}^{pH\ 13}$ 292, shoulder at 236 mμ.

Anal.—Calcd. for $C_{28}H_{28}N_4O_3S$: C, 62.7; H, 6.40; N, 12.7. Found: C, 62.9; H, 6.60; N, 12.6.

5 - (3 - Anilinopropyl) - 2 - dimethylamino - 6-methyl-4-pyrimidinol (XXII).—Treatment of 400 mg. (0.91 mmole) of XXI with sodium (0.15 Gm.) in liquid ammonia, as described for the conversion of *XVI* to *XIII*, gave upon neutralization of the hydrochloric acid solution, a solid that was collected on a filter, washed with water, and triturated with petroleum ether; yield, 218 mg. (81%), m.p. 120–121°. Recrystallization from petroleum ether (b.p. 60–110°) gave white crystals, m.p. 121–122°; $\nu_{max.}$ 3400 (NH or OH); 1630, 1575 (pyrimidine, C=C); 750, 690 cm.$^{-1}$ (aromatic CH); $\lambda_{max.}^{pH\ 1}$ 231 (ε 14,700), 271 mμ (ε 7700); $\lambda_{max.}^{pH\ 7}$ 234 (ε 26,800), inflection centering at 285 mμ (ε 5400); $\lambda_{max.}^{pH\ 13}$ 242 (ε 23,700), 290 mμ (ε 8100).

Anal.—Calcd. for $C_{16}H_{22}N_4O$: C, 67.1; H, 7.74; N, 19.6. Found: C, 67.3; H, 7.92; N, 19.5.

2 - Mercapto - 6 - methyl - 5 - (N - tosyl - 3-anilinopropyl)-4-pyrimidinol (XXIII).—To a solution of 5.6 Gm. of crude XX (73% pure by ultraviolet analysis) (15) in 30 ml. of reagent methanol was added 1.58 Gm. (29.2 mmoles) of sodium methoxide and 1.00 Gm. (13.1 mmoles) of thiourea. After being refluxed for 22 hours with magnetic stirring and protected from moisture, the mixture was spin-evaporated *in vacuo*. The residue was dissolved in 60 ml. of water and filtered from some insoluble by-product; acidification to about pH 4 with acetic acid gave 2.95 Gm. (71%) of product, m.p. 265–269°. Recrystallization from 2-methoxyethanol by addition of water gave 2.66 Gm. (64%) of pure product, m.p. 250–252°; $\nu_{max.}$ 3050 (NH or OH); 2980–2880 (acidic hydrogen); 1630, 1550 (pyrimidine, C=C); 1330, 1155 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1.7}$ 221, 278 mμ; $\lambda_{max.}^{pH\ 13}$ 313, plateau at 253–263, inflection centering at 235 mμ.

Anal.—Calcd. for $C_{21}H_{23}N_3O_3S_2$: C, 58.7; H, 5.40; N, 9.78. Found: C, 58.5; H, 5.35; N, 9.53.

6 - Methyl - 5 - (N - tosyl - 3 - anilinopropyl) - 4-pyrimidinol (XXIV).—To a solution of 1.00 Gm. (2.33 mmoles) of XXIII in 25 ml. of 2-methoxyethanol was added 4.5 Gm. of sponge nickel catalyst (Davison Co.). After being refluxed with stirring for 3 hours, the mixture was filtered through a Celite pad, then the insolubles were washed with hot 2-methoxyethanol (2 × 10 ml.). The combined filtrate and washings were spin-evaporated *in vacuo*,

leaving 0.54 Gm. (58%) of white solid m.p. 214–215°. Recrystallization from ethyl acetate gave 0.35 Gm. of white crystals, m.p. 214–215°; $\nu_{max.}$ 2950–2850 (acidic hydrogen); 1640, 1600 (C=O, pyrimidine, C=C); 1340, 1150 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 13}$ 265, with a shoulder at 233 mμ.

Anal.—Calcd. for $C_{21}H_{23}N_3O_3S$: C, 63.5; H, 5.83; N, 10.6. Found: C, 63.7; H, 5.91; N, 10.4.

4 - Chloro - 6 - methyl - 5 - (N - tosyl - 3 - anilinopropyl)pyrimidine (XXV).—A mixture of 200 mg. (0.50 mmole) of XXIV, 2 ml. of benzene, and 0.2 ml. of phosphorus oxychloride was heated under reflux for 30 minutes protected from moisture in an oil bath preheated to 85°. Within 10 minutes, the phosphate salt of XXV began to separate. The white crystals were collected on a filter and washed with benzene; yield, 0.252 Gm., m.p. 155–157°, which showed NH^+ bands at 2600–2350 and 2100–1900 cm.$^{-1}$ and a P → O band 1290 cm.$^{-1}$.

A mixture of the phosphate salt, chloroform (5 ml.), and 5% aqueous sodium bicarbonate (5 ml.) was shaken until solution was complete. The separated chloroform solution was washed with water, dried with magnesium sulfate, then spin-evaporated *in vacuo;* yield, 0.175 Gm. (84%) of white crystals, m.p. 124–125°. Recrystallization from ethanol-water gave white crystals of unchanged melting point; $\nu_{max.}$ 1580, 1540–1520 (C=N, C=C); 1320, 1150 cm.$^{-1}$ (−SO_2N−).

Anal.—Calcd. for $C_{21}H_{22}ClN_3O_2S$: C, 60.6; H, 5.33; N, 10.1. Found: C, 60.5; H, 5.30; N, 10.2.

4 - Amino - 6 - methyl - 5 - (N - tosyl - 3 - anilinopropyl)pyrimidine (XXVII).—A mixture of 465 mg. (1.12 mmoles) of XXV and 10 ml. of methanol saturated with ammonia at 0° was heated in a steel bomb at 140° for 5 hours. The cooled contents were clarified by filtration, and the solution was spin-evaporated *in vacuo.* The residual hydrochloride was dissolved in 30 ml. of 25% aqueous acetic acid, then the solution was adjusted to pH 12 with 30% sodium hydroxide. The product was collected on a filter and washed with water; yield, 470 mg. (100%), m.p. 169–172°. Recrystallization from ethanol-water gave 375 mg. (85%) of product, m.p. 170–172°. Further recrystallization raised the melting point to 173–175°; $\nu_{max.}$ 3450, 3300, 3180–3100 (NH); 1630, 1560 (NH, C=C, C=N); 1330, 1150 cm.$^{-1}$ (−SO_2N−).

Anal.—Calcd. for $C_{21}H_{24}N_4O_2S$: C, 63.6; H, 6.10; N, 14.1. Found: C, 63.8; H, 6.28; N, 13.9.

4 - Amino - 5 - (3 - anilinopropyl - 6 - methylpyrimidine (XXVI).—Reduction of 368 mg. (0.93 mmole) of XXVII with 177 mg. of sodium in liquid ammonia, as described for the conversion of XVI to XII, gave 170 mg. (76%) of product, m.p. 144–146°. Recrystallization from ethyl acetate–petroleum ether afforded 130 mg. (58%) of pure product as white crystals, m.p. 147–148°; $\nu_{max.}$ 3350, 3150 (NH); 1640, 1570, 1540 (NH, C=C, C=N); 790, 680 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}^{pH\ 1}$ 257 mμ (ε 12,300); $\lambda_{max.}^{pH\ 7}$ 242 (ε 17,600), 272 mμ (inflection, ε 8400); $\lambda_{max.}^{pH\ 13}$ 238 (ε 19,800), 275 mμ (ε 7600, shoulder).

Anal.—Calcd. for $C_{14}H_{18}N_4$: C, 69.4; H, 7.49; N, 23.1. Found C, 69.2; H, 7.32; N, 23.0.

RESULTS AND DISCUSSION

As stated in the introduction, the objective of this study was to shed further light on the remarkable increase in binding by aminopterin (XXVIII) and other 2,4-diaminoheterocycles to the enzyme, (dihydro)folic reductase.

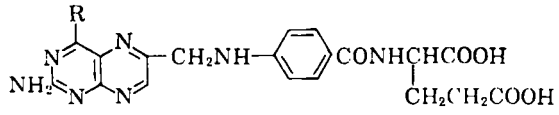
The early proposal by Baker (3) that greater protonation of the 2-amino group of the strongly basic aminopterin (XXVIII) (compared to folic acid, XXIX) could lead to increased binding to an anionic site was untenable with the later studies by Zakrzewski (4), who proposed that stronger binding could be accounted for by binding of the 4-amino group and the N-3 nitrogen.

That the 4-amino group does give increased binding is demonstrated clearly by comparing the binding of the 4-pyrimidinol (B) with the 4-aminopyrimidine (XXVII) (Table I); there is a greater than fiftyfold increase in binding to dihydrofolic reductase by this structural change, an indication that the 4-amino group binds to the enzyme. That the 2-amino group also contributes to binding to dihydrofolic reductase is seen clearly by comparing the 2-amino-4-pyrimidinol (I*b*) to the 4-pyrimidinol (B) (Table I). Removal of the 2-amino group led to a greater than eightfold loss in binding. The 2,4-diaminopyrimidine (XIII) binds 350-fold stronger than the 2-amino-4-pyrimidinol (I*b*), thus indicating that both the 2-amino and 4-amino are involved in binding to the enzyme, probably by hydrogen bonds as proposed by Zakrzewski (4). To this point, Zakrzewski's explanation is satisfactory that (*a*) four hydrogen bonds exist between the 2,4-diaminopyrimidine moiety and the enzyme (XXX) and (*b*) the energy change necessary to convert the normal 4-oxo tautomer (XXXII) of folic acid to the 4-hydroxy tautomer (XXXI) leads to a net decrease in binding compared to XXX.

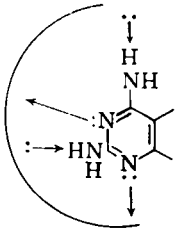
The first discrepancy appears when one considers the extent of binding of the 2-aminopyrimidine (A) compared to the 2,4-diaminopyrimidine (XIII) and the 2-amino-4-pyrimidinol (I*b*) (Table I). The 2-aminopyrimidine clearly has its N-3 nitrogen available for binding to the enzyme without a tautomeric shift. Thus, if Zakrzewski's explanation of N-3 and 4-amino binding (XXX) were correct, then the 2-aminopyrimidine (A) with its available N-3 should have been bound to the enzyme considerably stronger than the 2-amino-4-pyrimidinol (I*b*), but not so strong as the 2,4-diaminopyrimidine (XIII). At least a tenfold tighter binding should have been seen. These results indicate that the N-3 of the 2,4-diaminopyrimidyl moiety does not bind to folic reductase as previously indicated in XXX. There are a number of other discrepancies which are not completely compatible with Zakrzewski's formulation of XXX as the mode of binding of the 2,4-diaminopyrimidyl moiety of aminopterin (XXVIII) and other 2,4-diaminoheterocycles.

To make comparisons more valid between the compounds in Table I and the binding of aminopterin to rat liver folic reductase, the 2,4-diaminopyrimidine (XIII) was measured as an inhibitor of this enzyme system using folic acid as substrate; XIII had[1] $K_i = 2.4 \times 10^{-8}$. Compared to I*b*, which has $K_i = 6.3 \times 10^{-5}$ in this system (8), the 2,4-diaminopyrimidine (XIII) binds to rat liver folic reductase 2600-fold more tightly than I*b;* in contrast, aminopterine (XXVIII) binds to rat liver

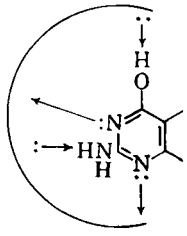
[1] The authors thank Dr. W. C. Werkheiser, Roswell Park Memorial Institute, for this determination on rat liver folic reductase.



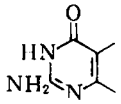
XXVIII, R = NH_2
XXIX, R = OH



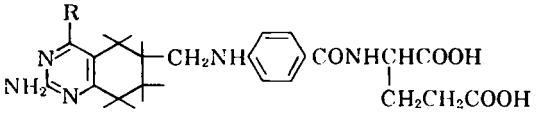
XXX



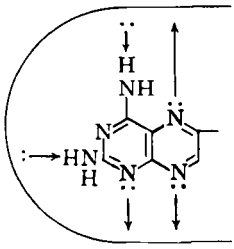
XXXI



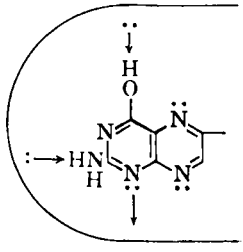
XXXII



XXXIII, R = NH_2
XXXIV, R = OH



XXXV



XXXVI

folic reductase in the same assay system 100,000-fold more tightly than folic acid (XXIX) (2). Thus, a discrepancy of 100,000/2600 = 38-fold remains when one compares the pair XIII–I*b* with the pair XXVIII–XXIX. Furthermore, comparison of folic acid (XXIX, $K_m = 1 \times 10^{-5}$) to the corresponding pyrimidyl analog (I*a*, $K_i = 2 \times 10^{-6}$) (8) shows that the pyrimidyl portion of folic acid accounts for all of the binding of its pteridine moiety. These discrepancies can be accounted for by some binding of the pyrazine portion of the 2,4-aminopteridine system in XXVIII but no binding by the pyrazine portion of the 2-amino-4-hydroxypteridine system of folic acid (XXIX). Support for this additional binding point of 2,4-diaminopteridines can be gleaned from the literature.

(*a*) 2,4-Diamino-5-methylpteridine ($K_i = 4.7 \times 10^{-7}$) binds sixtyfold tighter than 2,4-diamino-5,6-dimethylpyrimidine ($K_i = 2.8 \times 10^{-5}$) (4).

(*b*) Aminopterin (XXVIII) binds to folic reductase 10-to 100-fold stronger than its tetrahydroquinazoline analog (XXXIII) (18), whereas the tetrahydroquinazoline analog (XXXIV) (19) of folic acid binds five times stronger than folic acid (XXIX) (20).

(*c*) 5-(*p*-Chlorophenyl)-2,4-diamino-6-ethylpyrimidine ($K_i = 7 \times 10^{-9}$)[1] binds much stronger than 2,4-diamino-6-methylpyrimidine ($K_i = 2.6 \times 10^{-5}$) (4), an indication that the chlorophenyl group is an additional binding point (8).

(*d*) 2-Amino-5-(3-anilinopropyl)-4-pyrimidinol with a 6-phenyl or 6-benzyl substituent has $K_i = 1.8 \times 10^{-6}$ and 4.0×10^{-6}, respectively (15), compared to the 6-methyl substituent (I*b*) with $K_i = 6.3 \times 10^{-5}$ (8). These data indicate that the 6-phenyl or 6-benzyl group have an additional point of binding to folic reductase.

It is probable that the extra binding point needed for (*a*)–(*d*) is the same, providing this binding point is a locus of fairly broad dimensions (21). A logical possibility for this locus can be surmised when one considers what is known about the mechanism of some dehydrogenases (or hydrogenases if the reaction is a reduction).

In lactic dehydrogenase, where pyruvate is reduced by DPNH to lactate, it has been reasonably well established that the imidazole ring of a histidine is present at the active site (22). The imidazole in its protonated form not only acts as a proton donor during the reduction, but also is probably a binding point for the ketone oxygen atom (23). This has been represented diagrammatically by XXXVII (24), and the mechanism of reduction proceeds through the transition state, XXXVIII, to the products XXXIX. If the reaction is reversible, the electron flow shown in XXXIX will return the components to the transition state, XXXVIII. It is logical to assume that all enzymic hydrogenases and dehydrogenases would have an imidazole group at the active site as a proton donor or acceptor, thus resulting in a more facile reaction than if the substrate had to pick up a proton from the solvent.

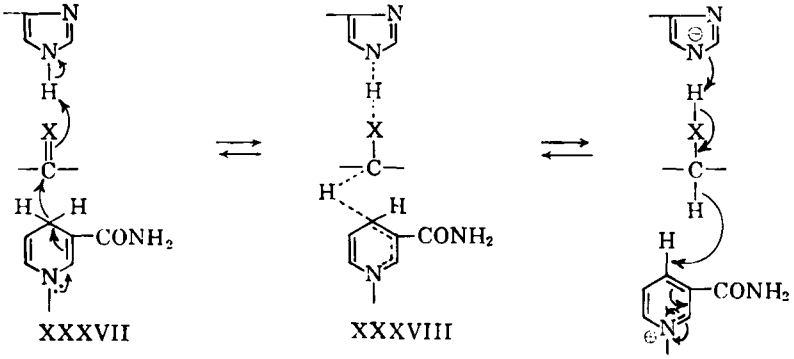
Therefore, it is logical to invoke the presence of an imidazole ring in the active site of folic reductase to aid in the reduction of the double bonds of the pyrazine ring. Molecular models show that the imidazole can be juxtapositioned more or less face-to-face with the pyrazine ring so that in one form (XL) the imidazole proton could easily be transferred to the 7- or 8-position of the pteridine; in the other form (XLII) a proton could easily be transferred to the 6- or 5-position of the pteridine, thus accounting for any possible structure for the intermediate dihydrofolate. The TPNH for hydride transfer can be juxtapositioned on the opposite side of the pteridine from the imidazole so that it can readily transfer a hydride anion to either C_6 or C_7 of the pteridine.

The supposed presence of the imidazole ring at the active site of folic reductase juxtapositioned face-to-face with the pyrazine moiety will now allow explanation of the binding discrepancies (*a*)–(*d*) cited earlier. Aminopterin, which is about 1000 times more basic than folic acid (6), could bind at either N-8 (XLI) or N-5 (XLIII) with the acidic NH group of the imidazole to give a quasi-ionic bond. Even though N-5 or N-8 may not be the first point of protonation in free solution (6), when aminopterin is complexed with the enzyme the juxtapositioning of the imidazole NH to N-5 or N-8 could certainly protonate one of these two groups first. Hydroxylation of the pteridine, as in folic acid (XXVIII), decreases basicity of the ring system by 1000-fold, which would be sufficient to negate appreciable binding to the imidazole of the type indicated in XL and XLII.

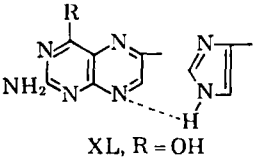
The extra point of binding of certain 6-phenyl and 6-benzyl pyrimidines (15) can be depicted as a charge-transfer complex between the imidazole and the benzene ring where the latter is an electron donor (XLIV). Studies on the mode of binding of the 6-phenyl group are so far in agreement with this concept (24). The binding of the phenyl group of the 5-phenylpyrimidines can also be depicted as a charge-transfer complex of either the π-type (XLV) or the n-type.

Whether an imidazole is in the juxtaposition at the active site of folic reductase as depicted (XL–XLV) is being vigorously pursued in this laboratory by construction of the proper type of active-site-directed irreversible inhibitor that could label the imidazole (25, 26).

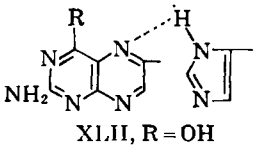
The relatively tight fit of the 2,4-diaminopyrimidyl moiety to folic reductase, as depicted by Zakrzewski in structure XXX (4) but modified as in structure XXXV, was studied by synthesis and enzymic



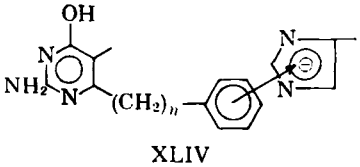
XXXVII XXXVIII XXXIX



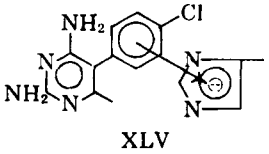
XL, R = OH
XLI, R = NH_2



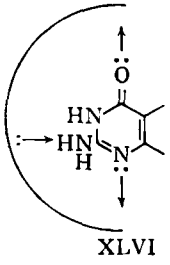
XLII, R = OH
XLIII, R = NH_2



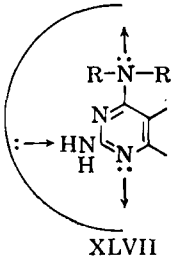
XLIV



XLV



XLVI



XLVII

evaluation of the 2-dimethylaminopyrimidine (XXII) and the 4-dimethylaminopyrimidine (XIX*b*). A greater than thirtyfold loss in binding occurred with the 2-dimethylamino-4-pyrimidinol (XXII) compared to the 2-amino-4-pyrimidinol (I*b*) (Table I). This loss in binding can be attributed either to steric hindrance to binding by the two bulky methyl groups or the loss of the necessary NH for hydrogen bonding or both.

The 2-amino-4-dimethylaminopyrimidine (XIX*b*) suffered a 26-fold loss in binding compared to the 2,4-diaminopyrimidine (XXVII) (Table I). Actually, one could have expected a greater loss in binding since XIX*b* still binds eightfold stronger than the 2-aminopyrimidine (A), which does not have a substituent at the 4-position. These results indicate that there is no steric hindrance to binding created by the two bulky methyl groups, but that the 4-NH group necessary for binding is no longer present. That the 2-amino-4-dimethylaminopyrimidine (XIX*b*) binds eightfold stronger than the 2-aminopyrimidine (A) suggests that the dimethylamino group causes the N-1 and C_2-NH to bind more tightly due to the increased basicity in the dimethylamino system.

Although the compounds in Table I were synthesized to study the mode of binding of 2,4-diaminopyrimidines to dihydrofolic reductase, the fact that I*b* was also an inhibitor of thymidylate synthetase suggested that these compounds also be investigated as inhibitors of thymidylate synthetase for what information could be gleaned on the mode of binding of the 2-amino-4-pyrimidinol moiety to this enzyme.

In a previous paper (9) it was observed that both the 2-amino group and 4-hydroxyl (or 4-oxo) group contributed to binding to thymidylate synthetase and that the 4-hydroxyl contributed to binding to the latter enzyme relatively more than to dihydrofolic reductase. The results shown in Table I are compatible with the binding depicted in XLVI. The 2,4-diaminopyrimidine (XIII) has about the same amount of binding as the 2-amino-4-pyrimidinol (I*b*). This indicates that the 4-oxo group of I*b* (depicted in XLVI) binds equally as well as the N: → H-E bond depicted in XLVII. Thus, the 3-NH of XLVI and the 3-N of XLVII are not likely to be binding points. Furthermore, the 2-amino-4-dimethylaminopyrimidine (XIX*b*) binds almost as well as the 2,4-diaminopyrimidine (XIII). Therefore, (*a*) the 4-NH of XIII cannot be a binding point, (*b*) the two bulky methyl groups are tolerated, and (*c*) the mode of binding of XIII and XIX*b* at the 4-position is most probably *via* an N: → H-E bond, as depicted in XLVII.

In contrast, the 2-dimethylamino-4-pyrimidinol (XXII) shows a greater than ninefold loss in binding compared to the 2-amino-4-pyrimidinol (I*b*). These results indicate that either the loss of the 2-N-H destroys the N-H ← E type of binding depicted in XLVI or there is no room for the two bulky methyl group or both.

Although 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (I*b*) can inhibit both thymidylate synthetase and dihydrofolic reductase, being slightly more effective on the former enzyme, the mode of binding of I*b* to the two enzymes is different; the differences and similarities are summarized as follows.

(*a*) The 2-amino group apparently binds to both enzymes by an N-H ← E bond, and binding is considerably decreased if the 2-amino group is converted to a 2-dimethylamino group.

(*b*) The 4-hydroxyl group binds to dihydrofolic reductase by an O-H ← E bond but to thymidylate synthetase by an = O: → H-E bond of the 4-oxo tautomer. As a result, conversion of the 4-hydroxyl group to a 4-amino group (XIII) causes a 350-fold increase in binding to dihydrofolic reductase, but no increase in binding to thymidylate synthetase.

(*c*) A 6-phenyl group, instead of the 6-methyl group on I*b*, causes a thirtyfold increase in binding to dihydrofolic reductase—presumably by binding to an imidazole on the protein surface—but gives no change in binding to thymidylate synthetase (15).

(*d*) The binding of the anilino moiety to dihydrofolic reductase is presumably through a charge-transfer complex and is readily influenced by change in the electronegativity of the benzene ring; this influence on binding to thymidylate synthetase is much less pronounced (13).

(*e*) The bridge length between the pyrimidyl and anilino moieties of I*b* is more restrictive on dihydrofolic reductase than on thymidylate synthetase, *i.e.*, the locus on thymidylate synthetase binding the anilino group seems to have larger dimensions than the locus on dihydrofolic reductase (27). As a result, compounds more selective on thymidylate synthetase have been obtained (27).

Studies to obtain greater selectivity of inhibition of thymidylate synthetase than dihydrofolic reductase and vice versa, dependent upon the differences cited above, are continuing.

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Oxidation of Aldehydes Solubilized in Nonionic Surfactants I

Solubility of Benzaldehyde and Methylbenzaldehyde in Aqueous Solutions of Polyoxyethylene Glycol Ethers

By A. G. MITCHELL* and LUCY S. C. WAN

The solubilities of benzaldehyde and *p*-methylbenzaldehyde in aqueous solutions of polyoxyethylene glycol ethers of varying polyoxyethylene chain length have been measured at 25°. Solubility increases with surfactant concentration and is greater for benzaldehyde than *p*-methylbenzaldehyde. The start of the solubility curve neither corresponds to the critical micelle concentration (CMC) of the surfactant alone nor to that of the ternary system. On a molar basis, solubility increases with an increase in hydrophilic chain length but decreases if solubility is expressed in terms of the amount solubilized per ethylene oxide unit. The solubility of benzaldehyde in a lauryl derivative is the same as in a cetyl derivative of the same polyoxyethylene chain length, whereas the solubility of methylbenzaldehyde is greater in the surfactant with the longer hydrocarbon chain. From the solubility data and an examination of the ultraviolet absorption spectra in aqueous surfactant solutions and other solvents, it is suggested that the solubilization of benzaldehyde occurs in the polyoxyethylene region of the micelle, while *p*-methylbenzaldehyde is distributed between this region and the hydrocarbon interior.

SURFACTANTS are widely used in the preparation of emulsions and solutions; increased attention is being directed to the stability of these systems toward hydrolysis (1–6) and oxidation (7–13). For certain systems, the rate of reaction depends on the degree of saturation, where this is expressed as a ratio of the amount of reactant present to its solubility (5, 6, 12). This paper reports the solubilities of benzaldehyde and *p*-methylbenzaldehyde in nonionic surfactants of the polyoxyethylene ether type and examines the effect on solubility of variation in chain length of the polyoxyethylene and hydrocarbon groupings as a preliminary to oxidation studies.

EXPERIMENTAL

Materials.—Benzaldehyde (British Drug Houses, analytical reagent grade) and *p*-methylbenzaldehyde (Eastman Organic Chemicals, practical grade) were distilled at low pressure under oxygen-free nitrogen using a Towers fractional distillation unit fitted with automatic reflux ratio control. The aldehydes were packed in clear glass ampuls under oxygen-free nitrogen and stored protected from light in a refrigerator. The ampuls had been cleaned previously in concentrated sulfuric acid, washed in water, and dried. The absence of impurities and decomposition products in each aldehyde was confirmed by infrared spectroscopy in carbon tetrachloride and vapor-phase chromatography on a Celite-silicone column, using an argon ionization detector.

The nonionic surfactants used were Texofors A16, A24, A30, A60, and B23 (Glovers Chemicals Ltd.). Texofors in the A series are polyoxyethylene ethers of cetyl alcohol, represented by the formula $CH_3\cdot(CH_2)_{15}(O\cdot CH_2\cdot CH_2)_n\cdot OH$, where n is approximatey 16, 24, 30, or 60. Texofor B23 is a polyoxyethylene ether of lauryl alcohol, where n is approximately 23. The values of n as given were used in calculating the molecular weight. For convenience, the cetyl alcohol derivatives will be designated C16, C24, C30, and C60; similarly, the lauryl derivative will be designated L23. Some physical constants of the surfactants are given in Table I. Concentrated stock solutions were prepared, stored in the dark, and diluted as required. Double distilled water from an all-glass still was used throughout. Polyethylene glycols having molecular weights corresponding to those of the polyoxyethylene chain of each surfactant were prepared by mixing appropriate amounts of polyethylene glycol 600 and polyethylene glycol 4000 (Union Carbide Co.).

Measurement of Solubility of Aldehyde in Water.—A saturated solution of aldehyde in water at 25° was prepared. The amount of aldehyde was determined gravimetrically as the 2:4-dinitrophenylhydrazone, according to the method of Iddles and Jackson (14). Assays of different known concentrations of aldehyde in water gave 100% precipitation as the 2:4-dinitrophenylhydrazone.

Measurement of Solubilities of Aldehydes in Texofor Solutions.—Varying amounts of aldehyde were weighed into a series of 25-ml. graduated glass-stoppered cylinders containing the required concen-

TABLE I.—PHYSICAL CONSTANTS OF NONIONIC SURFACTANTS

Texofor	Designation	n^{30} [a]	d^{30} [b]	M.p.[c]	CMC$^{25°}$ [d] $M \times 10^{-5}$
A16	C16	1.448	1.093	37	6–7
A24	C24	1.449	1.123	38	8–10
A30	C30	1.451	1.155	48	16–18
A60	C60	1.455	1.191	58	32–40
B23	L23	1.449	1.148	41	100–160

[a] Abbe refractometer. [b] Displacement technique in dry *n*-heptane. [c] Micro melting point apparatus. [d] From surface tension measurements using the Du Nouy tensiometer.

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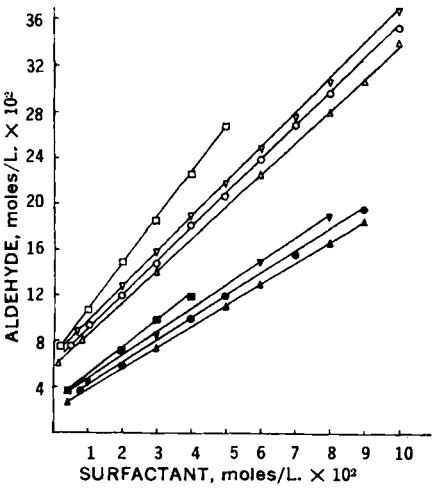


Fig. 1.—Solubilities of benzaldehyde and *p*-methylbenzaldehyde in aqueous solutions of polyoxyethylene glycol ethers of varying polyoxyethylene chain length at 25°. Open symbols, benzaldehyde; closed symbols, *p*-methylbenzaldehyde. Key: △, C16; ○, C24; ▽, C30; □, C60.

tration of Texofor. The cylinders were rotated in a thermostatically controlled water bath at 25 ± 0.01° for 24 hours. The end point was estimated visually and taken as the mean between a clear and cloudy dispersion.

Ultraviolet Spectra Measurements.—The ultraviolet spectra of benzaldehyde and *p*-methylbenzaldehyde in various solvents were determined with a Bausch & Lomb spectronic 505 recording spectrophotometer using short-path length cells.[1] Absorption by the solvent system was eliminated by using it in the comparison cell in all cases. Comparison between the various solvent systems was made on the basis of the relative positions of absorption maxima. Differences in the height of the curves are due to differences in solution concentration and path length.

RESULTS AND DISCUSSION

Solubilities of Aldehydes.—The water solubilities of benzaldehyde and *p*-methylbenzaldehyde at 25° were 6.55 Gm./L. (0.0617 *M*) and 2.27 Gm./L. (0.0189 *M*), respectively. The figure for benzaldehyde is considerably higher than previous literature values which range from 3.0 to 3.5 Gm./L. (15–18). The value in this report was therefore confirmed by a gas chromatographic technique (19). The solubilities of benzaldehyde and *p*-methylbenzaldehyde in surfactants C16, C24, C30, and C60 are shown in Fig. 1. Aldehyde solubilities are proportional to surfactant concentration and increase with the number of polyoxyethylene units in the surfactant molecule. The solubility of *p*-methylbenzaldehyde is less than that of benzaldehyde. Since each surfactant has the same hydrocarbon grouping, increase in aldehyde solubility on ascending the series C16 through C60 can be attributed to the increase in the number of polyoxyethylene units. Figure 2 shows the solubilities of benzaldehyde in solutions of polyethylene glycols. Solubility increases with molar concentration and with increase in the molecular weight of the polyethylene glycol but is much less than in solutions of their surface-active derivatives. Polyethylene glycols are predominantly hydrophilic in character, and low solubilities in their solutions compared to surfactants may be due to the absence of micelles. Solubility in aqueous solutions of polyethylene glycols is thought to involve complex formation (20) and, although Kato (21, 22) has shown the presence of a micelle-like structure in aqueous solutions of a number of glycols, it would appear that the presence of micelles formed from molecules with both hydrophilic and hydrophobic properties is necessary for marked solubilization.

The solubilities of benzaldehyde and *p*-methylbenzaldehyde in low concentrations of C24 are shown in Fig. 3. There is a marked increase above the solubility in water in the region 1–10 × 10^{-3} *M* surfactant. This is well in excess of the CMC of C24 which, from surface tension measurements, was 8–10 × 10^{-6} *M*. Increase in solubility usually

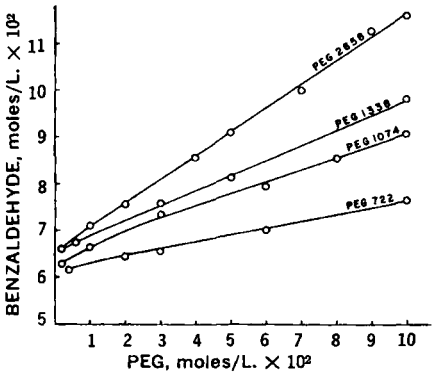


Fig. 2.—Solubility of benzaldehyde in aqueous solutions of polyethylene glycols (PEG) of varying molecular weight at 25°. In each case the number after PEG refers to the approximate molecular weight of a blend of PEG 600 and PEG 4000.

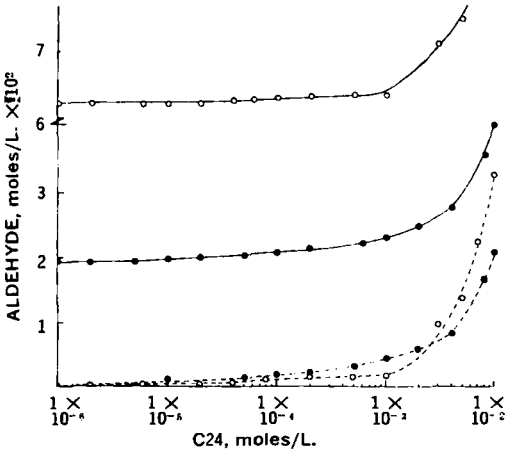


Fig. 3.—Solubilities of aldehydes in dilute aqueous solutions of C24 at 25°. Open symbols, benzaldehyde; closed symbols, *p*-methylbenzaldehyde. Key: - - - , corrected for water solubility; ———, uncorrected for water solubility.

[1] Research and Industrial Instruments Ltd., London, England.

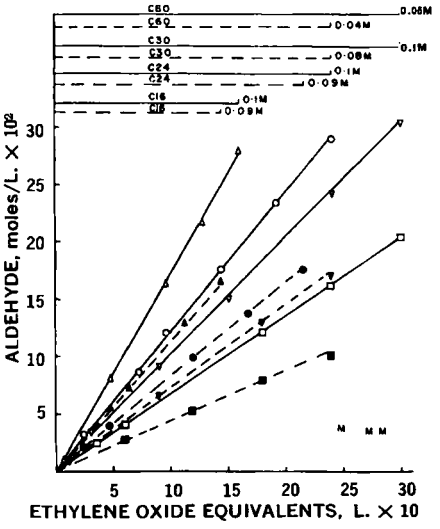


Fig. 4.—Solubilities of aldehydes in aqueous solutions of polyoxyethylene glycol ethers at 25° corrected for water solubilities, where the surfactant concentration is expressed as ethylene oxide equivalents per liter. Open symbols, benzaldehyde; closed symbols, *p*-methylbenzaldehyde. Key: Δ, C16; O, C24; ▽, C30; □, C60.

begins at the CMC; for this reason, the onset of solubilization has been used as a method to estimate the CMC (23). A possible explanation for the discrepancy between the CMC and the start of the solubility curve is that the presence of aldehyde has increased the CMC. Both increases and decreases in the CMC of anionic and cationic surfactants have been found, depending on the nature and concentration of added solubilizate. These effects have been reviewed by Lawrence (24) and Hyde (25). Little evidence is available on the effect of solubilizates on the CMC of nonionic surfactants. Mulley and Metcalf (26) found that various solubilizates had little effect, even where strong interaction occurred between the solubilizate and nonionic surfactant. They suggested that large shifts in the CMC occur only where ionic effects are important, as shown by the effect of electrolytes (23, 27, 28). The CMC of solutions of C24 saturated with benzaldehyde was 32–40 × 10^{-6} *M*. This concentration is slightly greater than that of the surfactant alone but far less than the concentration of surfactant at which solubilization starts. It seems likely that the micelles must reach a certain degree of complexity before solubilization of benzaldehyde and *p*-methylbenzaldehyde can take place.

Figure 4 shows the solubility curves plotted as aldehyde concentration in moles per liter (corrected for water solubility) against surfactant concentration expressed as equivalents of ethylene oxide per liter. It is apparent from this method of expressing solubility that the efficiency of solubilization becomes less as the number of ethylene oxide units in the Texofor chain is increased. Goodhart and Martin (29) have reported a similar pattern for the solubilization of benzoic acid derivatives in nonionic surfactants. From light scattering (30–32) and vapor pressure measurements (33) several workers have shown that the micellar weight and the number of surfactant molecules per micelle decreases as the ethylene oxide chain lengthens. It has been suggested that the origin of this effect lies in the increasing affinity of the monomer for water as the hydrophilic chain length increases, the hydrocarbon part of the molecule remaining constant. Furthermore, there appears to be little change in the radius of the micelle on increasing the number of ethylene oxide units. It appears that considerable curling of the monomer must occur when incorporated into the micelle, and this presumably takes place in the polyoxyethylene chain. Therefore, it seems likely that the decrease in efficiency of solubilization with an increase in the number of ethylene oxide units is associated with the accompanying decrease in micellar weight and number of surfactant molecules per micelle.

The effect of variation in hydrocarbon chain length of surfactant on solubilization was examined by comparing the solubilities in C24 and L23. From the solubility curves (Fig. 5) it is apparent that the solubility of benzaldehyde is not affected by variation in hydrocarbon chain length, while the solubility of *p*-methylbenzaldehyde is greater in C24, *i.e.*, the surfactant with the longer hydrocarbon chain. These results, together with the effect of variation in polyoxyethylene chain length on solubility, suggest that benzaldehyde is solubilized in the hydrophilic polyoxyethylene region of the micelle, while *p*-methylbenzaldehyde is distributed between both the hydrophilic and hydrophobic regions. Further evidence for these suggestions was obtained from a study of the ultraviolet absorption spectra of solutions of aldehydes in surfactants and other solvents.

Ultraviolet Absorption Spectra.—Riegelman and co-workers (34) have suggested that the ultraviolet spectrum of a solubilizate affords a sensitive method for determining its position within the micelle.

Spectra of benzaldehyde in various solvents are shown in Fig. 6. In solutions of C24 (curves 4–6) $\lambda_{max.}$ is at a wavelength longer than in *n*-heptane

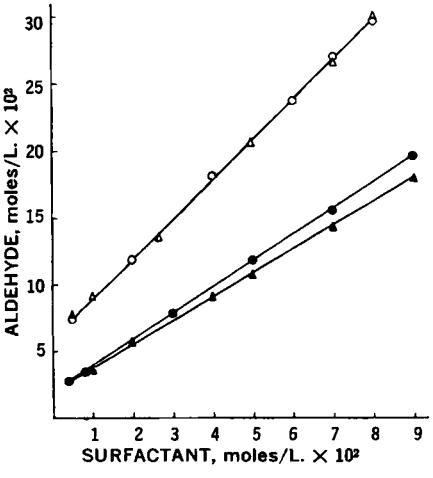


Fig. 5.—Effect of variation in hydrocarbon chain length of polyoxyethylene glycol ethers on the solubilities of benzaldehyde and *p*-methylbenzaldehyde at 25°. Open symbols, benzaldehyde; closed symbols, *p*-methylbenzaldehyde. Key: Δ, L23; O, C24.

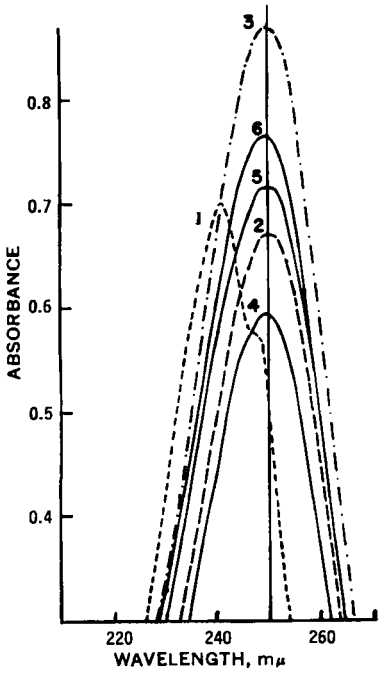


Fig. 6.—Ultraviolet spectra of benzaldehyde in various solvents. Key: 1, heptane—0.00005 moles/L.; 2, water—0.00005 moles/L.; 3, PEG 1074 (0.02 *M*)—0.0685 moles/L.; 4, C24 (0.02 *M*)—0.00005 moles/L.; 5, C24 (0.02 *M*)—0.0685 moles/L.; 6, C24 (0.005 *M*)—0.0685 moles/L.

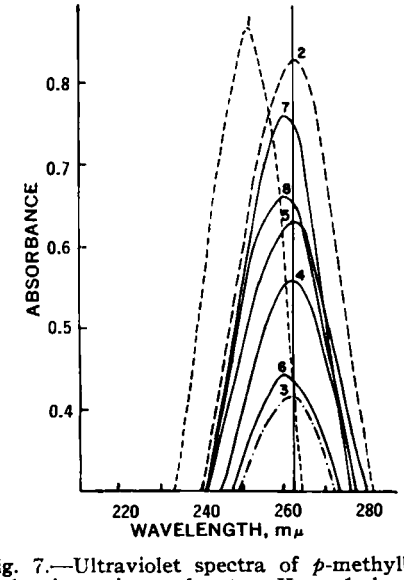


Fig. 7.—Ultraviolet spectra of *p*-methylbenzaldehyde in various solvents. Key: 1, heptane—0.00005 moles/L.; 2, water—0.00005 moles/L.; 3, PEG 1074 (0.02 *M*)—0.02 moles/L.; 4, C24 ($1 \times 10^{-7} M$)—0.00005 moles/L.; 5, C24 (0.0002 *M*)—0.0210 moles/L.; 6, C24 (0.002 *M*)—0.0248 moles/L.; 7, C24 (0.02 *M*)—0.00005 moles/L.; 8, C24 (0.02 *M*)—0.02 moles/L.

(curve 1) and is identical to that in water (curve 2) and solutions of polyethylene glycol (curve 3); $\lambda_{max.}$ is not affected by the concentrations of benzaldehyde or surfactant (curves 4–6). Figure 7 shows the spectral curves of *p*-methylbenzaldehyde. In surfactant solutions below the CMC (curve 4) and also greater than the CMC but below the concentration at which solubilization begins (curve 5), $\lambda_{max.}$ is the same as in water (curve 2) and solutions of polyethylene glycols (curve 3). In the concentration range of surfactant at which solubilization starts (curve 6) and above this concentration (curves 7–8), $\lambda_{max.}$ for *p*-methylbenzaldehyde shows a shift of 2 mμ toward a shorter wavelength, *i.e.*, toward heptane (curve 1). These findings, together with the solubility data, support the view that solubilization of benzaldehyde in aqueous surfactant solutions occurs in the polar polyoxyethylene region of the micelle, while *p*-methylbenzaldehyde is located in both the polyoxyethylene region and in the hydrocarbon interior of the micelle. It appears that *p*-methylbenzaldehyde is taken up in the hydrocarbon interior from the start of solubilization since the surfactant concentration at which the shift in $\lambda_{max.}$ first occurs (curve 6, Fig. 7) coincides with the beginning of the solubility curve. Furthermore, the shift is found even when the aldehyde concentration is below its water solubility (curve 7), a demonstration that the aldehyde is preferentially soluble in the micellar pseudophase.

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Effect of Complex Formation on Drug Absorption I

Complexes of Salicylic Acid with Absorbable and Nonabsorbable Compounds

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The effect of complex formation on drug absorption (gastric absorption by rats) has been studied by means of two model systems: (*a*) a rapidly absorbed drug (salicylic acid) complexed with a hydrophilic, surface-active, nonabsorbed, macromolecular substance (polysorbate 60), and (*b*) a rapidly absorbed drug (salicylic acid) complexed with a much more slowly absorbed drug (caffeine). In both studies, ethanol was used as a noncomplexed marker to afford recognition of possible changes in permeability of the gastric mucosa. Results of these studies were correlated with kinetic analyses based on the stability or binding constants of the respective complexes, the difference in diffusivity between free drug and drug complex, and the absorption rate of the free drug. It is shown that the absorption rate of drugs can be modified by formation of complexes which differ from the free drug mainly in size or in lipoid-water partition coefficient.

COMPLEX FORMATION of drugs has been the subject of extensive physicochemical studies in recent years. These studies have demonstrated the potential utility of complexation as a means of increasing the solubility and stability of drugs. It is also appreciated now that unintended complex formation may occur between drugs that are administered simultaneously either in a single or in separate dosage forms. These considerations warrant an investigation of the effect of complex formation on drug absorption. Moreover, it is desirable to explore the potentialities of intentional complex formation as an approach to the modification of absorption characteristics of chemotherapeutic agents.

The physicochemical properties of drug complexes can differ in several respects from those of the respective free drugs; such differences can involve electrical charge, diffusivity, size, and lipoid-water partition coefficient, among others. These properties can have an appreciable effect on the rate of transfer of substances across biologic membranes. Studies in this laboratory, to be described in this and in subsequent reports, have been concerned with the use of model compounds and complexes to investigate various aspects of complex formation and its effect on drug absorption, as well as with the exploration of a number of potential applications of drug complexation in chemotherapy. This initial report deals with a study of the effect of complexation with an absorbable and nonabsorbable compound, respectively, on the absorption of salicylic acid from the stomach of the rat.

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EXPERIMENTAL

Experimental Procedure in Rats.—Female Wistar rats weighing 100–150 Gm. were used. The rats were starved for 15–20 hours prior to the experiment, and water was removed from the animals 0.5 hour prior to the operation. The animals were anesthetized with ether and were maintained under anesthesia for the entire course of the experiment. A midline incision was made in the abdominal region, and the stomach was exposed. The esophagus and the small intestine were then tightly ligated immediately adjacent to the cardiac sphincter and about 1 cm. from the pyloric sphincter, respectively. Care was taken not to occlude major blood vessels. Five milliliters of drug solution, previously warmed to 37°, was injected with a syringe and hypodermic needle by inserting the needle into the intestine between the pyloric valve and the ligature on the intestine and subsequently working the needle through the pyloric valve into the lumen of the stomach. A third ligature was tied around the intestine and needle to prevent leakage on injection. As the needle was withdrawn, this third ligature was tightened further to prevent any leakage of the drug solution. After completion of the procedure, the incision was closed with wound clips. At the end of 1 hour, the entire stomach was excised and homogenized. The homogenate was then assayed for unabsorbed salicylic acid and ethanol.

Determination of Temperature of Drug Solution within the Rat Stomach.—The surgical procedure was the same as described in the above paragraph, except that along with the hypodermic needle a thermistor probe was inserted through the pyloric valve into the stomach. The thermistor probe was left in the stomach for 1 hour after the drug solution, which had been preheated to 37°, was injected. The thermistor probe was part of a thermistor type temperature recorder[1] which provided a continuous record of the drug solution temperature over the 1-hour absorption period.

[1] Tri-R Tempcord-R, Tri-R Instruments, Jamaica, N. Y.

TABLE I.—EFFECT OF COMPLEX FORMATION ON SALICYLIC ACID ABSORPTION FROM RAT STOMACH

Compn. of Solution,[a] %	Animals, No.	Salicylic Acid Absorbed,[b] %	Half-Absorption Time,[c] Hr.	Initial Complexation of Salicylic Acid,[d] %
Salicylic acid, 0.1	10	50 (6)[e]	1.0	0
Salicylic acid, 0.1 Polysorbate 60, 2	11	33 (5)	1.7	62
Salicylic acid, 0.1 Caffeine, 2.75	10	37 (4)	1.5	84

[a] 0.1 *N* HCl served as the solvent in all solutions. [b] In 1 hour. [c] Assuming absorption to be a first-order process. [d] Based on *in vitro* determinations. [e] Standard deviation in parentheses.

Determination of Fluid Volume in the Rat Stomach.—Phenol red was used as a marker to determine the extent of volume change of the drug solution in the rat stomach due to water absorption or fluid secretion. The procedure was the same as that used in the absorption experiments, except for the following modifications. The stomach was first washed with four separate portions of a phenol red solution (0.02% in 0.1 *N* hydrochloric acid) before injecting the solution for the 1-hour absorption period. Instead of the entire stomach being homogenized, the solution was withdrawn from the stomach, and the concentration of phenol red was determined. A control experiment using the same procedure on excised stomachs was also performed in order to determine the extent of binding of phenol red to gastric contents and to the mucosa during the 1-hour absorption period. Determinations were made both with a 0.02% phenol red solution and a solution containing 0.02% phenol red and 0.1% salicylic acid.

Phenol red was assayed by diluting the withdrawn solution, adding sufficient sodium hydroxide to yield a 0.01 *N* concentration of this base, filtering through a filter (Millipore), and determining the absorbance at 560 mμ with a Bausch & Lomb spectronic 20 colorimeter. Any change in the volume of the drug solution in the stomach during the hour would be reflected by a corresponding change in phenol red concentration.

Assay for Salicylic Acid.—A modification of the method of Brodie *et al.* (1) for determination of salicylic acid in plasma was used. The rat stomach and its contents were homogenized with 20 ml. water in a Waring Blendor micro-jar. The homogenate was transferred to a separator, acidified with 5 ml. of 6 *N* hydrochloric acid, and extracted with one 30-ml. and three 20-ml. portions of ethylene dichloride. Each ethylene dichloride layer was separated by centrifuging. Enough ethylene dichloride was added to the combined extracts to obtain a total of 100 ml. A 20-ml. aliquot of the ethylene dichloride extract was shaken for 5 minutes with 10 ml. of an aqueous solution of ferric nitrate (1%) in 0.07 *N* nitric acid. After centrifuging, the aqueous phase was aspirated and assayed colorimetrically for salicylic acid at 530 mμ with a Bausch & Lomb spectronic 20 colorimeter.

Using the tissue extraction method described above, tissue blanks were analyzed and were found to yield essentially negligible salicylate blank values whether or not polysorbate 60 or caffeine was present. Extraction efficiency was determined by adding known amounts of salicylic acid solution to rat stomachs, homogenizing, and then extracting and assaying as described above. Recovery was found to be essentially constant over the concentration ranges encountered, regardless of the presence or absence of polysorbate 60 or caffeine. The average recovery was 91%. In the absorption experiments, the amount of salicylic acid remaining unabsorbed was calculated on the basis of the concentration of the assayed solution, its dilution, and the correction factor for recovery.

Assay for Ethanol.—Ethanol concentration was determined by the enzymatic method of Bücher and Redetzki (2), using a small aliquot of diluted rat stomach homogenate. In this method, ethanol is reacted with alcohol dehydrogenase (yeast) and diphosphopyridine nucleotide (DPN) in a buffered medium. The enzyme catalyzes the oxidation of ethanol by DPN. Ethanol concentration is reflected by the concentration of reduced DPN which was determined spectrophotometrically at 360 mμ with a Beckman model DU spectrophotometer. Tissue blanks were determined and, although significant, were quite constant. Recoveries were found to be essentially constant over the concentration range used, the average recovery being 87%. In the absorption experiments, the amount of ethanol remaining unabsorbed was calculated from the spectrophotometric results appropriately corrected for blank value, dilution factor, and recovery.

Determination of the Extent of Binding of Salicylic Acid by Polysorbate 60.—This determination was carried out by an equilibrium dialysis method. Ten milliliters of a 2% polysorbate 60 solution in 0.1 *N* hydrochloric acid was placed in a dialysis bag.[2] The bag was then placed into a 125-ml. conical flask which contained 110 ml. of a salicylic acid solution in the same solvent. The solutions were allowed to equilibrate at 33° for 7 days in a constant temperature water bath shaker. During this time the solutions were constantly agitated. At the end of 7 days, the solutions inside and outside the dialysis bag were assayed for salicylic acid concentration using the colorimetric procedure of Trinder (3). The increase in concentration of the inside solution over that of the outside solution is a direct indication of the amount of salicylic acid bound to polysorbate 60 contained in the bag. Similar experiments were carried out using distilled water and 0.005 *N* HCl (rather than 0.1 *N* HCl) as the solvent.

Determination of the Extent of Binding of Ethanol by Polysorbate 60.—This determination was carried out by an equilibrium dialysis method similar to the one described in the preceding paragraph. Ten milliliters of a 2% ethanol solution in distilled water was placed in a dialysis bag which was suspended in 110 ml. of 2% ethanol and 2% polysorbate 60 in distilled water. The solutions were equilibrated

[2] Tomac Nylon Bags, American Hospital Supply Corp., Evanston, Ill.

for up to 15 days and assayed by the method of Pawan and Hoult (4). Control experiments were carried out to verify the permeability of the dialysis membrane to ethanol, the impermeability of the membrane to polysorbate 60, and the absence of interference of polysorbate 60 with the ethanol assay.

Determination of the Stability Constant of the Caffeine–Salicylic Acid Complex.—The solubility method described by Higuchi and Zuck (15) was used to determine the stability constant of the caffeine–salicylic acid complex at 33°.

Determination of Partition Coefficients.—Partition coefficients were determined using 0.1 *N* hydrochloric acid and isoamyl acetate as the aqueous and organic phase, respectively. Equilibration was carried out at room temperature for at least 12 hours using an automatic shaking apparatus. Salicylic acid concentration was determined by the method of Trinder (3) and caffeine concentration by the method of Axelrod and Reichenthal (5).

RESULTS AND DISCUSSION

The model drug used in these studies, salicylic acid, is absorbed from the stomach and intestinal tract mainly by passive diffusion of nonionized molecules (6, 7). It was chosen because it is rapidly absorbed, and it forms complexes with many drugs and other compounds of pharmaceutical interest. It was decided to study gastric rather than intestinal absorption for the following reasons: the pH dependence of salicylic acid absorption and the feasibility of maintaining a low and constant pH in the stomach, the more rapid absorption of one of the complexing agents (caffeine) from the intestine which resulted in lethalities during preliminary intestinal perfusion experiments, the relative constancy of gastric volume under the experimental conditions, the possibility of using relatively large volumes of drug solution, and the ease of isolation and manipulation of the stomach.

The experimental procedures differed somewhat from the methodology used by many other investigators. In order to prevent damage to the delicate gastric mucosa, the rat stomach was not washed prior to the absorption experiment; the entire stomach and its contents (rather than gastric contents alone) were assayed at the end of the absorption period, so that only drug which had entered the circulation was considered as having been absorbed.

The two complexing systems used in this investigation represent different characteristics. The salicylic acid–polysorbate 60 system consists of a well-absorbed drug and a macromolecular substance which is not absorbed as such. It may be assumed, therefore, that the micellar complex of salicylic acid and polysorbate 60 will not pass across biologic membranes. On the other hand, both components of the salicylic acid–caffeine system are absorbed, but the latter at a much slower rate than the former (6). It may be expected that the complex itself would be absorbable but at a rate somewhat different from that of either of the component drugs.

Since the *in vivo* effects of complex formation were to be correlated with *in vitro* binding or stability data, the *in vivo* temperature of the drug solutions was determined, and *in vitro* measurements were carried out at the same temperature. Continuous temperature recording in the stomach of rats under conditions similar to those of actual absorption experiments indicated an average intragastric temperature of 34° initially, which decreased to 32° toward the end of 1 hour. Consequently, all physicochemical measurements were made at 33°.

The Salicylic Acid–Polysorbate 60 Complex.—The absorption of salicylic acid was decreased from 50% in 1 hour to 33% in 1 hour in the presence of 2% polysorbate 60 (Table I). This difference was found to be statistically significant ($p < 0.01$) by the Student *t* test. Absorption of salicylic acid from the stomach can be depicted satisfactorily as an exponential process (6, 8, 9), provided that adequate corrections are made for gastric emptying or that gastric volume can be maintained essentially constant. In general agreement with the observations of Schanker *et al.* (6), we found (by using phenol red as a marker) that gastric volume in the ligated rat stomach had changed on the average by less than 7% at the end of 1 hour, under the experimental conditions. This permitted calculation of first-order rate constants and half-absorption times, although the values must be looked upon as approximations only.

Levy (10) has discussed various mechanisms by which surface-active agents may modify drug absorption. He cautioned that it is necessary to consider possible effects on the absorbing membranes, interaction of the surface-active agent with the drug, and modification of dosage form properties. Some surface-active agents can cause damage to the gastrointestinal epithelium (11), and it is therefore possible that the over-all effect of polysorbate 60 on the absorption of salicylic acid, as observed in the present study, may represent the sum of two effects: modification of membrane permeability characteristics and micellar complexation of the drug. To investigate this possibility, the absorption of ethanol without as well as in the presence of polysorbate 60 was determined. Ethanol is not bound to polysorbate 60 (as evidenced by the results of equilibrium dialysis), and modification in membrane permeability characteristics should consequently be reflected by changes in the absorption rate of ethanol. Two per cent polysorbate 60 had no measurable effect on ethanol absorption (Table II), and it may be concluded therefore that changes in salicylic acid absorption in the presence of polysorbate 60 are due solely (or at least predominantly) to complex formation.

TABLE II.—EFFECT OF SALICYLIC ACID, CAFFEINE, AND POLYSORBATE 60 ON ETHANOL ABSORPTION FROM RAT STOMACH

Compn. of Solutions,[a] %	Animals, No.	Ethanol Absorbed,[b] %	S.-D.
Ethanol, 2	5	48	8
Salicylic acid, 0.1 Ethanol, 2	4	46	6
Salicylic acid, 0.1 Polysorbate 60, 2 Ethanol, 2	5	42	7
Salicylic acid, 0.1 Caffeine, 2.75 Ethanol, 2	4	51	4

[a] 0.1 *N* HCl served as the solvent in all solutions. [b] In 1 hour.

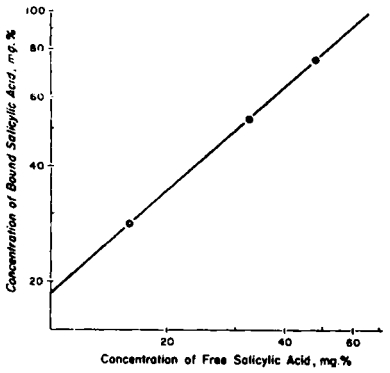


Fig. 1.—Relation between concentration of bound and free salicylic acid in solution of 2% polysorbate 60 in 0.1 *N* hydrochloric acid at 33°. Each point represents the average of four determinations.

The magnitude of interaction of salicylic acid with polysorbate 60 was determined by equilibrium dialysis and was found to be describable by the equation

$$C_b = KC_f^a \qquad \text{(Eq. 1)}$$

or

$$\log C_b = \log K + a \log C_f \qquad \text{(Eq. 2)}$$

where C_b is the concentration of bound drug, C_f is the concentration of free drug, and K and a are constants. This relationship, which is analogous to the Freundlich adsorption isotherm, applies also to the interaction of many drugs with plasma proteins (12). Results of the binding study (Fig. 1) showed that in the salicylic acid concentration range which existed in the absorption experiment (0.1% initially, decreasing to 0.067% at the end of 1 hour), the binding of drug with polysorbate 60 varied from 61.5 to 63.0%. Thus, the extent of drug complexation was essentially constant.

The gastric absorption of salicylic acid in the absence of complexing agents can be described by the exponential expression

$$\log A = \log A_0 - \frac{K}{2.3} t \qquad \text{(Eq. 3)}$$

where A_0 is the amount of drug introduced into the stomach, A is the amount remaining in the stomach at time t, and K is the first-order absorption rate constant.

When a constant fraction of drug is complexed and when it is assumed that only free drug is absorbed, absorption rate may be described by

$$\frac{dA}{dt} = -KfA \qquad \text{(Eq. 4)}$$

where dA/dt is the rate of absorption and f is the fraction of free drug. Upon integration, evaluation of constant of integration and change to common logarithms,

$$\log A = \log A_0 - \frac{K}{2.3} ft \qquad \text{(Eq. 5)}$$

The amount of salicylic acid remaining unabsorbed at 1 hour, upon administration of 5 ml. of a solution of 0.1% salicylic acid and 2% polysorbate 60, was calculated by Eq. 5. Using values of K = 0.69 hour^{-1} and f = 0.38, the theoretical amount A remaining unabsorbed is 3.8 mg. The actual average amount unabsorbed was 3.4 mg. The small difference between the theoretical and experimental value may be due to normal experimental variation, or it may be caused by competitive binding of polysorbate 60 by mucosal proteins or gastric contents. Lack of stability of polysorbate 60 in acidic gastric fluids was not a factor, since equilibrium dialysis of salicylic acid against polysorbate 60 in either 0.1 *N* HCl or distilled water yielded essentially equal results. It is suggested, therefore, that the effect of polysorbate 60 on the absorption of salicylic acid, under the experimental conditions, is due to a decrease in activity of the drug as a result of micellar complexation. It appears that the magnitude of the *in vivo* effects may be estimated satisfactorily on the basis of kinetic considerations based on *in vitro* binding constants and the absorption rate constant for the free drug.

The Salicylic Acid–Caffeine Complex.—The gastric absorption of salicylic acid is decreased from 50% per hour to 37% per hour in the presence of caffeine (Table I). The difference is statistically significant ($p < 0.01$). Since caffeine is ulcerogenic in rats [daily oral administration of caffeine for 5 days has caused superficial erosions of the glandular mucosa (13)], and since such breakdown of the mucosa:plasma barrier can result in more rapid drug absorption (14), ethanol was used as a noncomplexed marker to determine possible modification in membrane permeability characteristics. There was no evidence of increased permeability (Table II), and it may be concluded that acute administration of caffeine did not damage the gastric mucosa significantly during the 1-hour absorption period. The decreased absorption of salicylic acid cannot be ascribed to an increased pH of the solution containing caffeine, since the pH remained the same as that of the solution which did not contain caffeine.

The stability constant of the salicylic acid–caffeine complex in 0.1 *N* hydrochloric acid at 33°, determined by the solubility method (15), was found to be 40 (Fig. 2). This value compares favorably with that reported by Higuchi and Zuck (15) for the same complex at 30°—namely 44. A decrease in the stability constant at higher temperatures is to

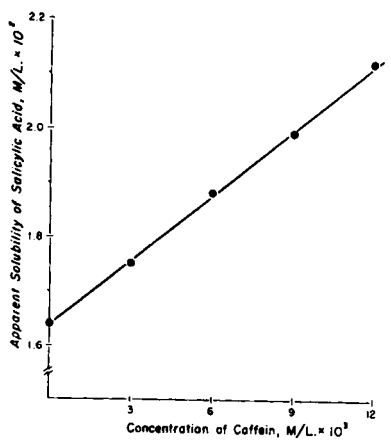


Fig. 2.—Apparent solubility of salicylic acid in 0.1 *N* hydrochloric acid at 33° as a function of caffeine concentration. Each point represents the average of at least four determinations.

TABLE III.—PARTITION COEFFICIENTS (ISOAMYL ACETATE, 0.1 *N* HCl) OF SALICYLIC ACID AND CAFFEINE ALONE AND PARTLY COMPLEXED

Initial Compn. of Aqueous Phase, %	Ratio, Organic: Aqueous Phase	Partition Coefficient
Salicylic Acid		
Salicylic acid, 0.1	5:100	163
Salicylic acid, 0.1 Caffeine, 2.75	5:100	28
Caffeine		
Caffeine, 0.2	10:1	0.27
Caffeine, 0.2 Salicylic acid, 0.275	10:1	0.37
Caffeine, 0.2 Salicylic acid,[a] 3.025	1:1	0.46
Caffeine, 0.2 Salicylic acid,[a] 8.525	1:1	0.84

[a] In these instances, most of the salicylic acid was dissolved in the organic phase prior to equilibration.

be expected. The partition coefficient (isoamyl acetate:0.1 *N* HCl) of salicylic acid is considerably greater than that of caffeine. Complexation of salicylic acid with caffeine decreases the apparent partition coefficient of the former and increases the apparent partition coefficient of the latter (Table III). This suggests one possible mechanism for the decreased absorption of salicylic acid in the presence of caffeine, as found in the present study: the salicylic acid–caffeine complex may be absorbed more slowly than salicylic acid itself and, since salicylic acid was 84% complexed in the administered solution, the over-all absorption retarding effect may be appreciable.

The theoretical rate of absorption of the salicylic acid–caffeine complex may be calculated by use of an equation developed by Higuchi and Lachman for an analogous *in vitro* system (16):

$$\frac{1}{t_{0.5}}(K_sC + 1) = \frac{1}{t_{0.5f}} + \frac{1}{t_{0.5c}}K_sC \quad \text{(Eq. 5)}$$

where K_s is the stability constant of the complex, C is the molar concentration of free caffeine, $t_{0.5}$ is the half-absorption time of salicylic acid in the presence of caffeine, $t_{0.5f}$ is the half-absorption time of free salicylic acid, and $t_{0.5c}$ is the half-absorption time of the salicylic acid–caffeine complex. Equation 5 applies in the strict sense only to systems where the complexing agent (caffeine in this case) is not absorbed, but the equation can be used satisfactorily in the present case since caffeine is relatively slowly absorbed and is used in high concentration.

The following information is used to solve Eq. 5: $t_{0.5} = 1.5$ hours and $t_{0.5f} = 1.0$ hours (both from data in Table I), $K_s = 40$ (from data depicted in Fig. 2), and $C = 0.135$ *M* or 0.101 *M* calculated from K_s using

$$(C) = \frac{(S - C)}{(S)K_s} \quad \text{(Eq. 6)}$$

where (C) is the concentration of free caffeine, (S) is the concentration of free salicylic acid, and $(S - C)$ is the concentration of the salicylic acid-caffeine complex. According to the data of Schanker *et al.* (6), caffeine is 24% absorbed in 1 hour. Our own data [based on 90 minute absorption experiments (17)] indicate an absorption rate of 27% per hour. If caffeine were not absorbed at all (which is the condition actually required for use of Eq. 5), $t_{0.5}$ is 6.3 hours. Going to the other extreme by assuming caffeine concentration to be 27% lower from the very beginning, $t_{0.5}$ is 5.0 hours. It is evident that (*a*) the equation can be used satisfactorily for estimating purposes (particularly in view of the limitations imposed by experimental variability of the *in vivo* system) and (*b*) it must be assumed that the complex itself is absorbed since the theoretical $t_{0.5}$ calculated above is much greater than the experimentally obtained value. Accepting this assumption and solving for $t_{0.5c}$ (the half-absorption time of the complex), yields a value of 1.7 hours (this value is the same if based on no absorption of free caffeine or on immediate 27% absorption). Interestingly, this value is intermediate between that of free salicylic acid (1.0 hour) and free caffeine (2.2–2.5 hours). The relative absorption rates are also consistent with the respective partition coefficients.

Despite the attractiveness of the hypothesis presented above, it is appropriate to consider an alternative mechanism to explain the experimental data. There is some indication that the gastric absorption of salicylic acid by the rat is, under the experimental conditions, rate limited by the rate of movement of drug molecules from the center of the gastric lumen to the periphery (6, 18). Assuming this to be mainly a diffusion process (rather than mixing and flow of gastric contents) and using Thovert's equation (19) to estimate the effect of molecular weight on diffusivity, it can be shown that the diffusivity of salicylic acid is 55% greater than that of the salicylic acid–caffeine complex:

$$\frac{\text{diffusivity of salicylic acid}}{\text{diffusivity of salicylic acid–caffeine complex}} = \frac{\sqrt{\text{mol. wt. of complex}}}{\sqrt{\text{mol. wt. of free acid}}} = 1.55$$

Correcting for the extent of salicylic acid complexation (84%) in the salicylic acid–caffeine system, the absorption rate constant of free drug should be about 42% greater than that in the presence of caffeine. This was indeed the case. Thus, the experimental data are equally compatible with an assumed decreased membrane permeation rate of the complex (compared with free salicylic acid) and a decreased rate of diffusion of the complex to the absorbing membranes. Further studies of this problem are in progress.

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Effect of Certain Drugs on Perfused Human Placenta IV

Detection of Specific Receptor Sites

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The effects of three adrenergic blocking agents (phentolamine, tolazoline, and hydralazine) on the action of epinephrine, norepinephrine, *l*-epinephrine, histamine, and 5-hydroxytryptamine in placental vessels were investigated. The results indicated that phentolamine not only diminished and/or blocked the vasoconstricting action of epinephrine, norepinephrine, and histamine but also caused a reversal of their effects. However, phentolamine could only diminish the vasopressor action of 5-hydroxytryptamine. From the results obtained in this investigation, it appears that the placental vessels have both *alpha* and *beta* receptors.

THE HUMAN BODY is composed of numerous organs consisting of various types of tissues which manufacture chemicals to perform specific functions. Whenever certain organs exhibit a variance in the amount of chemical substances normally produced, the homeostatic processes of the body controlled by these chemicals will usually be altered. However, when the same condition occurs in the pregnant state, not only normal body functions will be altered but also possibly fetal nourishment and development. Therefore, since it has been shown that 5-hydroxytryptamine (serotonin) (1), sympathomimetics, and histamine (2) constrict placental vessels directly and also that the placental barrier is permeable to substances below a molecular weight of 1000 (3), allowing their passage from maternal to fetal circulation, any condition which actually causes an increased level of these normal body chemicals could, *via* placental transfer, ultimately lead to teratogenic effects (4), decreased fetal oxygenation (1, 2), and even fetal death (5, 6).

Many drugs other than natural body chemicals have been shown to affect the placental vasculature. Such drugs include the narcotic analgesics, which cause constriction (morphine, meperidine, and codeine) (1) and certain vasodilators (isosorbide dinitrate, isoproterenol hydrochloride, nitroglycerin, and papaverine) (7). Because some of these agents cross the placental barrier (8, 9) and have been shown in isolated placental preparations to affect directly the placental vasculature (1, 2, 7), it is possible that when such drugs are administered to pregnant women they may disturb the homeostatic conditions of the placenta and exert serious effects on the fetus. Consequently, it is imperative to determine the action of all drugs on placental vasculature.

During the past 45 years, many perfusion experiments have been performed to test the effects of various neurohormones [epinephrine, norepinephrine, *l*-epinephrine, 5-hydroxytryptamine (serotonin)] and histamine on the placental vasculature (1, 2, 7, 10–13). It has been generally shown from these placental perfusion experiments that 5-hydroxytryptamine and histamine cause pronounced vasoconstriction, whereas the catecholamines (epinephrine, norepinephrine, and *l*-epinephrine) produce relatively slight vasoconstriction on isolated placental vasculature.

Recently, Astrom and Samelius (12) demonstrated that the vasoconstricting action of 5-hydroxytryptamine (serotonin) and adrenaline on the placental vasculature was blocked by the adrenergic blocking agent phentolamine (Regitine). This laboratory performed preliminary experiments to confirm these results. It was established that phentolamine not only blocked the action of epinephrine but also caused a reversal of its action on the placental vasculature. However, phentolamine only caused a slight diminution of the effects of 5-hydroxytryptamine.

The dual effect of epinephrine (vasoconstriction and vasodilatation) on the nerve-free placental vessels has raised the question of just how the catecholamines act. Is it possible to credit their action to the activation of specific receptor sites, to direct musculotropic action, or to endogenous release of the normal mediators? Therefore, the purpose of this investigation was to discern the effects of certain adrenergic blocking agents on the action of various catecholamines and attempt

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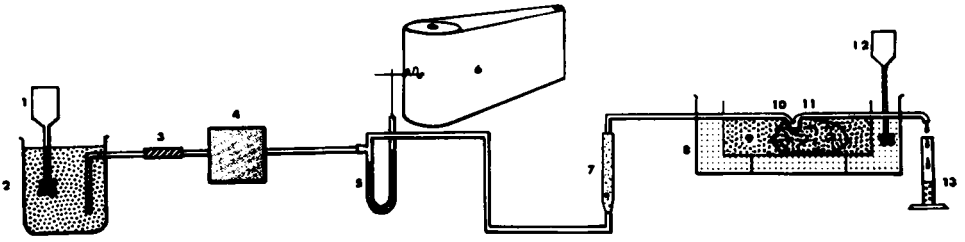


Fig. 1.—Schematic diagram of the apparatus for perfusion of placental vasculature. Key: 1, Bronwill thermoregulator; 2, reservoir, perfusion fluid (Tyrode's with P.V.P.); 3, rubber tubing (injection site); 4, Sigmamotor pump (model TS-8); 5, mercury manometer; 6, Livingston variable-speed kymograph; 7, RGI flowmeter; 8, outer Plexiglas chamber (water jacket); 9, inner Plexiglas chamber (perfusion chamber); 10, arterial cannula; 11, venous cannula; 12, Bronwill thermoregulator; 13, 100-ml. graduated cylinder.

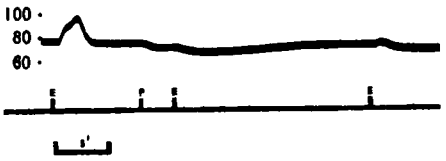


Fig. 2.—Effect of phentolamine on the action of epinephrine hydrochloride in placental vasculature. Key: E, 20 mcg. epinephrine hydrochloride; P, 3 mg. phentolamine methanesulfonate.

to identify the specific mechanism of action whereby these agents affect the placental vasculature.

MATERIALS AND METHODS

The full-term human placentas utilized in this experiment were obtained from the delivery room of the hospital 5–15 minutes after normal delivery. A glass container of Tyrode's solution heated to 38° was employed to transport the placental preparations.

The procedures concerned with the manipulation and preparation of the placentas for perfusion were described in a previous paper (2). A schematic diagram of the apparatus is shown in Fig. 1. Umbilical cords of placentas measuring more than 5.5 cm. in length were designated as long cords; those under 5.5 cm. were designated as short cords.

The placental outflow was measured directly at the onset of the perfusion by means of a 100-ml. graduated cylinder and never reperfused. If the volume of inflow greatly exceeded the volume of outflow or if the perfusion pressure could not be maintained at a constant level, the placenta was discarded. During this study, 51 placental preparations were utilized on which 132 successful experiments were performed.

The following drugs were injected into the arterial side of the perfusion in a volume of distilled water not exceeding 1.5 ml.: epinephrine hydrochloride, 0.1%; *l*-epinephrine bitartrate,[1] 0.1%; *l*-norepinephrine bitartrate (Levophed), 0.2% (0.1% as base); histamine acid phosphate, 0.055% (0.02% as base); 5-hydroxytryptamine creatinine sulfate (serotonin), 0.01%; phentolamine methanesulfonate (Regitine),[2] 0.2%; tolazoline hydrochloride (Priscoline), 2.5%; and hydralazine hydrochloride (Apresoline),[2] 0.4%.

[1] Supplied through the courtesy of Sterling-Winthrop Research Institute, Rensselaer, N. Y.

[2] Supplied through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

RESULTS

The following results were obtained on full-term human placental vasculature perfused at the pressure range of 50–90 mm. Hg. This range of perfusion pressure (50–90 mm. Hg) corresponds to 30–70 ml. of perfusion fluid inflow per minute.

Effects of Phentolamine on the Action of Certain Amines in the Placental Vasculature

Epinephrine.—Table I shows that prior to the administration of 3 mg. of the adrenergic blocking agent, phentolamine, 20 mcg. of epinephrine exhibited a moderate vasoconstriction in placentas with both short and long cords. However, the vasoconstrictor action of epinephrine in the placentas with long cords seemed to be more pronounced than in those with short cords.

When 20 mcg. of epinephrine was given subsequent to the administration of phentolamine, not only was the vasoconstrictor response blocked but vasodilatation (reversal) effect of epinephrine also became apparent (Fig. 2). This reversal effect of epinephrine appeared to be similar in placentas with short and/or long cords. A subsequent injection of epinephrine 15–20 minutes later produced a normal response. In one experiment, the placental vasculature did not exhibit a response to the action of epinephrine.

Norepinephrine.—Prior to the administration of phentolamine, 40 mcg. of norepinephrine exhibited a moderate vasoconstriction in the placental vasculature, irrespective of cord length (Table I). Also, when norepinephrine was administered approximately 3 minutes subsequent to the administration of phentolamine, a reversal of its normal vasoconstrictor response occurred (Fig. 3). When 40 mcg. of norepinephrine was administered 15–20 minutes subsequent to the administration of phentolamine, its normal vasoconstrictor response appeared again.

l-Epinephrine.—Table I shows that when 20 mcg. of *l*-epinephrine was perfused through the placental vascular system before the administration of 3 mg. of phentolamine, moderate vasoconstriction occurred in the placentas with short umbilical cords. However, in one experiment in which a long-cord placenta was utilized, the vasoconstricting effect of *l*-epinephrine appeared to be more pronounced than its vasoconstricting action on short-cord placentas.

When *l*-epinephrine was perfused through the placental vasculature following the administration of phentolamine, both vasoconstriction and vasodilatation occurred. However, the vasoconstrictor effect

TABLE I.—EFFECTS OF PHENTOLAMINE ON THE ACTION OF CERTAIN AMINES IN THE PLACENTAL VASCULATURE

Drug and Dose	Cord Length[a]	Range of Pressure Changes, mm. Hg: Increase	Av. Change	No. Expt.	Decrease	Av. Change	No. Expt.
Before Phentolamine							
Epinephrine, 20	S	+1.9 to +5.4	+3.3	3	...	...	..
mcg.	L	+1.2 to +19.6	+7.8	5	...	...	..
After Phentolamine							
Epinephrine, 20	S	+1.8	+1.8	1	−2.2 to −7.6	−4.5	4
mcg.	L	...	...	..	0.0 to −5.0	−2.3	6
Before Phentolamine							
Norepinephrine,	S	+11.2	+11.2	1	...	...	..
40 mcg.	L	+2.0 to +7.4	+4.3	4	...	...	..
After Phentolamine							
Norepinephrine,	S	...	...	..	−2.6 to −4.0	−3.3	3
40 mcg.	L	...	...	..	−2.8 to −6.4	−3.9	7
Before Phentolamine							
l-Epinephrine,	S	+3.4 to +22.0	+10.4	4	...	...	..
20 mcg.	L	+60.0	+60.0	1	...	...	..
After Phentolamine							
l-Epinephrine,	S	+0.8 to +1.0	+0.9	2	−2.0 to −6.0	−4.0	2
20 mcg.	L	...	...	..	−3.6	−3.6	1
Before Phentolamine							
Histamine, 20 mcg.	S	+2.0 to +64.0	+12.5	11	...	...	..
	L	+1.8 to +136.0	+41.9	16	...	...	..
After Phentolamine							
Histamine, 20 mcg.	S	+3.2 to +5.2	+4.7	9	−2.4 to −2.8	−2.6	2
	L	+1.0 to +110.0	+34.4	11	−1.6 to −4.0	−2.6	5
Before Phentolamine							
Serotonin, 50 mcg.	S	+4.0 to +108.0	+35.0	16	...	...	..
	L	+43.0 to +145.2	+83.2	15	...	...	..
After Phentolamine							
Serotonin, 50 mcg.	S	+1.4 to +93.4	+25.5	16	...	...	..
	L	+10.0 to +164.6	+62.2	15	...	...	..

[a] S, small cord; L, long cord.

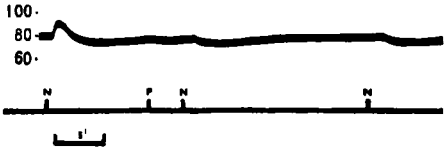


Fig. 3.—Effect of phentolamine on the action of norepinephrine bitartrate in placental vasculature. Key: N, 40 mcg. norepinephrine bitartrate; P, 3 mg. phentolamine methanesulfonate.

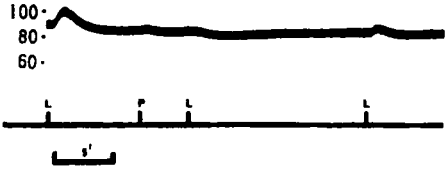


Fig. 4.—Effect of phentolamine on the action of *l*-epinephrine bitartrate in placental vasculature. Key: L, 20 mcg. *l*-epinephrine bitartrate; P, 3 mg. phentolamine methanesulfonate.



Fig. 5.—Effect of phentolamine on the action of histamine acid phosphate in placental vasculature. Key: H, 20 mcg. histamine acid phosphate; P, 3 mg. phentolamine methanesulfonate.

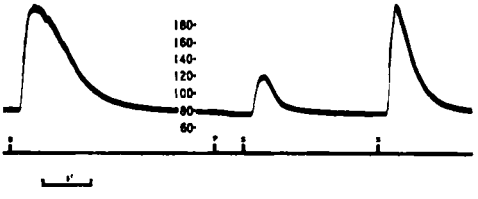


Fig. 6.—Effect of phentolamine on the action of serotonin in placental vasculature. Key: S, 50 mcg. serotonin; P, 3 mg. phentolamine methanesulfonate.

occurring after phentolamine was markedly reduced compared to the vasoconstriction produced originally. The vasodilatation or reversal effect occurring after phentolamine administration was approximately the same in placentas with short or long cords (Fig. 4).

Histamine.—When histamine (20 mcg.) was perfused through the placental vasculature, it exhibited a vasoconstrictor action greater than epinephrine, norepinephrine, or *l*-epinephrine, irrespective of cord length. Table I shows that when histamine was given prior to the administration of phentolamine, its vasconstrictor action generally appeared to be more pronounced in the placentas with long cords than in those with short cords.

When 20 mcg. of histamine was perfused through

TABLE II.—EFFECTS OF TOLAZOLINE ON THE ACTION OF CERTAIN AMINES IN THE PLACENTAL VASCULATURE

Drug and Dose	Cord Length[a]	Range of Pressure Changes, mm. Hg: Increase	Av. Change	No. Expt.	Decrease	Av. Change	No. Expt.
Before Tolazoline							
Epinephrine, 20 mcg.	S	+3.0 to +36.0	+15.7	3	...	...	..
After Tolazoline							
Epinephrine, 20 mcg.	S	+1.0 to +4.4	+2.8	3	...	...	..
Before Tolazoline							
Norepinephrine, 40 mcg.	S	+1.6 to +4.0	+2.5	3	...	...	..
After Tolazoline							
Norepinephrine, 40 mcg.	S	0.0 to +1.0	+0.5	2	...	...	..
	L	...	...		−7.0	−7.0	1
Before Tolazoline							
l-Epinephrine, 20 mcg.	S	+2.8 to +5.0	+3.9	3	...	...	..
After Tolazoline							
l-Epinephrine, 20 mcg.	S	+2.0 to +2.4	+2.1	3	...	...	..
Before Tolazoline							
Histamine, 20 mcg.	S	+4.6 to +52.0	+13.1	9	...	...	..
After Tolazoline							
Histamine, 20 mcg.	S	+2.8 to +15.0	+10.2	8	−3.0	−3.0	1
Before Tolazoline							
Serotonin, 50 mcg.	S	+26.0 to +88.0	+53.3	9	...	...	..
After Tolazoline							
Serotonin, 50 mcg.	S	+24.0 to +100.2	+57.6	9	...	...	..

[a] S, Small cord; L, long cord.

TABLE III.—EFFECTS OF HYDRALAZINE ON THE ACTION OF CERTAIN AMINES IN THE PLACENTAL VASCULATURE

Drug and Dose	Cord Length[a]	Range of Pressure Changes, mm. Hg[b]: Increase	Av. Change	No. Expt.	Decrease	Av. Change	No. Expt.
Before Hydralazine							
Epinephrine, 20 mcg.	S	+2.8 to +3.4	+3.1	2	...	...	..
	L	+20.0	+20.0	1	...	...	..
After Hydralazine							
Epinephrine, 20 mcg.	S	+3.0 to +3.6	+3.2	2	...	...	..
	L	+31.6	+31.6	1	...	...	..
Before Hydralazine							
Norepinephrine, 40 mcg.	S	+4.0 to +12.0	+6.3	3	...	...	..
After Hydralazine							
Norepinephrine, 40 mcg.	S	+3.4 to +11.4	+4.4	3	...	...	..
Before Hydralazine							
l-Epinephrine, 20 mcg.	S	+2.8	+2.8	1	...	...	..
	L	+2.0 to +4.0	+3.0	2	...	...	..
After Hydralazine							
l-Epinephrine, 20 mcg.	S	+2.8	+2.8	1	...	...	..
	L	+2.6 to +5.0	+3.5	2	...	...	..
Before Hydralazine							
Histamine, 20 mcg.	S	+3.4 to +101.0	+52.2	2	...	...	..
	L	+40.0	+40.0	2	...	...	..
After Hydralazine							
Histamine, 20 mcg.	S	+3.4 to +110.0	+56.7	2	...	...	..
	L	+20.0 to +30.0	+25.0	2	...	...	..
Before Hydralazine							
Serotonin, 50 mcg.	S	+24.0 to +118.0	+48.0	6	...	...	..
	L	+15.6 to +56.0	+40.4	3	...	...	..
After Hydralazine							
Serotonin, 50 mcg.	S	+12.0 to +109.0	+38.8	6	...	...	..
	L	+20.0 to +60.4	+40.2	2	...	...	..

[a] S, Small cord; L, long cord. [b] No decrease or average change in any of the experiments.

the placental vasculature subsequent to 3 mg. of phentolamine, its vasoconstrictor action was not only markedly diminished (Fig. 5), but also a reversal of its normal pressor action occurred in some experiments (Table I).

5-Hydroxytryptamine (Serotonin).—Of all of the agents used in this experiment, 5-hydroxytryptamine (serotonin) had the greatest vasoconstricting effect on the placental vasculature. Table I shows that the effect of 50 mcg. of 5-hydroxytryptamine on perfused placental vasculature before the administration of phentolamine on the average was greater than the vasoconstricting action exerted when it was administered after phentolamine. However, the vasoconstrictor action of 5-hydroxytryptamine was never completely blocked or reversed (Fig. 6).

TABLE IV.—COMPOSITE RESULTS OF COMPARATIVE ANTAGONISTIC ACTION OF PHENTOLAMINE, TOLAZOLINE, AND HYDRALAZINE ON THE ACTION OF CERTAIN AMINES IN THE PLACENTAL VASCULATURE

Drug	Dose, mcg.	Expt., No.	Degree of Antagonism			
			None	Partial	Complete	Reversal
Phentolamine						
Epinephrine	20	11	..	1	1	9
Norepinephrine	40	10	..	..	..	10
l-Epinephrine	20	5	..	2	..	3
Histamine	20	27	7	12	1	7
Serotonin	50	31	4	27	..	..
Tolazoline						
Epinephrine	20	3	..	3	..	..
Norepinephrine	40	3	..	1	1	1
l-Epinephrine	20	3	..	3	..	..
Histamine	20	9	5	3	..	1
Serotonin	50	9	6	3	..	..
Hydralazine						
Epinephrine	20	3	2	1	..	..
Norepinephrine	40	3	2	1	..	..
l-Epinephrine	20	3	3	..	..	..
Histamine	20	4	1	3	..	..
Serotonin	50	8	3	5	..	..

Effects of Tolazoline on the Action of Certain Amines in the Placental Vasculature

Epinephrine.—As shown in Table II, the vasoconstrictor effect of 20 mcg. of epinephrine in the perfused placental vasculature exhibited the same moderate vasoconstriction obtained under *Effects of Phentolamine*. However, following the perfusion of 5 mg. of tolazoline, 20 mcg. of epinephrine produced a vasoconstrictor response which was greatly diminished compared to the response obtained prior to the administration of tolazoline. It was also observed that tolazoline did not cause reversal of epinephrine effects on the placental vasculature (Table IV).

Norepinephrine.—Norepinephrine (40 mcg.) administered to the perfused placental vasculature prior to the administration of tolazoline exhibited a slight vasoconstrictor response. This vasoconstrictor effect of norepinephrine, in this part of the investigation was not so pronounced as its vasoconstricting effect produced under *Effects of Phentolamine*. When norepinephrine was administered subsequent to the administration of 5 mg. of tolazoline, its vasoconstrictor action was diminished; in one case, a reversal of its action occurred (Table IV).

l-Epinephrine.—*l*-Epinephrine (20 mcg.), perfused through the placental vasculature prior to the administration of tolazoline, exhibited a vasoconstrictor response comparable to that elicited by norepinephrine. When 20 mcg. of *l*-epinephrine was administered subsequent to the administration of 5 mg. of tolazoline, its vasoconstricting response was slightly reduced (Table II).

Histamine.—Histamine (20 mcg.), administered prior to tolazoline, exhibited a marked vasoconstrictor effect on the placental vasculature (Table II). However, this marked vasoconstrictor effect of histamine was slightly diminished when it was given subsequent to the 5 mg. of tolazoline. In only one experiment did a reversal of histamine's action occur—when it was administered subsequent to 5 mg. of tolazoline (Table IV).

5-Hydroxytryptamine (Serotonin).—Table II shows that 5-hydroxytryptamine again exhibited the greatest vasoconstrictor effect on the placental vasculature. When 5-hydroxytryptamine was perfused through the placental vasculature subsequent to 5 mg. of tolazoline, the vasoconstricting response of 5-hydroxytryptamine was, on the average, just slightly increased. At no time was there a reversal of 5-hydroxytryptamine's pressor action (Table IV).

Effects of Hydralazine on the Action of Certain Amines in the Placental Vasculature

Epinephrine.—The perfusion of 20 mcg. of epinephrine through the placental vasculature (Table III) prior to the administration of 4 mg. of hydralazine elicited a vasoconstrictor response comparable to that observed previously for epinephrine under *Effects of Phentolamine* and *Effects of Tolazoline*. Hydralazine did not appear to affect the vasoconstrictor action of epinephrine in short-cord placentas. However, in one experiment utilizing a placenta with a long cord, hydralazine actually caused an increase in the constrictor action of epinephrine (Table III).

Norepinephrine.—When 40 mcg. of norepinephrine was administered prior to the administration of 4 mg. of hydralazine, it produced moderate vasoconstriction on the placental vasculature (Table III). However, when 40 mcg. of norepinephrine was given subsequent to the administration of 4 mg. of hydralazine, there was a slight diminution in its vasoconstrictor action. No reversal of norepinephrine effects was observed with hydralazine (Table IV).

l-Epinephrine.—*l*-Epinephrine (20 mcg.) produced slight vasoconstriction in the placental vasculature when it was given prior to the administration of 4 mg. of hydralazine. This vasoconstrictor action of *l*-epinephrine was not altered to a significant degree by 4 mg. of hydralazine. It was also observed that no reversal response occurred with *l*-epinephrine when it was administered after hydralazine (Table IV).

Histamine.—Table III also shows that 20 mcg. of histamine had a marked vasoconstrictor effect on the placental vasculature when it was administered prior to the administration of 4 mg. of hydralazine. When 20 mcg. of histamine was administered subsequent to the administration of 4 mg. of hydralazine,

a diminution of its vasoconstricting effects occurred only in placentas with long cords. No reversal of histamine vasoconstrictor action was observed when it was administered after hydralazine (Table IV).

5-Hydroxytryptamine (Serotonin).—It can be seen from Table III that 5-hydroxytryptamine (serotonin) appeared to exhibit the greatest vasoconstrictor response of all of the agents used in this study. The vasoconstrictor action of 5-hydroxytryptamine was moderately diminished in those placentas with short cords when it was administered subsequent to the administration of 4 mg. of hydralazine. No reversal of 5-hydroxytryptamine's vasoconstricting action was observed using 4 mg. of hydralazine (Table IV).

DISCUSSION

In the majority of the experiments, the agonist was administered before the antagonist to establish a standard response. This standard response served as a comparison for the agonist's action when it was administered 3 minutes subsequent to any of the adrenergic blocking agents employed. When the agonists were administered 15–20 minutes subsequent to the administration of an adrenergic blocking agent, their responses began to approach those initially produced. This method was utilized to rule out the possibility of tachyphylaxis occurring due to the action of the agonist (Figs. 2–6).

The antagonists (phentolamine, tolazoline, and hydralazine) employed in this investigation exhibited their greatest blocking effect on the action of the catecholamines (epinephrine, norepinephrine, and *l*-epinephrine) and least blocking effect on 5-hydroxytryptamine and histamine. In most experiments, epinephrine, norepinephrine, and *l*-epinephrine usually exhibited a reversal action when given subsequently to phentolamine. However, the occurrence of the reversal action decreased markedly when tolazoline was employed; no reversal occurred when hydralazine was utilized. Thus, as seen from Table IV, the ability of adrenergic blocking agents to antagonize the vasoconstricting action of the agonists employed in this investigation is in decreasing order of potency phentolamine, tolazoline, and hydralazine.

The possibility of endogenous release of normal mediators being the specific mechanism of action of the amines employed can be eliminated, since full-term human placentas have been shown to be devoid completely of nervous tissue (14–17). Also, since the vasoconstricting action of epinephrine, norepinephrine, and *l*-epinephrine can be blocked by the *alpha* receptor adrenergic blocking agent phentolamine, as shown in this study, this would tend to eliminate the factor of general direct musculotropic action. Hence, the only remaining mechanism of action is the activation of specific receptor sites.

In recent years different receptor site theories have been postulated for the action of sympathomimetic amines. Of the theories advanced, the one which seems to be the most popular is that postulated by Ahlquist (18, 19). He suggested two receptor sites, classified as *alpha* and *beta*. The *alpha* adrenergic receptor site, when stimulated, exhibits an excitatory or vasoconstrictor action (with the possible exception of intestinal relaxation), and stimulation of the *beta* adrenergic receptor site exhibits an inhibitory or vasodilator action.

The vasoconstricting action which is produced by the agonists (epinephrine, norepinephrine, *l*-epinephrine, histamine, and 5-hydroxytryptamine) possibly corresponds to the stimulation of the *alpha* adrenergic receptors in Ahlquist's classification. This is substantiated because the vasoconstricting action of these drugs can either be diminished, blocked, or reversed with the use of an *alpha* receptor blocking agent, such as phentolamine. Consequently, because of the blocking of the *alpha* adrenergic receptor site with phentolamine, the secondary effect (vasodilatation or reversal) of epinephrine, norepinephrine, *l*-epinephrine, and histamine also appears. This possibly corresponds to *beta* receptor stimulation, since according to the Ahlquist's theory, vasodilatation is the result of stimulation of *beta* adrenergic receptor sites. Therefore, it appears at this time that *alpha* and *beta* adrenergic receptor sites are present in the placental vasculature. Thus, the hypothesis previously presented by Dornhorst and Young (10), in which they stated that adrenaline and noradrenaline may act on placental vasculature by a simple direct action, must now be modified to a specific action on adrenergic receptors.

Astrom and Samelius (12), in a previous investigation, indicated that the vasoconstrictor action of 5-hydroxytryptamine and adrenaline on placental vessels could be blocked by 3 mg. of phentolamine. This laboratory found that adrenaline could not only be blocked, but also its action could be reversed when it was administered subsequent to 3 mg. of phentolamine. However, it was observed that vasoconstrictor action of 5-hydroxytryptamine could only be diminished with 3 mg. of phentolamine and never blocked completely. Thus, the results obtained previously by Astrom and Samelius could not be confirmed. However, the results of this experiment support other investigations (2, 13), in which it was observed that the catecholamines, epinephrine and *l*-epinephrine, produced slight to moderate vasoconstriction, histamine marked vasoconstriction, and 5-hydroxytryptamine the most pronounced vasoconstriction on the placental vessels.

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Cardiac Effects of Angiotensin II

By EUGENE E. VOGIN* and JOSEPH P. BUCKLEY

The injection of angiotensin II, 1.0 mcg./Kg., into pentobarbital anesthetized dogs produced a significant increase in systolic and diastolic blood pressures and myocardial blood flow, a slight increase in the force of right ventricular contraction, and no significant effects on heart rate. Cardiac output was initially decreased; but within 80 to 100 seconds after angiotensin II administration, a secondary rise was noted. The rise in diastolic pressure was significantly greater than that of the systolic pressure at the 5 per cent level. The coronary flow increase appeared to be dependent entirely on the increase in diastolic blood pressure. In coronary perfusion studies, angiotensin II produced myocardial vasoconstriction when injected either intravenously or intra-arterially. These effects were mediated by both direct musculotropic actions and the sympathetic nervous system since acute sympathectomy reduced, but did not abolish, the activity of angiotensin II. The sympathetic component of the cardiovascular effects of angiotensin II appear to be *via* the alpha receptors since nethalide markedly potentiated the activity of angiotensin II. The effect of pretreatment with phenoxybenzamine, atropine, or dichloroisoproterenol on the cardiovascular responses to angiotensin II was also evaluated.

THERE HAVE BEEN several conflicting reports concerning the effects of angiotensin II on coronary blood flow. Investigators have reported that angiotensin decreases coronary blood flow (1), produces coronary vasoconstriction (2, 3), produces a transient reduction in coronary flow followed by a transient increase in flow (4), increases coronary vascular resistance without altering coronary flow (5), produces no alteration in coronary flow (6), and produces an increase in coronary blood flow (7). This paper reports a group of studies designed to facilitate a better understanding of certain cardiovascular actions of angiotensin II.

EXPERIMENTAL

Mongrel dogs of either sex, weighing between 10.2 and 18.6 Kg., were anesthetized by an intravenous injection of pentobarbital sodium (35 mg./Kg.) and the trachea cannulated. Femoral arterial blood pressure was recorded with a Statham pressure transducer (model P23AC) following cannulation of the right femoral artery. Heart rate was determined from the blood pressure tracing.

Cardiac output flow measurements were performed utilizing an electromagnetic flowmeter (Medicon FM-6), described by Olmsted (8). The animals were placed on artificial respiration with a Harvard respirator (model 606) utilizing a mixture of 95% O_2 and 5% CO_2. The thoracic cavity was entered at the fourth intercostal space using electrocautery to minimize bleeding. The lungs were retracted and the pericardium incised and secured to the chest wall with wound clips to create a pericardial sac whereby the heart and aorta were easily accessible. The ascending aorta was carefully isolated from the surrounding tissue, and Medicon flo-probe (12 or 15 mm. i.d.) was placed around the vessel. The left common carotid artery was cannulated and connected to the inflow tubing of a Shipley-Wilson rotameter; a 7- to 10-mm. segment of the left anterior descending coronary artery was isolated for subsequent cannulation and the animal heparinized (1000 units/Kg. i.v.). The left descending coronary artery was perfused as described by Buckley *et al.* (9).

In another series of animals, flow to the cannulated coronary artery was maintained constant by a Sigmamotor pump (TM10) and changes in coronary perfusion pressures measured as described by Fowler and Holmes (2). Changes in ventricular force were estimated with a Walton-Brodie strain gauge arch (10) sutured to the right ventricle.

All physiological measurements were recorded on an Offner dynograph or Grass polygraph. Coronary vascular resistance was calculated from the formula of Wegria *et al.* (11)

$$\text{Resistance} = \frac{\text{mean blood pressure (mm. Hg)}}{\text{mean coronary flow (ml./minute)}}$$

Acute cardiac denervation was performed in two dogs according to the method described by Morrow *et al.* (12). This consisted of bilateral cervical vagotomy and excision of the stellate and upper four sympathetic ganglia. The following compounds were administered: synthetic angiotensin II,[1] 1.0 mcg./Kg.; phenoxybenzamine hydrochloride, 5.0 mg./Kg.; atropine sulfate, 0.25 mg./Kg.; dichloroisoproterenol hydrochloride (DCI), 5.0 mg./Kg.; and nethalide hydrochloride,[2] 5.0 mg./Kg.

STATISTICAL METHODS

Because of the wide variation in control values, all results were calculated as their percentage change from control, then analyzed statistically by the following methods. To compare the effects of angiotensin prior to surgical or drug treatment, the mean percentage difference was calculated and probabilities determined using the Student *t* test for

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[1] Kindly supplied as Hypertensin by Dr. W. Wagner, Ciba Pharmaceutical Co., Summit, N. J.

[2] Kindly supplied as Ay 6204 by Dr. A. Sahagian-Edwards, Ayerst Laboratories, New York, N. Y.

TABLE I.—EFFECTS OF ANGIOTENSIN (1 mcg./Kg.) ON CARDIAC ACTIVITY

	Systolic Pressure	Diastolic Pressure	Coronary Blood Flow	Heart Rate	Right Ventricular Force	Coronary[a] Perfusion Pressure	Cardiac Output
Intravenous Administration							
Mean % change	48	70	63	6	39	27	−23
S.E.[b]	4	6	8	5	4	3	3
N[c]	21	21	15	21	6	5	21
P	<0.001	<0.001	<0.001	>0.05	<0.001	<0.001	<0.001
Intra-arterial Administration							
Mean % change	40	72	...	3	37	33	−23
S.E.[b]	4	11	...	5	7	3	3
N[c]	6	6	...	6	6	6	5
P	<0.001	<0.001	...	>0.05	<0.01	<0.001	<0.001

[a] Flow to coronary bed was maintained constant by a Sigmamotor pump. [b] ± Standard error. [c] Number of animals.

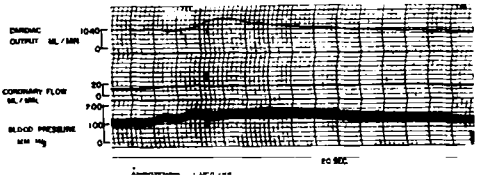


Fig. 1.—The effect of intravenous injection of angiotensin II (1 mcg./Kg.) on blood pressure, coronary flow, and cardiac output.

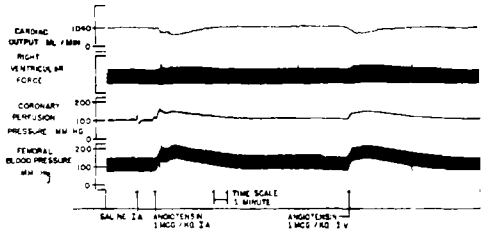


Fig. 2.—The effect of intra-arterial and intravenous injections of angiotensin II on blood pressure, coronary perfusion pressure, right ventricular force, and cardiac output.

paired results (13). To determine the effects of surgical sympathectomy or drug treatment on the response to angiotensin, the data were analyzed for homogeneity (13). All homogeneous results were then subjected to an analysis of variance (13); if significant differences existed, the means were compared using Harter's modification of Duncan's multiple-range test (14–16).

RESULTS

The effects of angiotensin II injected either intravenously or intra-arterially are summarized in Table I (Figs. 1 and 2). These results show that there were significant increases in systolic pressure, diastolic pressure, coronary blood flow, right ventricular force, and coronary perfusion pressure. The coronary flow increase occurred concomitantly with the increase in diastolic pressure throughout its entirety. Heart rate increased in several animals while in others it decreased; no definite pattern of effect could be established in these pentobarbital anesthetized animals when monitoring this parameter.

During the first minute following angiotensin injections, cardiac output decreased. However, in several animals cardiac output increased between the second and third minutes after injection. Coronary vascular resistance calculations showed that in the autoperfusion studies, angiotensin neither increased nor decreased this value. However, the increase in coronary perfusion pressure indicated that the myocardial vessels were constricted as a result of angiotensin administration. The rise in diastolic pressure was significantly greater than that of the systolic pressure at the 0.1% level following intravenous injections and at the 5% level following intra-arterial injections of angiotensin.

Systolic and Diastolic Blood Pressure.—Tables II and III depict the results of surgical sympathectomy or various drug pretreatments on the blood pressure responses to injected angiotensin. Only in the surgical sympathectomized animals were changes in the systolic blood pressure response noted. The reduction in responsiveness to angiotensin was significant at the 0.1% level.

Nethalide at a dose which blocked the chronotropic effect of isoproterenol (1 ml. of 10^{-6} solution) potentiated the rise in diastolic blood pressure to an extent greater than any of the other treatments. This potentiation was significant at the 0.1% level following intravenous angiotensin. Neither surgical sympathectomy nor other drug pretreatments were able to alter significantly the diastolic blood pressure response to intravenous angiotensin; nor did nethalide significantly alter the response to intra-arterial injections of this polypeptide. As in the untreated animals, the increase in diastolic pressure was significantly greater than that of the systolic pressure in the sympathectomized, atropine pretreated, and nethalide pretreated animals at the 1% level and at the 5% level in the DCI pretreated animals. Phenoxybenzamine pretreated animals did not show this greater increase in diastolic pressure.

Coronary Blood Flow, Coronary Vascular Resistance, and Coronary Perfusion Pressure.—Nethalide potentiated the effect of angiotensin in increasing coronary blood flow to an extent greater than any of the other treatments (Tables II and III). The increase in coronary blood flow following this agent differed from the nontreated animals at the 0.1% level. None of the other agents altered the coronary blood flow response to angiotensin significantly. It should be noted that in the surgically sympathectomized animals, the coronary blood flow response to angiotensin was significantly reduced ($p < 0.001$) when compared to the nontreated animals.

TABLE II.—EFFECT OF SURGICAL SYMPATHECTOMY OR DRUG PRETREATMENT ON THE CARDIOVASCULAR RESPONSES TO ANGIOTENSIN (1 mcg./Kg. i.v.)

Treatment	Systolic Pressure	Diastolic Pressure	Coronary Blood Flow	Cardiac Output
Surgical Sympathectomy				
Mean % change	18	48	18	−18
S.E.[a]	8	2	2	6
N[b]	2	2	2	2
Phenoxybenzamine, 5 mg./Kg.				
Mean % change	46	64	49	−13
S.E.[a]	9	13	6	8
N[b]	5	5	5	5
Atropine, 0.25 mg./Kg.				
Mean % change	52	80	62	−10
S.E.[a]	4	1	2	9
N[b]	2	2	2	2
Dichloroisoproterenol, 5 mg./Kg.				
Mean % change	52	87	56	+19
S.E.[a]	4	8	5	2
N[b]	3	3	3	3

[a] ±, Standard error. [b] Number of animals.

TABLE III.—EFFECT OF NETHALIDE (5 mg./Kg. i.v.) PRETREATMENT ON THE CARDIOVASCULAR RESPONSES TO ANGIOTENSIN (1 mcg./Kg.)

	Systolic Pressure	Diastolic Pressure	Coronary Blood Flow	Right Ventricular Force	Coronary Perfusion Pressure	Cardiac Output
Intravenous Angiotensin						
Mean % change	50	125	103	25	21	−20
S.E.[a]	6	20	5	3	4	5
N[b]	7	7	2	5	4	7
Intra-arterial Angiotensin						
Mean % change	53	106	...	27	26	−25
S.E.[a]	9	35	..	6	11	7
N[b]	5	5	...	5	3	2

[a] ± Standard error. [b] Number of animals.

As in the untreated animals, neither surgical sympathectomy nor drug pretreatment significantly altered the effects of angiotensin on coronary vascular resistance. Nethalide pretreatment did not alter significantly the increase in coronary perfusion pressure produced by either intravenous or intra-arterial injections of angiotensin.

Heart Rate.—Similar to the results obtained in untreated animals, neither surgical sympathectomy nor drug pretreatment significantly altered the effects of angiotensin on heart rate.

Right Ventricular Force.—Nethalide pretreatment attenuated the increase in intravenous or intra-arterial right ventricular force of contraction produced by angiotensin to a slight extent, but this antagonistic activity was not statistically significant ($p > 0.05$).

Cardiac Output.—The effect of surgical sympathectomy or drug pretreatment on the cardiac output response to angiotensin is summarized in Tables II and III. Following surgery or nethalide pretreatment, the decrease in cardiac output to intravenous angiotensin was still statistically significant ($p < 0.05$). Pretreatment with phenoxybenzamine or atropine, however, attenuated this effect. Nethalide pretreated animals showed only a decrease in cardiac output following angiotensin with no secondary increase as seen in the untreated animals. DCI pretreatment caused subsequent angiotensin injections to produce an initial highly significant elevation in cardiac output ($p < 0.01$) and only a negligible decrease at the end of 3 minutes. Figures 3 and 4 illustrate the difference in the cardiac output response to angiotensin in the nethalide and DCI pretreated animals.

DISCUSSION

The pressor effect of angiotensin has been reported to depend on arteriolar constriction largely in renal and splanchnic beds (17). This pressor response was characterized by Page *et al.* (18) as an initial sharp rise, followed by a small compensatory fall, then by a sustained rise lasting from 4 to 6 minutes. In addition to these observations, our results showed that the diastolic pressure rose significantly greater than the systolic pressure. While a slight vagal bradycardia has been reported to accompany the pressor response (19), this effect was not evident in our studies. A biphasic response in cardiac output was noted in a majority of our animals. The initial decrease was shown by Fowler and Holmes (2) to correspond to an increase in left atrial pressure and a decrease in left ventricular force during the initial impairment of cardiac function. These investigators also have shown that, following the initial depression, an improvement in cardiac performance persisted for 30 to 120 seconds. During this interval, their results showed that cardiac output increased above control values.

Since the right ventricle encountered a reduced resistance to outflow of blood (20), the initial depression noted in the studies of Fowler and Holmes were not seen in these experiments. On isolated cat papillary muscles, these investigators consistently obtained increases in inotropic force of contraction following addition of angiotensin to the bath. Similar inotropic responses were observed by Bianchi *et al.* (1) and Meier *et al.* (21).

Although angiotensin produced an increase in coronary blood flow, there was no change in coronary vascular resistance. Mandel and Saperstein (22) found no evidence of alteration in coronary flow during angiotensin infusion in rats. Forte *et al.* (7), using the nitrous oxide method, demonstrated an increase in coronary blood flow. Hill and Andrus (23) and Lorber (3) showed that angiotensin decreased coronary flow in the isolated perfused cat's heart, and Maxwell *et al.* (5) found that angiotensin increased coronary vascular resistance but did not alter coronary blood flow. Since in the present study the increase in myocardial blood flow was analogous to the increase in diastolic pressure, it was possible that the elevation in blood pressure and the increased perfusion pressure resulting therefrom masked the increase in coronary vascular resistance.

In those experiments in which coronary perfusion pressure was controlled and not dependent on the cardiac performance, both intravenous and intra-arterial injections of angiotensin demonstrated the vasoconstricting property seen in other vascular beds. These results were in agreement with those reported by Fowler and Holmes (2).

Bickerton and Buckley (24) reported that a pressor response to angiotensin could be mediated centrally *via* sympathetic pathways. Zimmerman (25) further showed that the peripheral vasoconstrictor effects of angiotensin could be reduced following surgical sympathectomy or cervical spinal transection, in which case there was a passive vasodilation due to the loss of sympathetic tone. Recently, Benelli *et al.* (26) demonstrated the involvement of peripheral sympathetic nerves in the action of angiotensin. They advanced the hypothesis that angiotensin acts at the peripheral nerve endings by promoting a greater output of norepinephrine. Following acute surgical sympathectomy, it was seen that although angiotensin still increased systolic and diastolic blood pressure and coronary blood flow, these effects occurred to a lesser extent than in the intact animal. Since there is some evidence (27) that the coronary flow is dependent to an extent on the sympathetic system, then it is probable that the decrease in cardiac activity following destruction of that system would result in a decreased responsiveness to angiotensin.

Bickerton and Buckley (24) reported that administration of the alpha adrenergic blocking agent, piperoxan, to the recipient's peripheral circulation prevented the central pressor response in the recipient animal elicited by the injection of angiotensin into the recipient's arterial inflow in the dog cross-circulation experiment. However, when angiotensin was given peripherally to the recipient animal, it still produced the potent pressor effect. Page and Bumpus (17) recently stated that sympathicolytic drugs in pentobarbital anesthetized dogs have little or no effect on the pressor action of angiotensin. Although our data obtained with angiotensin in phenoxybenzamine-treated animals are in agreement with the above reports, it should be noted that phenoxybenzamine pretreatment did prevent the decrease in cardiac output following angiotensin. This may be attributed to the fact that phenoxybenzamine possesses sympathomimetic activity of its own (28).

Both DCI and nethalide have been reported to be beta adrenergic blocking agents (29, 30); however, nethalide differs from DCI because it apparently lacks sympathomimetic activity (30). These differences were noted in the present study since DCI-produced positive chronotropic activity as reported by Furchgott (31), whereas nethalide induced bradycardia in all instances as reported by Black and Stephenson (30). DCI also increased coronary blood flow, and nethalide did not significantly alter myocardial flow. Thus, these results could explain the difference encountered by the administration of angiotensin to either DCI or nethalide pretreated animals. Nethalide potentiated the vascular responses to angiotensin to an extent greater than did DCI. In addition, DCI

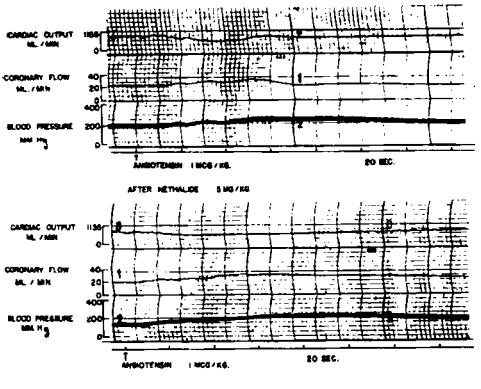


Fig. 3.—Effect of intravenous injection of angiotensin II (1 mcg./Kg.) on blood pressure, coronary flow, and cardiac output. Upper tracing shows the control response; lower tracing, after nethalide, illustrates the prolonged decrease in cardiac output and the prolonged increase in coronary flow.

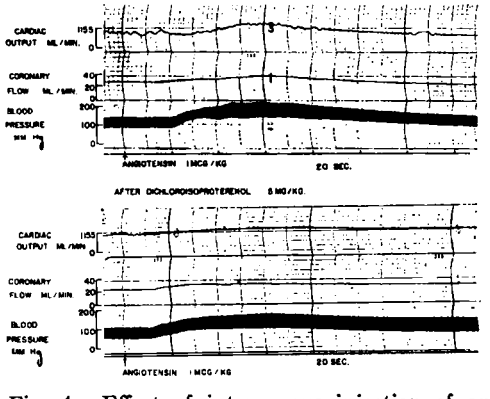


Fig. 4.—Effect of intravenous injection of angiotensin II (1 mcg./Kg.) on blood pressure, coronary flow, and cardiac output. Upper tracing depicts the control responses; lower tracing, after DCI, illustrates the prolonged increase in cardiac output. Coronary flow tracing retouched.

pretreatment caused an increase in cardiac output subsequent to angiotensin, whereas nethalide caused a further reduction in this parameter.

From these results, it seemed apparent that, in addition to direct musculotropic effects, the neurogenic component of the angiotensin-induced pressor response may be mediated in part *via* the alpha adrenergic receptor.

SUMMARY

1. The injection of angiotensin into pentobarbital anesthetized animals resulted in an increase in systolic and diastolic pressures (with the latter of greater magnitude), right ventricular force, and coronary perfusion pressure. The increase in myocardial blood flow was of approximately the same magnitude as that of the diastolic pressure; however, angiotensin had no effect on coronary vascular resistance or heart rate.

2. Angiotensin elicited a biphasic response on the cardiac output of the animals. There was an initial decrease, followed by an apparent compensatory increase.

3. The above effects were mediated *via* both direct musculotropic actions and the sympathetic nervous system since acute surgical sympathectomy reduced, but did not abolish, the effects of angiotensin.

4. The sympathetic component of the cardiovascular effects of angiotensin appears to be *via* the alpha receptors since nethalide greatly potentiated the activity of angiotensin.

5. The effect of pretreatment with phenoxybenzamine, atropine, DCI, or nethalide on the cardiovascular responses to angiotensin are discussed in an effort to clarify its mechanism of action.

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Aspirin Formulation and Absorption Rate I

Criteria for Serum Measurements with Human Panels

By S. V. LIEBERMAN, S. R. KRAUS, J. MURRAY, and JOHN H. WOOD

Replication of serum salicylate level distribution in a human panel is obtained by rigorous adherence to properly selected protocols. The panel must be sufficiently large to represent all types of absorption variability after aspirin ingestion. The inherent variability of the human group can be measured. Thus, it is possible to calculate panel sizes required to provide the sensitivity, in terms of least significant differences, needed to evaluate factors of aspirin absorption. With the panels studied, random crossover testing offers no advantage over sequential testing. During the initial absorption period, serum salicylate levels existing at the time of ingestion, whether small artifacts or real and appreciable levels, may be treated as deductible blanks for the study of aspirin absorption.

IN RECENT medical and pharmaceutical literature there have been several papers related to some aspect of blood salicylate levels (1–10) or urinary recovery levels (11, 12) resulting from aspirin ingestion. There has been general agreement that the rate of absorption of drugs is reflected in the rate of increase of blood level and that in turn this level is reflected in the rate of urinary recovery. The results have not been in complete agreement, particularly on the blood salicylate levels resulting from different aspirin preparations; but not all the reasons for different levels have been established. The large varia-

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1. The injection of angiotensin into pentobarbital anesthetized animals resulted in an increase in systolic and diastolic pressures (with the latter of greater magnitude), right ventricular force, and coronary perfusion pressure. The increase in myocardial blood flow was of approximately the same magnitude as that of the diastolic pressure; however, angiotensin had no effect on coronary vascular resistance or heart rate.

2. Angiotensin elicited a biphasic response on the cardiac output of the animals. There was an initial decrease, followed by an apparent compensatory increase.

3. The above effects were mediated *via* both direct musculotropic actions and the sympathetic nervous system since acute surgical sympathectomy reduced, but did not abolish, the effects of angiotensin.

4. The sympathetic component of the cardiovascular effects of angiotensin appears to be *via* the alpha receptors since nethalide greatly potentiated the activity of angiotensin.

5. The effect of pretreatment with phenoxybenzamine, atropine, DCI, or nethalide on the cardiovascular responses to angiotensin are discussed in an effort to clarify its mechanism of action.

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Aspirin Formulation and Absorption Rate I

Criteria for Serum Measurements with Human Panels

By S. V. LIEBERMAN, S. R. KRAUS, J. MURRAY, and JOHN H. WOOD

Replication of serum salicylate level distribution in a human panel is obtained by rigorous adherence to properly selected protocols. The panel must be sufficiently large to represent all types of absorption variability after aspirin ingestion. The inherent variability of the human group can be measured. Thus, it is possible to calculate panel sizes required to provide the sensitivity, in terms of least significant differences, needed to evaluate factors of aspirin absorption. With the panels studied, random crossover testing offers no advantage over sequential testing. During the initial absorption period, serum salicylate levels existing at the time of ingestion, whether small artifacts or real and appreciable levels, may be treated as deductible blanks for the study of aspirin absorption.

IN RECENT medical and pharmaceutical literature there have been several papers related to some aspect of blood salicylate levels (1–10) or urinary recovery levels (11, 12) resulting from aspirin ingestion. There has been general agreement that the rate of absorption of drugs is reflected in the rate of increase of blood level and that in turn this level is reflected in the rate of urinary recovery. The results have not been in complete agreement, particularly on the blood salicylate levels resulting from different aspirin preparations; but not all the reasons for different levels have been established. The large varia-

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bility in early serum levels among individuals requires fuller consideration and a study of the factors which contribute to variability.

Some of the factors can be defined and standardized arbitrarily without experimental studies of alternative standardizations. These include a number of protocols for dealing with a panel of humans. Others can be standardized but require experimental justification. These include analytical procedures, treatment of salicylate levels encountered in serum blanks, and the selection from alternatives of two panel protocols, either random coadministration or sequential administration of test materials and either fasted or nonfasted subjects. A variability associated with the process of measuring aspirin absorption still remains, due to factors beyond simple definition or control by standardized procedures. This variability is amenable to statistical procedures, so that its magnitude and effect can be measured and considered.

This investigation has dealt with these factors; this and succeeding papers will establish the necessary protocols and criteria, study the *in vivo* absorption process as reflected by serum levels, develop suitable *in vitro* test procedures for various formulative changes, and provide suitable *in vivo–in vitro* correlations. With this basis, the role of additives in accelerating absorption will be examined for the mechanisms involved. In addition, the granulating and tableting procedures, press operation, role of excipients, disintegrating and wetting agents, humidity, and crystal habit of the aspirin will be examined for their relative contributions to better absorption. As a consequence of the correlations obtained, a general review of the kinetics of over-all drug absorption will be developed, including the relative contributions of *in vivo* dissolution, inherent absorptive process, hydrolysis kinetics, and clearance.

Relative to potent prescription drugs, aspirin is very innocuous. Therefore, it provides an excellent vehicle for an *in vivo* study of those factors which can affect the rate of drug uptake. Accordingly, much of the ensuing report should be considered as a general prototype of what may be done to study the absorptive capabilities in humans of any other drug with more restrictions.

It became apparent early in this study that the variability of *in vivo* measurements was greater than the variability of the *in vitro* measurements. Therefore, it was necessary to establish first a panel protocol to minimize variability and to determine a minimum panel size, so that the differences observed between two tests would be of high significance statistically. This paper reviews the problems of using the human panel, the precision of the analytical data, and the statistical treatment to delineate panel performance, so that the least significant difference for various tests with panels of different sizes could be determined.

TABLE I.—ANALYSIS OF SALICYLATE STANDARDS BY THE BRODIE PROCEDURE

Present, mcg.	Found, mcg.	Difference, mcg.
0.5	1.0	+0.5
1.4	1.5	+0.1
1.5	1.8	+0.3
2.6	2.0	−0.6
3.6	3.3	−0.3
3.9	4.0	+0.1
4.7	4.3	−0.4
8.7	7.5	−0.8
9.5	9.0	−0.5
19.5	17.3	−2.2
27.7	26.0	−1.3
48.7	51.0	+2.3

EXPERIMENTAL AND RESULTS

The blood samples were held at room temperature for a minimum of 2 hours to ensure complete hydrolysis of aspirin to salicylic acid. The analysis was by the Brodie procedure for serum levels (13, 14), modified slightly in transfer volumes to increase sensitivity. The volumes used were 2 ml. of serum mixed with 0.5 ml. of 6 *N* HCl and 30 ml. of specially purified ethylene dichloride for the first extraction. From this, 25 ml. of ethylene dichloride was taken for mixing with 5 ml. of ferric reagent. The color of the aqueous layer was then read on a Beckman DU

TABLE II.—INTERLABORATORY COMPARISONS OF SERUM SALICYLATE ANALYSES

Lab. *A* mcg./ml.	Lab. *B* mcg./ml.	Difference, *A*–*B* mcg./ml.
7.5	8.3	−0.8
7.5	8.0	−0.5
5.0	5.5	−0.5
4.0	4.5	−0.5
45.0	47.0	−2.0
1.5	3.5	−2.0
23.0	25.0	−2.0
4.0	5.3	−1.3
55.0	54.5	+0.5
7.5	7.5	0.0
62.5	68.0	−4.5
15.2	15.0	+0.2
36.0	32.0	+4.0
51.0	48.6	+2.4
14.0	11.8	+2.2
5.5	3.0	+2.5
27.5	28.0	−0.5
27.0	26.8	+0.2
7.5	8.0	−0.5
7.0	7.5	−0.5
27.0	28.5	−1.5
53.5	56.0	−2.5
28.5	30.0	−1.5
18.0	18.0	0.0
45.0	44.8	+0.2
18.0	21.0	−3.0
23.0	22.0	+1.0
55.5	53.0	+2.5

TABLE III.—SALICYLATE SERUM LEVELS (mcg./ml.) FOR SIX SAMPLES ADMINISTERED REPETITIOUSLY TO THE PANEL

Aspirin Prepn.	Test Code	Mo./Yr.	No.[a]	10-Min. Levels $\bar{x}$[b]	10-Min. Levels S.D.[c]	20-Min. Levels $\bar{x}$	20-Min. Levels S.D.
1	L	3/58	40	6.92	5.99	18.14	11.34
1	SC	3/59	39	7.69	6.16	18.71	9.95
2	EA	3/58	40	4.89	6.19	15.67	14.25
2	ER	3/58	39	5.39	7.30	14.78	14.52
3	AA	3/58	40	9.25	8.70	30.33	17.09
3	AR	4/58	39	10.47	12.17	27.87	17.89
4	BD	10/58	35	10.21	9.26	32.64	16.81
4	BF	11/58	39	14.37	13.33	32.17	19.29
5	BE	10/58	37	14.24	12.19	34.59	16.54
5	BG	11/58	38	14.67	13.55	35.53	16.51
6	SA	10/58	42	4.63	5.29	16.66	12.57
6	SB	2/59	39	8.10	9.23	20.15	15.58
6	SX	3/59	38	7.21	9.55	17.01	15.14
6	SY	3/59	39	7.43	6.84	21.55	12.44
6	SZ	1/60	39	9.19	7.67	21.10	11.36

[a] Number of subjects. [b] $\bar{x}$, Average level. [c] S.D., Standard deviation of individual observation.

spectrophotometer against a reagent blank. Analysis results for a series of standards in serum carried through the procedure are given in Table I. Analyses were performed in two different laboratories. The data for interlaboratory comparisons on random samples run during normal routine in both laboratories are given in Table II.

The serum blank usually ranged from zero to a small finite value. In all work reported the serum blank was subtracted from the observed readings of that individual. In one test, the panel was predosed with 10 gr. of aspirin 10–12 hours prior to the test to provide large finite zero time levels for all subjects. These large serum blanks were subtracted. The results are given in Table III, and the analyses for individual paired differences with (test SX) and without (test SB) predosing are given in Table IV.

The subjects ate normal light breakfasts at home about 2 to 3 hours before the test and took nothing in the interim. For one test (SY) of a reference lot of tablets, they were strictly fasted after the previous evening meal. This result (Table III) was compared (Table IV) to test SB of the same reference lot and employed normal panel conditions.

The panel was used over a long term, with infrequent changes in personnel, for sequential testing. Tests of reference and some other tablets were repeated at intervals as checks during the course of the investigation. The results and comparisons are listed in Tables III and IV. Tests SA, SB, SX, SY, and SZ are repetitions of one reference lot of tablets, although there were special conditions with test SX (predosed subjects for high initial level) and test SY (fasted subjects). There were a number of duplicate tests: EA and ER, AA and AR, BD and BF, BE and BG, and L and SC. Some were preparations of aspirin alone, and some had an antacid in the tablet. Six formulations are involved; these are identified by number to show replications in Table III. All tests have involved ingestion of two tablets, each containing 5 gr. of aspirin.

Because of economy of time and effort, sequential testing was the procedure chosen; a comparison of this procedure to random coadministration procedure was obtained by scheduling four of the desired repetitions as two pairs of random coadministration tests. Tests AR and ER were paired random coadministration repetitions of sequential test AA and EA, respectively. Tests BF and BG were sequential repetitions of BD and BE, respectively, done earlier as a random coadministration pair.

To obtain a least significant difference smaller than that observed with other panels, our initial panels were 30 subjects per test, increased to 40 later. The data required for computing statistical values are included in Table III.

A number of protocols were fixed at the outset and have been continued with no further consideration. Subjects were paid volunteers selected only for their availability; participation by subjects was weekly, using a fixed day and hour for each subject. There was infrequent rescheduling; if a subject had to be scheduled for a second participation within 1 week,

TABLE IV.—STATISTICAL DATA FOR COMPARISONS OF TESTS FOR TABLE III BY PAIR DIFFERENCES

Test Codes	No.[a]	10-Min. Levels $\bar{d}$	10-Min. Levels S.D.[b]	10-Min. Levels t[c]	20-Min. Levels $\bar{d}$	20-Min. Levels S.D.	20-Min. Levels t
SB–SX	37	0.62	10.56	0.36	3.20	17.08	1.14
SB–SY	38	0.05	8.66	0.035	−2.06	16.26	0.78
EA–ER	39	−0.39	7.81	0.31	1.18	13.92	0.53
SA–SB	37	−3.44	9.80	2.13	−3.55	15.31	1.41
SB–SZ	31	−2.22	8.50	1.46	−3.37	13.96	1.35
AA–AR	39	−1.17	13.29	0.55	2.56	15.77	1.01
BD–BF	30	−3.45	17.53	1.09	2.72	25.52	0.59
BE–BG	29	1.15	15.00	0.40	1.78	21.38	0.45
(BD–BE)-(BF–BG)	28	−4.23	23.1	0.971	2.95	30.0	0.521
(ER–AR)-(EA–AA)	39	−1.38	16.5	0.523	1.40	21.4	0.407

[a] Number of individuals participating in *both* tests for whom data were paired. [b] S.D., Standard deviation of individual pair differences. [c] t, Student t value for the null hypothesis.

the minimum interval was 3 days. All were instructed to avoid medications for 3 days prior and fatty foods for 1 day prior to preclude analytical difficulties caused by interference or by cloudy serum. They were questioned routinely on these points. Rescheduling for any reason entailed no financial penalties, and cooperation was excellent. The sampling time was the time of stopping blood circulation in the arm with a cuff, with 10–12 ml. of blood withdrawn promptly (usually within 30 seconds) by venipuncture in the cubital area. Prior to test ingestions, a blood sample was drawn to provide a (zero time) serum blank. The test tablets were taken with 100 ml. of room temperature water.

DISCUSSION

The precision and accuracy of the slightly modified analytical procedure were considered adequate for the measurements to be made. The initial trial of recovery of known amounts added to serum, shown in Table I, was evaluated by regression analysis. For these 12 pairs, the slope of the line was 1.02, not significantly different from 1; its intercept was −0.52, not significantly different from zero. Two laboratories were involved in analyzing serum samples—simultaneously at one time and singularly at other times. Some blood samples, randomly designated, were drawn to provide sufficient serum for both laboratories at the rate of four per week for 7 weeks, with one analyst unaware of the checking, and the second analyst aware of his function. The 28 results are shown in chronological sequence in Table II. The correlation coefficient was 0.995, the slope of the line 1.01, and its intercept 0.09. These do not differ significantly from unity and zero, respectively. For a group of such data containing a number of items, each with its slight error, the statistical treatment shows that the standard error of the average will be small.

The human variability makes the major contribution to total variability. This is so large that only statistical parameters can represent the data adequately. Examination of serum salicylate levels for individuals in repetitive tests shows ranges varying from small for some subjects to large for others. There are some people who show small variabilities between tests at the low, intermediate, and high levels. There are others whose variability is so great that it is impossible to assign them to a level category. The variability within a group is great. The distribution of the levels with a panel for any one test substance is somewhat skewed from the normal. The skewness is less apparent with higher average levels, but is remarkably reproducible in panels of sufficient size, even though individual variability is high. Figure 1 shows the shape and reproducibility of distributions of levels for repetitions of the same preparation under codes SA, SB, and SZ. The reproducibility of the distributions is also evident in the reproducibility of the averages and medians (Table III). Since the population of levels is a skewed distribution, there is the choice of a suitable measure of the panel. The average is, of course, the most readily determined parameter, but it is the most sensitive to a few outlying values in the distribution. The median is another useful parameter for comparisons. Approximately two-thirds of the population have the average level or lower in

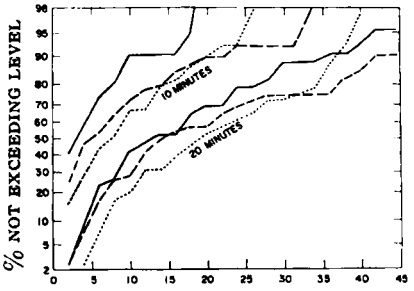


Fig. 1.—Cumulative per cent plots on probability scale for aspirin preparation 6 studied three times (panel essentially invariant). Key: ——, SA; - - -, SB; · · ·, SZ.

time periods reflecting the early absorption process. The use of distributions and medians in assessing test results can render an insight into affects of formulation and processing changes but lacks the utility of averages and standard deviations in statistical comparisons.

In comparing two tests, the Student t test can be applied to the difference between the two averages, using a standard deviation resulting from pooling the two variances. Another procedure is available if the same panel subjects were used in both tests. The average and its standard deviation of the individual differences between the two tests (pair differences) can be used in the t test to measure the significance of difference from zero of the average pair difference. Depending on the variances and correlation coefficient encountered, either procedure may be favored in various circumstances. In this paper, the method of pair differences has been chosen arbitrarily for all comparisons; these appear in Table IV.

The magnitudes of the variations within and between individuals for a useful differentiation during early absorption require a panel larger than has been used in previous studies. The smaller panels suffered from the disadvantage of not representing the categories now observed in the population. The ability to differentiate two test products is related inversely to the standard deviation of average paired differences and directly with the square root of the size of the panel. Finer differentiations require either reduced standard deviation or more subjects.

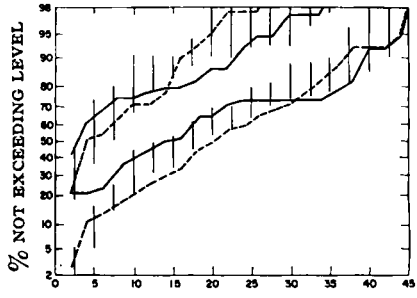


Fig. 2.—Cumulative per cent plots on probability scale resulting from subtraction of large zero levels (SX, ——) and for complete fasting (SY, - - -). Vertical lines indicate range of normal repeats from Fig. 1.

Only the latter can be manipulated, and the number selected controls the least significant difference (l.s.d.) attaining statistical significance. After the first panels of 30, a panel of 40 was considered optimum for balancing the volume of work with the ability to differentiate. This size, with individuals occasionally unavailable plus panel membership attrition, permitted a sufficient number to be available for the method of paired differences to give the desired l.s.d. Thus, in Table V are tabulated average l.s.d. for a 95% confidence for typical 10- and 20-minute levels for slowly and rapidly absorbed products. These are calculated for panels of various sizes using representative standard deviations observed for the panels and the appropriate Student t value (15). The l.s.d. for other confidence levels may be calculated similarly. Note that Table V refers to pair differences. For comparison of data from two different panels, a pooled standard deviation is required, and the l.s.d. is increased further by the square root of 2.

During the early absorption from tablets, the standard deviations for the individual runs or for the paired results for the several duplicates show similar magnitudes for corresponding averages. This similarity in magnitude is purely coincidental, and no

Table V.—Least Significant Difference for Pair Data (95% Confidence) Resulting from Panel Size at Three Typical Average Salicylate Levels

Panel, No.	l.s.d. $\bar{x}$[a] = 7 mcg./ml. (S.D.[b] = 8)	l.s.d. $\bar{x}$ = 17 mcg./ml. (S.D. = 13)	l.s.d. $\bar{x}$ = 32 mcg./ml. (S.D. = 18)
3	19.9	32.3	44.7
5	9.1	16.1	22.3
10	5.7	9.3	12.9
20	3.7	6.1	8.4
30	3.0	4.8	6.7
40	2.6	4.2	5.9
50	2.3	3.7	5.1
100	1.6	2.6	3.6

[a] $\bar{x}$, Average serum salicylate level at 10 or 20 minutes. [b] S.D., Typical standard deviation of average level (taken from Fig. 3).

significance should be drawn. It has been noticed that the standard deviation appears to increase with an increase in average blood levels during the absorption period. Any test shows both fast and slow absorbers giving high and low levels; but as the average level increases, the proportion of high levels increases at the expense of the number of slow absorbers. The result is an increase in variance as average increases (Fig. 3) during early absorption. The time and serum level dependency of variance will be treated in the next paper.

An examination of the data for tests L and SC in Table III shows the closeness of agreement in averages. This is especially evident in Fig. 4 which gives the two distributions involved, an indication that if the group is sufficiently large, data replication dose not require all individuals common to both tests. There were only 25 subjects common to test L and SC. The pair comparison is omitted from Table IV.

Reproducibility of results with the panel of subjects was achieved over a long period. Table IV shows repeat runs made 1 week and 1 year apart and

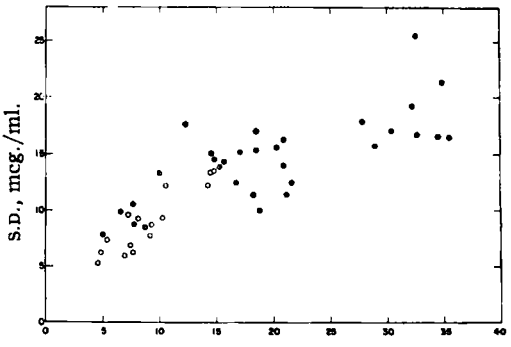


Fig. 3.—Variation of standard deviations with salicylate serum levels after absorption. Key: O, 10-minute average and ●, 20-minute average level; ◐, 10-minute pair difference and ◑, 20-minute pair difference plotted at the average of the pair.

some intermediate intervals. Credit for this achievement must go to rigid adherence to the fixed protocols to limit the experimental variability and the use of sufficient numbers of subjects to cope with human variability. There are eight pair difference comparisons in Table IV of both 10- and 20-minute serum levels. The pairs are each duplicate tests, and only one of the 16 comparisons has a significant t value. With 16 comparisons expected to be nonsignificant differences at 95% confidence, the discovery of one contrary to expectations is not surprising.

Early consideration for conducting the program as either a series involving coadministration of multiple test materials with random sequence assignments, or a series of sequential single tests, favored the latter. This offers economy of time and effort with less repetitions of standards and, with *rigid adherence* to protocols combined with only slight changes in panel personnel, no loss in sensitivity of measurements. The former procedure (random crossover) has a wide vogue in biological testing. Rigid adherence to protocols is the only protection against the occasional unrecognized error by either mode. It was possible to schedule a number of the wanted repetitions, so that both procedures were utilized and a comparison became available. Four of the comparisons given in Table IV (EA–ER, AA–AR, BD–BF, and BE–BG) had one test of each pair performed as a single test in the long sequential series.

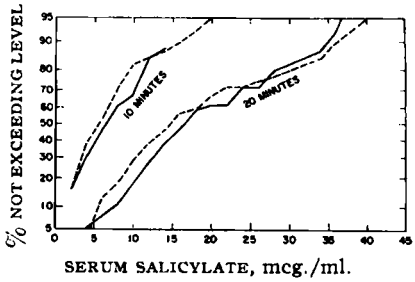


Fig. 4.—Cumulative per cent plots on probability scale for aspirin preparation 1 administered as L (- - -) and SC (——). (Only two-thirds of panel common to both tests.)

The four other tests of these four pairs were performed as two pairs of random crossover tests. None of the eight comparisons (four with 10-minute data and four with 20 minute data) performed as paired differences for individual subjects showed a significant difference from zero for the average pair difference. Another comparison is possible: that of the average individual difference between the two tests of the crossover period and the corresponding two tests of the sequential series—BD–BE of the crossover with BF–BG of the sequential and EA–AR of the crossover with EA and AA of the sequential. Table IV shows that none of the average differences were significantly different from zero. With both kinds of comparisons, the results of the carefully controlled sequential procedure were not different from the random crossover procedure.

Serum blanks (zero time samples) often exhibit low salicylate values which may be *bona fide* or artifacts. Occasionally, a subject showed a high zero time level which could be traced to recent ingestion of medications containing salicylates and, in one case of serendipitous sleuthing, wintergreen (methyl salicylate)-flavored candy lozenges. For some time, all data for subjects with a high zero time level were rejected, and the subject was rescheduled. The feasibility of subtracting serum blanks was demonstrated in one test (SX). All subjects were given two tablets (10 gr. of aspirin) to take the night before their scheduled test. As a result, everyone appeared with a high serum blank value which averaged 14.1 mcg./ml., with a range from 3.5 to 52.3. On correcting the data by subtracting the appropriate serum blanks, the 10- and 20-minute levels were like the results of test SB using the same tablets. For test SB, however, the serum blanks averaged 1.7 mcg./ml., with a range from 0.5 to 3.5. Subtraction of serum blanks of any level is now routine, although the injunction against medication during the 3 days prior is continued, and high serum blanks are rare. Where the duration of the test will result in any natural decay of the blank level, a correction for the clearance rate (16) is required.

Prior to initiation of this work in 1957, discussions with clinicians indicated a consensus that higher blood levels and more consistent results were expected for a fasting panel. Necessary to the reproducibility of the panel was the need for careful control and constancy of extraneous background. Figure 2 and Table IV, in which the panel distribution of a truly fasted panel (SY) is compared to the normal (SB), show that statistically no difference existed between the normal (semifasted) and fasted panel. A more positive assurance for this one test of a fasted panel is the observation that the distribution curves (Fig. 2) are obvious members of the family of curves from other tests of the same aspirin tablets. Thus, by waiting 2 to 3 hours after a light breakfast, it is possible to have the test advantage of the fasted panel without the personal disadvantages of periodic fasting over a long term.

SUMMARY

A modified Brodie serum salicylate analysis has been shown to have the necessary precision and accuracy for following serum salicylate levels in groups of 30 to 40 subjects.

Human variability is of such magnitude that the data are represented fairly by statistical parameters only.

Rigid compliance with proper protocols and large panels of subjects give statistically reproducible average blood levels.

The least significant difference, useful in differentiating test results, is controlled primarily by the number of subjects.

Carefully controlled sequential testing with adequate panels gives results indistinguishable from those obtained by random crossover testing. Panel replicability over prolonged periods is excellent and defined by statistical parameters.

Serum blanks, or zero time serum salicylate levels, can be subtracted from the serum levels of subsequent samples for 10- and 20-minute intervals.

A panel of semifasted subjects is as useful in results as a panel of fasted subjects and more amenable to a long-term testing program.

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Aspirin Formulation and Absorption Rate II

Influence on Serum Levels of Tablets, Antacids, and Solutions

By S. V. LIEBERMAN and JOHN H. WOOD

Serum salicylate levels were measured for seven aspirin preparations alone and with a mixture of dihydroxyaluminum glycinate and magnesium carbonate. A twofold increase in serum levels at 10 and 20 minutes after ingestion was observed with the mixture present. With the aspirin preparations, the salicylate serum levels clearly reflected the technology of the aspirin granulation preparation. An optimum level of antacid for rapid absorption was found. A study of the salicylate levels resulting with time from ingestion of solutions and tablets showed that more rapid absorption was accompanied by earlier and higher serum levels.

THE PREVIOUS paper in this series (1) has established criteria for the sensitivity, reproducibility, and range of applicability of a human panel to assess serum salicylate levels accurately after ingestion. It is now possible to apply the human panel to a study of factors involved when aspirin is absorbed from tablets or solutions. Paul and Routh (2) reported that a tablet in which aspirin was combined with a mixture of dihydroxyaluminum glycinate and magnesium carbonate gave a build-up of blood salicylate levels more rapid than a tablet of aspirin alone. Subsequently, there has been disagreement in the literature; some works (3–5) have shown enhanced early absorption, while others (6–8) have questioned this finding. Work dealing with blood levels has not clearly established the reason for the enhancement. Levy (5) has shown a correlation between more rapid dissolution of tablets and early high urinary levels equated to enhanced absorption and has also stated (9) that the added antacid accelerated dissolution. Also it has been suggested (10) that the difference, if real, could be ascribed to aspirin technology rather than to the antacid component of the formulation.

With the sensitivity of the human panel defined by the preceding paper, a valuable tool exists to measure the influence of the antacid combination and of the aspirin technology on the early salicylate blood levels and thus assess the relative roles of each. Additionally, the panel permits a determination of the optimum level of antacid for salicylate absorption enhancement.

Salicylate plasma levels represent the composite result of several discrete and simultaneous processes. The aspirin must dissolve before any absorption can occur in the gastrointestinal tract. After absorption, distribution occurs to the tissues, and the elimination or clearance process begins. The faster the dissolution of the tablet, the larger the concentration gradient, and the faster should be the absorption. Earlier and higher plasma levels must result from higher concentration gradients since absorption and distribution are processes faster than elimination (11).

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EXPERIMENTAL AND RESULTS

Table I lists the panel codes and describes the composition of a series of different aspirin tablets and the corresponding two-layer tablets, with aspirin in one layer and antacid in the other. The aspirin was in each case 5.0 gr. per tablet with 0.56 gr. of cornstarch. The antacid layer was a gelatin granulation (0.16 gr.) containing 0.75 gr. of aluminum glycinate and 1.50 gr. of magnesium carbonate to which 0.23 gr. of cornstarch was added prior to tableting.

Two human panels, identified as panels *1* and *2*, were used concurrently. Aspirin of code N was used in both panels to serve as a link. Each subject received two tablets; the protocol was described previously (1). The data for 10- and 20-minute levels are given in Table II; the pairs are the aspirin alone and its corresponding combination with antacid.

Two different two-layer tablets were prepared using one aspirin granulation with two concentrations of the active antacids—1.5 gr. (code AC) and 3.0 gr. (code AB). These are one-third less and one-third more than the 2.25 gr. antacid used in the previous set of tablets. Using a different aspirin granulation, the relative specificity of the ratio of antacid to aspirin was again examined using 2.25 gr. antacid with 5.0 gr. (code QD) and 6.25 gr. (code QE) of aspirin. The data for these four tablets are given in Table III.

The serum levels from three aspirin preparations—a commercial tablet L, aspirin of code E from the first series, and its corresponding antacid combination, D—were studied as a function of time from 30 to 150 minutes after administration. Since 10- and

20-minute data for D and E were available from panel *1*, L was run separately for these two times. In addition, values for E as EA and ER are also available in the previous paper (1).

Two solutions were used. The salicylate time profile to 90 minutes was determined for a solution prepared by neutralizing salicylic acid with choline so that 100 ml. contained 868 mg. choline salicylate (code NA). This amount is equivalent in salicylate to 10 gr. of aspirin. In addition, the equivalent amount of sodium salicylate, 576 mg. (code NB), was studied for serum levels at 10 and 20 minutes. The results are recorded in Table IV.

DISCUSSION

The data of the two panels in Table I are related through sample N which is common to both. The difference in 10-minute serum salicylate levels between the two panels was small. The difference in 20-minute serum level averages was considerable, although it missed being significant at 95% confidence. This large difference in average was not reflected in the medians which were closer. An examination of the cumulative per cent distribution curves lead to the conclusion that a small number of rapid absorbers in panel *1* were not matched in panel *2*, and the use of larger panels would have eliminated this discrepancy.

This slight possible mismatch between panels does not affect the comparison of the pairs of buffered and unbuffered tablets since each pair involved the same participants. All preparations with the antacid additive show faster acetylsalicylate absorption as reflected in 10- and 20-minute salicylate levels. Except for the comparison B–W at the 10-minute level, all tablet pairs at both 10 and 20 minutes show enhancement, which is statistically significant (95% confidence), for the antacid combination over the aspirin alone. The differences in the averages for the pairs of levels are so great that the ratio of the enhancement in level is approximately twofold.

If one considers median salicylate levels instead of averages, the difference between the pairs is even more striking, showing about a threefold enhancement. As discussed earlier (1), this is a consequence of the asymmetric distribution of levels that occurs during the early absorption. Figure 1 shows median and average levels from early absorption data. The asymmetry of the distribution of level results in a number average appreciably greater than the median level, with a maximum deviation at an average of approximately 20 mcg./ml. of salicylate in the serum. During the early absorptive process, two-thirds of the population may have the average level or less. At low levels and for averages above 40 mcg./ml., the two parameters coincide. The median level is possibly more characteristic than the average for the population and can be treated by nonparametric methods. The average and its statistical significance are the more familiar tools of the investigator.

Figure 2 shows the cumulative per cent distributions for one pair, N and K. This pair is typical of others in the set. It is apparent that the distribution of salicylate levels at 10 minutes with the antacid combination is similar to that of 20 minutes with plain aspirin. The agreement is closer than for the pairs of repeats studied in the previous paper (1). The presence of the antacid has shifted the whole population, not just a small portion of it. Since enhancement of salicylate serum levels was present in every pair, the effect of the antacid layer must be relatively independent of any technology involved in the preparation of the aspirin granulation.

TABLE I.—ASPIRIN DESCRIPTIONS FOR TABLETS USED IN PANELS *1* AND *2*

Aspirin Code	Antacid Combn. Code	Panel	Aspirin Layer Description
B	W	*2*	20-Mesh crystals of manufacturer *A*
E	D	*1*	Aspirin-starch granulation of supplier *A* (1)
N	K	*1*	Aspirin-starch granulation of supplier *B* (2)
S	H	*1*	1/3 (1)–2/3 (2)
T	A	*1*	1/3 (1)–2/3 20-mesh crystals of manufacturer *A*
R	Z	*2*	1/3 (1)–2/3 20-mesh crystals of manufacturer *B*
P	X	*2*	1/3 (1)–2/3 20-mesh crystals of manufacturer *C*

TABLE II.—SERUM SALICYLATE LEVELS OBTAINED WITH THE TABLETS IN TABLE I

Code Designation	Subjects, No.	10-Min. Levels, mcg./ml. Av.	S.D.	Median	20-Min. Levels, mcg./ml. Av.	S.D.	Median
B	28	2.59	1.91	2.0	5.76	5.39	3.7
W	28	3.93	5.11	2.2	18.90	12.28	16.0
E	27	6.13	6.88	2.5	16.76	15.65	12.8
D	27	13.68	10.75	11.0	32.95	17.03	27.5
N (Panel *1*)	27	3.63	4.46	2.0	12.31	11.06	9.0
K	27	10.00	7.69	8.5	30.16	15.56	28.4
S	27	4.31	4.47	1.9	13.52	13.45	7.0
H	27	10.82	9.85	6.0	28.36	17.04	22.2
T	27	3.32	3.44	1.9	9.28	7.15	7.8
A	27	9.49	7.86	6.8	25.64	14.96	23.0
R	28	4.95	5.23	3.4	10.79	10.00	7.0
Z	28	8.69	7.56	7.0	25.91	13.58	26.0
P	28	3.44	4.62	1.8	9.05	12.15	4.0
X	28	7.71	6.61	6.0	24.80	13.11	22.5
N (Panel *2*)	28	3.44	3.77	3.0	7.57	6.83	6.8

TABLE III.—SERUM SALICYLATE LEVELS RESULTING FROM VARIATIONS IN CONCENTRATIONS OF ACTIVE INGREDIENTS WITH BUFFERED TABLETS[a]

Code Designation	Tablet Distinction	10-Min. Levels, mcg./ml. Av.	S.D.	Median	20-Min. Levels, mcg./ml. Av.	S.D.	Median
AC	1.5 gr. antacid	10.11	9.36	6.0	28.24	15.03	20.0
AB	3.0 gr. antacid	11.01	13.74	6.0	29.03	17.28	32.0
QD	Normal	17.3	10.5	15.0	36.5	14.1	36.8
QE	6.25 gr. aspirin	19.8	12.4	17.0	42.8	17.2	40.0

[a] Thirty-eight subjects.

TABLE IV.—SERUM SALICYLATE LEVELS AS A FUNCTION OF TIME FOR TABLET AND SOLUTION PREPARATIONS, mcg./ml.

Sample and Measure		Time Period 10 Min.	20 Min.	30 Min.	40 Min.	60 Min.	90 Min.	120 Min.	150 Min.
D	Av.	...	...	43.56	...	48.05	46.44	43.68	40.33
	No.	...	...	39	...	39	39	40	39
	S.D.	...	...	15.16	...	10.33	9.64	8.99	8.56
	Median	...	...	45.0	...	48.0	46.4	43.8	40.0
E	Av.	...	...	28.36	...	41.53	46.24	45.07	43.11
	No.	...	...	39	...	39	39	39	39
	S.D.	...	...	17.95	...	16.44	13.85	11.96	9.75
	Median	...	...	29.2	...	43.7	48.0	44.3	43.0
L	Av.	6.92	18.14	28.01	...	39.56	42.52	43.72	44.34
	No.	40	40	39	...	39	39	39	39
	S.D.	5.99	11.34	17.98	...	17.20	16.19	12.40	9.85
	Median	5.2	14.3	26.0	...	40.5	44.5	44.0	43.4
NA	Av.	48.01	57.41	...	54.99	52.57	48.22	...	...
	No.	39	38	...	39	38	38	...	...
	S.D.	22.37	11.87	...	10.43	7.41	6.65	...	...
	Median	53.5	64.0	...	55.0	52.0	47.0	...	...
NB	Av.	54.70	63.66	...	...	...	...	...	...
	No.	37	37	...	...	...	...	...	...
	S.D.	20.66	14.81	...	...	...	...	...	...
	Median	49.5	58.5	...	...	...	...	...	...

This technology does, however, contribute to the levels obtainable. The combination B and W, comprising all 20-mesh aspirin crystals, is notably inferior to either of the two commercial granulations. Indeed, the differences for this pair verge on significance with 95% confidence against mixtures of commercial granulation and crystals. Similarly, the differences between the tablets containing only commercial aspirin granulations and the tablets with mixtures border on significance. Thus, the technology of the aspirin preparation is an important factor. Indeed, for the preparations studied, the enhancement possible for the best aspirin, E, compared to the poorest aspirin, B, is more than two-fold—about the same magnitude as the enhancement that aspirin receives in the presence of the antacid combination.

Using urinary salicylate recovery, Levy (5), studying a number of commercial preparations, concluded that aspirin was absorbed more rapidly from the antacid combination. He found lesser differences occurring between different aspirin preparations without antacids.

Table III shows no difference in the average levels between preparations AB and AC which differ in being, respectively, one-third more and one-third less antacid than used in the formulations of Table I. This amount of antacid gives the maximum effect measured by the average level, and no significant additional enhancement was achieved by using additional antacid. The shape of the distribution in the case of AC, the 1.5 gr. of antacid, is somewhat distorted, so that its median is appreciably lower than that of the 3.0 gr. case, AB. This suggests the possibility that 1.5 gr. represents the lower limit for optimal antacid enhancement, an example of the advantage of considering both distributions and medians as well as the statistically based average level in studying differences between tablets.

The traditional aspirin dosage has been 10 gr. The use of additional aspirin should be expected, on a concentration mass action basis, to yield more drug in an absorbable form at any given time. This is confirmed by the serum levels found with the pair manufactured QD–QE, which differ only in the amount of aspirin present (10 *versus* 12.5 gr.) with the antacid layer. The difference for 20-minute serum levels for 12.5 gr. is so great that the method of individual pair difference yields a Student t of 2.6 (99% confidence).

An examination of Table IV and its graphical representation in Fig. 3 shows that, as predicted theoretically, earlier peaking of levels results in higher levels and that the earliest peaking is for the salicylate solutions. Hogben (12) and Smith (13) have shown that salicylate is absorbed faster than acetylsalicylate. Choline salicylate solution (14) has been advocated for extremely rapid absorption. These data give higher levels at 10 and 20 minutes for sodium salicylate (NB) over choline salicylate (NA). The difference is not significant statistically at the 10-minute level, but it is significant statistically with 95% confidence for 20 minutes.

The tablet data show that tablet D, the aspirin with dihydroxyaluminum glycinate and magnesium carbonate, has significant enhancement of salicylate

serum levels over aspirin alone for periods up to and including 60 minutes. The buffered preparation clearly peaks in level in about 45 minutes, while the corresponding aspirin alone, E, reaches its peak in about 90 minutes. The differences in levels between these two at 90, 120, and 150 minutes, although not significant statistically, should be real from the nature of the curves. The commercial aspirin, L, appears to give a peak at 150 minutes or even later, as judged from average levels. The median levels, however, are more in line with tablet E. The average levels at 90 and 120 minutes are not statistically significantly different from those of D. At 150 minutes, the difference is just significant. The set of experimental data confirms the theoretical prediction that earlier peaking leads to higher maximum serum salicylate levels.

In the preceding paper (1), it was shown for aspirin absorption from tablets that the standard deviation of average salicylate serum levels increased almost directly with the level attained. This was true during the early portion of the absorption cycle. It is apparent from Table IV that this does not hold as absorption continues. As the maximum level is approached, the standard deviation decreases markedly and continues to decrease even after the maximum average has been attained.

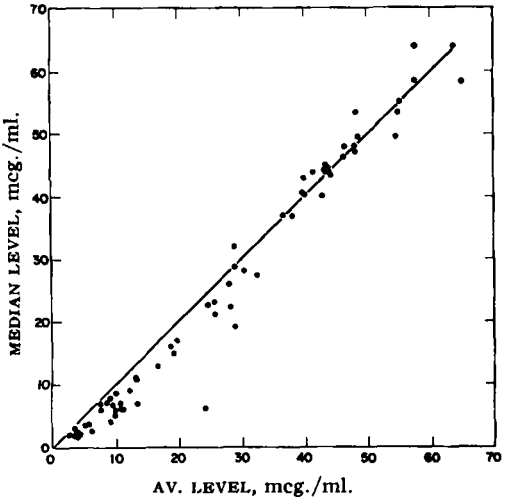


Fig. 1.—Variation of median and average level of serum salicylate during absorption.

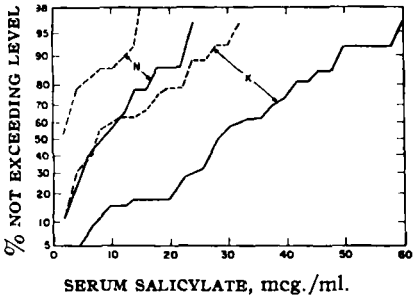


Fig. 2.—Cumulative per cent plot on probability scale of levels obtained with aspirin (N) and its corresponding tablet with antacids (K). Key: - - -, 10-minute levels; ——, 20-minute levels.

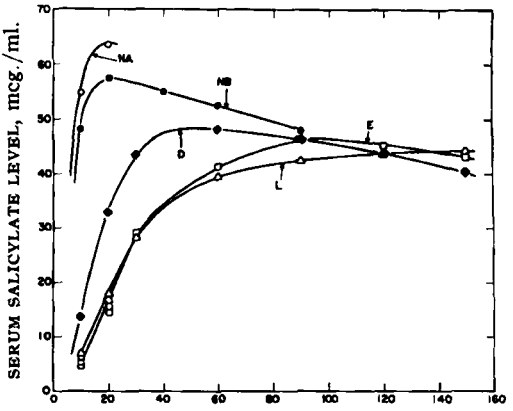


Fig. 3.—Serum salicylate levels resulting from solutions and tablets. Key: NA, sodium salicylate solution; NB, choline salicylate solution; E, aspirin alone; D, with buffer components; L, a commercial aspirin.

This is expected since the fast absorbers have virtually completed their absorption, while the slow absorbers, which contribute notably to the spread of the distribution, begin to build up appreciable levels and the distribution becomes tighter. This is a necessary condition resulting from the fact that the clearance process is slower than the absorption and less variable (11).

SUMMARY

Faster absorption of asprin leads to earlier and higher peak salicylate serum levels. This is shown by tablets of aspirin with a mixture of dihydroxyaluminum glycinate and magnesium carbonate relative to those of aspirin alone. Still earlier and higher peak levels are obtained by a solution. Sodium salicylate solution yields an early level slightly higher than that of choline salicylate solution.

The presence of the cited antacid mixture in an aspirin tablet leads to a twofold enhancement in the average serum salicylate levels attained at 10 and 20 minutes. The median level is increased approximately threefold.

There is an optimum amount of antacid for maximum effect of salicylate serum levels. Variations about this optimum produce no change in levels.

Technology of aspirin granulation can lead to appreciable differences in observed salicylate serum levels.

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Isolation of Coumestrol and Other Phenolics from Alfalfa by Countercurrent Distribution

By E. M. BICKOFF, A. L. LIVINGSTON, S. C. WITT, B. E. KNUCKLES, JACK GUGGOLZ, and R. R. SPENCER

Countercurrent distribution (CCD) procedures are described for the isolation of coumestrol and 12 other phenolic compounds from alfalfa. Three of these were identified as trifoliol, tricin, and salicylic acid. Elemental analysis indicated empirical formulas for four of the other compounds. R_f values on paper chromatograms and fluorescent characteristics under ultraviolet light are presented for all the compounds isolated.

IN RECENT YEARS, a tremendous interest has developed in the use of synthetic hormones to stimulate rate and efficiency of gain in animals. Alfalfa meal, rich in the estrogenlike compound coumestrol (1), has been shown to produce increased growth rates when fed to sheep (2, 3) or cattle (4). This increased growth rate may be attributed either to coumestrol or to factors presently unidentified. Because of inadequate supplies of the pure plant estrogens, their effectiveness on animal growth has not yet been ascertained.

Work was recently undertaken to obtain enough coumestrol as a concentrate or in crystalline form to permit evaluation of its potential as a growth promoter. An earlier paper (5) reported the preparation from alfalfa of approximately 2500 Gm. of a crude crystalline product consisting of about 70% coumestrol. This paper reports the procedures employed to obtain the coumestrol in pure crystalline form.

It also seemed of interest to investigate the accompanying compounds, many of which fluoresced strongly under ultraviolet light. The techniques employed for the isolation of a number of them by countercurrent distribution are also presented.

EXPERIMENTAL

Equipment.—Two Craig countercurrent distribution (CCD) instruments were used for the separations. Both instruments are 100-tube models, robot-operated, and equipped with fraction collectors. The smaller (20-ml. tube capacity) was mainly used for exploratory development of solvent systems, while the larger (200-ml. tube capacity) was used for most of the isolation work. The single withdrawal procedure (6) was used.

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Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Paper Chromatography.—The progress of each CCD run was followed by spotting solutions from selected tubes, both from the instrument and from the fraction collector on Whatman No. 1 paper. The system which gave maximum resolution of the compounds under investigation was isopropyl alcohol/ammonium hydroxide (28–30% NH_3) (2/1), descending (solvent system 1). The various components could be detected on the developed chromatogram as colored fluorescent spots under ultraviolet light. Exposure to ammonia vapor or spraying with dilute sodium carbonate solution usually intensified the fluorescence and often changed the color of the spot. A chromatogram made at the completion of each CCD run served as a permanent record of that separation (Fig. 1). The composition of the various fractions obtained during processing could be evaluated more accurately by two-dimensional paper chromatography. Solvent system 2, employed for the second dimension, consisted of 50% acetic acid, ascending.

Many compounds were present in minute amounts and were not detected by paper chromatography in the original crude extract or in the initial fractions. However, as the major components were removed by CCD, the minor ones became visible on the developed chromatograms. Table I gives approximate R_f values and fluorescent color for the compounds under investigation. The R_f values of the compounds are averages from a number of chromatograms of the various fractions. Since these compounds move a little differently when chromatographed as mixtures, these data only locate the relative position of each compound approximately.

Distribution of the Crude Crystalline Product.—One thousand grams of the coumestrol preparation, isolated in the large-scale processing of alfalfa (5), was the starting material for this study. Coumestrol and a number of the other compounds have only limited solubility in most of the common sol-

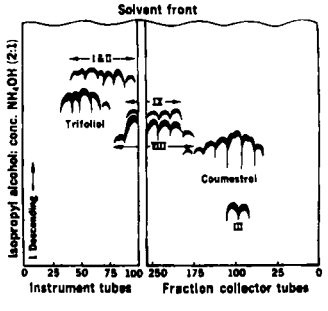


Fig. 1.—Schematic representation of the paper chromatogram of the CCD run for fraction *19* through solvent system D.

TABLE I.—FLUORESCENCE AND R_f VALUES OF PHENOLIC COMPOUNDS OF ALFALFA ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS

Compd.	R_f Values: First Dimension[a]	R_f Values: Second Dimension[b]	U.V. Fluorescence: Untreated	U.V. Fluorescence: plus NH_3 vapor
Coumestrol	0.50	0.52	Purple-white	Yellow-white
Salicylic acid	0.77	0.81	Blue-gray	Deep blue
Tricin	0.36	0.56	Black absorbing	Yellow
Trifoliol	0.66	0.57	Dull blue-gray	Blue-gray
I	0.75	0.60	Blue	Intense blue
II	0.75	0.60	Blue	Intense blue
III	0.36	0.36	Bluish-white	White
IV	0.44	0.69	Light tan	Yellow-green
V	0.17	0.55	Tan	Yellow
VI	0.16	0.34	Purple-gray	Yellow-gray
VII	0.26	0.43	Violet	Yellow-pink
VIII	0.58	0.42	Blue	Intense blue
IX	0.64	0.53	Blue	Intense blue

[a] Solvent system 1—isopropyl alcohol:concentrated ammonium hydroxide (2:1), descending. [b] Solvent system 2—50% glacial acetic acid, ascending.

TABLE II.—SOLVENT SYSTEMS EMPLOYED IN COUNTERCURRENT DISTRIBUTION OF ALFALFA PHENOLICS

Solvent System	Solvents and Proportions by Vol.	Starting Material	Total Solids, Gm.	Solids in Each Run, Gm.
A	Skellysolve B:ether:MeOH:H_2O:DMF[a] (2:5:3:1:0.4)	Crude coumestrol preparation	1000	40
B	Ethyl acetate:skellysolve B: Ether:MeOH:H_2O:DMF (12:12:10:5:4:8)	Fraction *2*	860	215
		Fraction *11*	56	28
		Fraction *12*	96	24
C	Acetone:skellysolve B:ether:H_2O (2:1:1:1)	Combined fractions *4* and *7*	200	10
D	H_2O:DMF:acetone:CCl_4 (2:4:3:5)	Fraction *19*	36	12

[a] DMF, dimethylformamide.

vents employed for the distributions. However, by using dimethylformamide as a carrier solvent, over 200 Gm. of material could be distributed in certain solvent systems, and adequate separations could be made.

Fifty-eight distributions employing five solvent systems (Table II) were required to separate coumestrol and 12 of the other phenolic compounds. Figure 2 presents the sequence of these distributions. The Roman numeral assigned to a particular compound depended upon its place in the sequence of purification. No number was assigned to a compound unless it was evident that it could be isolated. The first objective was to isolate the bulk of the coumestrol from the minor constituents in the mixture. This was necessary because the total amount of material to be processed greatly exceeded the normal capacity of the equipment.

Distribution in Solvent System A.—The material was first distributed in 40-Gm. batches (Fig. 2). Because of limited solubility, the material was introduced into the first 18 tubes of the instrument. After 260 transfers, the runs were divided into fractions *1–3*, determined by a paper chromatogram of selected tubes of the completed distribution. Comparable fractions from each of the 25 identical distributions were combined and taken to dryness *in vacuo*.

To date, no further work has been done on fraction *1*, which weighed about 60 Gm. and contained most of the dark brown waxy impurities and few fluorescing compounds. Fraction *2* weighed 860 Gm. and contained most of the coumestrol. Fraction *3* contained a small amount of coumestrol and three other compounds which were difficult to separate from one another. However, they could be separated more readily as their acetates. Repeated crystallization of the acetylated mixture from acetone yielded a pure preparation of the acetate of a compound identified as the coumestan, trifoliol. The latter compound was recently isolated from ladino clover (7). The other two compounds (compounds I and II) had similar solubility characteristics and were difficult to separate from one another. About 1 mg. of each as the acetate has been obtained by thin-layer chromatography (TLC) on silica gel using ether/skellysolve B (4/1) as the developer. However, they could not be separated in larger quantities by CCD, column chromatography, or recrystallization.

Distribution of Fraction 2.—This fraction was distributed in solvent system B (Table II) in four 215-Gm. batches. For each distribution, the starting material was introduced into the first 22 tubes of the instrument. After 400 transfers, three fractions (*4–6*,) were made as determined by a paper chromatogram of the completed run. The corresponding fractions from each distribution were combined and concentrated *in vacuo*. Fraction *4* (160 Gm.) contained most of the minor constituents and only a small amount of coumestrol. Fraction *6*, which amounted to about 10 Gm. and was mostly compounds I, II, and trifoliol, was acetylated and combined with fraction *3*.

Fraction *5* contained 80% of the weight of the starting material. When analyzed by a fluorometric method (8), this fraction contained about 90% coumestrol. The other compounds could not be

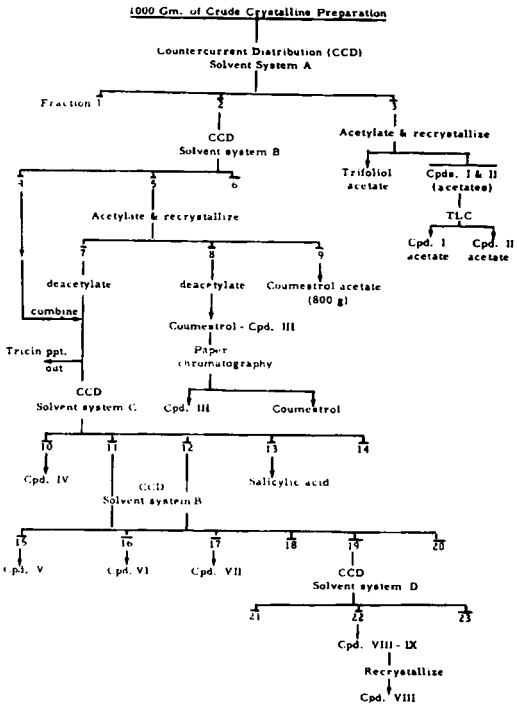


Fig. 2.—Flow sheet for separation procedures.

separated from the coumestrol by recrystallization. However, acetylation and repeated careful recrystallization from chloroform gave pure coumestrol acetate (fraction *9*) and two additional fractions, *7* and *8*. Fraction *7* appeared to be similar to fraction *4* in composition, except that it contained a small amount of the acetates of compounds I, II, and trifoliol. Therefore, fraction *7* was deacetylated, combined with fraction *4*, and these served as the starting material for the next series of distributions. Fraction *8*, after deacetylation, weighed about 55 Gm. and consisted of only two compounds (coumestrol and compound III). Compound III could be separated from coumestrol by paper chromatography employing solvent system I. To date, we have not succeeded in separating it by CCD or recrystallization.

Isolation of Coumestrol.—Fraction *9* consisted of approximately 800 Gm. of pure coumestrol acetate. It was deacetylated to yield 560 Gm. of coumestrol. For deacetylation, the coumestrol acetate was hydrolyzed in 40-Gm. batches. To a cold 1/2% potassium hydroxide solution in methanol (2 L.) was added 40 Gm. of the acetate. The mixture was stirred in an ice-bath for 1 hour; then the ice-bath was removed, and the reaction was slowly allowed to come to room temperature with application of heat. The stirring was continued for 5 to 6 hours until almost complete solution had occurred. The mixture was filtered and added to 6 L. of ice-water. The pH of the yellow solution was adjusted to about 5.5 with dilute hydrochloric acid to precipitate coumestrol. The heavy slurry was cooled for several hours; then the pH was adjusted to 2–4, and the coumestrol (30 Gm.) was collected. A whiter product could be obtained when the final pH adjustment was made just before filtering the product.

Isolation of Tricin.—When combined fractions *4* and *7*, weighing 225 Gm., were dissolved in acetone and mixed with about a half volume of skellysolve B, a copious precipitate was formed. Recrystallization from methanol gave 25 Gm. of pure tricin. This flavone has been previously isolated from alfalfa by another process (9).

Distribution of Combined Fractions 4 and 7.—The above filtrate was evaporated to dryness, then distributed in 10-Gm. batches in solvent system C (Table II) for a total of 200 transfers. Comparable tubes from each of the runs were combined and divided into five additional fractions (fractions *10–14*). Recrystallization of the solids obtained from fraction *10* gave 5 Gm. of compound IV. Fraction *14* consisted of a mixture of compounds I, II, and trifoliol and was combined with fraction *3*.

Isolation of Salicylic Acid.—Crystals were obtained from fraction *13* by extraction with hot skellysolve B, followed by recrystallization from water. About 5 Gm. of colorless needles, identified as salicylic acid, were obtained.

Distribution of Fractions 11 and 12.—Fractions *11* and *12* contained essentially the same group of compounds, although their relative proportions differed greatly. Therefore, each fraction was separately distributed in solvent system B for a total of 900 transfers. Based on paper chromatograms of the completed runs, comparable tubes were combined to give six fractions (fractions *15–20*). Although several of the fractions consisted predominantly of one compound, small amounts of other compounds were also present. However, by recrystallization of the solids in each fraction from methanol, the compound present in largest amount was obtained. In this way, compounds V, VI, VII were obtained from fractions *15*, *16*, and *17*, respectively.

Distribution of Fraction 19.—Fraction 19 was a mixture of six compounds. Distribution of this fraction in solvent system D for 365 transfers gave three additional fractions (fractions *21–23*) (Fig. 2). Fraction *22* contained only compounds VIII and IX. Compound VIII was obtained from this mixture by recrystallization from ethyl acetate.

TABLE III.—ANALYTICAL AND PHYSICAL DATA OF PHENOLIC COMPOUNDS ISOLATED FROM ALFALFA

Compd.	Empirical Formula	Anal. C Calcd., %	Anal. C Found, %	Anal. H Calcd., %	Anal. H Found, %	M.p., °C.
Coumestrol	$C_{15}H_8O_5$	67.1	66.8	3.09	3.10	385° dec.
Trifoliol	$C_{16}H_{10}O_6$	64.4	64.5	3.36	3.42	327° dec.
Tricin	$C_{17}H_{14}O_7$	61.8	61.8	4.24	4.38	291–294°
Salicylic acid	$C_7H_6O_3$	60.9	61.1	4.35	4.41	158°
IV	$C_{15}H_{10}O_4$	70.9	70.8	3.93	3.99	315–316°
VI	$C_{15}H_8O_6$	63.4	62.8	2.84	3.20	>350° (darkens)
VII	$C_{16}H_{10}O_6$	64.4	64.3	3.38	3.46	303° dec.
VIII	$C_{17}H_{12}O_6$	65.4	65.5	3.85	3.85	306°

Characterization.—For all 13 compounds, two-dimensional paper chromatograms were prepared, and the R_f values and fluorescence of each compound were ascertained (Table I). In addition, for eight of the compounds, melting points (uncorrected) were determined and the compounds subjected to elemental analysis. From the elemental analyses, the most probable empirical formulas have been calculated (Table III).

Identification of coumestrol, tricin, trifoliol, and salicylic acid was accomplished by comparison of their X-ray diffraction patterns and infrared spectra with those of authentic samples. In addition, mixed melting point determinations and two-dimensional paper chromatograms further confirmed their identity.

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Synthesis of Some Hexamine Derivatives as Potential Antispasmodics

By GARY OMODT

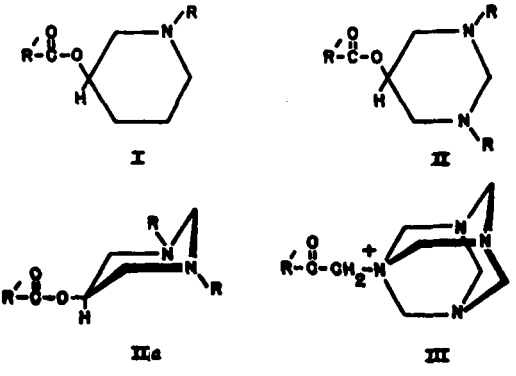
The activity of an antispasmodic of the atropine type appears to be related to the rigidity of the nitrogen-containing moiety. Hexamine contains a very rigid ring system, and it would seem that hexamine would furnish a promising starting point in the synthesis of a good antispasmodic. Some hexamine derivatives were synthesized by reacting the appropriate halomethyl amide or ketone with an excess of hexamine in refluxing chloroform. Preliminary pharmacological evaluation indicates that one of the hexamine derivatives possesses good antispasmodic activity.

THE LITERATURE indicates that a parasympatholytic antispasmodic containing a rigid nitrogen ring system is more active than when the nitrogen is not held in a conformation so rigid (1, 2). Perhaps the reason for this is due to a more or less fixed nitrogen-carbonyl distance *versus* a variable distance in the nonrigid compound. The foregoing hypothesis presumes that the fixed distance is optimum for combination with the potential acetylcholine receptor. Recent research has been directed along the lines of incorporating very rigid bridged nitrogen systems into synthetic antispasmodics (3–8).

Compounds with the general structure of I are on the market as useful antispasmodics (piperidolate, pipenzolate methylbromide, mepenzolate methylbromide) in which the R group is a small alkyl, and the R′ is a large, bulky blocking group. Considering only receptor combination and disregarding effects due to solubility and distribution, compounds relative to structure II might possibly possess activity equal to or better than compounds relative to structure I. This is the case because of the additional nitrogen located in an identical position (with relation to the carbonyl) to the first nitrogen, and thus providing an additional receptor combination site; *i.e.*, the chances of the compound combining with the potential acetylcholine receptor would be increased. This is illustrated better by structure IIa. This is actually structure II from a "45° side view," whereas structure II is a view from the "top."

In reference to compound III, three nitrogens of equal distance from the carbonyl are present, the distance being approximately equal to the nitrogen-carbonyl distance in structure I. In addition, structure III is very symmetrical with respect to the nitrogen ring system. Since all three nitrogens are of equal distance from the carbonyl (this distance being rigidly fixed) and the ring system is quite symmetrical, it would appear that a compound with a structure similar to that of III would be apt to combine with the potential acetylcholine receptor.

The quaternized nitrogen in compounds possessing type III structure may affect the ability to



I II IIa III

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Synthesis of Some Hexamine Derivatives as Potential Antispasmodics

By GARY OMODT

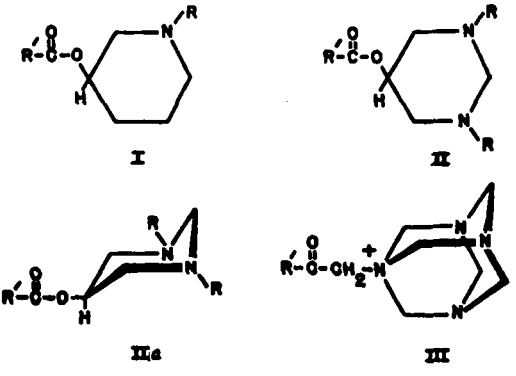
The activity of an antispasmodic of the atropine type appears to be related to the rigidity of the nitrogen-containing moiety. Hexamine contains a very rigid ring system, and it would seem that hexamine would furnish a promising starting point in the synthesis of a good antispasmodic. Some hexamine derivatives were synthesized by reacting the appropriate halomethyl amide or ketone with an excess of hexamine in refluxing chloroform. Preliminary pharmacological evaluation indicates that one of the hexamine derivatives possesses good antispasmodic activity.

THE LITERATURE indicates that a parasympatholytic antispasmodic containing a rigid nitrogen ring system is more active than when the nitrogen is not held in a conformation so rigid (1, 2). Perhaps the reason for this is due to a more or less fixed nitrogen-carbonyl distance *versus* a variable distance in the nonrigid compound. The foregoing hypothesis presumes that the fixed distance is optimum for combination with the potential acetylcholine receptor. Recent research has been directed along the lines of incorporating very rigid bridged nitrogen systems into synthetic antispasmodics (3–8).

Compounds with the general structure of I are on the market as useful antispasmodics (piperidolate, pipenzolate methylbromide, mepenzolate methylbromide) in which the R group is a small alkyl, and the R′ is a large, bulky blocking group. Considering only receptor combination and disregarding effects due to solubility and distribution, compounds relative to structure II might possibly possess activity equal to or better than compounds relative to structure I. This is the case because of the additional nitrogen located in an identical position (with relation to the carbonyl) to the first nitrogen, and thus providing an additional receptor combination site; *i.e.*, the chances of the compound combining with the potential acetylcholine receptor would be increased. This is illustrated better by structure IIa. This is actually structure II from a "45° side view," whereas structure II is a view from the "top."

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The quaternized nitrogen in compounds possessing type III structure may affect the ability to



I II IIa III

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bind with the receptor but should not adversely affect the distribution in the body since quaternization invariably increases antispasmodic activity in similar compounds; in fact, pipenzolate methylbromide and mepenzolate methylbromide are both quaternized compounds. This paper is concerned with the synthesis and pharmacological evaluation of compounds relating to structure III.

The compounds that were used to react with hexamine were phenacyl bromide, 10-bromoacetyl phenothiazine, 9-bromoacetyl carbazole, *N*-bromoacetyl diphenylamine, and chloromethyl benzohydryl ketone.[1] All of the compounds possess large bulky groups that correspond to R′ in structure III, except phenacyl bromide where R′ would be phenyl. Apparently only three of the above compounds (phenacyl bromide, 10-bromoacetyl phenothiazine, and 9-bromoacetyl carbazole) reacted with hexamine to give the expected products. The other two (*N* bromoacetyl diphenylamine and chloromethyl benzohydryl ketone), when reacted with hexamine in refluxing chloroform, yielded crystalline material that analyzed for what was apparently the mono hydrohalide salts of hexamine. The reaction by which these hydrohalide salts were formed is in question.

The hexamine derivatives were unstable in the presence of active hydrogen containing polar solvents. This might be expected when one considers the Dele'pine (Sommelet) reaction, where a hexamine derivative is decomposed by heating with acidic alcohol or water to give an amine or aldehyde. It is also stated that compounds of this type are virtually impossible to recrystallize (9). Because of this, the compounds were purified by washing with chloroform, pulverization, washing with hot benzene, then drying at 100° *in vacuo*.

Methods of Preparation

The halomethyl amides were synthesized by modifying slightly the methods of Dahlbom and Ekstrand (10, 11).

The halomethyl ketone was synthesized by reacting diphenylacetyl chloride with diazomethane and treating the product with dry hydrogen chloride according to the method for the preparation of benzyl chloromethyl ketone (12). The diphenylacetyl chloride was prepared by heating diphenylacetic acid with thionyl chloride as in the synthesis of *n*-butyryl chloride (13). A general method was used in the preparation of diazomethane (14).

The hexamine derivatives were prepared according to the method of Mackie and Misra (15) with some modification of reaction conditions and amounts of reactants. The general procedure consisted in dissolving hexamine (7.0 Gm., 0.05 mole) in 85 ml. of refluxing chloroform in a three-necked, round-bottom flask equipped with a stirrer, dropping funnel, condenser, and drying tube. The halomethyl compound (0.025 mole) was dissolved in 40 ml. of chloroform and added dropwise over a period of 1.5 hours to the vigorously stirred, refluxing solution. After complete addition of the halomethyl compound, the mixture was stirred and refluxed for 1 hour, then allowed to cool to room temperature. The crystals were removed by suction filtration and washed two times with 50-ml. portions of chloroform. The crystals were then air-dried, pulverized, and placed in 100 ml. of boiling benzene for 30 minutes. The hot benzene was removed by suction filtration and the crystals air-dried. The crystals were further dried for 6 hours *in vacuo* at 100°. Different amounts of reactants were used at times, and these are specified under the separate headings with corresponding per cent yields

[1] This compound was never isolated in a pure form and did not yield the expected hexamine derivative; it is possible that it was never obtained.

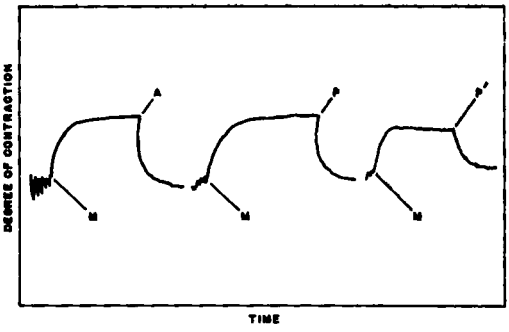


Fig. 1.—Pharmacological evaluation of 10-hexaminoacetyl phenothiazine bromide. Key: M, methacholine chloride solution; A, atropine sulfate solution; P, 10-hexaminoacetyl phenothiazine bromide solution; P′, 10-hexaminoacetyl phenothiazine bromide solution after standing 30 minutes.

Biological Results

A pharmacological evaluation was run on each compound. Hexaminomethyl phenyl ketone bromide showed no activity, and 9-hexaminoacetyl carbazole bromide showed a slight decrease in methacholine chloride-induced spasm. Definite antispasmodic activity was evidenced by 10-hexaminoacetyl phenothiazine bromide; the activity compared favorably to the atropine sulfate control (see Fig. 1). The solutions of the test compounds were very unstable and upon standing at room temperature for a few minutes became cloudy. Even after standing for 30 minutes at room temperature and becoming quite cloudy, 10-hexaminoacetyl phenothiazine bromide exhibited considerable antispasmodic activity (see P′, Fig. 1).

EXPERIMENTAL

All melting points recorded in this paper were taken with the Fisher-Johns melting point apparatus. The melting points are all uncorrected. The halide analyses (Volhard) and the nitrogen analyses (semimicro Kjeldahl) were performed in these laboratories.

10-Bromoacetyl Phenothiazine.—Phenothiazine (10.0 Gm., 0.05 mole) and bromoacetyl bromide (10.1 Gm., 0.05 mole) were refluxed in 100 ml. of anhydrous benzene for 2 hours. The hot solution was filtered and concentrated to 50 ml. Petroleum ether (30–60°, 25 ml.) was carefully layered on top

of the solution, and crystallization was allowed to take place at room temperature. After crystallization had appeared to cease, the mixture was placed in the refrigerator for 2 hours. The crystals were then removed by suction filtration and air-dried. The yield was 12.3 Gm. (81.5%) of light tan crystals, softening up to 119°, m.p. 119–121°.

Anal.—Calcd. for $C_{14}H_{10}BrNOS$: N, 4.38. Found: N, 4.27.

An additional crop of crystals was obtained by concentrating the filtrate to 25 ml. and repeating the crystallization procedure. The yield was 1.9 Gm. (12.6%) of brown crystals softening up to 98°, m.p. 98–100°.

9-Bromoacetyl Carbazole.—Carbazole (8.4 Gm., 0.05 mole) and bromoacetyl bromide (10.1 Gm., 0.05 mole) were refluxed in 100 ml. of anhydrous benzene for 2 hours. Upon admixture of the reactants, a precipitate formed. The precipitate slowly disappeared during reflux; a clear solution resulted after the reflux period. Crystallization was carried out as under *10-Bromoacetyl Phenothiazine*. The first crop of light tan crystals weighed 9.2 Gm. (68.1% yield), softening up to 83°, m.p. 83–85°.

Anal.—Calcd. for $C_{14}H_{10}BrNO$: N, 4.86. Found: N, 4.83.

The second crop of crystals (light tan) weighed 3.7 Gm. (27.4% yield), softening up to 83°, m.p. 83–85°.

N-Bromoacetyl Diphenylamine.—Diphenylamine (8.5 Gm., 0.05 mole) and bromoacetyl bromide (10.1 Gm., 0.05 mole) were refluxed in 100 ml. of anhydrous benzene for 2 hours. Upon admixture of the reactants, a precipitate formed. The precipitate slowly disappeared during reflux; a clear solution resulted after the reflux period. Crystallization of the product was carried out as under *10-Bromoacetyl Phenothiazine*. The first crop of white crystals slowly turned light blue on exposure to the atmosphere and weighed 11.5 Gm. (84.6% yield), softening up to 118°, m.p. 118–120°.

Anal.—Calcd. for $C_{14}H_{12}BrNO$: N, 4.83. Found: N, 4.60.

The second crop of crystals slowly turned dark blue on exposure to the atmosphere and weighed 1.1 Gm. (8.1% yield), softening up to 116°, m.p. 116–118°.

Chloromethyl Benzohydryl Ketone.—Diazomethane was prepared from 21.5 Gm. (0.10 mole) of *p*-tolysulfonylmethyl nitrosamide. The total distillate from this preparation (assumed to contain 2.7 Gm. of diazomethane based on a 64% yield) was transferred to a three-necked, round-bottom, 1000-ml. flask equipped with a magnetic stirrer, a dropping funnel, and a reflux condenser with a drying tube. To the stirred solution was added, over a period of 15 minutes, a solution of 6.9 Gm. (0.03 mole) of diphenylacetyl chloride (b.p. 168–171°/15 mm.). The final ether solution, after washing with two 50-ml. portions of 5% sodium carbonate solution, was dried with anhydrous calcium chloride and the solvent removed on a rotating vacuum evaporator. The residue weighed 5.5 Gm. (75.3% yield). The residue was used without purification.

Hexaminomethyl Phenyl Ketone Bromide.—Phenacyl bromide (5.0 Gm., 0.025 mole) was reacted with hexamine (7.0 Gm., 0.05 mole) by following the general procedure. The yield of light pink crystals was 7.8 Gm. (92.0%), m.p. 163–165° dec.

Anal.—Calcd. for $C_{14}H_{19}BrN_4O$: Br, 23.56; N, 16.52. Found: Br, 23.92; N, 16.66.

10-Hexaminoacetyl Phenothiazine Bromide.[2]—10-Bromoacetyl phenothiazine (7.6 Gm., 0.025 mole) was reacted with hexamine (7.0 Gm., 0.05 mole) by following the general procedure. The yield of white crystals was 8.8 Gm. (79.3%), m.p. 195–200° dec.

Anal.—Calcd. for $C_{20}H_{22}BrN_5OS$: Br, 17.46; N, 15.21. Found: Br, 17.36; N, 15.18.

9-Hexaminoacetyl Carbazole Bromide.—9-Bromoacetyl carbazole (6.8 Gm., 0.025 mole) was reacted with hexamine (7.0 Gm., 0.05 mole) by following the general procedure. The yield of white crystals was 8.6 Gm. (83.5%), m.p. 165–168° dec.

Anal.—Calcd. for $C_{20}H_{22}BrN_5O$: Br, 18.66; N, 16.35. Found: Br, 18.52; N, 16.27.

Pharmacological Evaluation

The pharmacological evaluation was carried out by utilizing a constant-temperature muscle bath and kymograph. Locke's solution (200 ml.) was used to bathe the muscle strip. When the temperature of the Locke's solution had reached 38°, a flow of oxygen was bubbled through the solution; a 5–7-cm. section of freshly excised rabbit ileum was attached to the muscle hooks so that the muscle section was completely submerged in the warm Locke's solution. The kymograph drum was slowly rotated, and 0.5 ml. of a 6.67×10^{-3} *M* solution of methacholine chloride was added to the Locke's solution. After the muscle strip had responded to the methacholine chloride, 0.5 ml. of a 6.67×10^{-3} *M* solution of atropine sulfate[3] was added. The same procedure was employed with each of the test compounds,[4] utilizing a fresh strip of ileum and 200 ml. of fresh Locke's solution each time. All test solutions were prepared immediately before use by dissolving the compounds in cold Locke's solution to inhibit decomposition. The results of the evaluation are shown in Fig. 1.

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[2] 10-Hexaminoacetyl phenothiazine chloride has previously been prepared by Mackie and Misra for use as an anthelmintic agent (15).

[3] The concentration of the atropine sulfate solution should have been 3.33×10^{-3} to insure comparison on a molecular basis.

[4] When preparing solutions of the test compounds, 10-hexaminoacetyl phenothiazine bromide and 9-hexaminoacetyl carbazole bromide would not dissolve completely. Therefore, 6.67×10^{-3} does not indicate the correct molar concentrations for these compounds.

Chromatographic Investigation of the Essential Oil of *Achillea fragrantissima*

By AHMED F. SHALABY* and GÜNTHER RICHTER

Achillea fragrantissima oil was obtained by steam distillation in a yield of 0.83 per cent (v/w)—d_{23}^{23} 0.9001, $[\alpha]_D^{20}$ + 8.25°, n_D^{23} 1.4640. It was light yellow and had a fresh aromatic odor. The oil was free from azulene, proazulene, aldehydes, and acids; but it contained the following: terpene hydrocarbons—α-pinene, *d*-myrcene, and sabinene; esters of formic, acetic, and butyric acids (the alcoholic parts of the esters were *l*-linalool, α-terpineol, *n*-hexen(3)-ol(1), and a small amount of nerol); ketones—ethyl *n*-amylketone and a short-chain unsaturated, unidentified ketone; phenols—eugenol and carvacrol; alcohols—*l*-linalool, *n*-hexen(3)-ol(1), 3-nonanol, and the possible presence of methanol, ethanol, and *n*-butanol is indicated.

ALTHOUGH MANY of the *Achillea* species have been investigated extensively, there have been no reports on the chemical constituents of *A. fragrantissima* (Forsk.) Sch. Bip., a xerophyte growing in the Egyptian desert.

The present work was carried out to determine the physical constants of the oil, to investigate the azulene and proazulene present, to separate the other oil components, and to identify the terpene hydrocarbons, esters, carbonyl compounds, phenols, and alcohols.

EXPERIMENTAL

The plant material used in the present investigation was collected by A. F. Shalaby from Wadi Hoff near Helwan, about 25 Km. South of Cairo, and was determined systematically (1).

Volatile Oil

The plant material, cut into small pieces, was subjected to water distillation in B.P. apparatus. The oil yield, 7 ml., was 0.83% (v/w) on the fresh-weight basis. The oil was neutral to pH paper. On cooling to −23°, there was no deposit from it. On treating the oil with EP-reagent (2), a negative result was obtained, an indication of the absence of azulenes.

The oil was pale yellow with a fresh aromatic odor. After drying over anhydrous sodium sulfate, the oil had the following physical constants: d_{23}^{23} 0.9001; n_D^{23} 1.4640; $[\alpha]_D^{20}$ + 8.25°.

Chromatographic Separation of the Oil Components

Thin-Layer Chromatogram.—The oil (1:9 in benzol) was spotted on a chromatoplate covered with Kieselgel G (3), and the chromatograph was developed with benzol:ethyl acetate (95:5). The developed plate was dried at room temperature, sprayed with antimony pentachloride, and dried at 105°. Dark spots formed with terpene hydrocarbons, esters, and alcohols, and a reddish spot with phenols (Fig. 1). The plate was then sprayed with 2,4-dinitrophenylhydrazine (DNPH), and a yellow spot formed with the carbonyl compounds.

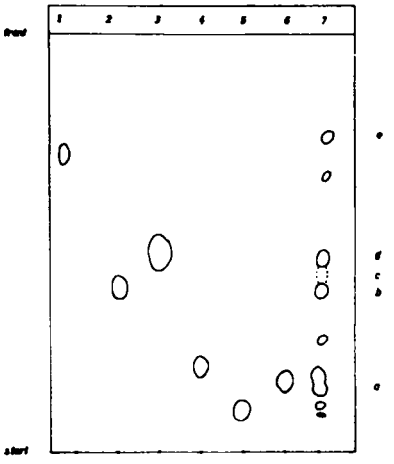


Fig. 1.—Chromatoplate of 1, α-pinene; 2, ethyl *n*-amylketone; 3, terpinyl acetate; 4, *l*-linalool; 5, *n*-hexen(3)-ol(1); 6, 3-nonanol; 7, achillea oil; a, alcohols; b, ketones; c, phenols; d, ethers; e, terpenes.

Column Chromatography.—The different components of the oil were separated on a silica gel column as follows: 0.5 ml. of oil was put on top of a silica gel[1] column. The packed column was 26.5 cm. high, with a diameter of 0.8 cm. About 100 ml. of petroleum ether (b.p. 30–50°) was added to the column, and twelve 7.5-ml. fractions were collected. These fractions contained the terpene hydrocarbons. The column was then eluted with benzol–petroleum ether (1:1), and 81 fractions were collected. A plate chromatogram was developed with each fraction, using the aforementioned system. For detection of the terpene hydrocarbons, esters, alcohols, and phenols, the plate was sprayed with antimony pentachloride reagent. For detection of ketones, the plate was then sprayed with DNPH. The results are shown in Fig. 2 and indicated in Table I.

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[1] Kieselgel Merck, 0.2–0.5 mm., activated at 150° for 3 hours.

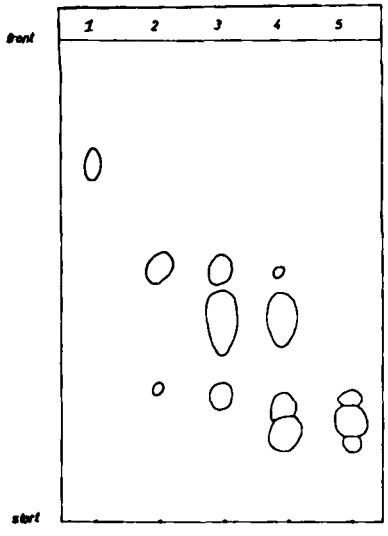


Fig. 2.—Plate chromatogram of different fractions from *A. fragrantissima* oil obtained by separation on kieselgel column: 1, fractions 1–12: terpenes; 2, fractions 13–16: esters and alcohols; 3, fractions 17–20: esters, ketones, and alcohols; 4, fractions 21–37: esters, ketones and alcohols; 5, fractions 38–93: alcohols.

Terpene Hydrocarbons

Gas Chromatographic Analysis.—Details of the two columns used for identification of the terpene hydrocarbons fraction, liquid phase, and operating conditions are summarized in Table II. The pure terpene fractions (from 1–12, Table I) were evaporated *in vacuo* at room temperature, and 10 μl. of the residual terpenes was injected on each of the two columns. Figures 3 and 4 show the results obtained with experiments *A* and *B*, respectively, which indicated the presence of α-pinene, *d*-myrcene, and sabinene. These results were also confirmed by references and by mixing them with achillea terpenes.

TABLE I.—CONTENT OF COLLECTED FRACTIONS[a]

Fraction No.	Terpenes	Esters	Ketones	Alcohols
1–12	+++	...	...	...
13–16	...	+++	...	+
17–20	...	+++	+++	++
21–37	...	+	++	++
38–93	...	...	...	+++

[a] Amounts of the substances: +, small; ++, moderate; +++, large.

Esters

To obtain the ester fraction free from other constituents, another column was prepared as described previously but using 1 ml. of oil. The prepared column was eluted with petroleum ether (b.p. 30–50°) and 33 fractions of 8 ml. each were obtained. The first 20 fractions contained the terpene hydrocarbons, and fractions 21 to 33 contained the esters. The ester fractions were mixed, and the petroleum ether was distilled off. The acidic and alcoholic parts of the esters were identified as follows.

Acidic Part.—The acids of the separated esters were identified in the form of hydroxamates by treating part of the ester with alcoholic hydroxylamine hydrochloride (4). The hydroxamate derivatives were then chromatographed in the presence of test substances on Schleicher & Schüll 2048 MGl paper. Development was done in the system of amyl alcohol–acetic acid–water (4:1:5), using the ascending technique. The developed chromatogram was dried, then the spots were detected with alcoholic solution of 0.1% $FeCl_3$. Formic, acetic, and butyric acids were identified (see Fig. 5).

Alcoholic Part.—The other part of the ester fraction was saponified with 5 ml. of 5% KOH in methanol at room temperature. After 2 hours, the mixture was diluted with 50 ml. of water, then shaken with ether to extract the alcohols. The ether phase was washed with water, then dried with anhydrous sodium sulfate. The ether was distilled, and the residual alcohols were identified by the following methods.

Paper Chromatogram.—Ascending chromatography (5) utilized Ederol 208/P paper, with the system of methyl isopropyl ketone–*n*-heptane (5:20 v/v), and detection was made in a saturated jar with osmium tetroxide reagent. Three spots were identified as linalool, terpineol, and nerol.

Gas Chromatography.—The operating conditions are summarized in Table II. The result (Fig. 6) confirmed the presence of *l*-linalool, α-terpineol, *n*-hexen(3)-ol(1), and a small amount of nerol.

Phenols

Two milliliters of oil in 10 ml. of petroleum ether (b.p. 30–50°) were shaken with 5% NaOH solution. The sodium phenolate solution was acidified with sulfuric acid, the phenols were extracted with ether, the ether phase was dried with anhydrous sodium sulfate. The ether was then removed, and the residual phenols were investigated by the following methods.

Paper Chromatogram.—The ascending procedure utilized Ederol 208/P paper and the following two solvent systems: (*a*) *n*-hexane–*n*-heptane–acetic

TABLE II.—OPERATING CONDITIONS[a]

Conditions	Terpene Hydrocarbons Expt. *A*	Terpene Hydrocarbons Expt. *B*	Alcohols of Esters and Free Alcohols
Column liquid phase	Reoplex 400	β,β′-Oxydipropionitrile	Reoplex 400
Ratio liquid:support (w/w)	20:100	15:100	20:100
Column temperature (°C.)	75.0	65.0	145.0
Hydrogen flow rate (ml./min.)	60.0	60.0	50.0
Gas inlet pressure (ata)	0.25	1.5	0.5
ma.	250	250	200
Philips writer (mv.)	2.5	2.5	2.5

[a] The following conditions apply to all experiments: apparatus, Gasofract 300 with Katharometer; solid support, Chromo sorb W; column, 3-mm. length, 4-mm. diameter, copper coil; paper speed, 0.5 cm./minute; sample size, 10 μl.

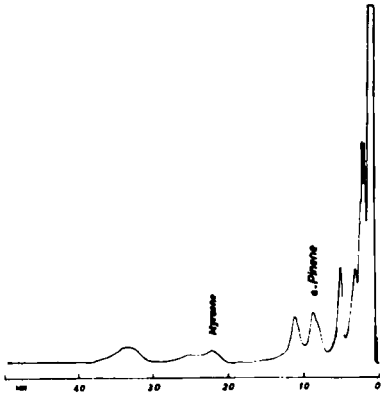


Fig. 3.—Gas chromatographic separation of the monoterpene hydrocarbons of achillea oil on Reoplex 400. (For operating conditions see Table II.)

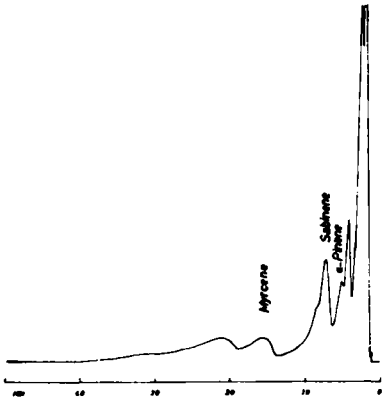


Fig. 4.—Gas chromatographic separation of the monoterpene hydrocarbons of achillea oil on β,β'-oxydipropionitrile. (For operating conditions see Table II.)

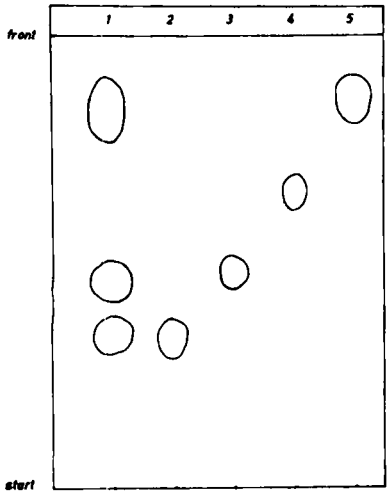


Fig. 5.—Paper chromatogram of 1, hydroxamate derivatives of the acidic part of the esters; 2, formyl hydroxamate; 3, acetyl hydroxamate; 4, propionyl hydroxamate; 5, butyryl hydroxamate.

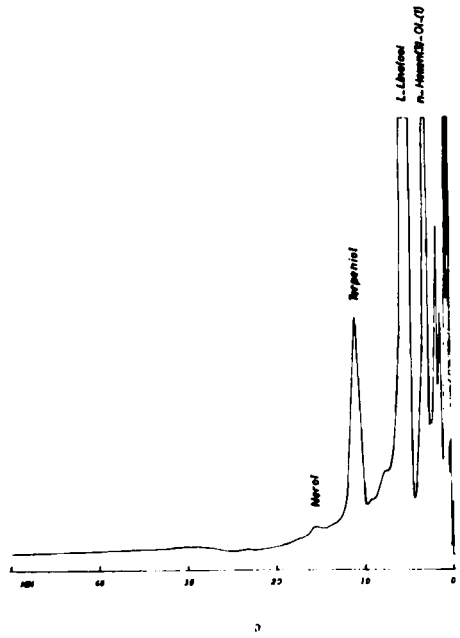


Fig. 6.—Gas chromatographic separation of the alcoholic part of the ester fraction of achillea oil on Reoplex 400. (For operating conditions see Table II.)

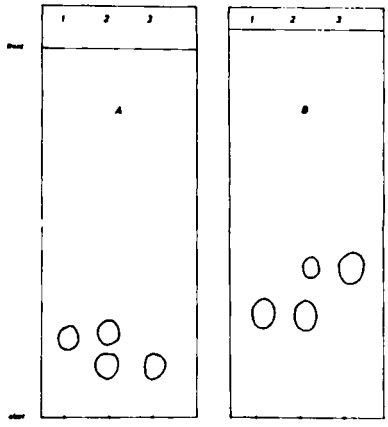


Fig. 7.—Paper chromatogram of 1, eugnol; 2, achillea phenols; 3, carvacrol. Solvent in A: *n*-hexane–*n*-heptane–acetic acid (15:15:2 v/v). Solvent in B: methyl isopropyl ketone—*n*-heptane (5:20 v/v).

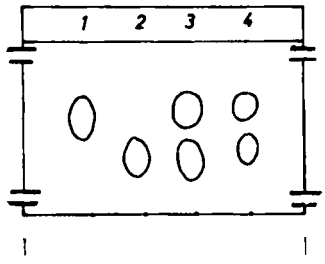


Fig. 8.—Part of a chromatoplate of 1, eugenol; 2, carvacrol; 3, achillea phenols; 4, mixture of achillea phenols, eugenol, and carvacrol.

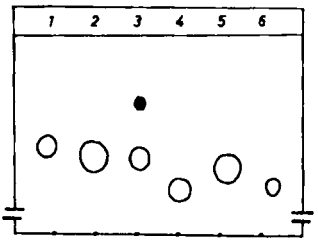


Fig. 9.—Part of a chromatoplate of the ketone-hydrazone derivatives of 1, 3-octanone; 2, ethyl-*n*-amylketone; 3, achillea ketones; 4, 3-decanone; 5, 3-nonanone; 6, 2-decanone; ●, unsaturated ketone.

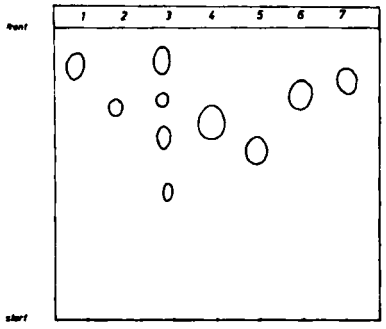


Fig. 10.—Paper chromatogram of the alcohols 1, *l*-linalool; 2, citronellol; 3, achillea alcohols; 4, farnesol; 5, geraniol; 6, terpineol; 7, santalol.

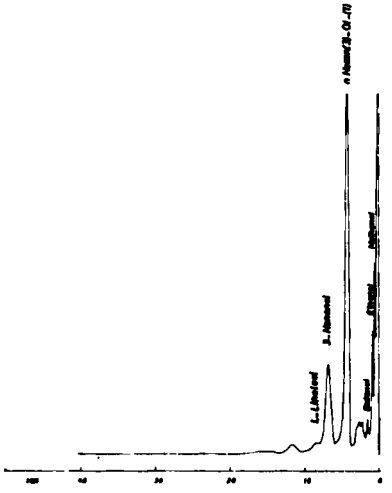


Fig. 11.—Gas chromatographic separation of the free alcohols in achillea oil on Reoplex 400. (For operating conditions see Table II.)

acid (15:15:2 v/v); (*b*) methyl isopropyl ketone–*n*-heptane (5:20 v/v). The antimony pentachloride reagent yielded a brown color with eugenol and a red-violet with carvacrol (Fig. 7).

Plate Chromatogram.—A chromatoplate covered with Kieselgel G was used with chloroform as the developing solvent. The results are indicated in Fig. 8.

Ketones

The ketones were identified in the form of 2,4-DNPH-derivatives (6). The crystalline derivatives were dissolved in benzol, and the solutions were spotted on a plate covered with silica gel which was impregnated with 5% paraffin in benzene. The solvent system of dimethylformamide–methanol–water (4:1:1) was used for development (7). Two ketonic hydrazones were detected (Fig. 9): a short-chain unsaturated ketone (+v result with OsO_4) with higher R_f value and ethyl *n*-amyl ketone.

Free Alcohols

Phthalate derivatives had been separated by Ruzicka (8), and Sabetay (9) separated the alcohols by means of 30% NaOH. The pure alcohols were identified in this work as follows.

Paper Chromatogram.—The method described in the investigation of the alcohols of the esters was used with the free alcohols. The result is indicated in Fig. 10, where it appears that there are at least three alcohols, and that one of them is *l*-linalool.

Gas Chromatography.—Gas chromatography, using the operating conditions summarized in Table II, indicated the presence of *l*-linalool, *n*-hexen(3)-ol(1), 3-nonanol, and the possible presence of methyl, ethyl, and butyl alcohols (Fig. 11).

DISCUSSION

Species other than *A. millefolium*, which does contain azulene and proazulene in its oil, have been reported to contain no azulene—*viz.*, *A. ageratum* (10), *A. atrata* (11), *A. clavenae* (12), *A. moschata* (11), *A. micrantha* (13, 14), *A. filipendulina* (15), and *A. coronopifolia* (16).

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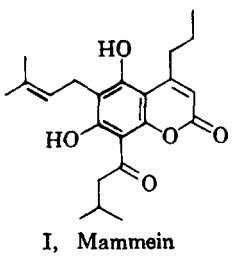
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Constitution of Mamey Wax

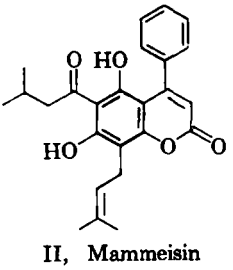
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Mamey wax, m.p. 79.5°, isolated from the seed oil of *Mammea americana* L. is shown to be a mixture of saturated, long straight-chain fatty esters with carbon contents ranging from C_{38} to C_{54}. The major constituent is the symmetrical C_{48} homolog, tetracosanyl tetracosanoate. After hydrolysis, in addition to even carbon-numbered species, small amounts of odd carbon-numbered acids were detected.

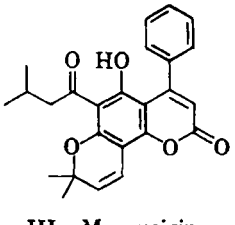
SCIENTIFIC INTEREST in various parts of the mamey tree (*Mammea americana* L., family *Guttiferae*) stems, at least in part, from the century old report of de Grosourdy (1) on its insecticidal activity. Several studies of this property of mamey seed extracts (2–5) led eventually to the isolation (6) of a crystalline active principle. Degradative and spectroscopic (7, 8) as well as synthetic evidence (9) was adduced (8) in support of I for this substance which was named mammein (7). Subsequently, a yellow toxic (10) compound was isolated from the fruit peelings and shown (11) to possess II. [Compound II was later named mammeisin (12).] Recently, a new coumarin with yet unknown physiological properties, mammeigin, was isolated from the seed oil and reported (13) to have the closely related structure III.



I, Mammein



II, Mammeisin



III, Mammeigin

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As part of a continuing study of the constituents of mamey seed oil, we have characterized the wax obtained by acetone precipitation from this oil. The present paper is concerned with these experiments.

RESULTS and DISCUSSION

In connection with their original isolation of mammein, Morris and Pagán (6) reported that a "nontoxic white, powdery solid, melting sharply at 78°," was obtained as a precipitate from an acetone solution of mamey oil. Although Morris and Pagán provide no additional details concerning this substance, there is no doubt that this material is identical to that which we have obtained by a similar procedure and named mamey wax. The melting point of the crude wax is 74–76° but can be raised by repeated recrystallization from dioxane, isopropanol, ethyl acetate, or chloroform to 79.5°.

The presence in the molecule of a saturated ester grouping was indicated by the bands in the infrared at 1733 (carbon-oxygen double bond stretching) and 1179 cm.$^{-1}$ (carbon-oxygen single bond stretching) (14). In addition to these bands and the strong absorption in the 2900 cm.$^{-1}$ region corresponding to carbon-hydrogen stretching modes, the spectrum showed relatively few more bands. A series of small evenly spaced peaks between 1340 and 1190 cm.$^{-1}$ indicative of band progression in long-chain fatty acid esters, suggested a chain length of at least 22 carbons (15). A pair of bands at 730 and 724 cm.$^{-1}$ associated with skeletal vibrations of methylene chains was present (16) as was a band at 1377 cm.$^{-1}$ arising from the carbon-hydrogen deformation frequency for a terminal methyl group. This latter band, however, was of significantly low intensity when compared to the band at 1471 cm.$^{-1}$ arising from the analogous vibrations of methylene groups. Finally, it was noted that absorption due to hydroxyl or carbon-carbon double bonded groupings was absent.

The conclusion that the wax was indeed saturated was supported by a negative tetranitromethane test (17) and by the absence of vinyl hydrogen absorption in the proton magnetic resonance spectrum. (These observations were also consistent with the complete absence of absorption in the ultraviolet region.) Although some difficulty was encountered in the determination of the NMR spectrum (low solubility), it did serve to confirm the features already indicated by the infrared measurements. The terminal methyl absorption at 9.13 τ was quite weak compared to the intense signal at 8.75 τ caused by the methylene groups. In addition, the unique pair of methylene groups which flank the ester carboxyl function gave rise to a pair of weak

TABLE I.—PARENT MASS PEAKS IN MAMEY WAX

Mass	Molecular Formula C	H	O	Peak Ht., Chart Divisions
564	38	76	2	4
592	40	80	2	11
620	42	84	2	18
648	44	88	2	28
676	46	92	2	47
704	48	96	2	69
732	50	100	2	30
760	52	104	2	12
788	54	108	2	4

TABLE II.—MASS SPECTRAL ANALYSIS OF MAMEY WAX SAPONIFICATION PRODUCTS

Carbon No.	Alcohols Wt., %	Acids (as Methyl Esters) Wt., %
18	...	0.2
19	...	...
20	5	1.5
21	...	...
22	6	11.1
23	...	0.7
24	62	67.8
25	Less than 1	1.2
26	15	15.1
27	...	0.2
28	8	1.8
29	...	0.1
30	4	0.3
31	...	0.02
32	...	0.01

triplets (J = 6.5 cps.) situated appropriately (18) at 5.95 τ (—$CH_2\underline{CH_2}O$—) and 7.72 τ (—$CH_2\underline{CH_2}$-CO_2—). With the presence of at least two oxygen atoms established spectroscopically, the microanalytical results led to the formulation of mamey wax as $C_{48}H_{96}O_2$. The Kuhn-Roth determination indicated the presence of two C—CH_3 groups. Although the microanalytical data might be accommodated by nearby homologs of the C_{48} formula when taken in combination with the data obtained from the wax hydrolysis products, the formula indicated represents the best fit.

Saponification of the wax could be effected by reaction with boiling ethanolic potassium hydroxide solution or, more conveniently, by reaction with sodium hydroxide in boiling aqueous dioxane solution. Both the alcohol and the acid moieties were isolated in high yield and identified as lignoceryl alcohol, $CH_3(CH_2)_{22}CH_2OH$, and lignoceric acid, $CH_3(CH_2)_{22}CO_2H$. These structural assignments were based on the microanalytical results, including Kuhn-Roth determinations, and on comparisons (melting points and infrared spectra) with authentic materials.[1] Furthermore, the acid was converted to its methyl ester and this in turn compared to an authentic specimen.[2] The combined chemical, microanalytical, and spectroscopic evidence then requires that mamey wax be represented as lignoceryl lignocerate, $CH_3(CH_2)_{22}CO_2(CH_2)_{23}CH_3$. The central location of the ester function was verified by lithium aluminum hydride reduction which provided lignoceryl alcohol in accordance with this formulation.

It is generally recognized that natural waxes usually consist of mixtures of homologous components whether they be esters, acids, alcohols, ketones, or hydrocarbons (19, 20). It seemed likely that mamey wax was similarly constituted; we therefore made use of mass spectrometric measurements to extend and complete the chemical definition of the wax. The wax itself produced a spectrum with parent ion peaks ranging from mass 564 ($C_{38}H_{76}O_2$) to mass 788 ($C_{54}H_{108}O_2$). The relative peak heights are shown in Table I, where it can be seen that the most abundant species is the one at mass 704 corresponding to $C_{48}H_{96}O_2$. The baselines show small bulges at the positions of the parent masses of the odd-carbon esters, with those at C_{47} and C_{49} being the most pronounced. Although these were too small (the order of one chart division) to measure unambiguously, their magnitude is entirely sufficient to account for the presence of the odd-carbon acids discussed below. No information could be derived from the fragment region of the spectrum with respect to the location of the ester group in the chain. Examination of the saponification products, however, did provide verification of this point. The acid resulting from hydrolysis of the wax was analyzed as its methyl ester. It can be seen in Table II that this acid consists of all the saturated even numbered homologs from C_{18} to C_{32}. By far the most abundant species is the C_{24} member, lignoceric acid, which is present to the extent of nearly 68%. The fragmentation pattern was virtually identical to that observed for authentic methyl lignocerate.[2]

A most interesting feature of this analysis was the detection of the odd-numbered acids from C_{23} to C_{31}. The older view that natural fats and waxes consisted exclusively of even-numbered homologs[3] has been eroded gradually by the application of the sensitive analytical techniques of gas chromatography and mass spectrometry. The "unnatural" homologs since first being detected in certain animal fats (21–24), have been observed to occur in such diverse materials as human hair fat (25), soil extracts (26), human skin wax (27), petroleum reservoir waters (28), seawater (29), bituminous (montan) wax (30–32), wool wax (33), and beeswax (34). The first report of the occurrence of the odd-numbered (unnatural)[3] species in a plant wax (sugar cane cuticle wax) appeared in 1960 (35); it seems that their widespread occurrence will depend only on the sensitivity of the analytical method (36, 37).

Also listed in Table II is the distribution of alcohols obtained by saponification of mamey wax, which clearly shows the major constituent (62%) to be lignoceryl alcohol. The analysis of the alcohol, however, is subject to uncertainties greater than that of the methyl esters. In the first place, parent ion peaks do not appear for long-chain alcohols (38). Peaks of high sensitivity do appear at masses 18

[1] Lignoceric acid was obtained from the Applied Science Laboratories, Inc., State College, Pa. The corresponding alcohol was prepared from this material by lithium aluminum hydride reduction. The acid was stated to have a purity of 99+%.

[2] Methyl lignocerate was also obtained from the Applied Science Laboratories (see *Footnote 1*) and was stated to have a purity of 99.8+%. Our mass spectral analysis indicated a purity of 99.2% (by weight); the contaminants were the methyl esters of the homologous C_{25} (0.6%) and C_{26} (0.2%) acids.

[3] Hydrocarbon waxes, of course, consist predominately of odd-numbered homologs, presumably arising by decarboxylation of the even-numbered acids. Recent years have brought the detection of the rare even-numbered hydrocarbons, however.

units (loss of H_2O) and 20 units (additional loss of two hydrogens) below the parent mass number. In addition, a fraction of the long-chain alcohol molecules simultaneously lose one or more methylene groups, and therefore significant contributions appear at mass numbers corresponding to the principal peaks of other members of a mixture of homologous alcohols—the contribution is especially large for the loss of two methylene groups (38). The value for the alcohol at C_{22} is subject to the most uncertainty due to the large contribution resulting from the loss of two methylene groups from the main constituent at C_{24}. The spectra of two pure compounds, lignoceryl alcohol[1] and behenyl alcohol [$CH_3(CH_2)_{20}CH_2OH$], were used to establish contribution factors for the analysis of the mixture. Within the accuracy of these factors, the observed peaks corresponding to the odd-carbon alcohols (except the C_{25} member) were accounted for by contributions from even-carbon alcohols of higher molecular weight. The most abundant odd-carbon alcohol is apparently the C_{25} homolog which, incidentally, corresponds in chain length to the most abundant odd-carbon acid. Table III shows the distribution of alcohols obtained by lithium aluminum hydride reduction of mamey wax. This distribution, corrected for contributions, reflects the superposition of the alcohol and acid distributions shown in Table II. None of the specimens examined showed evidence for branching in the hydrocarbon chains.

In 1922, Brigl and Fuchs reported the isolation of lignoceryl lignocerate from beech tar wax and proved its structure by a combination of degradative and synthetic means (39). The chemical and physical properties which we have observed for mamey wax and its hydrolysis products accord well with those reported by Brigl and Fuchs for their ester. More recently, Bell and Harvey have shown that lignoceryl lignocerate is an important constituent of the wax from the heartwood of *Phyllocladus trichomanoides* (40).

EXPERIMENTAL[4,5]

Isolation of Mamey Wax.—Thirty-four kilograms of dried and ground seeds of *M. americana* L. were extracted with a total of 12 gal. of *n*-hexane. The residue remaining after removal of the solvent by distillation was treated with 1 gal. of technical grade acetone. A yellow semisolid material precipitated and was collected on a filter. A single recrystallization from isopropanol or dioxane provided 20 Gm. of slightly yellow solid, m.p. 74–76°, (0.06% yield based on the amount of dried seed). Treatment with charcoal, followed by several recrystallizations from dioxane, isopropanol, or ethyl acetate provided pure white material melting sharply at 79.5°. The literature melting point for lignoceryl lignocerate is 79° (39).

Anal.—Calcd. for $C_{48}H_{96}O_2$: C, 81.74; H, 13.72; O, 4.54; 2 C—CH_3, 4.26. Found: C, 82.27, 81.78, 82.10, 81.98; H, 13.44, 13.40, 13.43, 13.46; O, 4.09, 4.48; C—CH_3, 2.25, 2.58, 3.09.

Table III.—Mass Spectral Analysis of Alcohols Derived from Mamey Wax by Reduction

Carbon No.	Alcohol from Reduction, Peak Ht., Divisions
18	7
20	27
22	183
24	252
26	97
28	35
30	28
32	13

The infrared spectrum (KBr) of mamey wax showed strong bands at 2933, 2841, 1733, 1471 (doublet), 1179, 730, and 724 cm.$^{-1}$. The material was transparent in the ultraviolet (20.3 mg. in 10 ml. $CHCl_3$) and gave a negative tetranitromethane test for unsaturation (17). The NMR spectrum was determined in deuterochloroform solution using tetramethylsilane as an internal standard. Signals were observed at 5.95 τ (triplet, J = 6.5 cps.), 7.72 τ (triplet, J = 6.5 cps.), 8.75 τ (singlet), and 9.13 τ. Mass spectra were determined on two samples, m.p. 77.5–78.5° and m.p. 79.5°, with essentially identical results.

Saponification of the Wax.—One gram of ester, m.p. 79.5°, was heated at reflux in dioxane solution (50 ml.) with 20 ml. of aqueous sodium hydroxide (20%). After 48 hours, an additional 50 ml. of dioxane was added, and the mixture again heated at reflux for 1 hour. The hot mixture was then filtered and the white salt washed three times with 25 ml. of hot dioxane. The combined dioxane solutions were concentrated to about 100 ml. and cooled in an ice bath. The alcohol crystallized from solution and was collected on a filter, 380 mg., m.p. 75°. Two additional recrystallizations from chloroform provided 345 mg. of product, m.p. 76°. The literature melting point of lignoceryl alcohol is 76° (39). Microanalyses were performed on samples with m.p. 74–75° and 76°.

Anal.—Calcd. for $C_{24}H_{50}O$: C, 81.28; H, 14.21; O, 4.51; mol. wt., 354.6; C—CH_3, 4.23. Found: C, 81.28, 81.41; H, 14.08, 13.81; mol. wt., 385; C—CH_3, 3.04.

The infrared spectrum (KBr) showed prominent bands at 3584, 3106, 3040, 1484, 1064, 728, and 718 cm.$^{-1}$ and was similar to that of an authentic sample of tetracosanol, m.p. 76.5–77°; literature[6] m.p. 77.5° (39). No absorption was observed in the NMR spectrum at lower field than the triplet (J = 6 cps.) at 6.36 τ indicating the absence of vinyl protons. The mass spectral measurements were carried out on two samples, one with m.p. 75–75.5° and another (less extensively purified) with m.p. 69–73°.

The salt obtained from the saponification mixture described above was heated on a steam bath with 5 ml. of 2 *N* hydrochloric acid. After 30 minutes, the mixture was diluted with 15 ml. of water, and the acid was collected on a filter, washed with water until the washings were neutral, and dried in a

[4] The present work was initiated by E. J. Eisenbraun while in the Department of Chemistry, Wayne State University, and continued by R. A. Finnegan while in the Department of Chemistry, The Ohio State University, Columbus.

[5] Melting points were determined on a Fisher-Johns block and are uncorrected. Microanalyses were performed by Dr. A. Bernhardt, Mulheim, Germany. The infrared spectra were recorded on Baird model B and Perkin-Elmer model 137 Infracord instruments. The ultraviolet measurement was made on a Perkin-Elmer Spectracord, and the NMR spectra were determined with the Varian Associates A 60 spectrometer.

[6] The melting points of the synthetic materials are invariably somewhat higher than the corresponding naturally derived materials. This is known to be caused by the presence of homologs in the latter.

vacuum oven. This material weighed 428 mg., m.p. 74–76°. A single treatment with charcoal and three recrystallizations from ethyl acetate raised the melting point to 78.5°. Lignoceric acid is reported to melt at 79° (39). Microanalyses were carried out on samples with m.p. 77.5–79.5° and 78.5°.

Anal.—Calcd. for $C_{24}H_{48}O_2$: C, 78.19; H, 13.13; O, 8.68; C—CH_3, 4.08. Found: C, 78.37, 77.91, 78.30; H, 12.97, 12.88, 12.95; O, 8.22; C—CH_3, 0.68.

The infrared spectrum (KBr) showed bands at 3100, 2900, 2850, 2600, 1700, 1455, 925, 725, and 717 cm.$^{-1}$. The spectrum of an authentic specimen of tetracosanoic acid differed from that of the mamey wax acid only in the number of low intensity bands which were better resolved.

The saponification was also carried out in a more usual fashion by heating a solution of the wax in ethanolic potassium hydroxide at reflux for 24 hours. The properties of the products obtained in this way were the same as for the products described above.

Methylation of the Wax Acid.—A solution in anhydrous methanol (50 ml.) of 231 mg. of the acid, m.p. 73–76°, and a few drops of concentrated sulfuric acid was heated at reflux temperature for 14 hours. The white precipitate obtained when the reaction mixture was poured onto ice, was dissolved in ether, and washed several times with aqueous potassium bicarbonate solution. After being dried, the solvent was removed. The residual methyl ester weighed 200 mg. and had m.p. 54–56°. The infrared spectrum of this sample was similar to that of an authentic sample[2] of methyl tetracosanoate, m.p. 57–58°, differing only in the degree of resolution of the less intense bands. The sample was used for mass spectral measurements without further purification.

Reaction of the Wax with Lithium Aluminum Hydride.—A 3.50-Gm. sample of mamey wax, m.p. 76–78°, was added to a Soxhlet extraction apparatus mounted on a 2000-ml., three-necked flask containing 1000 ml. of anhydrous ether. The ether was stirred vigorously with a mechanical stirrer, and 4 Gm. of lithium aluminum hydride was added in portions. The suspension was stirred and heated at reflux temperature for two 8-hour periods until all of the wax had been dissolved and carried to the reaction vessel. Refluxing was continued for an additional 2 to 3 hours, the heating mantle was removed, the reaction allowed to cool, and water was added dropwise until the stirred suspension turned from gray to completely colorless.

The suspension was filtered through a layer of magnesium sulfate in a sintered-glass funnel. The ether was removed from the filtrate by evaporation to give 2.5 Gm. of a colorless solid. Sodium hydroxide (10%) was added to the salts, and the resulting suspension was extracted successively with ether and chloroform. The ether and chloroform extracts were combined, dried over anhydrous magnesium sulfate, filtered, and the solvent evaporated to give 1.05 Gm. of colorless solid which was combined with the earlier fraction. The crude product melted at 67–68.5° with some softening at 60°. A fraction (500 mg.) of this material was recrystallized twice from ethyl acetate to give 360 mg. of the alcohol, m.p. 73.5–74°. Additional recrystallizations from the same solvent did not raise the melting point. The infrared and NMR spectra of this material were virtually identical to those of the alcohol obtained by saponification.

Mass Spectrometric Determinations.—The instrument used is a Consolidated Electrodynamics Corp. model 21-103 (modified) mass spectrometer. The operating conditions were as follows: ionizing voltage, 70 v.; ionizing temperature, 270°; inlet temperatures, 250–275° for the alcohols and methyl esters, 340° for mamey wax.

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Enzyme Inhibitors III

Syntheses of *cis*-(6-Substituted-9-purinyl)cycloalkylcarbinols as Adenosine Deaminase Inhibitors

By HOWARD J. SCHAEFFER, D. D. GODSE, and GEORGIANA LIU

The syntheses of *cis*-4-(6-chloro-9-purinyl)cyclohexylcarbinol (VI) and *cis*-3-(6-chloro-9-purinyl)cyclopentylcarbinol (XVII) have been accomplished by the condensation of 5-amino-4,6-dichloropyrimidine with the appropriate amino alcohol, followed by ring closure of the resultant substituted pyrimidine to give the desired purines. Nucleophilic displacement of the 6-chloro group in VI and XVII gave the following 6-substituted derivatives: (*a*) hydroxy, (*b*) mercapto, (*c*) amino, (*d*) methylamino, and (*e*) dimethylamino. Evaluation of these substrate analogs as inhibitors of adenosine deaminase revealed that those compounds with an amino or methylamino group at the 6-position of the purine nucleus were inhibitory and that the hydroxymethyl group on the cyclopentyl or cyclohexyl nucleus makes only a small contribution to binding of the inhibitor to the enzyme.

THE UTILIZATION of chemicals for the treatment of disease has been the goal of much research since ancient times. During the past 20 years, tremendous progress has been made in our understanding of cellular chemistry and biochemistry. For example, in the area of purine metabolism the biosyntheses and many cellular transformations of purines, their nucleosides, and nucleotides have been elucidated (1). In cancer chemotherapy it is apparent that one of the areas of fruitful investigation is the inhibition of one or more stages of purine biosynthesis or purine transformations.

Many important enzymes utilize purine nucleosides or nucleotides as their normal substrates, but the mode of binding of these compounds to the various enzymes has not been studied extensively. Because of the interest in determining which atoms and functional groups of purine nucleosides and nucleotides are important in binding to various enzymes, the authors have begun a study of the inhibition of certain enzymes by a variety of substrate analogs. As the first enzyme for such an investigation, adenosine deaminase has been selected. Previous studies on the inhibition of adenosine deaminase by purine nucleoside analogs gave information concerning the importance to binding by the 2′- and the 3′-hydroxyl group of the ribose moiety and the types of groups which are necessary at the 6-position of the purine nucleus for binding to the enzyme (2, 3). This paper describes the synthesis and enzymatic evaluation of *cis*-3-(6-substituted-9-purinyl)cyclopentylcarbinols and *cis*-4-(6-substituted-9-purinyl)cyclohexylcarbinols as inhibitors of adenosine deaminase.

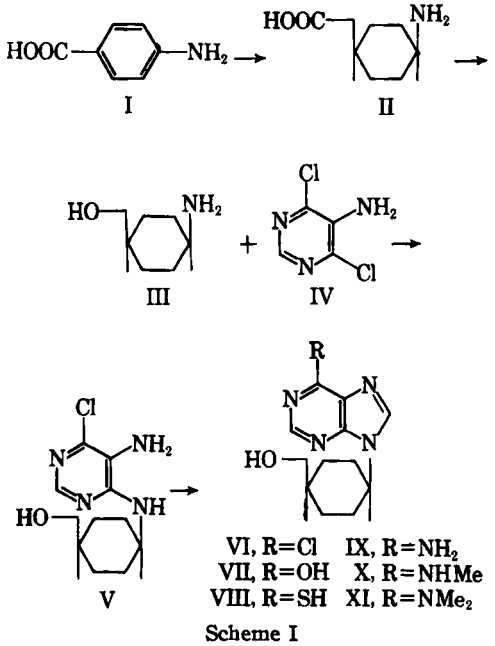
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Chemistry

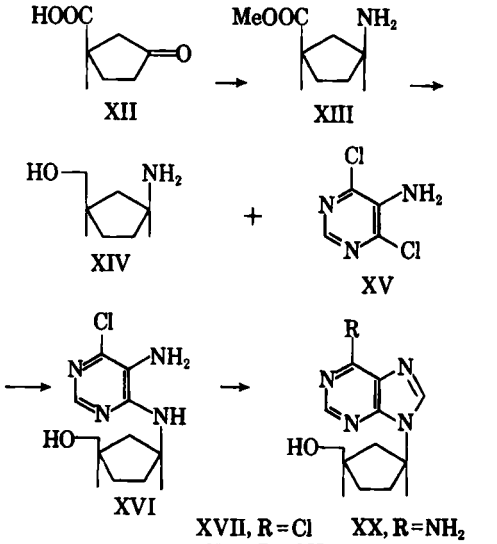
The general synthetic procedure for the synthesis of the desired compounds is a modification of the method of Montgomery and Temple (4) and involves the preparation of a 6-chloro-9-substituted purine, which can be converted into a variety of 6-substituted analogs by reaction with appropriate nucleophilic reagents. The synthesis of the 6-chloro-9-substituted purines involved the preparation of an appropriate hydroxymethylcycloalkylamine which, on condensation with 5-amino-4,6-dichloropyrimidine followed by ring closure, generated the desired purine. For the synthesis of the hydroxymethylcyclohexylpurines (Scheme I), *p*-aminobenzoic acid (I) was reduced by catalytic hydrogenation to *cis*-4-carboxycyclohexylamine (II) by a modification of the literature procedure (5, 6). Conversion of II into its ethyl ester, followed by reduction with lith-



Scheme I

ium aluminum hydride, gave the desired *cis*-4-hydroxymethylcyclohexylamine (III). Condensation of III with 5-amino-4,6-dichloropyrimidine (IV) resulted in the formation of V which, on reaction with triethyl orthoformate and acetic anhydride, gave *cis*-4-(6-chloro-9-purinyl)cyclohexylcarbinol (VI) in moderate yield; the 6-hydroxy analog (VII) was also formed during the cyclization of V. Nucleophilic displacement of the 6-chloro group of VI by thiourea, ammonia, methylamine, and dimethylamine gave the corresponding 6-substituted compounds (VIII–XI) in good yields.

The synthesis of the hydroxymethylcyclopentylpurines is outlined in Scheme II. 3-Oxocyclopentanecarboxylic acid (XII) (7) was converted to its oxime which, on catalytic hydrogenation, gave *cis*-3-carboxycyclopentylamine (8). Esterification of the acid gave the corresponding methyl ester (XIII). Conversion of XIII to XIV was accomplished by reduction with lithium aluminum hydride. Condensation of *cis*-3-hydroxymethylcyclopentylamine (XIV) with XV gave in good yield the desired pyrimidine (XVI), which underwent ring closure with

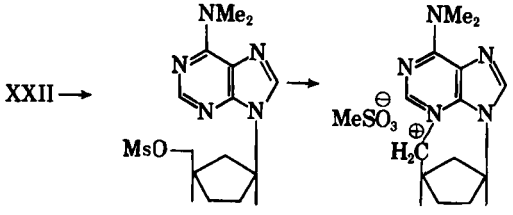


XVII, R=Cl XX, R=NH_2
XVIII, R=OH XXI, R=NHMe
XIX, R=SH XXII, R=NMe_2

Scheme II

triethyl orthoformate and acetic anhydride to generate the key intermediate (XVII). This intermediate was converted to its 6-substituted derivatives by modification of known procedures; the details are described under *Experimental*.

Concerning the stereochemistry of the hydroxymethylcyclopentylpurines (XVII–XXII), it is believed that the substituents on the cyclopentyl nucleus are *cis*. Our assignment of the *cis* configuration is based on the following observations. When the 6-dimethylamino derivative (XXII) was allowed to react with methanesulfonyl chloride in pyridine solution, a product was obtained which possessed the characteristic spectral properties of the methanesulfonate (XXIII). When XXIII was heated under reflux in an acetone or chloroform solution, a new isomeric product was isolated which, in contrast to XXIII, was soluble in water and insoluble in acetone or chloroform. In addition, the ultraviolet spectrum of XXIV exhibited a maximum at 291 mμ, whereas XXII and XXIII exhibited maxima at 270 and 272 mμ, respectively, in acidic solution. The saltlike character of the product and the shift in the ultraviolet maximum can be readily explained on the basis of the quaternary salt (XXIV). In the



XXIII XXIV

nucleoside area, Clark, *et al.* were the first to prepare cyclo-nucleosides and stated that only those ribonucleosides with a β-configuration (*i.e.*, the purine and the hydroxymethyl groups are *cis*) are capable of forming the quaternary salt (9). Later, Baker and Joseph prepared a similar quaternary salt using an appropriately blocked derivative of puromycin (10). Based on these results, it is logical to assign a *cis* relationship of the hydroxymethyl group and the purine nucleus in the cyclopentyl analogs XVII–XXII.

EXPERIMENTAL[1]

cis-4-Hydroxymethylcyclohexylamine (III).—A solution of 976 mg. (4.69 mmoles) of *cis*-4-ethoxycarbonylcyclohexylamine hydrochloride (11) in 3 ml. of water was stirred at room temperature with 10 ml. of a saturated solution of sodium bicarbonate for 30 minutes. The mixture was extracted with chloroform (5 × 10 ml.); the combined extracts were dried over sodium sulfate and concentrated *in vacuo*. The crude *cis*-4-ethoxycarbonylcyclohexylamine (650 mg., 3.82 mmoles) was dissolved in 10 ml. of ether and slowly added to a suspension of 220 mg. (5.8 mmoles) of lithium aluminium hydride in 20 ml. of ether at 0°. The mixture was stirred for 1 hour at 0°, then heated under reflux for 1 additional hour. The excess lithium aluminium hydride was destroyed with ether saturated with water (20 ml.) and then with water (4–5 drops). The ether layer was decanted, and the solid inorganic residue was triturated with chloroform (2 × 15 ml.) and filtered. The combined organic extracts were dried over sodium sulfate, concentrated *in vacuo*, and gave on distillation 317 mg. (58%) of III, b.p. 84–85°/0.5 mm. $\bar{\nu}$ in $cm.^{-1}$: 3400–3300 (OH and NH_2).

Anal.[2]—Calcd. for $C_7H_{15}NO$: C, 65.07; H, 11.70; N, 10.84. Found: C, 64.83; H, 11.58; N, 10.65.

The hydrochloride of III, prepared in the usual manner, was obtained as a white crystalline compound in a 78% yield, m.p. 164–166°.

Anal.—Calcd. for $C_7H_{16}ClNO$: C, 50.80; H, 9.67; Cl, 21.40; N, 8.45. Found: C, 50.55; H, 9.81; Cl, 21.27; N, 8.40.

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra and enzyme rates were determined on a Perkin-Elmer model 4000-A spectrophotometer. The melting points were determined on a Kofler Heizbank and are corrected.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

cis - 4 - (5 - Amino - 6 - chloro - 4 - pyrimidinylamino)cyclohexylcarbinol (V).—A solution of 9.03 Gm. (70 mmoles) of III, 10.90 Gm. (66.5 mmoles) of 5-amino-4,6-dichloropyrimidine (IV), and 9.0 Gm. (89 mmoles) of triethylamine in 120 ml. of *n*-butanol was heated under reflux for 16 hours; then the volatile materials were removed *in vacuo*. The residue on crystallization from water, gave a yellowish solid (V); yield, 8.36 Gm. (48.4%). One recrystallization of the crude product from water gave the analytical sample, m.p. 201°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 305 (12.8); pH 7, 290 (8.83), 262 (8.69); pH 13, 290 (9.47), 262 (9.32). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3420 (OH), 3290 (NH_2).

Anal.—Calcd. for $C_{11}H_{17}ClN_4O$: C, 51.46; H, 6.68; Cl, 13.82; N, 21.82. Found: C, 51.56; H, 6.69; Cl, 14.01; N, 21.74.

cis-4-(6-Chloro-9-purinyl)cyclohexylcarbinol (VI).—A solution of V (1.005 Gm., 3.91 mmoles) in 30 ml. of triethyl orthoformate was refluxed for 48 hours. Acetic anhydride (0.8 ml.) was then added to the solution, and the mixture was refluxed for an additional 3 hours. The volatile materials were removed *in vacuo;* the residue (1.18 Gm.) was treated with methanolic ammonia (20%, 15 ml.) at 0° overnight, after which time the volatile materials were removed *in vacuo* at 25°. The residue was dissolved in chloroform (30 ml.), washed with water (3 × 5 ml.), dried with sodium sulfate, and concentrated to dryness. The crude product (VI, 998 mg.) was crystallized from chloroform-hexane; yield, 507 mg. (48.5%), m.p. 160°. Recrystallization of the crude VI from chloroform-hexane gave the analytical sample, m.p. 166°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$) pH 1, 264 (9.78); pH 7, 264 (9.96); pH 13, 266 (9.78). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3400 (OH); 1590, 1560 (C=C and C=N).

Anal.—Calcd. for $C_{12}H_{15}ClN_4O$: C, 54.03; H, 5.67; Cl, 13.29; N, 21.01. Found: C, 54.25; H, 5.46; Cl, 13.42; N, 21.17.

The aqueous washings obtained after the methanolic ammonia treatment were concentrated to dryness. The residue (170 mg.) was dissolved in aqueous sodium hydroxide (0.1 *N*, 10 ml.), decolorized with charcoal, and acidified to pH 3 with glacial acetic acid. On cooling, a crystalline material was obtained, collected by filtration, and washed with cold water. Recrystallization of the crude material from water gave the analytical sample of VII, m.p. 326–328°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 250 (11.65); pH 7, 251 (10.87); pH 13, 254 (12.50). $\bar{\nu}$ in cm.$^{-1}$: 3400 (OH), 1680 (C=O); 1590, 1550 (C=C and C=N).

Anal.—Calcd. for $C_{12}H_{16}N_4O_2$: C, 58.04; H, 6.49; N, 22.56. Found: C, 58.11; H, 6.39; N, 22.33.

cis - 4 - (6 - Mercapto - 9 - purinyl)cyclohexylcarbinol (VIII).—A solution of 153 mg. (0.576 mmoles) of VI and 40 mg. (0.526 mmoles) of thiourea in 4 ml. of *n*-propanol was heated under reflux for 1 hour, then cooled in an ice bath. The solid which precipitated was collected by filtration, washed with cold *n*-propanol (3 ml.), and dried; yield, 134 mg. (96%), m.p. 320°. Two crystallizations of the crude material from aqueous methyl cellosolve gave the analytical sample (VIII), m.p. 327–327.5° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 227 (7.98), 325 (20.0); pH 13, 232 (13.7), 311 (22.3). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3370 (OH); 2800–2000 (acidic hydrogen); 1585 and 1525 (C=C and C=N).

Anal.—Calcd. for $C_{12}H_{16}N_4OS$: C, 54.50; H, 6.10; N, 21.18; S, 12.13. Found: C, 54.23; H, 6.18; N, 20.99; S, 12.27.

cis-4-(6-Amino-9-purinyl)cyclohexylcarbinol (IX).—A mixture of 286 mg. (1.08 mmoles) of VI and 3 ml. of liquid ammonia was heated in a stainless steel bomb at 55° for 24 hours. The ammonia was allowed to evaporate, and the residual solid (IX) was recrystallized from aqueous ethanol; yield, 178 mg. (66.9%), m.p. 177°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 260 (14.5); pH 7, 261 (14.8); pH 13, 261 (14.8). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3450, 3350 (OH and NH); 1690 (NH); 1605 and 1560 (C=C and C=N).

Anal.—Calcd. for $C_{12}H_{17}N_5O \cdot H_2O$: C, 54.32; H, 7.22; N, 26.40. Found: C, 54.47; H, 7.24; N, 26.27.

cis - 4 - (6 - Methylamino - 9 - purinyl)cyclohexylcarbinol (X).—To 204 mg. (0.76 mmole) of VI in 3.5 ml. of ethanol was added 10 ml. of 40% methylamine in water; the reaction mixture was refluxed for 3 hours. The reaction mixture was evaporated to dryness *in vacuo*, and the residual solid was extracted with hot benzene (3 × 10 ml.). The benzene extracts, on cooling, gave a crystalline product which was collected by filtration. One recrystallization of the crude material from benzene gave the pure product (X), m.p. 212°; yield, 40.5 mg. (20.4%). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (OH and NH): 1620 (C=C and C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 265 (15.9); pH 7, 267 (15.5); pH 13, 269 (15.3).

Anal.—Calcd. for $C_{13}H_{19}N_5O$: C, 59.75; H, 7.33; N, 26.80. Found: C, 60.01; H, 7.47; N, 26.69.

cis - 4 - (6 - Dimethylamino - 9 - purinyl)cyclohexylcarbinol (XI).—A solution of 123 mg. (0.462 mmole) of VI in 2.5 ml. of ethanol and 2.5 ml. of aqueous dimethylamine (25%) was heated under reflux for 1 hour. The volatile materials were removed *in vacuo*, and the residue was recrystallized twice from benzene and hexane to give the analytical sample (XI); yield, 90 mg. (70.8%), m.p. 144°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 269 (18.9); pH 7, 277 (19.6); pH 13, 277 (19.6). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3350 (OH); 1590, 1550 (C=C and C=N).

Anal.—Calcd. for $C_{14}H_{21}N_5O$: C, 61.09; H, 7.68; N, 25.43. Found: C, 61.27; H, 7.75; N, 25.43.

3-Methoxycarbonylcyclopentylamine (XIII).—Methane sulfonic acid (1.0 ml., 11.0 mmoles) was slowly added to a suspension of 1.24 Gm. (9.6 mmoles) of 3-carboxycyclopentylamine (8) in absolute methanol (50 ml.). The mixture was refluxed for 12 hours, then stirred at 0° with 1.5 Gm. of solid sodium carbonate for 30 minutes. The solvent was evaporated at 25–30°. The residue was triturated with chloroform (3 × 30 ml.); the chloroform extract was filtered through a Celite pad, and the solvent was evaporated *in vacuo*. The residual crude product (XIII) was purified by distillation; yield, 1.06 Gm. (76.8%), b.p. 73–74°/2.5 mm. $\bar{\nu}$ in cm.$^{-1}$ (film): 3300 (NH); 1720 (C=O); 1640 (NH).

Anal.—Calcd. for $C_7H_{13}NO_2$: C, 58.73; H, 9.15; N, 9.78. Found: C, 58.69; H, 9.39; N, 9.61.

3-Hydroxymethylcyclopentylamine (XIV).—A solution of XIII (790 mg., 5.52 mmoles) in ether (40 ml.) was added dropwise at 0° to a suspension of lithium aluminum hydride (650 mg., 17.1 mmoles) in ether (40 ml.). After stirring the mixture for 1 hour at room temperature, it was refluxed for 1 more hour. The excess of lithium aluminum hydride was

decomposed with ether saturated with water, then with water (2.5 ml.). The ethereal layer was decanted, and the insoluble residue was triturated with chloroform (3 × 20 ml.). The combined ethereal and chloroform extracts were dried with sodium sulfate and concentrated *in vacuo*. The crude product (XIV) was purified by distillation; yield, 465 mg. (73%), b.p. 90–91°/2.5 mm.

Anal.—Calcd. for $C_6H_{13}NO$: C, 62.43; H, 11.35; N, 12.14. Found: C, 62.38; H, 11.50; N, 11.97.

cis - 3 - (5 - Amino - 6 - chloro - 4 - pyrimidinylamino)cyclopentylcarbinol (XVI).—A solution of 4,6-dichloro-5-aminopyrimidine (149 mg., 0.908 mmole), 3-hydroxymethylcyclopentylamine (XIV, 105 mg., 0.913 mmole), and triethylamine 144 mg., 1.42 mmoles) in *n*-butanol (5 ml.) was refluxed for 6.5 hours. The volatile materials were removed *in vacuo*, and the residue (353 mg.) was extracted with benzene (5 × 10 ml.). Concentration of the benzene extract gave a gum (164 mg.) which was crystallized from benzene. The crude product (66 mg., 33%, m.p. 154°), after recrystallization from benzene, gave the analytical sample (XVI), m.p. 162°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$); pH 1, 305 (13.1); pH 7, 290 (10.1), 262 (9.70); pH 13, 290 (10.1).

Anal.—Calcd. for $C_{10}H_{15}ClN_4O$: C, 49.38; H, 6.13; Cl, 14.61; N, 23.09. Found: C, 49.28; H, 6.34; Cl, 14.33; N, 22.85.

cis - 3 - (6 - Chloro - 9 - purinyl)cyclopentylcarbinol (XVII).—A solution of XVI (163 mg., 0.672 mmole) in triethyl orthoformate (10 ml.) was refluxed for 68 hours. Acetic anhydride (0.2 ml.) was then added to the solution, and the mixture was heated under reflux for an additional 3 hours. The volatile materials were removed *in vacuo;* the residue (185 mg.) was treated with methanolic ammonia (20%, 3 ml.) at 0° overnight. The volatile materials were removed *in vacuo* at 25°; the residual gum was dissolved in chloroform (20 ml.) and filtered through a short alumina column. The filtrate was concentrated, and the residue was crystallized from chloroform-hexane; yield, 84 mg. (50%), m.p. 104°. Recrystallization of the crude product from chloroform-hexane gave the analytical sample (XVII), m.p. 112°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 264 (9.38); pH 7, 265 (9.28); pH 13, 266 (9.07). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3360 (OH); 1580, 1545 (C=C and C=N).

Anal.—Calcd. for $C_{11}H_{13}ClN_4O$: C, 52.37; H, 5.14; Cl, 14.03; N, 22.69. Found: C, 52.16; H, 5.36; Cl, 14.31; N, 22.44.

cis - 3 - (6 - Hydroxy - 9 - purinyl)cyclopentylcarbinol (XVIII).—To a mixture of 258 mg. (1.02 mmoles) of XVII in 12 ml. of water was added 2 ml. of 1.1 *N* sodium hydroxide; the mixture was heated under reflux for 4 hours, after which time the reaction mixture was decolorized with charcoal, and the filtrate was acidified at 0° with acetic acid. The white precipitate (120 mg.) was collected and recrystallized from water, m.p. 274–276°; yield of XVIII, 71 mg. (29.7%). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3440 (OH); 2800–2300 (acidic hydrogen); 1680 (C=O); 1590, 1540 (C=C and C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 251 (12.4); pH 7, 251 (14.7); pH 13, 255 (15.5).

Anal.—Calcd. for $C_{11}H_{14}N_4O_2$: C, 56.40; H, 6.02; N, 23.92. Found: C, 56.38; H, 5.86; N, 23.78.

cis - 3 - (6 - Mercapto - 9 - purinyl)cyclopentylcarbinol (XIX).—To a suspension of 263 mg. (1.04 mmoles) of XVII in 10 ml. of *n*-propyl alcohol was added 81.8 mg. (1.07 mmoles) of thiourea. The mixture was heated under reflux for 2 hours, then cooled in an ice-bath. The solid was collected by filtration, washed with 3 ml. of cold *n*-propyl alcohol, and dissolved in 8 ml. of 5% sodium hydroxide. The solution was filtered, chilled in an ice-bath, and acidified to pH 5 with 1.3 ml. of glacial acetic acid. The white product was collected by filtration and dried. The crude material, after recrystallization from methanol, gave the pure product (XIX), m.p. 295–300°; yield, 74.8 mg. (28.8%). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3500 (OH); 2700–2300 (acidic hydrogen); 1600 (C=C and C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 325 (18.3); pH 7, 323 (25.0); pH 13, 310 (19.7).

Anal.—Calcd. for $C_{11}H_{14}N_4OS$: C, 52.78; H, 5.64; N, 22.38. Found: C, 52.54; H, 5.79; N, 22.20.

cis - 3 - (6 - Amino - 9 - purinyl)cyclopentylcarbinol (XX).—To 277 mg. (1.10 mmoles) of XVII in a stainless steel bomb was added 10 ml. of liquid ammonia, and the reaction mixture was heated at 60° for 17 hours. On evaporation of the ammonia, there was obtained a yellow semisolid mass, which was dissolved in ethanol. Addition of acetone caused the precipitation of ammonium chloride, which was removed by filtration. The filtrate was evaporated to dryness *in vacuo* and gave a white residue (189 mg.). One recrystallization of the crude material from chloroform and hexane gave the pure product (XX), m.p. 129–131°; yield, 85.5 mg. (33.4%). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3400 (OH); 3230 (NH); 1670 (NH); 1600, 1570 (C=C and C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 261 (8.96); pH 7, 261 (9.91); pH 13, 261 (9.67).

Anal.—Calcd. for $C_{11}H_{15}N_5O$: C, 56.63; H, 6.48; N, 30.03. Found: C, 56.37; H, 6.46; N, 30.22.

cis - 3 - (6 - Methylamino - 9 - purinyl)cyclopentylcarbinol (XXI).—A mixture of 203 mg. (0.800 mmole) of XVII, 10 ml. of 40% methylamine in water, and 10 ml. of ethanol was heated in a stainless steel bomb at 85° for 2.5 hours. Concentration of the reaction mixture gave a residue which was extracted with a 1:1 mixture of benzene and chloroform (3 × 3 ml.). Evaporation of the extracts gave a crude XXI which could not be crystallized as its free base. The hydrochloride of XXI was prepared by adding concentrated hydrochloric acid to a methanolic solution of crude XXI and evaporating the mixture *in vacuo*. Recrystallization of the residual solid from methanol and ether, then from methanol and ethyl acetate gave 85 mg. (37.5%) of the hydrochloride of XXI, m.p. 189.5–192°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (OH); 2650, 1690 (C=N—H$^+$); 1610 (C=C and C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 265 (12.9); pH 7, 269 (12.9); pH 13, 268 (11.9).

Anal.—Calcd. for $C_{12}H_{18}ClN_5O$: C, 50.79; H, 6.39; Cl, 12.49; N, 24.69. Found: C, 50.54; H, 6.26; Cl, 12.45; N, 24.65.

cis - 3 - (6 - Dimethylamino - 9 - purinyl)cyclopentylcarbinol (XXII).—A mixture of 212 mg. (0.838 mmole) of XVII, 11 ml. of 25% dimethylamine in water, and 10 ml. of ethanol was refluxed for 20 hours, after which time the volatile materials were removed *in vacuo*. The residue was extracted with a 1:1 mixture of benzene and chloroform (3 × 30 ml.). Evaporation of the organic extract gave the crude XXII which could not be crystallized as the

free base. Thus, the crude XXII was converted to its hydrochloride by adding concentrated hydrochloric acid to a methanol solution of XXII, followed by evaporation *in vacuo*. The residual solid was recrystallized from methanol and ethyl acetate and gave 144 mg. (53.3%) of the hydrochloride of XXII, m.p. 195–198.5°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3400 (OH); 2750 and 1670 (C=$\overset{+}{\text{N}}$—H); 1590 (C=C and C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 270 (17.4); pH 7, 277 (18.1); pH 13, 277 (17.9).

Anal.—Calcd. for $C_{13}H_{20}ClN_5O$: C, 52.43; H, 6.77; N, 23.52. Found: C, 52.20; H, 6.64; N, 23.37.

Conversion of XXII to Its Quaternary Salt by Means of Its Mesylate Ester.—To a solution of 965 mg. (3.24 mmoles) of XXII in 6 ml. of pyridine cooled in an ice-bath was added 0.8 ml. of methanesulfonyl chloride with stirring. After 18 hours at 3°, the reaction mixture was poured into 40 ml. of ice-water, and the mixture was extracted with chloroform (4 × 30 ml.). The combined organic extracts were washed with sodium bicarbonate solution, water, dried with anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to dryness to give 682 mg. (62.0%) of the crude mesylate (XXIII) as an oil. $\bar{\nu}$ in cm.$^{-1}$ (film): 1590 (C=C and C=N); 1340 and 1170 (sulfonate ester). $\lambda_{max.}^{pH\ 1}$ 272 mμ.

A solution of 309 mg. of crude XXIII in 4 ml. of acetone containing 5 drops of methanol was heated under reflux for 4 hours, then the volatile materials were removed *in vacuo*. The residue was triturated with 1 ml. of acetone, and the resulting crystalline solid was collected by filtration; yield, 200 mg. (63.6%), m.p. 202–203°. One recrystallization of the crude product from methanol and acetone gave the pure product (XXIV), m.p. 202–203°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 1620, 1595, and 1555 (C=C and C=N); 1190 (sulfonate). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 290 (15.8).

Anal.—Calcd. for $C_{14}H_{21}N_5O_3S$: C, 49.53; H, 6.24; N, 20.64; S, 9.42. Found: C, 49.83; H, 6.39; N, 20.40; S, 9.15.

Reagents and Assay Procedure

Adenosine and adenosine deaminase were purchased from the Sigma Chemical Co. The general method of assay employed is described in Colowick and Kaplan (12) and involves measuring the rate of disappearance of the absorption band of adenosine at 265 mμ. All reactions were run in 0.05 *M* phosphate buffer at pH 7.6 at 25°. The stock solutions of all reagents were prepared in 0.05 *M* phosphate buffer at pH 7.6. For the assay, the cell contained a total volume of 3.1 ml., which was 0.066 m*M* with respect to adenosine. In those cases where inhibition was studied, varying amounts of the buffer were replaced by the appropriate volume of a solution of the inhibitor in buffer. A sufficient amount of enzyme was used, so that the initial rate of reaction gave a change of approximately 0.8–1.0 absorbance units per minute.

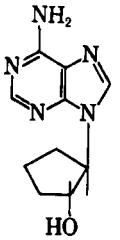
Results

Enzymatic evaluation of these groups of compounds revealed that only those compounds with an amino or methylamino group at the 6-position of the purine nucleus (IX, X, XX, and XXI) were inhibitory at concentrations two to three times that of substrate. The percentage inhibition of adenosine deaminase by a 0.12 m*M* solution of inhibitor is given in Table I. For comparison, the percentage inhibition of two closely related structural analogs has also been included in Table I. These analogs are *trans*-2-[9-(6-aminopurinyl)]cyclopentanol (XXV) and *trans* - 2 - [9 - (6-aminopurinyl)]cyclohexanol (XXVI).

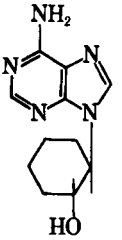
TABLE I.—PARTIAL INHIBITION OF ADENOSINE DEAMINASE BY ANALOGS OF PURINE NUCLEOSIDES

Compd.	Concn., m*M*[a]	*Vo/V*	% Inhibition
IX	0.12	1.25	20
X	0.12	1.30	23
XX	0.12	1.28	22
XXI	0.12	1.20	16
XXV	0.12	1.54	35
XXVI	0.12	1.53	35

[a] The concentration of adenosine in all experiments was 0.066 m*M*.



XXV



XXVI

DISCUSSION

Only those purine nucleoside analogs with an amino or methylamino group at the 6-position of the purine nucleus were inhibitory. Those compounds with a chloro, hydroxy, mercapto, or dimethylamino group at the 6-position were essentially noninhibitory at concentrations two to three times that of adenosine. In previous investigations 6-aminopurine nucleoside analogs have been prepared which were substituted at the 9-position by cyclopentyl, *cis*- and *trans*-2-hydroxycyclopentyl, *cis*- and *trans*-2-hydroxycyclohexyl, and *cis*- and *trans*-3-hydroxycyclohexyl groups. These studies (2) showed that the 2′-hydroxy group of the cycloaliphatic nucleus in both the cyclopentyl and cyclohexyl analogs makes a significant contribution to binding to the enzyme; the *trans* compounds are slightly more effective than the *cis* isomers. Furthermore, the 3′-hydroxy group on the cyclohexyl nucleus made little or no contribution to binding to the enzyme since these compounds were no more effective in inhibiting the enzymatic reaction than the unsubstituted cyclopentyl analog (3).

The present study gives information concerning the contribution to binding made by the hydroxymethyl group on the substituent at the 9-position of the purine. An examination of Table I reveals that IX, X, XX, and XXI are inhibitory, but that they are less inhibitory than the corresponding compounds with a 2′-hydroxy group (XXV and XXVI). Therefore, the effect of the hydroxymethyl group on binding to the enzyme is small. Consequently, if it is assumed that XX binds to the same place on the enzyme that adenosine does, the hydroxymethyl group of adenosine makes only a small contribution to binding. The study of the bulk tolerance at the

6-position of the purine nucleus and of the steric requirements for binding to adenosine deaminase is continuing.

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Sterol Metabolism in Larvae of the Confused Flour Beetle, *Tribolium confusum*

By EDWARD E. SMISSMAN, NEIL A. JENNY, and STANLEY D. BECK

Dehydroepiandrosterone, pregnenolone, and progesterone were isolated as metabolic products during normal insect metabolism of dietary sterols in the confused flour beetle, *Tribolium confusum*. Their identity was established by elemental analysis, thin-layer chromatography, gas chromatography, ultraviolet absorption, optical rotation, and 2,4-dinitrophenylhydrazone derivatives compared to authentic samples. A proposed metabolic pathway of dietary sterols in *T. confusum* larvae is outlined.

THE INDISPENSABILITY of sterols for the growth of insects has been amply established by numerous nutritional studies on a variety of species (1–3). Higher animals are able to synthesize sterols from simple compounds such as acetate, while the lack of sterol synthesis seems to be a metabolic defect common to all insects (4).

The requirement for specific sterols varies widely among the insecta, and most larvae exhibit only moderate specificity with respect to their need for dietary sterols. Several dietary sterols of the larvae of *Tribolium confusum* are converted to cholesterol and 7-dehydrocholesterol which are the principal tissue sterols of the insect (5). The present and related metabolic studies (5–7) indicate the ability of insects to metabolize dietary sterols to the extent of altering the side chain and the degree of saturation in the B ring of the sterol nucleus. The objective of this study is to demonstrate the molecular alteration of side-chain substituted sterols during normal insect metabolism by isolation and characterization of the ketosteroid metabolic products.

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EXPERIMENTAL AND RESULTS

Since the results obtained in a previous study indicated that dietary sterols are converted into cholesterol and 7-dehydrocholesterol (6), it was decided to grow the *T. confusum* larvae on a natural diet supplemented with a dietary sterol that could be converted to cholesterol. The beetle larvae were reared from egg to larval maturity using a basic diet of 4 parts white flour, 4 parts graham wheat flour, and 1 part yeast with the additional dietary sterol, ergosterol, present in the yeast. Before isolation of lipids, the larvae were held for 24 hours on a sterol-free diet to minimize interference from sterols contained in the gut contents.

Extraction.—In previous studies of steroid isolation, many types of solvent systems have been used. Weakly polar solvents are used most commonly. Since it was difficult to determine which procedure would be best for this study, several extraction procedures were used in an attempt to separate the steroids from the numerous other constituents of the insect body. In all cases the larvae were first washed with diethyl ether and skellysolve B to remove any diet material on the surface of the insects and any steroids present in the insect epicuticle.

Skellysolve B Extraction.—After washing the larvae, 250 Gm. of larvae in 100 ml. of skellysolve B was homogenized in a Waring Blendor for 5 seconds. This homogenate of larvae was extracted with 1.5 L. of skellysolve B in a Soxhlet apparatus for 48 hours. The skellysolve B extract was concentrated, redissolved in 100 ml. of 5% alcoholic potassium hydroxide solution, and refluxed for 1 hour. The hydrolyzate was poured into 300 ml. of distilled water, and the nonsaponifiable material was extracted three times with 400 ml. of skellysolve B. The organic layer was washed several times with water and dried over anhydrous magnesium sulfate.

6-position of the purine nucleus and of the steric requirements for binding to adenosine deaminase is continuing.

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Sterol Metabolism in Larvae of the Confused Flour Beetle, *Tribolium confusum*

By EDWARD E. SMISSMAN, NEIL A. JENNY, and STANLEY D. BECK

Dehydroepiandrosterone, pregnenolone, and progesterone were isolated as metabolic products during normal insect metabolism of dietary sterols in the confused flour beetle, *Tribolium confusum*. Their identity was established by elemental analysis, thin-layer chromatography, gas chromatography, ultraviolet absorption, optical rotation, and 2,4-dinitrophenylhydrazone derivatives compared to authentic samples. A proposed metabolic pathway of dietary sterols in *T. confusum* larvae is outlined.

THE INDISPENSABILITY of sterols for the growth of insects has been amply established by numerous nutritional studies on a variety of species (1–3). Higher animals are able to synthesize sterols from simple compounds such as acetate, while the lack of sterol synthesis seems to be a metabolic defect common to all insects (4).

The requirement for specific sterols varies widely among the insecta, and most larvae exhibit only moderate specificity with respect to their need for dietary sterols. Several dietary sterols of the larvae of *Tribolium confusum* are converted to cholesterol and 7-dehydrocholesterol which are the principal tissue sterols of the insect (5). The present and related metabolic studies (5–7) indicate the ability of insects to metabolize dietary sterols to the extent of altering the side chain and the degree of saturation in the B ring of the sterol nucleus. The objective of this study is to demonstrate the molecular alteration of side-chain substituted sterols during normal insect metabolism by isolation and characterization of the ketosteroid metabolic products.

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EXPERIMENTAL AND RESULTS

Since the results obtained in a previous study indicated that dietary sterols are converted into cholesterol and 7-dehydrocholesterol (6), it was decided to grow the *T. confusum* larvae on a natural diet supplemented with a dietary sterol that could be converted to cholesterol. The beetle larvae were reared from egg to larval maturity using a basic diet of 4 parts white flour, 4 parts graham wheat flour, and 1 part yeast with the additional dietary sterol, ergosterol, present in the yeast. Before isolation of lipids, the larvae were held for 24 hours on a sterol-free diet to minimize interference from sterols contained in the gut contents.

Extraction.—In previous studies of steroid isolation, many types of solvent systems have been used. Weakly polar solvents are used most commonly. Since it was difficult to determine which procedure would be best for this study, several extraction procedures were used in an attempt to separate the steroids from the numerous other constituents of the insect body. In all cases the larvae were first washed with diethyl ether and skellysolve B to remove any diet material on the surface of the insects and any steroids present in the insect epicuticle.

Skellysolve B Extraction.—After washing the larvae, 250 Gm. of larvae in 100 ml. of skellysolve B was homogenized in a Waring Blendor for 5 seconds. This homogenate of larvae was extracted with 1.5 L. of skellysolve B in a Soxhlet apparatus for 48 hours. The skellysolve B extract was concentrated, redissolved in 100 ml. of 5% alcoholic potassium hydroxide solution, and refluxed for 1 hour. The hydrolyzate was poured into 300 ml. of distilled water, and the nonsaponifiable material was extracted three times with 400 ml. of skellysolve B. The organic layer was washed several times with water and dried over anhydrous magnesium sulfate.

Skellysolve B Followed by Continuous Ether Extraction.—The skellysolve B extract of nonsaponifiable material (see above) was concentrated and mixed with 200 ml. of aqueous 5% sodium carbonate. This mixture was extracted with 1 L. of diethyl ether in a liquid-liquid continuous extractor for 4 days. The diethyl ether extract was washed several times with water and dried over anhydrous magnesium sulfate.

Butyl Alcohol Extraction.—After washing the larvae, 100 Gm. of larvae was homogenized in a Waring Blendor for 5 seconds with 50 ml. of butyl alcohol. To a solution of 150 ml. of butyl alcohol, 20 Gm. of sodium hydroxide, and 100 ml. of water was added the larval homogenate, and the mixture was refluxed for 2 hours. Ten grams of celite filter aid was added and the mixture filtered. The filter cake was extracted with 50 ml. of hot butyl alcohol and the fractions combined. The solvent was removed by distillation, and the residue was extracted with 250 ml. of skellysolve B. Finally, the skellysolve B extract was washed several times with water and dried over anhydrous magnesium sulfate.

Although these three different extraction procedures were effective, the skellysolve B and butyl alcohol methods were more efficient.

Chromatography.—Heilbron (8) has succeeded in separating steroidal material from nonsaponifiable matter by aluminum oxide chromatography. A modification of his procedure, using deactivated neutral aluminum oxide, grade III (9), and a similar solvent sequence, proved useful for chromatographic separation of the metabolic products.

Adsorption Chromatography.—The residue from several skellysolve B extractions was placed on a chromatographic column (3 × 50 cm.) containing 5 Gm. of deactivated neutral aluminum oxide, grade III (Woelm, suitable for chromatography) for every 50 mg. of nonsaponifiable materials (see Table I). Since only the ketosteroid metabolic products would be isolated and characterized in this study, infrared spectra of all the fractions were performed to determine which fractions contained the desired material. Fraction *7* contained most of the carbonyl material, although the infrared spectra of fractions *1*, *5*, and *9* also indicated their presence in small amounts.

Before work was initiated on characterization of these carbonyl containing fractions, attempts were made to improve the separation of various components of the nonsaponifiable material. One solution of this problem was to reduce the tailing of bands during elution. This can be accomplished by using gradient elution technique (10).

Gradient Elution Adsorption Chromatography.—The residue from five skellysolve B extractions was placed on a chromatographic column (2 × 40 cm.) containing 5 Gm. of acid-washed aluminum oxide (Merck, suitable for chromatography) for every 200 mg. of nonsaponifiable material. The following eluent sequence was used: 500 ml. of skellysolve B, 500 ml. of benzene, 1000 ml. of diethyl ether, and 500 ml. of absolute ethanol. Ten-milliliter fractions were collected using an automatic fraction collector, and a total of 218 fractions were collected (see Table II). Again infrared spectra of the fractions were used to determine the location of the desired carbonyl material. Ultraviolet absorption spectra of tubes 81 to 145 were determined to give an indication of the ketosteroid products present. These ultraviolet maxima correspond favorably to the known maxima of progesterone and androstenedione at 240 and 241 μ. Fractions 81 to 126 contained most of the carbonyl material, although fractions 127 to 145 also contained a small amount.

Table I.—Adsorption Chromatography of Nonsaponifiable Material (1.06 Gm.)

Fraction	Amt., ml.	Eluent	Product
1	150	Skellysolve B	655 mg. white, waxy solid
2	200	Skellysolve B–benzene, 10:1	31 mg. yellow oil
3	250	Skellysolve B–benzene, 8:1	Nothing
4	150	Skellysolve B–benzene, 6:1	Nothing
5	150	Skellysolve B–benzene, 2:1	120 mg. yellow semisolid
6	150	Benzene	Nothing
7	150	Benzene–diethyl ether, 1:1	279 mg. tan solid
8	150	Diethyl ether	Nothing
9	200	Diethyl ether–ethanol, 3:1	29 mg. white solid
10	100	Diethyl ether–ethanol, 1:1	Nothing
11	100	Ethanol	Nothing

Table II.—Gradient Elution Adsorption Chromatography of Nonsaponifiable Material (3.8 Gm.)

Chromatographic Tubes	Product, mg.
1–4	Nothing
5–9	White, waxy solid, 100
10–18	White crystals, 220
19–24	Nothing
25–29	Yellow oil, 60
30–52	Colorless oil, 360
53–59	Colorless oil, 230
60–80	Colorless oil, 340
81–92	Colorless oil, 250
93–105	Light yellow oil, 310
106–126	Yellow oil, 520
127–130	Orange oil, 60
131–145	Yellow oil, 280
146–152	Colorless oil, 100
153–154	Orange oil, 30
155–157	Colorless oil, 40
158–160	Orange oil, 20
161–167	Colorless oil, 80
168–173	Yellow oil, 60
174–218	Colorless oil, 100

Thin-Layer Chromatography.—The analytical separation of slightly polar steroids has been realized by paper partition chromatography on acetylated or impregnated paper (11). These methods have some drawbacks, such as the long time of development. Van Dam (12) described a modification using adsorbent-coated glass plates which allow microadsorption chromatography on a flat surface. This procedure was used to separate further and identify partially the metabolic products in this study.

The chromatoplates were prepared according to the general procedure in the Desaga thin-layer apparatus manual (13). An amount of 10–100 mcg. of sample was placed on the Desaga silicic acid

TABLE III.—APPROXIMATE R_f VALUES FROM THIN-LAYER CHROMATOGRAPHY

Sample	Benzene		Solvent: Benzene:2 Ethyl Acetate:1		Benzene:3 Ethyl Acetate:2		Toluene:9 Ethyl Acetate:1	
7-Dehydroepiandrosterone	...		0.65		0.69		0.45	
Cholesterol	0.11		0.53		0.59		0.28	
Ergosterol	0.09		0.65		0.69		0.43	
β-Sitosterol	0.11		0.44		...		0.33	
Testosterone	0		0.28		...		0.12	
Androsterone	0		0		...		0	
Dehydroepiandrosterone	0.02		0.38		0.42		0.14	
Pregnenolone	...		0.42		0.46		0.16	
Progesterone	...		0.50		0.53		0.25	
Androstenedione	...		0.53		0.56		0.28	
Fraction 7	0.02	0.20	0.09	0.62	...		0.15	0.49
	0.14	0.62	0.38	0.78			0.24	0.74
			0.48					
Tubes 81–92	...		0.29	0.52	0.24	0.42	0.06	0.2
			0.43		0.51		0.17	
Tubes 93–105	...		0.02	0.33	0.02	0.36	0.07	
			0.19	0.41	0.18	0.51	0.14	
			0.25	0.52	0.22			
Tubes 106–126	...		0.02	0.39	0.02	0.37	0.03	0.22
			0.16	0.51	0.16	0.49	0.06	0.37
			0.24		0.21	0.57	0.14	

chromatoplates and then were developed. After thorough drying at 105°, they were sprayed with a saturated solution of antimony trichloride in chloroform and heated for 5 minutes at 105°. The reading of the spots was aided using long-wave ultraviolet light. An outline of the R_f values found in the different experiments is given in Table III. Known steroids were included in each experiment as internal standards. Fraction 7 had R_f values corresponding to pregnenolone and/or dehydroepiandrosterone, androstenedione and/or progesterone, and ergosterol. Tubes 81 to 92 had R_f values corresponding to androstenedione and/or progesterone and pregnenolone. Tubes 93 to 105 and tubes 106 to 126 had R_f values corresponding to pregnenolone and/or dehydroepiandrosterone and androstenedione and/or progesterone.

TABLE IV.—RELATIVE RETENTION TIMES FROM GAS CHROMATOGRAPHY

Sample	Relative Retention Times 50–300°[a]		100–300°[a]	
Cholestane	1.00[b]		1.00[c]	
Progesterone	0.818		0.801	
Pregnenolone	0.788		0.764	
Dehydroepiandrosterone	0.754		0.733	
Androstenedione	0.732		0.705	
Fraction 7	0.141	0.685	...	
	0.301	0.775		
	0.517			
Tubes 81–92	0.219	0.631	0.240	0.713
	0.560	0.726	0.555	0.774
			0.616	
Tubes 93–105	...		0.555	0.780
			0.616	0.818
			0.700	
Tubes 106–126	0.685	0.772	0.700	
	0.732	0.819	0.780	

[a] Column, 6 ft. × 1/4 in. O.D., 3% SE-30 on Chromosorb W. 80/100 mesh; programmer switch, 11°/min.; block temperature, 280°; injection port temperature, 240°; bridge current, 150; helium flow, 100 ml./minute; reference flow, 20 ml./minute; and helium pressure, 20 p.s.i. on the F and M model 500 programmed temperature gas chromatograph. [b] Time, 32.5 minutes. [c] Time, 29.2 minutes.

Gas Chromatography.—Numerous studies on the separation and identification of steroids by gas chromatography have been performed, and a recent review article (14) summarizes most of the work in this field. Although it has been reported that steroids decompose at temperatures over 260° to give broad peaks, gas chromatography studies were performed using programmed temperatures to 300°. Reproducible results were obtained for a number of known steroids, including the suspected metabolic steroids; therefore, these conditions were used for partial identification of the isolated metabolic products. A summary of the relative retention times is given in Table IV. Fraction 7 and tubes 81 to 92 had relative retention times comparable to androstenedione and pregnenolone. Tubes 93 to 105 had relative retention times comparable to progesterone, pregnenolone, and androstenedione. Tubes 106 to 126 had relative retention times comparable to progesterone, androstenedione, and dehydroepiandrosterone.

2,4-Dinitrophenylhydrazones.—Along with the small amounts of ketosteroids, large amounts of hydrocarbons are present in the nonsaponifiable material (15). All compounds with double bond systems absorb strongly in the far ultraviolet region but this is the region of decreased sensitivity in laboratory spectrophotometers and where interference from solvent occurs. To solve these problems, a study was initiated that dealt with the formation and isolation of steroidal 2,4-dinitrophenylhydrazones, (2,4-DNPs), as well as a determination of their absorption characteristics in the visible range. The 2,4-DNPs of the ketosteroids in the fractions containing ethanol-soluble components were prepared according to the method of Reich (16). The colored solid which resulted was purified by chromatography on an acid-washed aluminum oxide column (1 × 20 cm.). (See Table V.)

Another method of 2,4-DNP preparation was employed for fractions not completely soluble in ethanol (17). After drying, the 2,4-DNP derivative was purified by chromatography on an acid-washed aluminum oxide column. (See Table V.)

TABLE V.—ADSORPTION CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES

Fraction or Tubes	Eluent	2,4-DNP Product
81–92	Benzene	Pregnenolone
81–92	Benzene–chloroform, 5:1	Progesterone (bis 2,4-DNP)
93–105	Benzene	Dehydroepiandrosterone
93–105	Benzene–chloroform, 3:1	Progesterone (20-mono-DNP)
106–126	Benzene	Dehydroepiandrosterone
106–126	Benzene	Progesterone (3-mono-DNP)
106–126	Benzene–chloroform, 3:1	Progesterone (20-mono-DNP)
7	Skellysolve B–benzene-diethyl ether, 80:10:5	Progesterone (bis-2,4-DNP)
7	Benzene	Pregnenolone
7	Benzene	Dehydroepiandrosterone
7	Benzene	Progesterone (3-mono-DNP)

All of the pure 2,4-DNP products in Table V were identified by ultraviolet spectral studies, melting point, mixed melting point with authentic samples, and analysis.

The bis-2,4-DNP of progesterone was recrystallized from a mixture of chloroform and absolute ethanol to give dark red plates, m.p. 270° dec. [lit. m.p. 282–283° (16)], which did not depress the melting point when mixed with an authentic sample of bis-2,4-DNP of progesterone. $\lambda_{max.}^{CHCl_3}$ 380 mμ, ϵ = 51,800 (lit. $\lambda_{max.}^{CHCl_3}$ 380 mμ) (16).

Anal.—Calcd. for $C_{33}H_{38}N_8O_8$: C, 58.74; H, 5.68; N, 16.60. Found: C, 58.52; H, 5.73; N, 16.60.

The 2,4-DNP of pregnenolone was recrystallized from a mixture of chloroform and absolute ethanol to give bright yellow plates, m.p. 253–254° [lit. m.p. 253–254° (16)], which did not depress the melting point when mixed with an authentic sample of the 2,4-DNP of pregnenolone. $\lambda_{max.}^{CHCl_3}$ 369 mμ, ϵ = 25,300 [lit. $\lambda_{max.}^{CHCl_3}$ 368 mμ (16)].

Anal.—Calcd. for $C_{27}H_{36}N_4O_5$: C, 65.29; H, 7.31; N, 11.28. Found: C, 65.37; H, 7.40; N, 11.30.

The 2,4-DNP of dehydroepiandrosterone was recrystallized from a mixture of chloroform and absolute ethanol to give bright yellow plates, m.p. 235–237° dec., which did not depress the melting point when mixed with an authentic sample of the 2,4-DNP of dehydroepiandrosterone. $\lambda_{max.}^{CHCl_3}$ 366 mμ, ϵ = 25,300 [lit. $\lambda_{max.}^{CHCl_3}$ 366 mμ (16)].

Anal.—Calcd. for $C_{25}H_{32}N_4O_5$: C, 64.08; H, 6.89; N, 11.96. Found: C, 63.73; H, 6.90; N, 12.18.

Several general methods for the regeneration of the corresponding carbonyl compounds have been reported (18–20). The Mattox-Kendal method (18, 19) which utilizes pyruvic acid and hydrobromic acid and the Demaeker-Martin method (20) which utilizes acetone, hydrochloric acid, and stannous chloride were employed to cleave the above isolated 2,4-DNPs.

The bis-2,4-DNP of progesterone was cleaved to give a white solid product. Recrystallization from dilute ethanol gave white needles, m.p. 127.5–128° [lit. m.p. 128.5° (21)], which did not depress the melting point when mixed with an authentic sample of progesterone. $[\alpha]_D^{18}$ + 191.1° in ethanol [lit. $[\alpha]_D^{18}$ + 192° in ethanol (21)]. $\lambda_{max.}^{EtOH}$ 240 mμ, ϵ = 19,600 [lit. $\lambda_{max.}^{EtOH}$ 240 mμ) (21)].

Anal.—Calcd. for $C_{21}H_{30}O_2$: C, 80.20; H, 9.54. Found: C, 80.68; H, 9.44.

The 2,4-DNP of pregnenolone was cleaved to give a white solid product. Recrystallization from dilute ethanol gave white needles, m.p. 188.5–190° [lit. m.p. 189–190° (21)], which did not depress the melting point when mixed with an authentic sample of pregnenolone. $[\alpha]_D^{18}$ + 30.8° in ethanol [lit. $[\alpha]_D^{18}$ + 30° in ethanol (21)].

Anal.—Calcd. for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: C, 79.43; H, 10.04.

The 2,4-DNP of dehydroepiandrosterone was cleaved to give a white solid product. Recrystallization from a mixture of chloroform and skellysolve B gave white needles, m.p. 140–142° [lit. m.p. 140–141° (21)], which did not depress the melting point when mixed with an authentic sample of dehydroepiandrosterone. $[\alpha]_D^{18}$ + 11.3° in ethanol [lit. $[\alpha]_D^{18}$ + 10.9° in ethanol (21)].

Anal.—Calcd. for $C_{19}H_{28}O_2$: C, 79.12; H, 9.79. Found: C, 79.38; H, 10.32.

DISCUSSION

The isolation and characterization of dehydroepiandrosterone, pregnenolone, and progesterone as metabolic products of normal sterol metabolism in *T. confusum* larvae indicates that certain insects possess enzyme systems capable of altering the side chain of dietary sterols. As part of a study concerned with the metabolic degradation of cholesterol, mammalian tissue was used to cleave the side chain

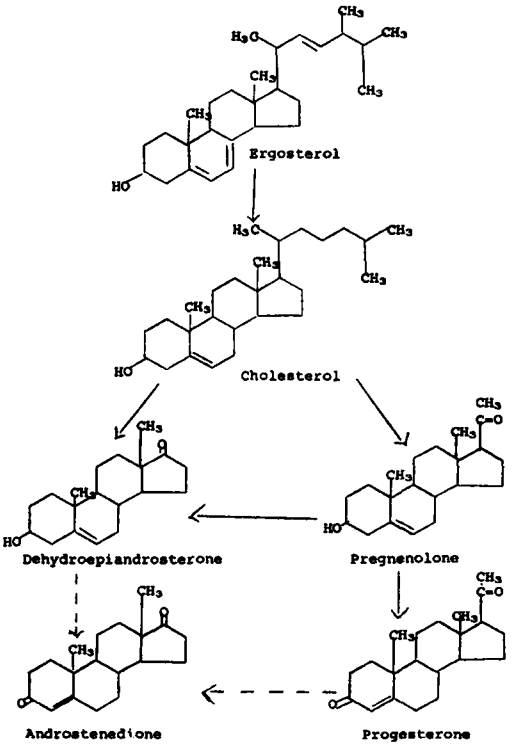


Fig. 1.—Proposed biogenetic breakdown of dietary sterols in the larvae of the confused flour beetle, *T. confusum*.

of cholesterol to yield isocaproic acid and pregnenolone (22) which is further oxidized to progesterone (23). Thus, mammalian enzymes capable of cleaving the cholesterol side chain are known. The action of this insect's enzyme system parallels that of enzyme systems in higher animals (24), and the proposed biogenetic breakdown of dietary sterols by *T. confusum* larvae is outlined in Fig. 1. Since it has been shown that many dietary sterols, including ergosterol, are converted to cholesterol and 7-dehydrocholesterol in *T. confusum* larvae, the conclusion may be made that alteration of the side chain of dietary sterols occurred after conversion to cholesterol.

During the isolation of these metabolic products, a number of other products was obtained whose structures could not be elucidated due to the paucity of material. Androstenedione may be included among these unidentified products, since its presence is indicated by physical methods of characterization, and it would be a normal conversion product from progesterone and dehydroepiandrosterone.

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Synthetic Polymers as Potential Sustained-Release Coatings

By JOHN W. KLEBER, J. FRANK NASH, and CHENG-CHUN LEE*

The characteristics of the absorption of prednisolone were studied both in intact dogs and in ligated segments of the intestinal tract. Absorption of uncoated prednisolone was essentially complete in 2 hours. Following the administration of copolymer-coated pellets, absorption was extended over a period of 10–12 hours.

FOLLOWING THE oral administration of a given drug, effective concentrations in the blood and target tissue are dependent on several factors—solubility, site of absorption, stability in the gastrointestinal tract, rate of metabolism, and excretion. In many instances maintenance of therapeutic effect requires repeated administration at 4–6-hour intervals. Longer duration of a single dose is often desirable to permit uninterrupted rest during the night or to diminish the possibility of missed dosage.

Various coatings have been investigated to delay the release of drug into the intestinal tract. The use of a pH-dependent coating to obtain sustained release of prednisolone has been reported (1); however, the chemical nature of the coating substance was not disclosed. Several copolymers have been discovered (2) that differ in their rate of solution in water at different pH values. An earlier report (3) on these synthetic copolymers described their use as enteric coatings on acetylsalicylic acid tablets. The current study also provides information about the potential of these copolymers to delay the absorption of a drug. Prednisolone served as the model drug.

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EXPERIMENTAL

Materials

Nonpareil sugar pellets, 16–18 mesh, prednisolone U.S.P., prednisolone acetate U.S.P., cetyl alcohol N.F., magnesium stearate-talc dusting powder (15:85), 1/2 *n*-butyl ethylene maleic acid copolymer (butyl EMA), 1/2 isopropyl polymethylvinylether/maleic acid copolymer (isopropyl PVM/MA), and 1/2 *n*-butyl polymethylvinylether/maleic acid copolymer (butyl PVM/MA) were utilized.

of cholesterol to yield isocaproic acid and pregnenolone (22) which is further oxidized to progesterone (23). Thus, mammalian enzymes capable of cleaving the cholesterol side chain are known. The action of this insect's enzyme system parallels that of enzyme systems in higher animals (24), and the proposed biogenetic breakdown of dietary sterols by *T. confusum* larvae is outlined in Fig. 1. Since it has been shown that many dietary sterols, including ergosterol, are converted to cholesterol and 7-dehydrocholesterol in *T. confusum* larvae, the conclusion may be made that alteration of the side chain of dietary sterols occurred after conversion to cholesterol.

During the isolation of these metabolic products, a number of other products was obtained whose structures could not be elucidated due to the paucity of material. Androstenedione may be included among these unidentified products, since its presence is indicated by physical methods of characterization, and it would be a normal conversion product from progesterone and dehydroepiandrosterone.

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Synthetic Polymers as Potential Sustained-Release Coatings

By JOHN W. KLEBER, J. FRANK NASH, and CHENG-CHUN LEE*

The characteristics of the absorption of prednisolone were studied both in intact dogs and in ligated segments of the intestinal tract. Absorption of uncoated prednisolone was essentially complete in 2 hours. Following the administration of copolymer-coated pellets, absorption was extended over a period of 10–12 hours.

FOLLOWING THE oral administration of a given drug, effective concentrations in the blood and target tissue are dependent on several factors—solubility, site of absorption, stability in the gastrointestinal tract, rate of metabolism, and excretion. In many instances maintenance of therapeutic effect requires repeated administration at 4–6-hour intervals. Longer duration of a single dose is often desirable to permit uninterrupted rest during the night or to diminish the possibility of missed dosage.

Various coatings have been investigated to delay the release of drug into the intestinal tract. The use of a pH-dependent coating to obtain sustained release of prednisolone has been reported (1); however, the chemical nature of the coating substance was not disclosed. Several copolymers have been discovered (2) that differ in their rate of solution in water at different pH values. An earlier report (3) on these synthetic copolymers described their use as enteric coatings on acetylsalicylic acid tablets. The current study also provides information about the potential of these copolymers to delay the absorption of a drug. Prednisolone served as the model drug.

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EXPERIMENTAL

Materials

Nonpareil sugar pellets, 16–18 mesh, prednisolone U.S.P., prednisolone acetate U.S.P., cetyl alcohol N.F., magnesium stearate-talc dusting powder (15:85), 1/2 *n*-butyl ethylene maleic acid copolymer (butyl EMA), 1/2 isopropyl polymethylvinylether/maleic acid copolymer (isopropyl PVM/MA), and 1/2 *n*-butyl polymethylvinylether/maleic acid copolymer (butyl PVM/MA) were utilized.

TABLE I.—SOLUBILITY pH VALUES FOR COATED FRACTIONS

Copolymer Coating	Amt. of Coating, Gm./100 Gm. of Pellets	Solubility pH Values
Butyl EMA	5.0	5.6
	7.5	5.8
	10.0	5.8
	12.5	6.0
Isopropyl PVM/MA	4.1	5.4
	10.5	5.6
	17.4	6.4
	21.3	>7.5
Butyl PVM/MA	5.4	6.0
	11.3	6.4
	16.2	7.5
	21.9	>7.5

Procedures

In Vitro.—Prednisolone or the acetate was applied to the sugar pellets using a light syrup as the adhesive. The pellets retained their spherical shape after the application of this material. The copolymer coatings were sprayed onto these pellets using 2 to 5% solutions of the copolymer and cetyl alcohol in acetone. Cetyl alcohol was used with each copolymer in a ratio of 1:4 and served to prevent tackiness, thus permitting the pellets to be coated evenly. In addition, when the butyl EMA copolymer was sprayed onto the pellets, the magnesium stearate–talc (15:85) dusting powder was required to prevent the pellets from sticking to each other. The ratio of dusting powder to this copolymer was 7:5. The use of this dusting powder reduced the amount of this copolymer needed to achieve the desired solubility pH. The coating solutions were applied to the pellets as they were being rotated in standard 8-in. coating pans revolving at 60 r.p.m. using a De Vilbiss spray gun (type EGA, series 501) at a constant air pressure of 5 p.s.i.

To standardize the preparation of the copolymer-coated pellets, an *in vitro* control method was necessary. Since the water solubility of the copolymers is pH dependent, an *in vitro* solubility test based on time and pH was developed. Aqueous solutions of pH 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 7.0, and 7.5, respectively, were prepared using a citrate buffer system (4). Twenty-milliliter portions of each buffer solution were placed in 40-ml. test tubes and warmed at 37° in a constant-temperature water bath. Two-hundred and fifty milligrams of each coated fraction of pellets was added to each tube of the various buffers. The pellets in each tube were agitated by inverting the tube and observing the pellets at 7.5, 15, 30, 45, and 60-minute intervals. Each coated pellet fraction was then assigned a solubility pH value, defined as that pH at which 75% of the pellets dissolved in 30 minutes as determined by visual observation. The solubility pH values for each of the coated fractions are reported in Table I.

In Vivo.—*Site of Absorption in Anesthetized Dogs.*—Eight female mongrel dogs weighing between 10 and 14 Kg. were anesthetized with phenobarbital. Each dog (except the two controls) was given 5 mg./Kg. of prednisolone dispersed in an acacia suspension. In two animals the drug was administered into the stomach, which had been ligated at both ends. The second pair received their dose through a ligated 8-in. segment of the duodenum. The third pair received prednisolone through an 8-in. ligated segment of the ileum. The fourth pair of dogs were subjected to a sham operation but received no prednisolone. Fifteen-milliliter blood samples were taken from each dog 1, 2, and 4 hours after the administration of the steroid. The plasma samples were assayed for free 17-hydroxycorticosteroid (17-OHCS) levels as Porter-Silber chromogens using the method of Eik-Nes (5).

Absorption Studies in Conscious Dogs.—Six female mongrel dogs weighing 10 to 20 Kg. were used. Food, but not water, was withheld from these dogs 16 hours prior to the administration of the steroid. Prednisolone was administered orally in a dose of 5 mg./Kg. in the form of sugar pellets coated with the steroid in capsules. Hourly blood samples were taken from the jugular vein without anesthesia. Feeding of the dogs was not resumed until after the last blood sample was taken. Plasma free 17-OHCS levels were determined as previously.

Because the results of this experiment showed that there were wide variations in the onset of absorption of the prednisolone in these dogs, this study was modified in the following manner.

After the animals had been fasted for 16 hours, each was fed about 200 Gm. of canned meat (Pard) 1 hour prior to the administration of the steroid. Blood samples, taken at hourly intervals, were assayed for free 17-OHCS as previously. The results are given in Table II. A comparison of the results obtained in this study to the one without feeding meat indicates that the steroid was absorbed more promptly and with less variation.

Since the ultimate objectives of this work might include the use of copolymer-coated pellets for prolonged absorption of other steroids, some of which might be in the ester form, it was of interest to compare the absorption of prednisolone acetate with that of the free alcohol. Prednisolone acetate, in a

TABLE II.—AVERAGE PLASMA 17-OHCS CONCENTRATIONS (mcg./100 ml.) AND THE STANDARD ERROR OF THE MEAN OBTAINED IN DOG STUDIES WITH PREDNISOLONE AND PREDNISOLONE ACETATE

Hr. After Administration	Prednisolone Dogs Fasted Only		Prednisolone Dogs Fasted, Then Meat-Fed		Prednisolone Acetate Dogs Fasted, Then Meat-Fed	
	Av.	S.E.	Av.	S.E.	Av.	S.E.
1	68	17.4	164	20.6	71	16.4
2	92	12.1	150	5.0	140	19.2
3	103	13.4	98	5.4	96	9.8
4	77	10.5	70	4.0	67	12.2
5	45	5.4	41	3.1	38	6.7
6	35	5.2	30	2.6	24	4.5
7	...	...	22	3.4	17	3.4

TABLE III.—SITE OF ABSORPTION

Site of Absorption	Plasma 17-OHCS Values (Av. of Two Dogs) (mcg./100 ml.) 1 Hr.	2 Hr.	4 Hr.
Stomach	16	20	19
Duodenum	57	60	59
Ileum	46	42	33
Sham operated	9	12	11

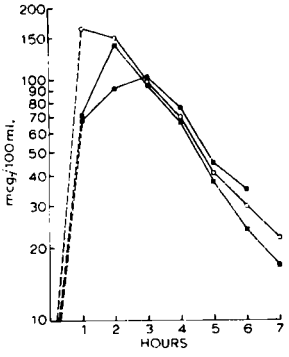


Fig. 1.—Plasma 17-OHCS levels in dogs following the oral administration of prednisolone or prednisolone acetate. The dose was 5 mg./Kg. Key: ●, prednisolone to fasted dogs; O, prednisolone to fasted, meat-fed dogs; ■, prednisolone acetate to fasted, meat-fed dogs.

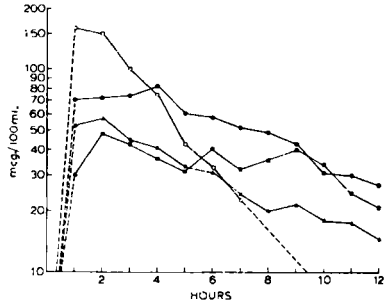


Fig. 2.—Plasma 17-OHCS levels in fasted, meat-fed dogs following the oral administration of prednisolone. The dose was 5 mg./Kg. Key: O, uncoated pellets; ●, pellets coated with isopropyl PVM/MA; ▲, pellets coated with *n*-butyl PVM/MA; ■, pellets coated with *n*-butyl EMA.

dose of 5 mg./Kg., was given in a manner similar to the same six dogs that received the free alcohol. Plasma 17-OHCS levels were determined as previously described.

Each of the six dogs used in the previous experiment was given three formulations of prednisolone, expected to produce a prolonged absorption of this steroid. The dose of prednisolone was 5 mg./Kg. Each formulation consisted of a mixture of 20% uncoated pellets and 80% coated pellets. The coated pellets were composed of equal parts of each of the four coating levels for each copolymer listed in Table I. These dogs were fasted, then meat-fed as previously. Uncoated prednisolone was also given to these dogs as a control. Plasma concentrations of free 17-OHCS were determined hourly for 12 hours.

RESULTS AND DISCUSSION

To study the ability of any pH dependent coating to prolong the absorption of a medicinal agent, it is necessary that the model drug be well absorbed throughout the small intestine. That prednisolone is well absorbed from the entire small intestine of the dog was shown by the study utilizing the ligated segments of the gastrointestinal tract. The results of this experiment are given in Table III.

A second requirement for a model drug is that it can be measured in the plasma by a simple analytical procedure. Detectable levels of this substance should be attained by the administration of nontoxic doses to the test animal. Plasma prednisolone levels were easily measured when a dose of 5 mg./Kg. was administered to the dogs. No dog showed symptoms of untoward effects at this dose.

Since the protective coating used in this study is pH dependent, it was necessary to insure that the stomach acidity of the dogs be controlled uniformly. This was achieved by stimulating the gastric secretion with meat 1 hour prior to the administration of the steroid dose. Meat-feeding the dogs also may have caused a more consistent emptying of the stomach to the site where absorption of prednisolone takes place.

A comparison of the absorption of prednisolone and prednisolone acetate in Fig. 1 shows that they follow a similar pattern. The quantitative difference in the plasma levels may be explained by the difference in the molecular weights of the two steroids. The apparent lag of the prednisolone acetate in reaching peak plasma levels 2 hours after dosing may be due to hydrolysis of the acetate group prior to absorption. Because their absorption patterns are similar, these steroid forms may be used interchangeably.

The results obtained with the coated formulations of prednisolone plotted in Fig. 2 indicate that significant alterations of the absorption pattern of this steroid were obtained. The formulation utilizing the $^1/_2$ isopropyl PVM/MA appears to have the best utilization of the prednisolone. The steroid does not appear to have been so efficiently absorbed from the $^1/_2$ *n*-butyl PVM/MA coated formulation, since the more heavily coated portions have a higher dissolution pH.

The *in vitro* testing of the coated pellets was intended as an aid to the reproducibility of the coating procedure. No conclusions regarding sustained-release of drug can be drawn from these data.

SUMMARY

Prednisolone is well absorbed throughout the small intestine of the dog.

Fasting, then meat-feeding the dogs 1 hour prior to the administration of the drug formulation results in greater uniformity in the plasma prednisolone levels attained.

A sustained-release formulation of prednisolone has been prepared by coating pellets containing this steroid with variable quantities of the copolymers reported in this study.

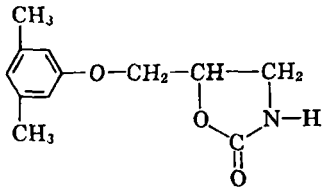
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Qualitative and Quantitative Tests for Metaxalone

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

5-(3,5-DIMETHYLPHENOXYMETHYL)-2-oxazolidinone; $C_{12}H_{15}NO_3$; mol. wt. 221.26. The structural formula of metaxalone may be represented as



Physical Properties.—Metaxalone occurs as a white, odorless, bitter, crystalline powder, m.p. 121–125°, U.S.P. Class I. It is freely soluble in chloroform, soluble in alcohol and in propylene glycol, and very slightly soluble in water.

Identity Tests.—Dissolve about 10 mg. of metaxalone in 10 ml. of sulfuric acid solution (65 in 100). To 1 ml. of this solution add 2 ml. of a 1 in 1000 solution of vanillin in sulfuric acid solution (65 in 100): a cherry-red color develops within about 10 minutes.

A 1 in 10,000 solution of metaxalone in alcohol exhibits ultraviolet absorbance maxima at about 280 [absorptivity (1%, 1 cm.) about 55] and 272 mμ [absorptivity (1%, 1 cm.) about 52] and a minimum at about 277 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.3% dispersion of metaxolone in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests.—Dry about 1 Gm. of metaxalone, accurately weighed, under vacuum at 80° for 3 hours: it loses not more than 0.5% of its weight.

Char about 1 Gm. of metaxalone, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.3%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of metaxalone in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U.S.P. XVI heavy metals test, method I: the heavy metals limit for metaxalone is 20 p.p.m.

Determine the nitrogen content by the U.S.P. XVI nitrogen determination, method II, using about 300 mg. of metaxalone, previously dried under vacuum at 80° for 3 hours and accurately weighed, and 0.1 *N* sulfuric acid for the titration. Each milliliter of 0.1 *N* sulfuric acid is equivalent to 1.401 mg. of nitrogen (N). The amount of nitrogen found is not less than 6.20% and not more than 6.46% of the weight of the sample taken.

Assay.—Transfer about 100 mg. of metaxalone, previously dried under vacuum at 80° for 3 hours and accurately weighed, to a 100-ml. volumetric flask, dissolve in alcohol, dilute to volume with alcohol, and mix. Transfer 10.0 ml. of this solution to a second 100-ml. volumetric flask, dilute to volume with alcohol, and mix. Prepare a solution of metaxalone reference standard in alcohol by dissolving a suitable quantity, previously dried and accurately weighed, and diluting quantitatively and stepwise to a concentration of about 100 mcg./ml. Concomitantly determine the absorbance of the two solutions in 1-cm. cells with a suitable spectrophotometer at the wavelength of maximum absorbance at about 280 mμ, using alcohol as the blank. Calculate the weight of $C_{12}H_{15}NO_3$, in milligrams, in the amount of metaxalone taken by the formula $C(A_u/A_s)$, where C is the exact concentration, in micrograms per milliliter, of metaxalone in the solution of the reference standard, A_u is the absorbance of the solution of the sample, and A_s is the absorbance of the solution of the reference standard. The amount of metaxalone found is not less than 98.0% and not more than 102.0% of the weight of the sample taken.

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A. H. Robins Co., Inc., Richmond, Va., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

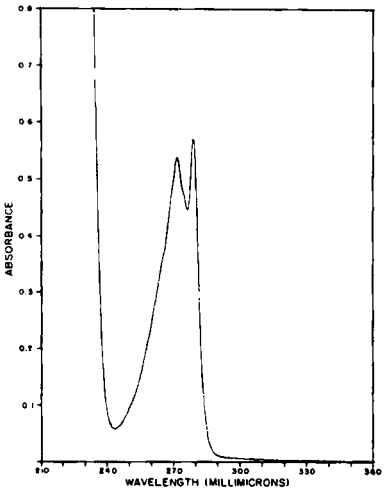


Fig. 1.—Ultraviolet absorption spectrum of metaxalone in alcohol (100 mcg./ml.). Beckman model DK-2A spectrophotometer.

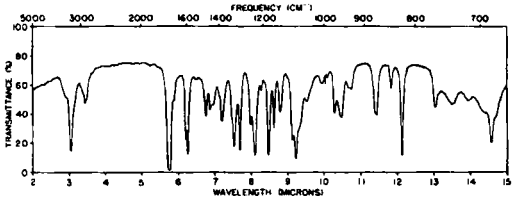


Fig. 2.—Infrared spectrum of metaxalone in potassium bromide disk (0.3%). Perkin-Elmer model 21 spectrophotometer; sodium chloride prism.

DOSAGE FORMS OF METAXALONE

Metaxalone Tablets

Identity Tests.—The ultraviolet absorption spectrum of the chloroform solution obtained in the *Assay* shows absorbance maxima (280 and 272 mμ) and a minimum (277 mμ) at the same wavelength as that of the *Standard Preparation*. The spectrum of metaxalone in chloroform is similar to the spectrum in alcohol (Fig. 1).

Evaporate to dryness 1 ml. of the initial chloroform solution obtained in the *Assay*. Dissolve the residue in 1 ml. of sulfuric acid solution (65 in 100) and add 2 ml. of a 1 in 1000 solution of vanillin in sulfuric acid solution (65 in 100): a cherry-red color develops within about 10 minutes.

Assay.—*Standard Preparation.*—Transfer about 100 mg. of metaxalone reference standard, previously dried under vacuum at 80° for 3 hours and accurately weighed, to a 100-ml. volumetric flask, dissolve in chloroform, dilute to volume with chloroform, and mix. Transfer 10.0 ml. of this solution to a second 100-ml. volumetric flask, dilute to volume with chloroform, and mix.

Procedure.—Weigh and finely powder not less than 20 metaxalone tablets and transfer a portion of the powdered tablets equivalent to about 200 mg. of metaxalone to a 60-ml. separator. Add 10 ml. of water, agitate to suspend the powder, and extract with five 25-ml. portions of chloroform. Filter the chloroform extracts through a pledget of chloroform-washed purified cotton into a 200-ml. volumetric flask, dilute to volume with chloroform, and mix. Transfer 10.0 ml. of this solution to a 100-ml. volumetric flask, dilute to volume with chloroform, and mix. Concomitantly determine the absorbance of this solution and that of the *Standard Preparation* in 1-cm. cells with a suitable spectrophotometer at the wavelength of maximum absorbance at about 280 mμ, using chloroform as the blank. Calculate the weight of $C_{12}H_{15}NO_3$, in milligrams, in the amount of powdered tablets taken by the formula $2C(A_u/A_s)$, where C is the exact concentration, in micrograms per milliliter, of metaxalone in the *Standard Preparation*, A_u is the absorbance of the solution from the tablets, and A_s is the absorbance of the *Standard Preparation*. The amount of metaxalone found is not less than 95.0% and not more than 105.0% of the weight of the sample taken.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever possible.

Metaxolone,[1] synthesized by Lunsford *et al.* (1), is a skeletal muscle relaxant for treatment of acute muscle spasm related to sprains and strains, fractures, dislocations, and trauma to tendons and ligaments.

Identity Tests.—Metaxalone reacts with certain aldehydes in acid medium to form colored products. Formaldehyde and *p*-dimethylaminobenzaldehyde gave yellow colors, while vanillin gave a bright cherry-red.

Alcohol is a particularly convenient solvent for the ultraviolet absorbance identity test and the assay test for bulk metaxalone. On the other hand, it is more convenient to use the extracting solvent, chloroform, directly for the absorbance measurement of metaxalone in the tablet assay, thereby avoiding the necessity of chloroform evaporation and subsequent solution in alcohol.

Quantitative Methods.—Analysis for both the bulk metaxalone and metaxalone tablets is based on ultraviolet absorption properties. Analysis of commercial tablets (400 mg.) gave an average value of 99.4 ± 0.2%[2] of the labeled amount.

REFERENCE

(1) Lunsford, C. D., *et al.*, *J. Am. Chem. Soc.*, **82**, 1166 (1960).

[1] Marketed as Skelaxin by A. H. Robins Co., Inc., Richmond, Va.

[2] Maximum deviation from the mean value.

New Preparative Technique for Effervescent Products

By VINCENT COLETTA and LLOYD KENNON

An air suspension coating-reacting technique has been applied to the preparation of effervescent products. Unlike the present method, traditional preparative processes employ cumbersome many-step procedures. The new technique consists essentially of suspending all or certain of the desired materials in a stream of air, then spraying the suspended mass with water or with a solution of the remaining components. Detailed examples are given to illustrate the versatility and advantages of the process described.

EFFERVESCENT salts, ordinarily in the form of powders, granules, or compressed tablets, constitute a well-known class of pharmaceutical preparations. They generally consist of medicinal ingredients, tartaric and/or citric acids, and a gas-generating base such as sodium bicarbonate. Usually they are administered after being put into water, where they effervesce to form a carbonated saline drink. Specific examples of this type of preparation can be found in the official compendia of pharmacy, the "United States Pharmacopeia" and the "National Formulary." The following are examples of such effervescent salts: sodium phosphate, compound salt of potassium bromide, artificial Carlsbad salt, artificial Kissingen salt, artificial Vichy salt, potassium citrate, salt of lithium citrate, salt of magnesium sulfate, and compound effervescent powders (Seidlitz powders).

The following are examples of commercial effervescent products: Bromionyl[1] and Citrocarbonate,[1] Bromo Seltzer,[2] Alka Seltzer,[3] Sal Hepatica,[4] and Brioschi.[5]

Standard processes used to prepare effervescent products consist of mixing the salts, adding a small amount of water to start the effervescence reaction and so obtain a workable mass, quickly drying the mass in ovens or heated dishes to stop the reaction, grinding the mass under dry conditions to form powder or granules, then bottling. Obviously, throughout the procedure the reaction, which liberates carbon dioxide, can be permitted only to proceed minimally, lest the effervescent character of the final product be destroyed.

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Accepted for publication August 20, 1964.
Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.

[1] Trademarked by The Upjohn Co.
[2] Trademarked by Warner-Lambert Products, Division of Warner-Lambert Pharmaceutical Co.
[3] Trademarked by Miles Laboratories, Inc.
[4] Trademarked by Bristol-Myers Products Division.
[5] Trademarked by Ceribelli & Co.

Control of the amount of water, which is intentionally and unintentionally added to the materials, and hence control of the extent of the reaction has always been difficult to achieve. Techniques such as moistening the materials with 5% water in alcohol, using the water of hydration of citric acid by liberating it *in situ* by heating the salt mix, or using bursts of steam to moisten the material have been used to try to control the extent of the reaction. These techniques have necessarily made the preparation of effervescent pharmaceuticals quite cumbersome.

It is the purpose of this communication to describe a method of preparing effervescent products to facilitate the attainment of control of the reaction just noted; the method also embodies other improvements over standard processes. The new technique consists essentially of suspending the dry sodium bicarbonate and citric acid (or other or all of the composition's ingredients) in a stream of air and spraying the suspended material with water or with a solution of sodium biphosphate or fruit acids. A device such as the Wurster air suspension apparatus (1, 2) or a modified form of it is suitable for this purpose. A satisfactory product can be produced because the following variables can be efficiently, *i.e.*, effectively and rapidly, controlled: the volume and velocity of the air passing through the device, the air temperature and humidity (hence drying capacity), and the liquid feed rate (hence the amount of water and/or other solvent added). Such positive control makes possible complete regulation of the effervescent reaction.

EXPERIMENTAL

The following illustrates the basic technique described. The example shows how the process functions when all of the ingredients are fluidized together, after which water is added to them. The

composition of the formulation made was: sodium biphosphate, 860 Gm.; tartaric acid, 260 Gm.; citric acid, 100 Gm.; flavor, 5 Gm.; sweetener, 13 Gm.; and sodium bicarbonate, 762 Gm.

The 2 Kg. of material was put into the 6-in. Wurster air suspension apparatus chamber as dry solids. Only distilled water was used to cause the ingredients to react slightly and thus form expanded granules. Running characteristics were: air flow = 30–90 c.f.m.; spray nozzle opening = 20/1000 in.; air nozzle opening = 70/1000 in.; atomization pressure = 60 psig; inlet temperature = 120–140 °F.; exhaust temperature = 100–110°F.; inlet fluid pressure = 2–5 psig with a flow rate of 60–70 ml./minute.

The yield was 95%; the granules obtained were in the range 10–30 mesh. The total time needed to process the 2 Kg. was 10 minutes, and the heat cost was about 0.05 cents/lb. of product. Steam could have been used instead of the distilled water to cause the formation of the granules.

The following formula is an illustration of one of the other techniques possible: citric acid, 140 Gm.; sodium bicarbonate, 860 Gm.; and sodium biphosphate, 1000 Gm.

The 6-in. chamber of the air suspension apparatus was charged with the citric acid and sodium bicarbonate; the sodium biphosphate was sprayed as a 40% w/w solution. Running characteristics were: airflow = 30–60 c.f.m.; spray nozzle opening = 20/1000 in.; air nozzle opening = 70/1000 in.; atomization pressure = 60 psig; inlet temperature = 140–160°F.; exhaust temperature = 110–125°F.; inlet fluid pressure = 2–7 psig with a flow rate of 30–40 ml./minute.

The yield was 95%; the granules obtained were in the range 10–30 mesh. Heat costs would be about 0.2 cents/lb. of product.

A similar formula example, but one which illustrates the making of a more complete formula in a larger chamber is: tartaric acid, 1560 Gm.; citric acid, 600 Gm.; sodium bicarbonate, 4572 Gm.; sodium cyclamate, 78 Gm.; water-soluble flavor, 30 Gm.; and sodium biphosphate, 5160 Gm.

The 12-in. diameter Wurster air suspension apparatus chamber was charged with the two acids and the bicarbonate; the sweetener and flavor were dissolved along with the biphosphate, which was sprayed as a 40% w/w solution. Running characteristics were: airflow = 180–300 c.f.m.; spray nozzle opening = 28/1000 in.; air nozzle opening = 70/1000 in.; atomization pressure = 70 psig; inlet temperature = 160–180°F.; exhaust temperature = 130–140°F.; inlet fluid pressure = 10 psig with a flow rate of 60–80 ml./minute.

The yield was 96%; the granules obtained were in the range 10–20 mesh. Heat costs would be about 0.2 cents/lb. of product.

DISCUSSION

Examination of the processes described for making effervescent products with the Wurster air suspension apparatus seems to indicate that the technique has many advantages over the standard preparative processes usually employed for such pharmaceuticals. That the Wurster device can function as a granulator, mixer, fluidized bed drier, and fluid energy mill is central to the attainment of these benefits.

The extent of the effervescent reaction can be controlled during preparation of the mass; this makes possible the regulation and distribution of the water in the mass. Also, the size of the particles can be controlled, and they can be made without the necessity of grinding a dried mass; this eliminates the familiar ovens and trays in addition to the grinders. In addition, segregation of the initial active ingredients is prevented, and fines can be eliminated or minimized.

Since some of the components can be used as filtered solutions, it is possible to eliminate extraneous materials such as specks or fibers, which may contaminate raw materials, even though they meet U.S.P. specifications. With regard to the final product, the stability may be increased because a protective layer of, *e.g.*, biphosphate can be built around the granules. Further, additional ingredients, such as therapeutically active substances or flavors, may be conveniently and uniformly added to the effervescent granules either before or after they are formed. Naturally, the granules can be compressed into tablets after the addition of suitable lubricants.

Advances in air suspension and other fluidized bed technology, such as that recently noted by Scott *et al.* (3, 4), suggest that a method like one described here may be made continuous. This could make the old space and time-consuming batch processes obsolete and create substantial time and cost savings for users of the new method.

SUMMARY

The Wurster air suspension apparatus has been used successfully to prepare effervescent pharmaceutical products. Use of the apparatus for this purpose was illustrated with examples. The advantages inherent to the suggested new method were discussed.

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Tablet Acceptance by a Double Sampling Plan

By R. A. GRUNDMAN and BERNARD ECANOW

A double sampling plan for tablet acceptance is presented and analyzed. The answers to three fundamental questions are presented: (*a*) How will the plan operate with respect to batches of tablets which vary in quality from batch to batch? (*b*) What will be the quality of tablets passed into stock by the plan? (*c*) How much inspection on the average will the plan require?

IN THE PRODUCTION of coated aspirin tablets intended to be used for sustained-release action, lot sizes of 500 tablets were produced. A double sampling plan was selected ($n_1 = 50$, $n_2 = 100$, $c_1 = 0$, $c_2 = 2$). The authors were interested in analyzing the ability of this plan with respect to the three fundamental features listed below.

(*a*) How will the plan operate with respect to batches of tablets which vary in quality from batch to batch?

(*b*) What will be the quality of tablets passed into stock by the plan?

(*c*) How much inspection on the average will the plan require?

The first question is answered by the operating characteristic (OC) curve for the plan. The second question is answered by the average outgoing quality (AOQ) curve for the plan and the third question by the average sample number (ASN) curve and the total amount of inspection (TAI) curve for the plan.

This paper is intended to show how to construct, by approximate methods, these curves for our double sampling plan.

DISCUSSION

Because sampling is a problem involving the laws of chance, the development of good sampling acceptance schemes requires consideration of the mathematics of probability.

Three probabilities must be noted:

(*a*) The probability of the joint occurrence of two independent or dependent events is the *product* of their respective probabilities. Thus, if a coin *and* a die are tossed simultaneously, the probability of a head *and* an ace is $1/2 \times 1/6 = 1/12$.

(*b*) The probability that one or the other of a set of mutually exclusive events will occur on an occasion where any of them may occur is the *sum* of their respective probabilities. Thus, the probability of a 7 *or* an 11 at one toss of two dice is $1/6 + 1/18 = 2/9$.

(*c*) The Poisson exponential will be used to approximate the more rigorously correct probabilities given by the binomial formula.

The probability (1) of finding m defects in a random sample of n pieces drawn from an infinite universe (general output of uniform product) in which the fraction defective is p, is given exactly by the m + 1st term of the expansion of the binomial, $[(1 - p) + p]^n$, $P_{m,n,p} = C_m^n(1 - p)^{n-m}p^m$ (Eq. 1)

When $p < 0.10$, a good approximation to Eq. 1 is given by the m + 1st term of the Poisson exponential distribution

$$P_{m,n,p} \approx P_{m,pn} = \frac{e^{-pn}(pn)^m}{m!}$$

The first study concerned the tablet weight (a nondestructive test) for which we constructed OC, AOQ, ASN, and TAI curves. The second study was for tablet disintegration, for which only the OC and ASN curves are used since this is a destructive test.

Operating Characteristic (OC) Curve.—(Table I, Fig. 1).—In our plan a 50-tablet sample ($n_1 = 50$) was taken. In testing for tablet weight on the 50-tablet sample, the batch was acceptable if all tablets passed the weight test ($c_1 = 0$). If all the tablets did not pass the weight test a 100-tablet sample ($n_2 = 100$) was tested, and the batch was acceptable if two tablets ($c_2 = 2$) or less did not pass the weight test in the first and second sample of 150. In the second study, on running U.S.P. XVI disintegration tests for coated tablets on a 50-tablet sample, the batch was acceptable if all tablets dissolved ($c_1 = 0$). If all the tablets did not dissolve (within the meaning of the test) a 100-tablet sample ($n_2 = 100$) was tested, and the batch was acceptable if two tablets ($c_2 = 2$) or less remained undissolved in the first and second sample of 150.

Let $P_a(p)$ = probability that a lot of quality p will be accepted. The lot will be accepted (*a*) if $c_1 = 0$ defectives appear in the first sample of $n_1 = 50$ *or* (*b*) if $c_2 = 2$ or less defectives appear in the first and second sample of 150.

The probability of (*a*) happening is $P_p(50,0)$, where we take

$$P_p(50, x) = \frac{(50p)^x e^{-50p}}{x!}$$

The probability of (*b*) happening consists of (*i*) the probability of 1 in 50 *and* 1 or less in 100 or (*ii*) the probability of 2 in 50 *and* 0 in 100.

These probabilities may be symbolized as

$$(i)\, P_p(50, 1) \sum_{x=0}^{1} P_p(100, x) \quad (ii)\, P_p(50, 2)\, P_p(100, 0)$$

Hence

$$P_a(p) = P_p(50, 0) + P_p(50, 1) \sum_{x=0}^{1} P_p(100, x) + P_p(50, 2)\, P_p(100, 0)$$

A work sheet now may be arranged, entries made

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TABLE I.—OPERATING CHARACTERISTIC CURVE FOR DOUBLE SAMPLING INSPECTION PLAN

$n_1 = 50, n_2 = 100, c_1 = 0, c_2 = 2$

$P_a(p) = P(50,0) + P(50,1)[P(100,0) + P(100,1)] + P(50,2)P(100,0)$

p	$P(50,0)$	$P(50,1)$	$P(50,2)$	$P(100,0)$	$P(100,1)$
.002	.905	.090	.005	.819	.164
.005	.779	.195	.024	.607	.303
.006	.741	.222	.033	.549	.329
.01	.607	.303	.076	.368	.368
.02	.368	.368	.184	.135	.271
.03	.223	.335	.251	.050	.149
.04	.135	.271	.271	.018	.074
.048	.091	.218	.261	.008	.040
.05	.082	.205	.257	.007	.034
.06	.050	.149	.224	.002	.015
.07	.030	.106	.185	.001	.006
.08	.018	.074	.146	.000	.003
.09	.011	.050	.112	.000	.001
.10	.007	.033	.085	.000	.000

$$
\begin{aligned}
P_a(.002) &= .905 + .090(.819 + .164) + .005(.819) = .998\\
P_a(.005) &= .779 + .195(.607 + .303) + .024(.607) = .971\\
P_a(.006) &= .741 + .222(.549 + .329) + .033(.549) = .954\\
P_a(.01) &= .607 + .303(.368 + .368) + .076(.368) = .858\\
P_a(.02) &= .368 + .368(.135 + .271) + .184(.135) = .542\\
P_a(.03) &= .223 + .335(.050 + .149) + .251(.050) = .302\\
P_a(.04) &= .135 + .271(.018 + .074) + .271(.018) = .165\\
P_a(.048) &= .091 + .218(.008 + .040) + .261(.008) = .103\\
P_a(.05) &= .082 + .205(.007 + .034) + .257(.007) = .092\\
P_a(.06) &= .050 + .149(.002 + .015) + .224(.002) = .053\\
P_a(.07) &= .030 + .106(.001 + .006) + .185(.001) = .031\\
P_a(.08) &= .018 + .074(.000 + .003) + .146(.000) = .018\\
P_a(.09) &= .011 + .050(.000 + .001) + .112(.000) = .011\\
P_a(.10) &= .007 + .033(.000 + .000) + .085(.000) = .007
\end{aligned}
$$

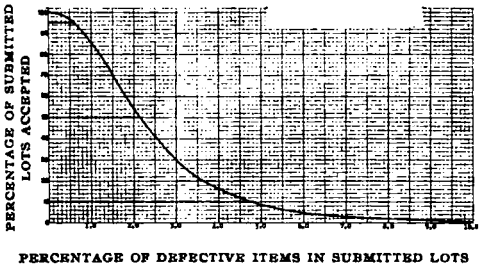


Fig. 1.—Operating characteristic curve for double sampling inspection plan. $n_1 = 50$, $n_2 = 100$, $c_1 = 0$, $c_2 = 2$. Sublot size 500.

from Molina's (2) tables for $p = 0$ to $p = .10$, and $P_a(p)$ computed.

Key features of our plan are (*a*) 95% of submitted lots accepted at about $p = .6\%$, (*b*) 10% of submitted lots accepted at about $p = 4.8\%$, and (*c*) 50–50 chance of acceptance at $p = 2.1\%$.

The slope of the tangent to the OC curve at and in a small neighborhood of $p = 0$ is of interest because a slope of zero implies no rejections. Obviously, $P_a(0) = 1.00$ for *any* plan, but the slope of the OC curve as $p \to 0$ indicates the power of the plan to detect small deviations from perfection in submitted lots. As a practical matter, one would generally prefer an OC curve which has essentially a zero slope for most values of p on the interval $0 \leq p \leq$ AQL. A plan whose OC curve approaches (0,1.00) with nonzero slope would operate to reject some lots of superior quality.

In the case of our double sampling plan, this property of slope of the OC curve near $p = 0$ may be studied as follows.

$$
\begin{aligned}
P_a(p) &= P(50,0) + P(50,1)\,[P(100,0) + P(100,1)] + P(50,2)P(100,0)\\
&= \frac{50!}{0!(50-0)!}\,p^0(1-p)^{50-0} + \frac{50!}{1!(50-1)!}\,p^1(1-p)^{50-1}\\
&\quad \times \left[\frac{100!}{0!(100-0)!}\,p^0(1-p)^{100-0} + \frac{100!}{1!(100-1)!}\,p^1(1-p)^{100-1}\right]\\
&\quad + \frac{50!}{2!(50-2)!}\,p^2(1-p)^{50-2} \times \frac{100!}{0!(100-0)!}\,p^0(1-p)^{100-0}\\
&= (1-p)^{50} + \frac{50!}{49!}\,p(1-p)^{49} \times \left[(1-p)^{100} + \frac{100!}{99!}\,p(1-p)^{99}\right]\\
&\quad + \frac{50!}{2!48!}\,p^2(1-p)^{48}(1-p)^{100}\\
&= (1-p)^{50} + 50p(1-p)^{149} + 5000p^2(1-p)^{148} + 1225\,p^2(1-p)^{148}\\
P_a'(p) &= -50(1-p)^{49} + 50[p^0(1-p)^{149} - 149\,p(1-p)^{148}] + 5000[2p(1-p)^{148}\\
&\quad - 148\,p^2(1-p)^{147}] + 1225[2p(1-p)^{148} - 148\,p^2(1-p)^{147}].
\end{aligned}
$$

If $p = 0$, $P_a'(p) = -50 + 50 = 0$.

This is an example of the case $c_1 = 0$, $c_2 \neq 0$, $p = 0$ gives $P_a'(p) = 0$. This case should not be taken as typical for all double sampling plans where $c_1 = 0$. There are double sampling plans for which $c_1 = 0$ and $P_a'(p) \neq 0$ as $p \to 0$.

Average Outgoing Quality (AOQ) Curve.—If all rejected lots are detailed, cleared of defectives, and

all defectives replaced by good pieces, the equation for the AOQ curve becomes

$$\mathrm{AOQ}(p) = P_a(p)p. \qquad \text{(Eq. 2)}$$

Derivation:

N = number of pieces in each lot
k lots at p
$NkP_a(p)$ accepted
$Nk[1 - P_a(p)]$ rejected
$NkP_a(p)p$ defective pieces accepted
$Nk[1 - P_a(p)]p$ defective pieces rejected

$$\mathrm{AOQ}(p) = \frac{NkP_a(p)p + Nk[1 - P_a(p)](0)}{Nk}$$
$$= P_a(p)p$$

The curve may be constructed from values already in the OC table.

If the rejected lots are detailed, cleared of defectives, and only the good pieces passed into stock, then

$$\mathrm{AOQ}(p) = \frac{P_a(p)p}{1 - p[1 - P_a(p)]} \qquad \text{(Eq. 3)}$$

Derivation:

$$\frac{kN_p\,P_a(p)}{kN - kNp[1 - P_a(p)]} = \frac{P_a(p)p}{1 - p[1 - P_a(p)]}$$

This curve may be constructed similarly from values available in the OC table.

TABLE II.—RELATIONSHIP BETWEEN INCOMING QUALITY, OUTGOING QUALITY, AND AOQL

Assume all rejected lots are cleared of defectives and restored as 100% lots	If rejected lots are cleared of defectives but not restored
$\mathrm{AOQ}(p) = P_a(p)p$	$\mathrm{AOQ}(p) = \frac{P_a(p)p}{1 - p[1 - P_a(p)]}$
$n_1 = 50, n_2 = 100, c_1 = 0, c_2 = 2$	
AOQ(.002) = .002	AOQ(.002) = .002
(.005) = .005	(.005) = .005
(.006) = .0057	(.006) = .0057
(.01) = .0086	(.01) = .0086
(.02) = .0108 ← AOQL	(.02) = .0109 → AOQL
(.03) = .0091	(.03) = .0093
(.04) = .0066	(.04) = .0068
(.048) = .0049	(.048) = .0051
(.05) = .0046	(.05) = .0048
(.06) = .0032	(.06) = .0034
(.07) = .0022	(.07) = .0024
(.08) = .0014	(.08) = .0015
(.09) = .0010	(.09) = .0011
(.10) = .0007	(.10) = .0008

An important feature of these curves is the existence of a unique maximum point called the average outgoing quality limit (AOQL) and defined as the poorest average quality level which can occur in the long run after inspection, no matter what quality lots are submitted for inspection.

For our plan the curve of Eq. 2 is similar to the curve for Eq. 3. They are identical up to AOQL; at that point Eq. 3 exceeds Eq. 2. This relationship continues to the end of the curve. AOQL for Eq. 2 is 1.08% for $p \approx 2\%$; AOQL for Eq. 3 is 1.09% for $p \approx 2\%$. (See Table II and Figs. 2 and 3.)

Average Sample Number (ASN) Curve.—The expected number of pieces inspected to reach a decision regarding disposition of a lot will consist of

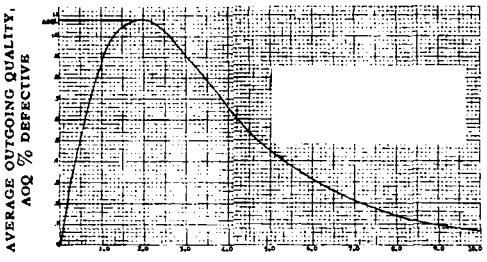


Fig. 2.—Relationship between incoming quality, outgoing quality, and AOQL. (Assume all rejected lots are cleared of defectives and restored as 100% lots.) $n_1 = 50, n_2 = 100, c_1 = 0, c_2 = 2$. AOQL = 1.08%. Sublot size 500.

three parts. (*a*) The average number required to accept the lot on the first sample, (*b*) the average number required to reject the lot on the first sample, (*c*) the average number required to reach a decision by virtue of inspecting a second sample.

If $P_{a_1}(p)$ = probability of accepting a lot of quality p on the first sample = $P_p(n_1, x \leq c_1)$ probability of c_1 or less in first n_1 observations from lot at level p.

If $P_{r_1}(p)$ = probability of rejecting a lot of quality p on first sample = $1 - P_p(n_1, x \leq c_2)$ probability of $c_2 + 1$ or more in first n_1 observations from lot at level p.

If $P_s(p)$ = probability of being required to take a second sample to reach a decision concerning disposition of the lot at level p = $P_p(n_1, x \leq c_2) - P_p(n_1, x \leq c_1)$ because

$$\sum_{x=0}^{c_1} P_p(n_1,x) + [1 - \sum_{x=0}^{c_2} P_p(n_1,x)] + P_s(p) = 1$$

Then

$$\mathrm{ASN}(p) = n_1P_{a_1}(p) + n_1P_{r_1}(p) + (n_1 + n_2)P_s(p)$$

This supposition is that for each lot requiring a second sample, inspection of the second sample is carried to completion, even though an action decision is reached before $n_1 + n_2$ pieces have been inspected.

We shall show how this is constructed for our particular plan.

$$\mathrm{ASN}(p) = 50[P_{a_1}(p) + P_{r_1}(p)] + 150P_s(p)$$

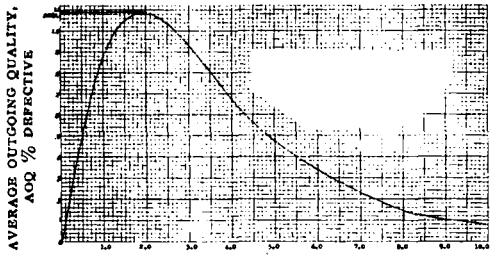


Fig. 3.—Relationship between incoming quality, outgoing quality, and AOQL. (If rejected lots are cleared of defectives but not restored.) $n_1 = 50$. $n_2 = 100, c_1 = 0, c_2 = 2$. AOQL = 1.09%. Sublot size 500.

TABLE III.—AVERAGE SAMPLE NUMBER CURVE FOR DOUBLE SAMPLING

$n_1 = 50, n_2 = 100, c_1 = 0, c_2 = 2$

$ASN(p) = n_1[P_{a_1}(p) + P_{r_1}(p)] + (n_1 + n_2)P_s(p)$

p	$P_{a_1}(p)$	$P_{r_1}(p)$	$P_s(p)$	$n_1[P_{a_1}(p) + P_{r_1}(p)]$	$(n_1 + n_2)P_s(p)$	ASN
.002	.905	.000	.095	45.45	14.25	59.70
.005	.779	.002	.219	39.05	32.85	71.90
.006	.741	.004	.255	37.25	38.25	75.50
.01	.607	.014	.379	31.05	56.85	87.90
.02	.368	.080	.552	22.40	82.80	105.20
.03	.223	.191	.586	20.70	87.90	108.60
.04	.135	.323	.542	22.90	81.30	104.20
.048	.091	.430	.479	26.05	71.85	97.90
.05	.082	.456	.462	26.90	69.30	96.20
.06	.050	.577	.373	31.35	55.95	87.30
.07	.030	.679	.291	35.45	43.65	79.10
.08	.018	.762	.220	39.00	33.00	72.00
.09	.011	.826	.163	41.85	24.45	66.30
.10	.007	.875	.118	44.10	17.70	61.80

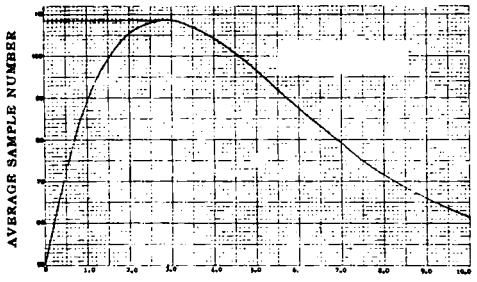


Fig. 4.—Average sample number curve for double sampling inspection plan. $n_1 = 50$, $n_2 = 100$, $c_1 = 0$, $c_2 = 2$.

Now

$$P_{a_1}(p) = P_p(n_1, x \leq c_1) = P_p(50,0)$$

$$P_{r_1}(p) = 1 - P_p(n_1, x \leq c_2) = 1 - \sum_{x=0}^{2} P_p(50,x)$$

$$P_s(p) = P_p(n_1, x \leq c_2) - P_p(n_1, x \leq c_1) = \sum_{x=0}^{2} P_p(50,x) - P_p(50,0)$$

Values of $P_{r_1}(p)$ and $P_s(p)$ may be approximated from Molina's tables for $p \leq .10$, but the binomial is preferable for $p > .10$. Values of $P_{a_1}(p)$ are available from the OC table.

A plan should reach a decision early concerning when either material of very high quality or very poor quality is under inspection. Since first sample sizes in double sampling are always smaller than the corresponding fixed sample size in single sampling, double sampling achieves the objective of less inspection for both exceptionally high and exceptionally poor incoming quality. On the other hand, for material of intermediate quality the forcing of a second sample leads to more inspection than would be required by the corresponding single sampling plan. Therefore, the ASN curve as a function of continuously varying p must and does have a maximum value for some unique value of p.

We shall now show the approximate maximum ASN compared to the single sampling plan corresponding respectively to our plan.

The corresponding single sampling plan means here the plan that would be used for comparable incoming quality range and lot sizes. Strictly speaking, two sampling plans correspond with respect to their discriminatory power only when they have essentially congruent OC curves. Correspondence as used in this discussion only approximates this ideal.

ASN is 109 at $p \approx 3\%$ when $n_1 = 50$, $n_2 = 100$, $c_1 = 0$, $c_2 = 2$. Single sampling plan is $n = 75$, $c = 1$.

The ASN curves assume that all second samples are completely inspected, an assumption not likely to be realized in practice. The result of terminating the inspection on a second sample after a decision to reject is obtained will, of course, materially decrease the ASN values over the right half of the curve. (See Table III and Fig. 4.)

Total Amount of Inspection (TAI) Curve.—When acceptance procedures involve 100% detailing of all rejected lots, the question of what will be the expected total amount of inspection at each quality level arises.

The TAI figure is the sum of three components: (a) expected number of pieces inspected to accept on the first sample, (b) expected number of pieces inspected to accept on the second sample, (c) expected number of pieces inspected by virtue of detailing rejected lots. Thus, TAI is a function of two independent variables, p and N. If N = lot size, $P_s(p)$ = probability of taking second sample, and $P_2^a(p)$ = probability of acceptance on second sample. Hence

$$TAI(p,N) = n_1 P_{a_1}(p) + (n_1 + n_2)P_s(p)P_{a_2}(p) + N[1 - P_s(p)P_{a_2}(p) - P_{a_1}(p)]$$

because

$$P_{a_1}(p) + P_s(p)P_{a_2}(p) + P_r(p) = 1.$$

For our plan $n_1 = 50$, $n_2 = 100$, $c_1 = 0$, $c_2 = 2$, and a lot size of 500 we have

$$TAI(p,500) = 50\,P_{a_1}(p) + 150\,P_s(p)P_{a_2}(p) + 500[1 - P_s(p)P_{a_2}(p) - P_{a_1}(p)]$$

Now

$$P_s(p)P_{a_2}(p) = P_a(p) - P_{a_1}(p)$$

so this and all other terms involved here are available from the OC table.

If the receiving staff does the detailing and uses the plan studied in this paper, it can expect to have

TABLE IV.—TOTAL AMOUNT OF INSPECTION CURVE

$n_1 = 50,\ n_2 = 100,\ c_1 = 0,\ c_2 = 2$

$N = 500$

$$TAI(p) = n_1 P_{a_1}(p) + (n_1 + n_2) P_s(p) P_{a_2}(p) + N[1 - P_s(p) P_{a_2}(p) - P_{a_1}(p)]$$

p	$P_{a_1}(p)$	$n_1 P_{a_1}(p)$	$P_s(p) P_{a_2}(p)$	$(n_1 + n_2) \cdot P_s(p) P_{a_2}(p)$	$N[1 - P_s(p) P_{a_2}(p) - P_{a_1}(p)]$	$TAI(p)$
.002	.905	45.25	.093	13.95	1.00	60.20
.005	.779	38.95	.192	28.80	14.50	82.25
.006	.741	37.05	.213	31.95	23.00	92.00
.01	.607	30.35	.251	37.65	71.00	139.00
.02	.368	18.40	.174	26.10	229.00	273.50
.03	.223	11.15	.079	11.85	349.00	372.00
.04	.135	6.75	.030	4.50	417.50	428.75
.048	.091	4.55	.012	1.80	448.50	454.85
.05	.082	4.10	.010	1.50	454.00	459.60
.06	.050	2.50	.003	.45	473.50	476.45
.07	.030	1.50	.001	.15	484.50	486.15
.08	.018	.90	.000	.00	491.00	491.90
.09	.011	.55	.000	.00	494.50	495.05
.10	.007	.35	.000	.00	496.50	496.85

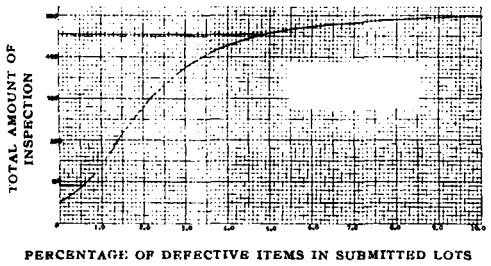


Fig. 5.—Total amount of inspection curve for double sampling inspection plan. $n_1 = 50$, $n = 100$, $c_1 = 0$, $c_2 = 2$. Lot size $N = 500$.

to inspect on the average 18% of every 500 piece lot that comes in at an AQL of .6%, and 91% of every 500 piece lot that comes in at a p_t of 4.8%. (See Table IV and Fig. 5.)

CONCLUSIONS

This paper has attempted to show how a given double sampling plan should be X-rayed to reveal its performance characteristics.

REFERENCES

(1) Dodge, H. F., and Romig, H. G., "Sampling Inspection Tables," John Wiley & Sons, Inc., New York, N. Y., 1959.
(2) Molina, E. C., "Poisson's Exponential Binomial Limit," D. Van Nostrand Co., Inc., Princeton, N. J., 1942.

Notes

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TABLE IV.—TOTAL AMOUNT OF INSPECTION CURVE

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$N = 500$

$$TAI(p) = n_1 P_{a_1}(p) + (n_1 + n_2) P_s(p) P_{a_2}(p) + N[1 - P_s(p) P_{a_2}(p) - P_{a_1}(p)]$$

p	$P_{a_1}(p)$	$n_1 P_{a_1}(p)$	$P_s(p) P_{a_2}(p)$	$(n_1 + n_2) \cdot P_s(p) P_{a_2}(p)$	$N[1 - P_s(p) P_{a_2}(p) - P_{a_1}(p)]$	$TAI(p)$
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.005	.779	38.95	.192	28.80	14.50	82.25
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.05	.082	4.10	.010	1.50	454.00	459.60
.06	.050	2.50	.003	.45	473.50	476.45
.07	.030	1.50	.001	.15	484.50	486.15
.08	.018	.90	.000	.00	491.00	491.90
.09	.011	.55	.000	.00	494.50	495.05
.10	.007	.35	.000	.00	496.50	496.85

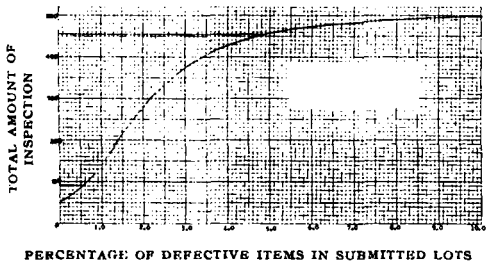


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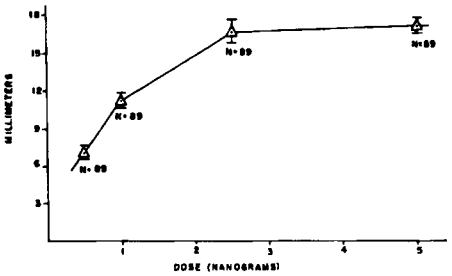


Fig. 1.—Response of the goldfish intestine to acetylcholine in the absence of cholinesterase inhibitors. Mean ± S.E.

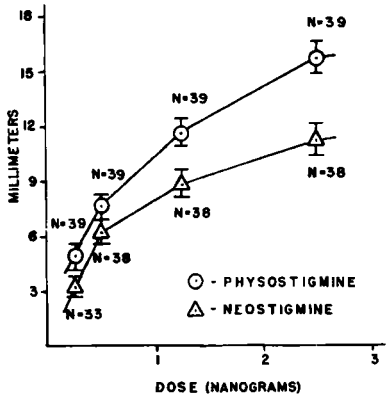


Fig. 2.—Response of the goldfish intestine to acetylcholine in the presence of 250 nanograms of either physostigmine or neostigmine. Mean ± S.E.

TABLE I.—STATISTICAL COMPARISON OF DRUG RESPONSES

Drug	b	S_b^{2a}	t Obtained	t Required at P = 0.05
Acetylcholine, *1*	10.38	1.28	...	...
Acetylcholine + physostigmine, *2*	10.67	2.36	0.152[b]	2.78
Acetylcholine + neostigmine, *3*	7.56	1.74	1.62[c]	2.78

[a] $S_b^2 = \frac{\Sigma[(x - \bar{x})(\text{S.E.})]^2}{[\Sigma(x - \bar{x})^2]^2}$ = estimated variance of slope. [b] Comparison between *1* and *2*. [c] Comparison between *1* and *3*.

to acetylcholine and because we are investigating the release of this substance from brain tissue where low concentrations would be encountered, the decision was made that the effects of both esterase inhibitors should be investigated to determine not only their effects on intestinal contractility but also their influence on intestinal sensitivity to acetylcholine.

EXPERIMENTAL

The microbath and goldfish intestinal preparation described by Gaddum and Szerb (3) was used for the quantitative estimation of acetylcholine. The effects of physostigmine and neostigmine on the acetylcholine response were also investigated. The intestinal contractions were recorded with a Sanborn linear differential transformer model FTA-1-1 and a Sanborn strain gauge amplifier model 140. The ranges of drug concentrations were: acetylcholine 0.25 to 5 nanograms; physostigmine and neostigmine 12 to 500 nanograms. (One nanogram equals 1×10^{-9} Gm.) All drug additions to the microbath were accomplished with a Kensco 50-μl. micropipet. The results were analyzed statistically by calculating the regression lines and comparing the slope values by the t test.

RESULTS

Exposure of the isolated goldfish intestine to acetylcholine resulted in a vigorous contraction with a response that was essentially linear over the dosage range of 0.5 to 2.5 nanograms. Higher doses gave no greater response (Fig. 1). The amount of acetylcholine detected with this preparation was increased when 250 nanograms of either physostigmine or neostigmine were mixed with the acetylcholine (Fig. 2), but the over-all response of the preparation to acetylcholine decreased. The increase in sensitivity was probably related to the inactivation of cholinesterase by the drugs and the decreased over-all response to the direct contractile effects of these drugs on the intestine. Both esterase inhibitors caused slight contractions of the intestine in the absence of exogenous acetylcholine, but the height of contraction was almost identical per dose over the dosage range of 12 to 500 nanograms. Furthermore, there was a tendency for the intestine to remain in a permanent contraction after exposure to doses above 250 nanograms. In all instances conversion to log dose-response curves and comparison of the slope values by the t test indicated no significant difference in the values obtained with or without the esterase inhibitors (see Table I).

DISCUSSION

The results obtained with the goldfish intestine following exposure to acetylcholine confirm those previously reported by others (1–3). The effects of physostigmine and neostigmine were similar to the observations of Dreyer (1). The authors have shown that this preparation gives a linear response over a dosage range of 0.5 to 2.5 $\times 10^{-9}$ Gm. of acetylcholine which is in agreement with Gaddum and Szerb (3). When either physostigmine or neostigmine are added to the solution, the lower limit drops to 0.25 $\times 10^{-9}$ Gm. Using the same technique and the cholinesterase inhibitor isopropylmethylphosphoryl fluoride (sarin), Szerb (4) showed that the leech muscle could detect 25 to 100 $\times 10^{-12}$ Gm. of acetylcholine. Thus, the leech muscle is approximately 10 times more sensitive than the goldfish intestine. However, the availability of the latter to most laboratories may more than compensate for the increased sensitivity of the former. Moreover, the addition of specific antagonists to the bathing fluid makes it possible to eliminate the effects produced by polypeptides, catecholamines, histamine, and other naturally occurring substances (3).

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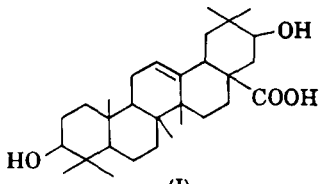
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Saponins and Sapogenins XVIII—Isolation of Proceric Acid, a New Triterpenic Acid, from Maharashtrian *Albizzia procera* Seeds

By I. P. VARSHNEY and S. Y. KHAN

A colorless saponin isolated from Maharashtrian *Albizzia procera* seeds on hydrolysis yields a new pentacyclic triterpenic acid, m.p. 268–270°, acetate, m.p. 288–290°, methyl ester, m.p. 224–225°, and acetyl methyl ester, m.p. 280–281°. The compound has been named proceric acid.

THE ALBIZZIA PROCERA, Benth. seeds, obtained from Madhya Pradesh, were studied earlier in these laboratories (1, 2) for their saponin and sapogenin contents. They contained a saponin, proceranin, which on hydrolysis yields an acid genin identified as machaerinic acid (3β, 21β, dihydroxy Δ^{12}-18β-oleanene-28-oic acid) (I). To carry out additional work on proceranin, a supply of *Albizzia procera* seeds was obtained from the Silviculturist, Maharashtra, Poona. But surprisingly, when the genin obtained from these seeds was acetylated in the usual manner, the acetate obtained was quite different from the machaerinic acid acetate. Its melting point and rotation differed considerably from the acetates of all known triterpenic acids. Therefore, a detailed study of the genin from these seeds was instigated.



(I)

The well-defatted seed powder was extracted with alcohol, and the usual treatment gave a colorless product which passed all tests for saponin. The saponin, on hydrolysis with sulfuric acid, gave a colorless sapogenin which was further purified by potassium salt formation and crystallization, m.p. 268–270°. The acid genin was then transformed into a methyl ester with diazomethane, yielding colorless needles, m.p. 224–227°. The genin, on acetylation with cold pyridine and acetic anhydride, gave an acetate, m.p. 288–290°. The acetylation of the methyl ester with cold pyridine and acetic anhydride easily gave the acetyl methyl ester, melting at 281–282°. But the methylation of the acetate with diazomethane did not yield an acetyl methyl ester. All these compounds gave a yellow color, with tetranitromethane showing the presence of at least one carbon-carbon double bond.

Although the methyl ester and acetyl methyl ester have the same melting points as those of the corresponding derivatives of machaerinic acid, the melting points of the acetate and the genin differed considerably (Table I). The rotations of all these derivatives also differed.

The infrared spectra of the acetate showed the presence of an acetyl group at 8.05 μ and of a γ-lactone at 5.68 μ, which showed that the second OH group is not acetylated and has formed a lactone with the acid group. The I.R. spectra of the methyl ester also differed from the spectra of the methyl ester of machaerinic acid. The infrared spectra of this acid (proceric acid), when taken in pyridine under the conditions described (3), showed characteristic bands in the B range at 1332, 1292, and 1249 cm.$^{-1}$. This does not fit well into the oleanolic acid or in the ursolic acid scheme but is similar to the spectra of Δ^{12}-28-acids, as other positions of the double bond or the carbonyl groups give rise to storing alterations of the mentioned triplet. The NMR spectra (Fig. 1) was also different. An 18α-structure for this acid might be possible. Since this acid may be new, it has been tentatively named proceric acid.

The NMR spectrum has the vinylic hydrogen absorption at 62–65 cycles per second (c.p.s.), which is rather strange. Usually such an absorption falls in the 75–79-c.p.s. region. That the compound shows low absorption may mean that the double bond is conjugated. For example, if there is a ketone at C-11 in addition to the double bond at C-12, such an absorption is obtained. It may be possible that the double bond is not at C-12 according to the findings of NMR spectrography.

The low absorption from 105–120 c.p.s. represents the alpha hydrogen of the C-3 acetate function. This absorption is about where it should be.

The doublet at 127.8 and 133.9 c.p.s. probably represents the alpha hydrogen to the alcoholic oxygen of lactone. But again this absorption is too high, *i.e.*, upfield. For example, in dumortierigenin diacetate, where a five-membered lactone is present (4), the alpha hydrogen occurs at 104–120 c.p.s. The peak at 211.5 c.p.s. represents the methyl group of the acetate function at C-3.

The peaks at 246.6, 250.4, 253.2, 255.7, and 259.6 c.p.s. represent saturated methyl functions. In other words, there is no vinylic methyl group of the type C:C $-CH_3$. The smaller peaks at the right-hand side of the spectrum 187.6, 198.7, 225.6 c.p.s., etc., stand for methylene or methine hydrogens.

This shows that the present genin acetate is a lactone between second OH and acid group. The 18α-structure for this acid, on the study of the molecular model and I.R. spectra, is not ruled out.

EXPERIMENTAL[1]

Defatting.—Well-powdered seeds (250 Gm.) were exhausted in a Soxhlet apparatus with light petroleum ether (40–60°). Recovery of the solvent gave a greenish-yellow oil with the typical odor of *Albizzia* seed oils.

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The authors are grateful to Professor R. Tschesche, University of Bonn, Bonn, Germany, and Professor M. Shamma, The Pennsylvania State University, for infrared and NMR spectra, respectively.
Previous paper: Varshney, I. P., and Khan, S. Y., *J. Sci. Ind. Res. India*, 21B, 401(1962).

[1] All melting points were taken on a Kofler hot microscopical stage and are corrected. The I.R. spectra were recorded on an Infracord model 137 and a Perkin-Elmer model 221 spectrophotometer by I. P. Varshney and Professor R. Tschesche, respectively. The NMR spectra were recorded by the Department of Chemistry, Whitmore Laboratory, The Pennsylvania State University. The rotations are in chloroform solution.

TABLE I.—MELTING POINTS

	Machaerinic Acid, m.p.	$[\alpha]_D$	Proceric Acid, m.p.	$[\alpha]_D$	Mixed M.p.
Genin	256–258	+82.4	268–270	...	263–267
Acetate	258–260	+88	288–290	+16.67	260–262
Methyl ester	224–225	+80.7	224–227	+67.16	225–227
Acetyl methyl ester	278–280	+90	281–282	+80.20	282–283
Acetyl bromolactone	276–278	+80	...	...	...

Extraction.—The defatted seed powder was exhausted with 95% alcohol in a Soxhlet apparatus, and the extract was concentrated to a brown syrupy liquid, which was extracted successively with petroleum ether, ether, chloroform, carbontetrachloride, and acetone to remove the impurities soluble in these solvents. The residue left was then dissolved in ethyl alcohol and filtered. The solution was added dropwise to a large amount of ether when a light brown precipitate was obtained. This operation of dissolution in alcohol and precipitation was repeated several times; finally, the precipitation occurred in acetone. The final precipitate was taken in alcohol and treated with activated charcoal. It was again precipitated in acetone. This gave a light cream-colored hygroscopic powder of saponin. This product gave all the tests for saponins—sneezing, abundant foam on shaking with water, toxicity to fishes in low concentrations, strong hemolytic effect in dilute concentrations, and the specific color reactions (5).

Isolation of Sapogenin.—The cream-colored powder of the saponin obtained by the ether/acetone precipitations was dissolved in a large amount of water and hydrolyzed with sulfuric acid (5–7%) by heating the solution first on a boiling water bath for 1 hour, then completing the hydrolysis by refluxing the solution for 1 more hour. After completion of the hydrolysis, the precipitate was filtered, washed with water until the washings were neutral, and finally dried. The genin thus obtained was dissolved in alcohol and decolorized with activated charcoal.

The crude genin was converted into potassium salt by heating with an alcoholic solution of caustic potash for 1 hour and thereafter distilling off half of the solvent. The solution was diluted with a large quantity of water and extracted three times with ether. The ethereal extracts were combined and washed free of alkali. On recovery of the solvent, an appreciable quantity of the neutral sapogenin was not obtained. The alkaline solution left after ether extraction was acidified with hydrochloric acid and left for 2 hours. The precipitate obtained was filtered and washed with water free of hydrochloric acid and crystallized from methanol, giving the acid genin, m.p. 268–270°.

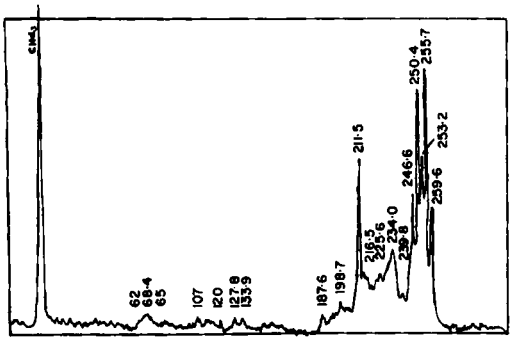


Fig. 1.—To convert to standard T values, divide by 40, then add 2.75 in every case. At 40 megacycles (Varian)—C_2Cl_4, solvent; $CHCl_3$, standard (internal).

Acetylation.—The acid genin was acetylated with acetic anhydride in the presence of pyridine by keeping the mixture overnight. It was poured in ice-water when the acetate was precipitated and filtered. The precipitate, after washing free of acid and pyridine, was crystallized as needles from methyl alcohol, m.p. 288–290°. It gave a positive reaction with tetranitromethane, $[\alpha]_D$ = +16.67°.

Anal.—Calcd. for $C_{32}H_{48}O_4$: C, 77.37; H, 9.74. Found: C, 77.08 and 77.52; H, 9.36 and 9.56.

Deacetylation.—Three-hundred milligrams of the acetate, m.p. 288–290°, was refluxed for 2 hours with methyl alcoholic potassium hydroxide (60 ml., 5%). The solution was diluted with a large amount of water and left overnight at room temperature, but no solid potassium salt separated out. The solution was acidified with hydrochloric acid which gave the precipitate of the acid sapogenin. The precipitate was filtered and washed with water free of acid and crystallized from methanol, m.p. 268–270°. It produced a yellow color with tetranitromethane and a positive Liebermann-Burchard reaction.

Anal.—Calcd. for $C_{30}H_{48}O_4$: C, 76.22; H, 10.23. Found: C, 76.43; H, 10.63.

Methylation.—The acid genin (100 mg.) was dissolved in ether, and an excess of the ethereal solution of diazomethane was added to it. The solution was left overnight, and the excess of diazomethane was then removed on a water bath. The residue left, after the evaporation of the ether, was crystallized as needles from methanol, m.p. 224–227°. This showed unsaturation with tetranitromethane, $[\alpha]_D$ = +67.16°.

Anal.—Calcd. for $C_{31}H_{50}O_4$: C, 76.49; H, 10.36. Found: C, 75.68; H, 10.38.

Acetyl Methyl Ester.—The methyl ester (100 mg.) was acetylated with acetic anhydride and pyridine in the usual manner. It crystallized as colorless needles from methyl alcohol, m.p. 281–282°, $[\alpha]_D$ = + 80.20°.

Anal.—Calcd. for $C_{35}H_{54}O_6$: C, 73.64; H, 9.53. Found: C, 74.00; H, 9.57.

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Metal-Acid Complexes with Members of the Tetracycline Family
II. Development of Stable Preconstituted Parenteral Formulations

By EDWARD G. REMMERS, WILLIAM C. BARRINGER, GEORGE M. SIEGER, and ALBERT P. DOERSCHUK

Stable preconstituted liquid formulations suitable for intramuscular and intravenous administration have been prepared using aluminum-calcium-gluconate complexes of tetracycline and 6-demethylchlortetracycline. These formulations are well tolerated at therapeutic levels and give adequate blood levels. The most desirable preparations contain the antibiotic-aluminum-calcium-gluconate complex of molar ratio 1:3:1:6 in 50 to 75 per cent propylene glycol at pH 8.5. Such formulations retain their initial potency at room temperature and elevated temperatures for prolonged periods.

IN THE FIRST publication in this series (1), the pharmaceutical properties of metal-acid complexes with members of the tetracycline family were described. These properties were considered of sufficient interest to develop suitable parenteral formulations of these complexes. The purpose of this publication is to describe the development of stable preconstituted parenteral formulations with this class of preparations.

EXPERIMENTAL

Preparation of Metal-Acid Complexes.—The method of preparing metal-acid complexes has been described (1).

Preparation of Aqueous Propylene Glycol Formulation.—The metal-acid complex was dissolved completely with stirring in a fraction of the total amount of distilled water to be used. The desired amount of propylene glycol was then added. Sodium formaldehyde sulfoxylate was dissolved in a small amount of distilled water and was added to the formulation. The pH was elevated to 8.0–8.5 with monoethanolamine, and the formulation brought to the proper volume with distilled water. The solution was then filtered through a fine sintered-glass filter and sealed in ampuls under a nitrogen atmosphere.

Blood Level Studies.—The procedure for intramuscular administration has been described (1); the cephalic vein was used for intravenous administration.

RESULTS AND DISCUSSION

Screening Studies.—A large number of metal-acid complexes with varying constituents and molar *ratios* were prepared for evaluation by criteria of complex formation previously described (1), solubility studies at neutral and slightly alkaline pH's, alkaline stability studies (2), and blood level and blood pressure depression studies. These screening experiments indicated the existence of the following important relationships for these preparations: (*a*) on intramuscular and intravenous administration, the aluminum-calcium-gluconate complexes of demethylchlortetracycline (DMCTC) and tetracycline (TC) exhibited high blood levels; (*b*) for good complex formation, it is necessary to use a minimum of 3 molar parts of aluminum; (*c*) the solubility of the complex under neutral or slightly alkaline conditions is reduced as the molar ratio of calcium is increased; and (*d*) a minimal amount of calcium (approximately 1 molar part) is needed to impart enhanced alkaline stability to the complex and to prevent blood-pressure depressions after intravenous administration.

Because of these relationships, most of the formulation studies were performed on the DMCTC and TC-aluminum-calcium-gluconate complexes having a molar ratio of 1:3:1:6.

Blood Levels After Intramuscular Administration.—Table I presents the results of a blood level experiment designed to investigate the effect of propylene glycol content in a formulation containing DMCTC-aluminum-calcium-gluconate (molar ratio 1:3:1:6) following intramuscular administration in dogs. The data indicate that no statistically significant differences in blood levels [measured by area under the 24-hour blood-level curve, AUC (3)] could be detected among formulations ranging from 0 to 75% propylene glycol.

No gross irritation was noted at 4 and 7 hours following intramuscular administration. At 24 hours after administration, one dog from each group receiving 25, 50, and 75% propylene glycol limped slightly.

A second blood level experiment was designed to determine the effect of pH of the aqueous propylene glycol formulation on the blood levels obtained with the DMCTC-aluminum-calcium-gluconate complex (molar ratio 1:3:1:6) when administered intramuscularly to dogs. The results of this experiment are summarized in Table II. The data indicate that the pH of the formulation exerts a statistically significant effect on blood levels. As the pH of the formulation is lowered from 8.5 to 4.0, blood levels tend to increase. The major portion of the increase occurs between pH 5.5 and 4.0.

In this experiment, gross irritation was noted in only one dog which received the formulation at pH 4.0. At 4 and 7 hours following intramuscular administration, the dog limped but had completely recovered 24 hours afterward.

Blood Levels After Intravenous Administration.—Table III compares the results obtained with DMCTC-aluminum-calcium-gluconate complexes in 56% propylene glycol with a DMCTC·HCl-ascorbic acid formulation in dogs. The data indicate that the metal-acid complexes in the aqueous propylene gly-

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Tetracycline and 6-demethylchlortetracycline are marketed as Achromycin and Declomycin, respectively, by the American Cyanamid Co., Pearl River, N. Y.

TABLE I.—EFFECT OF PROPYLENE GLYCOL CONTENT ON BLOOD LEVELS[a] IN DOGS FOLLOWING INTRAMUSCULAR ADMINISTRATION OF A DMCTC-METAL-ACID COMPLEX

DMCTC-Aluminum-Calcium-Gluconate Complex (1:3:1:6), pH[b] 8.3	Hr. After Injection[c] 1	4	7	24	AUC, mcg./Hr./ml.	95% Confidence Limits
Propylene glycol, 75%	6.24	3.87	2.10	0.74	50.0	38.5–65.0
Propylene glycol, 50%	5.64	4.77	3.18	0.95	63.2	48.6–82.1
Propylene glycol, 25%	5.40	3.28	2.03	0.73	48.2	37.1–62.7
No propylene glycol	6.60	3.60	2.24	0.68	52.5	40.4–68.3

[a] Based on microbiological assays expressed as micrograms per milliliter tetracycline·HCl. Average of three dogs per group. [b] pH adjusted with monoethanolamine after addition of 0.5% sodium formaldehyde sulfoxylate. [c] Dose = 1.5 mg. DMCTC·HCl/lb. body weight.

TABLE II.—EFFECT OF pH ON BLOOD LEVELS[a] IN DOGS FOLLOWING INTRAMUSCULAR ADMINISTRATION OF DMCTC-METAL-ACID COMPLEX

DMCTC-Aluminum-Calcium-Gluconate Complex (1:3:1:6) in 70% Propylene Glycol	Hr. After Injection[c] 1	4	7	24	AUC, mcg./Hr./ml.	95% Confidence Limits
pH[b] 4.0	11.2	7.1	7.2	1.17	112.5	83.1–152.2
pH 5.5	8.0	4.35	3.8	0.71	71.0	52.5–96.1
pH 7.0	6.56	4.2	3.85	0.64	69.0	51.0–93.4
pH 8.5	4.0	5.25	3.6	0.78	60.8	44.9–82.3

[a] Based on microbiological assays expressed as micrograms per milliliter tetracycline·HCl. Average of three dogs per group. [b] pH adjusted with monoethanolamine after addition of 0.5% sodium formaldehyde sulfoxylate. [c] Dose = 1.5 mg. DMCTC·HCl/lb. body weight.

TABLE III.—BLOOD LEVELS[a] IN DOGS FOLLOWING INTRAVENOUS ADMINISTRATION OF DMCTC-METAL-ACID COMPLEX AND ASCORBIC ACID FORMULATION

DMCTC-Aluminum-Calcium Gluconate Complex in 56% Propylene Glycol, pH[b] 8.5	Hr. After Injection[d] 1	4	7	24	AUC, mcg./Hr./ml.	95% Confidence Limits
Molar ratio 1:3:1:6	51.7	29.7	18.2	2.67	407.4	345.5–451.4
Molar ratio 1:4:1:12	48.0	28.8	18.2	1.98	381.7	323.7–450.0
Molar ratio 1:4:2:9	57.0	36.0	22.0	4.65	492.0	417.3–580.1
DMCTC·HCl-ascorbic acid formulation[c]	7.95	7.65	6.80	1.54	117.7	99.8–138.8

[a] Based on microbiological assays expressed as micrograms per milliliter tetracycline·HCl. Average of three dogs per group. [b] pH adjusted with monoethanolamine after addition of 0.5% sodium formaldehyde sulfoxylate. [c] DMCTC·HCl, 960 mg.; ascorbic acid, 2.40 Gm. (natural pH in distilled water, 2.2 to 2.4). [d] Dose = 4 mg. DMCTC·HCl/lb. body weight.

TABLE IV.—STABILITY DATA FOR AQUEOUS PROPYLENE GLYCOL FORMULATIONS

DMCTC-Aluminum-Calcium-Gluconate (1:3:1:6) in 80% Propylene Glycol[a]	mg. DMCTC·HCl/ml. (Microbio Assay)	% of Initial		TC-Aluminum-Calcium-Gluconate (1:3:1:6) in 70% Propylene Glycol[a]	mg. TC·HCl/ml. (Microbio Assay)	% of Initial
Theoret.	20.0	...	Theoret.		35.0	...
Initial	20.8	100	Initial		38.2	100
37°, 1 wk.	20.1	97	42°, 2 mo.		34.3	90
37°, 2 wk.	20.0	96	23°, 2 mo.		37.7	99
37°, 3 wk.	21.2	102	23°, 4 mo.		35.6	93
37°, 5 wk.	19.9	96	23°, 8 mo.		36.3	95
37°, 6 wk.	18.9	91	23°, 12 mo.		36.5	95
37°, 7 wk.	20.4	98	23°, 18 mo.		38.6	101

[a] pH 8.5, adjusted with monoethanolamine after addition of 0.5% sodium formaldehyde sulfoxylate.

col formulation resulted in significantly higher blood levels than those obtained with the DMCTC·HCl-ascorbic acid formulation. Although the molar ratios of the DMCTC-aluminum-calcium-gluconate complexes varied somewhat, these differences were not sufficient to result in blood levels significantly different. Blood level studies after intramuscular administration of DMCTC complexes having the same molar ratios used in this study also did not show blood levels significantly different. Within the range studied, the molar ratio of the complex did not exert a significant effect on blood level by either intravenous or intramuscular routes of administration.

There were no gross signs of irritation following intravenous administration in the four formulations used.

Stability Studies.—Ampuls held at various temperatures and for various periods were submitted at regular intervals for microbiological assays. Table IV presents the results of stability studies on DMCTC and TC-aluminum-calcium gluconate complexes in the aqueous propylene glycol formulation. These data indicate that the formulations are stable

with respect to microbiological potency under the conditions studied.

Studies on modifications of the aqueous propylene glycol formulation indicate that pH and propylene glycol content are critical factors. If the propylene glycol content is at a level of 50% or greater at pH 8.0–8.5, there is little or no loss of microbiological potency after 9 weeks at 37°. If the pH ranges from 8.0 to 8.5, the formulation retains its potency after 5 weeks at 37°. Below this pH range, the formulation slowly loses activity, presumably through C.4 epimerization, the kinetics of which have recently been described (4).

SUMMARY

Stability studies indicated that stable preconstituted formulations suitable for parenteral administration can be prepared with DMCTC and TC-aluminum-calcium-gluconate complexes in 50–80% propylene glycol at pH 8.0–8.5. Both pH and propylene glycol content are important factors affecting the stability of the formulation. The degree of blood-level enhancement is not altered significantly by the percentage of propylene glycol in the formulation or by the molar ratios of the complexes within the range studied following intravenous and intramuscular administration. By either route, the DMCTC complexes in the aqueous propylene glycol formulation appeared to be well tolerated in dogs when administered at therapeutic levels.

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Some Pharmacological Properties of Polymyxin B Sulfate

By DARRELL L. WITT and J. P. LONG

Polymyxin B sulfate produces diastolic standstill followed by contracture in the isolated perfused rabbit heart. No increase in cardiac output was noted in the perfused frog heart. Large doses inhibit splanchnic nerve innervation to the perfused superior mesenteric artery of the cat.

SYSTEMIC ADMINISTRATION of polymyxin B sulfate has a potent bactericidal effect on Gram-negative bacilli, particularly *Pseudomonas aeruginosa*. However, because of nephrotoxic and central nervous system effects, such as vertigo and paresthesias, it has generally been limited to topical application. Although it is generally realized that the systemic use of this antibiotic is hazardous, reports in the literature describe its use in this manner (1–4). Timmerman *et al.* (5) have demonstrated the neuromuscular blocking action of polymyxin in the rabbit.

The present work describes the effect of polymyxin B on the isolated rabbit heart, on cardiac output measured by the modified Howell and Clark (6) frog heart preparation, and on the vascular resistance of the perfused superior mesenteric artery of the cat.

METHODS

The effect of polymyxin B sulfate on the perfused isolated rabbit heart was studied using the preparation described by Langendorf (7). Dutch rabbits weighing 2–3 Kg. were sacrificed by a blow at the base of the skull, and the heart was removed *via* a midline chest incision. A glass cannula, with side arm tube, was placed in the aorta, and the coronary vessels were perfused with oxygenated Locke-Ringer's solution previously warmed to 37°. All drugs were administered through the side arm tube of the glass cannula. Isotonic recordings were made using an ink recorder on kymograph paper. The action of potassium chloride, calcium chloride, and magnesium chloride on the isolated rabbit heart was evaluated before and after the administration of polymyxin B sulfate. The results are expressed as per cent changes of the systolic contraction.

The effect of polymyxin B sulfate on cardiac output in the frog *in situ* was evaluated by a modification of the Howell and Clark technique (6). *Rana pipiens*, weighing 200–250 Gm., were double pithed and the hearts exposed. After ligation of the right aortic arch cannulae were placed in the inferior vena cava and cardiac output measured by collecting the perfusate in graduated cylinders for 1-minute periods. The heart rate was also recorded.

The action of polymyxin B sulfate on the perfusion of the cat gut was evaluated as follows. Anesthesia was induced by pentobarbital sodium (35 mg./Kg.), and a tracheotomy was performed. For intravenous administration of drugs, the inferior vena cava was cannulated below the renal vein. Heparin sodium (5 mg./Kg.) was administered intravenously. The abdominal aorta was cannulated with Tygon tubing and a Sigmamotor pump (model T-8) maintained a constant volume flow into the superior mesenteric artery. To measure changes in perfusion pressure, a pressure transducer was connected between the pump and the artery. The superior mesenteric plexus was isolated, and bipolar silver electrodes were placed on these nerves. Supramaximal stimulation with a frequency of 20/second for 30 seconds was employed. Systemic blood pressure was measured by cannulation of the common carotid and recorded using an Offner dynograph (type 542). The effect of intra-arterial doses of polymyxin B sulfate varying from 1 to 50 mg. (total dose) was evaluated in this preparation.

The data were evaluated statistically using a Student *t* test (8).

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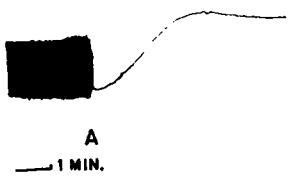


Fig. 1.—Effect of polymyxin B sulfate on the isolated rabbit heart. A, 10 mg. polymyxin B sulfate injected into the perfusing solution.

RESULTS

The effect of polymyxin B sulfate on the isolated rabbit heart was observed in 20 isolated heart preparations. Cardiac responses to polymyxin B sulfate are illustrated in Fig. 1. After the injection into the aortic cannula of 5 to 30 mg. of polymyxin B sulfate, a marked depression occurred within 30 seconds. The systolic contraction was 22 ±11 ($p < 0.01$) of the control contraction. After 3 to 8 minutes, a slowly increasing contracture occurred. The contracture was equal to 160 ±22 ($p < 0.01$) of the control systolic contraction.

After the administration of potassium chloride (0.1–0.3 ml. of a 30% solution) in a nonpolymyxin B sulfate treated heart, cardiac arrest was observed, and the heart became flaccid. Recovery was spontaneous in 20 experiments. The administration of 20 to 40 mg. of polymyxin B sulfate after recovery from potassium chloride produced cardiac arrest and contracture similar to that illustrated in Fig. 1.

At this time, repetition of the initial dose of potassium chloride produced an increase in contracture in 12 of 20 preparations and no change in the other eight. The average increase was 18%; this change was not significant ($p > 0.4$). In 10 experiments, 1 to 5 ml. of a 1:200 solution of magnesium chloride or 1 to 5 ml. of a 1:200 solution of calcium chloride also failed to produce relaxation or additional contraction after contracture had been produced by polymyxin B sulfate ($p > 0.5$).

In six isolated perfused frog hearts *in situ*, administration of increasing doses of polymyxin B sulfate (0.1–1.0%) produced only progressive depression, evidenced by the cardiac output. The average control heart rates were 52 ± 6.0, and the average cardiac output was 2.2 ± 0.5 ml./minute. After 5 minutes of infusion with polymyxin B sulfate prepared in the frog-ringer solution, the heart rates averaged 48 ± 8.0, and the average cardiac output was 0.7 ± 0.3 ml./minute. Difference in heart rate was not significant ($p > 0.5$), but the decrease in cardiac output was significant ($p < 0.01$). With continued infusion the hearts ceased to beat in diastole. These results suggest strongly that the mechanism of cardiac action is other than digitalis-like activity.

In 10 experiments using cats, when the superior mesenteric plexus was stimulated, an increase in perfusion pressure resulted. After the administration of 10 mg. of polymyxin B sulfate into the superior mesenteric artery, there was significant antagonism of the increase in vascular resistance produced by nerve stimulation. The increase in the mean blood pressure before polymyxin B was 109 ± 18 mm. Hg. After 10 mg. of the compound, nerve stimulation produced an elevation of 33 ± 14 mm. Hg. This reduction in response to nerve stimulation was significant ($p < 0.01$). The vascular response to 1 to 4 mcg. of norepinephrine was unaltered by this dose of polymyxin B sulfate.

DISCUSSION

The diastolic standstill and subsequent contracture produced by polymyxin B sulfate on the isolated rabbit heart may be due to the chelation of an essential ion by this polypeptide antibiotic. Loss of the ion may then account for the intracardiac block produced and subsequent cardiac arrest. However, the infusion of the calcium chloride, magnesium chloride, and potassium chloride failed to produce recovery after diastolic standstill and contracture had been produced by polymyxin B sulfate.

The possibility that polymyxin B sulfate may alter the permeability of the cellular membranes to sodium or other ions and thus prevent repolarization must also be considered. Finally, the contracture that takes place after diastolic standstill may be due to the direct action of polymyxin B sulfate on the contractile mechanism of the muscle cell.

The observation that polymyxin B sulfate did not antagonize the pressor response after the injection of norepinephrine into the superior mesenteric artery, but did depress the pressor response normally elicited by sympathetic nerve stimulation, suggests that polymyxin B sulfate may have an action at the presynaptic sympathetic nerve terminal. That the inhibition occurred within 1 minute after arterial injection of the antibiotic suggests that the mechanism is other than prevention of synthesis. A mechanism that prevents the release of norepinephrine from the nerve terminals would be consistent with the results reported above.

From the above discussion it is obvious that more experimental work must be done before any valid conclusion concerning the mechanism of action of polymyxin B on the isolated rabbit heart or on the sympathetic nerve endings to the cat vascular bed can be determined. However, these studies do illustrate that the polypeptide, polymyxin B sulfate, does possess various unusual cardiovascular properties.

SUMMARY

This report describes the effects of polymyxin B sulfate on the isolated rabbit heart, the perfused frog heart *in situ*, and the perfused superior mesenteric arterial bed. In the isolated rabbit heart, polymyxin B sulfate produces diastolic standstill and subsequent contracture; this is not reversed by the administration of potassium, calcium, or magnesium ions. Depression of cardiac output is noted *in situ* using perfused frog hearts. Vascular constriction induced by nerve stimulation was antagonized by polymyxin B sulfate.

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Method for Sterility Testing of Oils

By R. RUSSOMANNO and E. G. WOLLISH

A reliable procedure was needed for the sterility testing of oils since the present U.S.P. method, primarily designed for aqueous solutions, did not always yield unequivocal results. A membrane filtration method together with the necessary equipment was developed. The procedure, which is simple and rapid, is useful for the detection of contaminations of oils (except those of high viscosity) with viable organisms, even at low count levels.

THE STERILITY testing of oils used as a vehicle for drugs has presented problems for the bacteriologist for quite some time. Eisman *et al.* (1) have studied the preservation of vegetable oils by chemical agents and determined the number of surviving microorganisms by plate counts. In the present investigation, the U.S.P. XVI method (2), designed chiefly for aqueous preparations, has not proved very satisfactory for the sterility testing of oils; another procedure using detergents (3) has shown limitations due to poor growth conditions. In several publications, Gay (4) has demonstrated the advantages of membrane filtration for the sterility testing of injectables. Membrane filtration, proposed by Holdowsky (5), has now become the official procedure for the testing of antibiotics (6). At a November 1963 meeting in London, Gay presented details of a recent modification of a membrane filtration apparatus which completely eliminates manual transfers (7). Sokolski and Chidester (8) described a membrane filtration method for petrolatum-based ointments. This unique method utilizes isopropyl myristate as solubilizer for the ointment base.

EXPERIMENTAL

A simple method has been developed which permits the separation of viable microorganisms by membrane filtration. The filtration is followed by washing and removal of the oil with an aqueous solution of Triton X 100, a detergent that was found nontoxic in contact with several types of microorganisms. The membrane filter is punched out, and parts of it are incubated in both media fluid thioglycollate U.S.P. and fluid sabouraud U.S.P.

The method has been applicable to a variety of oils (sesame, peanut, mineral, and liquid coconut oil), except for those of high viscosity, like castor oil. The procedure has been found dependable in about two years of usage in our laboratory.

Equipment.—The assembled filter unit (Millipore or equivalent) (Fig. 1) without membrane is autoclaved for 30 minutes at 121° or 10 minutes at 132°. A Cornwall pipeting syringe (BD) (Fig. 1) is assembled as a pumping device to transfer oil samples into the filter unit. The pumping apparatus is sterilized in a manner as above. The sterile units are dried before use. (Our laboratory utilizes a high-vacuum sterilizer.) The bacteriological membrane filters (Millipore), 0.45-μ porosity, 47-mm. diameter, are sterilized for 10 minutes at 121°. Before the test, a filter membrane is aseptically placed in the unit. Membrane cutting devices and forceps are prepared

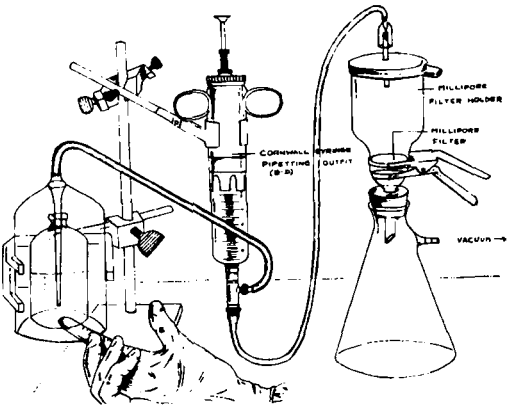


Fig. 1.—Assembled filtration unit.

and sterilized prior to testing. An approximate quantity of 100 ml. of fluid thioglycollate and sabouraud liquid medium (Difco or Baltimore Biological Labs.) with 0.1% polysorbate 80 U.S.P.[1] added is dispensed into 38 × 200-mm. test tubes. The tubes are closed with metal closures and sterilized for 20 minutes at 121°. Aqueous solutions of the detergent Triton X 100 (Rohm and Haas), 0.1%, and of peptone (Difco or BBL), 0.1%, are prepared and sterilized for 20 minutes at 121°.

Procedure.—A 200-ml. sample of oil is pumped aseptically (Cornwall syringe BD) into a filter unit (Fig. 1). A vacuum source (approximately 550 mm. Hg) is attached to the filter unit. The oil sample is then filtered through the bacteriological membrane. The membrane is washed by filtration with two 200-ml. portions of 0.1% Triton X 100 to remove residual oil. The Triton washings are immediately followed by a single wash with 200 ml. of 0.1% peptone. With a sterile, circular punch, the center area (approximately 17.5 mm. diameter) is cut out, as described by Holdowsky (5), and transferred aseptically to a 38 × 200-mm. tube containing approximately 100 ml. of fluid thioglycollate medium with 0.1% polysorbate 80.

The outer portion of the membrane is transferred aseptically into a similar tube containing approximately 100 ml. of sabouraud liquid medium with 0.1% polysorbate 80. A fluid thioglycollate medium tube containing the center portion of the membrane is incubated at 30–32° for 10 days. The tube containing the outer portion of the membrane in sabouraud liquid medium is incubated at 22–25° for 10 days. The culture tubes are gently agitated and observed for growth at regular intervals throughout the incubation period.

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Note added in proof: The following additional references are pertinent to the text: Avis, K. E. ,and Gershenfeld, L., THIS JOURNAL, 44, 682(1955); Avis, K. E., and Gershenfeld, L., *Am. J. Pharm.*, 129, 11,234(1957).

[1] Marketed as Tween 80 by the Atlas Chemical Industries Wilmington, Del.

TABLE I.—FILTRATION RESULTS OF OIL CONTAMINATED WITH LOW COUNT LYOPHILIZED CULTURES

Microorganism	Days 1	3	5	7
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[a] 0, No growth. [b] +, Growth.

DISCUSSION

Toxicity Studies on Triton X 100.—The detergent Triton X 100 at 0.1% concentration was nontoxic when in contact with vegetative forms of bacteria for several hours. The surfactant may be used at room or temperatures of 45 ±1° during the membrane washing phase. An additional wash with peptone 0.1% is used to remove the excess detergent and sustain viability of microorganisms that were impinged on the membrane, thereby maintaining the sensitivity of the method (9, 10).

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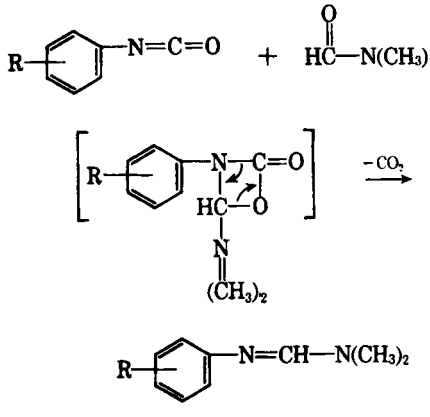
Convenient Synthesis of *N,N,N'*-Trisubstituted Formamidines

By JOHN L. NEUMEYER*

The applicability of a method for preparing trisubstituted formamidines by treating isocyanates with an excess of formamides under neutral conditions at 150° was examined. This amidine-forming reaction was limited to the use of aryl isocyanates when treated with *N,N*-dimethylformamide as the solvent.

RECENT DISCLOSURE of the reaction of *p*-toluenesulfonyl isocyanate (1) and phenyl isocyanate (2) with *N,N*-dimethylformamide (DMF) to form amidines prompted a further investigation into the scope of this facile method for the preparation of trisubstituted formamidines. The synthesis of amidines and formamidines by the interaction of substituted amides with anilines in the presence of phosphorus pentachloride (3–5) or phosphorus oxychloride (6) has been reported previously. More recently, symmetrical *N,N'*-diarylacetamidines were prepared by heating 2 moles of an aromatic amine with 1 mole of ethyl orthoacetate in the presence of *p*-toluenesulfonic acid (7) or, more effectively, acetic acid (8).

In the present investigation, the applicability of a method for preparing trisubstituted formamidines by treating isocyanates with an excess of formamides under neutral conditions at 150° was examined. This method was limited to the use of aryl isocyanates when treated with DMF as the solvent at 150° under neutral conditions. One mole of carbon dioxide was evolved per mole of isocyanate consumed in the reaction. By the removal of unreacted DMF, the *N,N*-dimethyl-*N'*-aryl formamidines could be isolated in 75–98% yield in a high state of purity.



The cyclic mechanism postulated by King (1) for the formation of the sulfonylamidine and by Weiner (2) for formamidine is supported by observations that groups on the benzene ring which tend to increase the electrophilicity of the isocyanate increase the rate of the reaction, whereas electron-donating groups have a tendency to decrease the rate of formation of the formamidine. 2,4,5,6-Tetrachlorophenylene diisocyanate readily formed the bis formamidine under the same reaction conditions. However, formamide failed to yield a formamidine with phenyl isocyanate under the conditions of our experiment, since formanilide was the isolated product.

Similarly, the treatment of alkyl isocyanates with *N,N*-dimethylformamide or aryl isocyanates with

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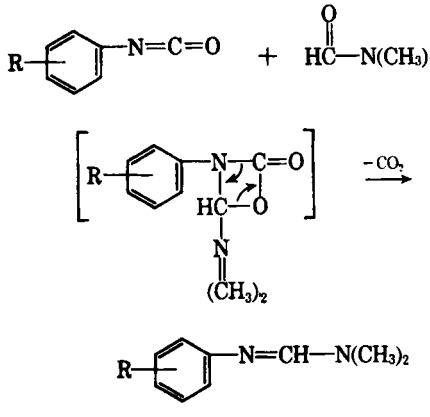
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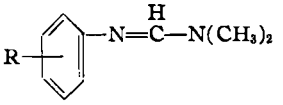


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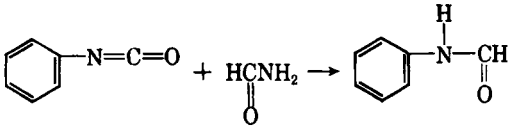
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TABLE I.—*N,N,N'*-TRISUBSTITUTED FORMAMIDINES



R	Salt	M.p., or B.p., °C., mm.	n_D^{25}	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
H	...	61–63 (0.01)[a]	1.5936	...	...	...	...	...	...
H	HCl	234–235[b]	...	...	...	...	...	...	...
H	CH_3I	143–144	...	...	...	...	...	...	...
3-Cl	...	120 (0.4)	1.6041	59.18	59.11	6.07	6.38	15.34	15.08
3-Cl	HCl	234–235.5	...	49.33	49.60	5.52	5.64	12.79	13.06
3-Cl	CH_3I	208–209	...	37.00	36.72	4.35	4.38	...	...
2-Cl	...	157 (14)	1.6009	59.18	59.18	6.01	6.04	...	...
2-Cl	HCl	202–203	...	49.33	49.48	5.52	5.33	...	...
2-OCH_3	...	136 (0.4)	1.5895	67.38	67.19	7.92	8.03	15.72	16.08
2-OCH_3	HCl	208–209	...	55.94	55.78	7.04	6.70	13.05	13.16
2-OCH_3	CH_3I	140–142	...	41.26	41.30	5.35	5.36	...	...
3-NO_2	...	153 (0.35)	1.6250	55.95	56.23	5.74	6.05	...	...
3-NO_2	HCl	260–261	...	47.05	47.05	5.27	5.47	...	...
4-Cl	...	160 (14)	1.6024	59.18	58.90	6.01	6.02	...	...
4-Cl	HCl	232–233	...	49.33	49.17	5.52	5.10	...	...
4-NO_2	...	77–78	...	55.95	55.70	5.74	5.78	...	...
2-NO_2	...	152–157 (0.4)	1.6153	55.95	55.78	5.74	5.74	...	...
3,4-diCl	...	140–148 (0.55)	1.6218	49.79	49.80	4.64	4.77	12.91	12.52
3,4-diCl	HCl	164–165	...	42.63	42.31	4.37	4.72	11.05	11.06
3,4-diCl	CH_3I	188–189	...	33.45	33.17	3.65	3.91	7.80	7.84
2,4,5,6-tetra Cl-3-N=CH—$N(CH_3)_2$	...	157.5–158.5	...	40.47	40.28	3.96	4.04	...	...
2,4,5,6-tetra Cl-3-N=CH—$N(CH_3)_2$	HCl	218–219[c]	...	31.00	31.34	4.34	4.24	...	...

[a] Lit. (2) b.p. 68–71° (0.05 mm.) and (4) 78–80° (0.1 mm.). [b] Lit. (6) m.p. 230–235°. [c] Dihydrate.



N,N-diethylformamide at 150° failed to yield the desired formamidines.

Table I summarizes the data on the reaction products obtained from the aryl isocyanates.

EXPERIMENTAL[1]

The following example is representative of the method employed for the preparation of the formamidines and their salts.

N,N-Dimethyl-N'-3-chlorophenylformamidine.—A mixture of 51.5 Gm. (0.336 mole) of *m*-chlorophenyl isocyanate (Eastman White Label) and 161 Gm. (2.21 moles) of redistilled DMF was refluxed for 4 hours in a 250-ml. flask equipped with a condenser; provision was made for the absorption of any evolved carbon dioxide in aqueous potassium hydroxide solution in a 250-ml. conical flask connected to the condenser with a short length of rubber tubing. The titration of the evolved CO_2 served as an indication of the rate of the reaction and as a determination of when the reaction had been completed. The major portion of unreacted DMF was removed by distillation. The residue was then distilled at reduced pressure to provide 54 Gm. (89% yield) of analytically pure product, b.p. 120° (0.4 mm.), n_D^{25} 1.6041.

The hydrochloride salt was prepared by treating an ethereal solution of the formamidine with gaseous hydrogen chloride for 5 minutes; filtration of the resulting precipitate and recrystallization from ethanol yielded a white powder, m.p. 234–235.5°.

The methiodide was prepared by treating 10.0 Gm. (0.055 mole) of the amine with 15.6 Gm. (0.11 mole) of methyl iodide in 30 ml. of isopropyl alcohol in a 100-ml. flask equipped with a reflux condenser and a drying tube and heated at reflux for 60 hours. To the flask was added 200 ml. of ether, and the resulting precipitate was filtered and recrystallized from isopropyl alcohol to yield 12.0 Gm. of white crystals, m.p. 208–209°. Concentration of the mother liquor yielded an additional 2.8 Gm. of product, m.p. 204–209°.

[1] Both the melting points and the boiling points were uncorrected. All melting points were obtained in a Thomas-Hoover silicone-filled capillary melting point apparatus. The technical assistance of Mr. Richard C. Pharo for the experimental portion of this work and of Mr. John E. Zarembo and his staff in carrying out the analyses reported is gratefully acknowledged.

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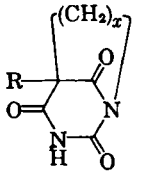
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Reduction of Barbiturates under Hydroboration Conditions

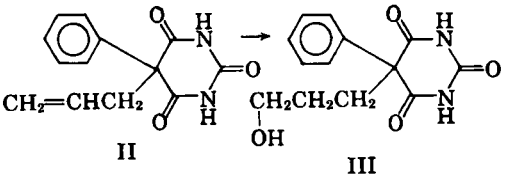
By EDWARD E. SMISSMAN, ALICE JEAN MATUSZAK, and CLINTON N. CORDER

An attempt to utilize hydroboration conditions on 5-phenyl-5-allyl barbituric acid afforded unexpected reduction of the barbituric acid nucleus. Further investigation of this reaction indicated sodium borohydride-boron trifluoride etherate will reduce 5-phenyl-5-ethyl barbituric acid to 5-phenyl-5-ethyl-2,4-hexahydropyrimidinedione (V) and 5-phenyl-5-ethyl-2-hexahydropyrimidinone (VI) in a stepwise manner.

During the synthesis of bridged barbiturates of the type I as potential anticonvulsant agents, an attempt was made to prepare 5-phenyl-5-(3-hydroxypropyl) barbituric acid (III) from 5-phenyl-5-allyl barbituric acid (II) by the hydroboration procedure of Brown (1).



I R = alkyl, aryl; $x = 0, 1, 2$, etc.



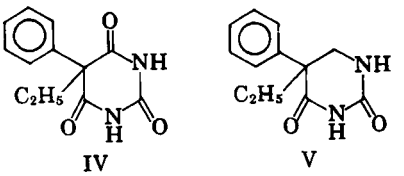
II III

The normal hydroboration procedure failed to give the desired product, and an investigation of this reaction was undertaken.

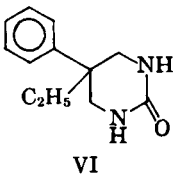
Phenobarbital (5-phenyl-5-ethylbarbituric acid) (IV) was subjected to hydroboration conditions and gave 5 - phenyl - 5 - ethyl - 2,4 - hexahydropyrimidinedione (V). The extension of this reduction to preparation of the mono- and diketo compounds from the corresponding barbiturates appeared feasible. The reduction of phenobarbital only proceeded using the conditions described by Brown (1). Sodium borohydride in absolute alcohol, tetrahydrofuran, or diglyme would not reduce the molecule, even though the liberation of hydrogen was observed. Sodium borohydride with hydrochloric acid in tetrahydrofuran was also ineffective (2). The possibility existed that diborane was the reducing agent; however, this agent in diglyme and tetrahydrofuran failed to reduce phenobarbital.

In the normal hydroboration procedure, oxidation to an alcohol is accomplished using sodium hydroxide and hydrogen peroxide. No reduction product was obtained until this procedure was invoked. If either sodium hydroxide or hydrogen peroxide was omitted, no product was obtained. If sodium phenobarbital rather than the acid was employed in the initial reaction, no reduction occurred. The application of heat to the reaction mixture had no effect on yield.

The stepwise reduction of phenobarbital proceeded smoothly to reduce two carbonyl groups but stopped with the formation of 5-phenyl-5-ethyl-2-hexahydropyrimidinone (VI). An excess of sodium borohydride and boron trifluoride-etherate did not increase the yield or result in the formation of the nonoxygenated product.



IV V

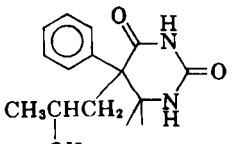


VI

Marshall (3) was able to prepare the 2-hexahydropyrimidinone (VI) utilizing lithium aluminum hydride.

The reaction was attempted on two barbiturates not containing a phenyl ring, 5,5-diethyl-1-methylbarbituric acid, and 5-ethyl-5-(1-methylbutyl) barbituric acid. Under a variety of conditions, including sodium borohydride in the presence of aluminum chloride (4), no reaction occurred.

The product obtained in the attempted hydroboration of allylphenylbarbituric acid (5-phenyl-5-allylbarbituric acid) (II) contained three atoms of oxygen rather than the predicted four atoms. The infrared spectrum of this compound showed a band at 7.27 μ attributable to C-methyl absorption, and the compound gave a positive iodoform test. It would be expected that side-chain hydroxylation and the reduction of the 6-keto function in the nucleus would give a compound capable of forming a dibenzoate. When the hydroboration product was treated with benzoyl chloride, a dibenzoate was obtained. Infrared analysis showed a band at 5.68 μ, which can be assigned to an ester carbonyl, and a band at 8.04 μ that could be due to the grouping C—O—C. This evidence leads to the conclusion that the product is 5-phenyl-5-(2-hydroxypropyl)-2,4-hexahydropyrimidinedione (VII).



VII

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The mechanism and unique specificity of this reaction will be investigated further.

EXPERIMENTAL[1]

5 - Phenyl - 5 - ethyl - 2,4 - hexahydropyrimidinedione (V).—To a stirred solution of 2.8 Gm. (0.073 mole) sodium borohydride in 85 ml. of diglyme maintained under nitrogen was added 23.2 Gm. (0.1 mole) of 5-phenyl-5-ethyl barbituric acid. Immediate effervescence was observed. A solution of 8.0 Gm. (0.056 mole) of boron trifluoride etherate in 15 ml. of diglyme was then added dropwise over a 40-minute period. The mixture was stirred 2.5 hours at room temperature and 0.5 hour at 100°. The clear solution was cooled and 20 ml. of water added in 5-ml. portions. Sodium hydroxide (4.8 Gm. in 40 ml. of water) was added to the frothing mixture as one portion. Hydrogen peroxide (30%, 40 ml.) was slowly added in a manner which caused gentle refluxing. The mixture was allowed to stand 12 hours at room temperature and crystals formed, m.p. 255–260°. The crystals were filtered, dissolved in 5% sodium hydroxide, and precipitated by the addition of 5% hydrochloric acid. The precipitate was recrystallized from ethanol to give 6.24 Gm. (28.6%) of a crystalline material, m.p. 268–269°. The product was identical to a known sample (5) as shown by infrared spectrum and mixed melting point.

Anal.—Calcd. for $C_{12}H_{14}N_2O_2$: C, 66.03; H, 6.47; N, 12.84. Found: C, 65.81; H, 6.69; N, 12.72.

5 - Phenyl - 5 - ethyl - 2 - hexahydropyrimidinone (VI).—Using the general procedure described above, 5.6 Gm. (0.147 mole) of sodium borohydride and 32.0 Gm. (0.224 mole) of boron trifluoride etherate was allowed to react with 23.2 Gm. (0.1 mole) of 5-phenyl-5-ethyl barbituric acid. The cooled reaction mixture was treated with 40 ml. of water, sodium hydroxide (9.16 Gm. in 80 ml. of water), and hydrogen peroxide (30%, 80 ml.) and allowed to stand 12 hours at room temperature. The mixture separated into two layers. The upper layer was decanted, diluted with water to the cloud point, and allowed to stand 24 hours at room temperature. The resulting precipitate was crystallized from ethanol-skellysolve B to give 5.6 Gm. (27.4%) of the desired product, m.p. 195–197°.

Anal.—Calcd. for $C_{12}H_{16}N_2O$: C, 70.56; H, 7.89; N, 13.71. Found: C, 70.34; H, 7.82; N, 13.28.

The product was identical in melting point and infrared spectrum to a sample prepared by the method of Marshall (3).

Hydroboration of 5-Phenyl-5-allylbarbituric Acid (II).—To a solution of 1.9 Gm. (0.05 mole) of sodium borohydride in 50 ml. of diglyme was added 2.62 Gm. (0.017 mole) of anhydrous aluminum chloride in 50 ml. of cold diglyme. A solution of 24.4 Gm. (0.1 mole) of allylphenylbarbituric acid in 50 ml. of diglyme was added dropwise over a period of 1 hour, after nitrogen was allowed to sweep through the apparatus for 15 minutes. The reaction mixture was allowed to stir at room temperature for 1 additional hour, then heated at 100° for 1 hour. After the reaction cooled to room temperature, 10 ml. of water was added in small portions. A solution of 2.4 Gm. of sodium hydroxide in 20 ml. of water was added, followed by 16 Gm. of 30% hydrogen peroxide. After standing overnight, the solution was filtered and the filtrate acidified with 85% phosphoric acid to pH 2. Two layers separated, with crystals forming at the interface. After standing, 5.31 Gm. (19%) of white crystalline material was obtained, m.p. 310° dec. Recrystallization from absolute alcohol gave crystals, m.p. 316–317° dec.

Anal.—Calcd. for $C_{23}H_{16}N_2O_3$: C, 62.89; H, 6.50; N, 11.28. Found: C, 63.35; H, 6.34; N, 11.25.

Principal absorbance bands in the infrared occurred at 5.85 μ (broad, single) and at 7.27 μ.

The filtrate was concentrated until it became viscous, water was added, and the mixture extracted with ether. The ether solution was dried over magnesium sulfate and distilled to leave a residue, which on crystallization, afforded 9.4 Gm. (0.039 mole) of allylphenylbarbituric acid.

Reaction of Allylphenylbarbituric Acid Hydroboration Product with Benzoyl Chloride.—A mixture of 0.213 Gm. (8.0×10^{-4} mole) of the allylphenylhydroboration product, 24 drops of benzoyl chloride, and 5 ml. of pyridine was heated at 100° for 1 hour and allowed to stand overnight. Distillation of the solvent *in vacuo* left an orange oil residue. Treatment of the oil with 5% sodium bicarbonate solution, followed by ether extraction and evaporation of the ether, produced an oil. Treatment of the oil with concentrated hydrochloric acid and extraction with ether, followed by distillation of the ether, afforded a solid. This solid, on recrystallization from ethanol, yielded 0.196 Gm. (54%) of crystals, m.p. 173.5–174.5°.

Anal.—Calcd. for $C_{27}H_{24}N_2O_5$: C, 71.04; H, 5.30; N, 6.14. Found: C, 71.16; H, 4.85; N, 6.03.

Principal bands in the infrared (Nujol mull) were at 5.68 μ (sharp), 5.85 μ (broad with a shoulder), and at 8.2 μ.

[1] Melting points were determined on a Thomas-Hoover apparatus and were corrected. Analyses were performed by Weiler and Strauss Microanalytical Laboratory, Oxford, England. The barbituric acids were dried *in vacuo* for 12 hours. The solvents used were carefully dried and distilled prior to use. Sodium borohydride and boron trifluoride-etherate were used without treatment.

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Coumarins III. Identification of the Lactone of *Leptotaenia multifida*

By PREM K. GUPTA and TAITO O. SOINE

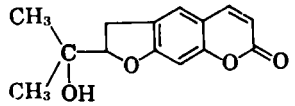
A lactone, m.p. 161.5°, originally isolated from *Leptotaenia multifida* Nuttall by Lloyd and Jenkins in 1942 and assigned the formula $C_{14}H_{14}O_4$ has been identified as the coumarinic aglycone, columbianetin.

THE ISOLATION of a white crystalline compound, m.p. 161.5°, from the roots of *Leptotaenia multifida* Nuttall was reported in 1942 by Lloyd and Jenkins (1). No structural assignment was made by these authors, although they suggested that it was a hydroxylactone with an empirical formula of $C_{14}H_{14}O_4$. These authors further suggested that it was possibly a coumarin—isomeric with, but not identical to, nodakenetin (I) the aglycone from the glycoside, nodakenin, which is obtained from *Peucedanum decursivum* (Miq.) (2). Inasmuch as the present authors were able to obtain a modest supply of the roots of this plant, the decision was made to examine the structure of the uncharacterized lactone.

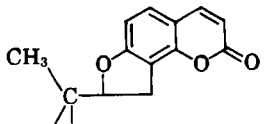
In the present study, the lactone reported by Lloyd and Jenkins was isolated only after acid hydrolysis of the aqueous extract, contrasted to the experience of the earlier authors, who obtained the same compound directly from the plant material. The conclusion must be made that the lactone exists in the plant as a glycoside and not as the free aglycone; it appears that their isolation had been of an artefact resulting from hydrolysis during some stage of collection, storage, or extraction. The aglycone has been identified as columbianetin (II), recently described by Willette and Soine (3), by a variety of comparisons with an authentic sample. The D-glucoside of columbianetin, columbianin, also described by Willette and Soine (3), has been shown to be the glycoside present in the roots in the present study by comparing it and its acetate with authentic samples.

The acetate of the aglycone exhibited dimorphism, evidenced by the fact that the lower melting acetate, reported by the earlier authors and Willette and Soine, was converted to the higher melting one simply by melting a sample of the former. The plant also contained a fair amount of sucrose in conformity to the findings of Lloyd and Jenkins. However, the sucrose present forms an interesting molecular complex with columbianin in the proportion of 7.5 parts sucrose to 1 part columbianin. This was not separable by crystallization but, on acetylation, both columbianin acetate and sucrose octaacetate were obtained. It appears that during the slow concentration of an extract containing both components, excess sucrose crystallizes out without columbianin but that, as a critical concentration of both sucrose and columbianin is reached, the molecular complex crystallizes preferentially until the sucrose concentration is reduced sufficiently to permit the columbianin to crystallize alone. The character of the molecular complex was confirmed by comparing it to a mixture of sucrose and columbianin in the requisite proportions which had been crystallized from methanol. The synthetic complex behaved in a manner identical to the one obtained from the natural source.

It is interesting that columbianetin, the angular isomer of nodakenetin and marmesin, had escaped detection until the studies of Willette and Soine (3) on *Lomatium columbianum* Math. and Const. Now, however, it has been isolated in the present study as well as from *Lomatium nuttallii* (A. Gray) (4), and it appears that it may be a common constituent in the *Lomatium* genus. We are examining other *Lomatium* species to confirm this.



I



II

EXPERIMENTAL

Plant Material.—The plant material was obtained as tuberous roots,[1] which were ground to a suitable state of subdivision in a Jacobsen laboratory grinder designed to keep frictional heat to a minimum.

Extraction.—The ground plant material (600 Gm.) was exhaustively extracted (10 days) in a Soxhlet apparatus with 95% ethanol, after which the solvent was removed from the extract under reduced pressure. The dark oily residue was then extracted several times with boiling water (4 L.). The aqueous extract was filtered and allowed to concentrate slowly on a steam bath. No crystalline material was obtained, in contrast to the experience of Lloyd and Jenkins (1).

One-fourth of the above aqueous extract was extracted thoroughly with ether, and the combined ethereal extracts were then concentrated to a convenient volume. The ethereal solution was then extracted with 5% aqueous sodium hydroxide solution, which was subsequently acidified and exhaustively re-extracted with ether. Although a small amount of fluorescent material apparently was present in the ethereal extract, it could not be induced to crystallize by slow evaporation and chilling of the solution. The remaining portion of the concentrated extract was combined with the aqueous phase from the above ether extraction and

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Previous paper: Willette, R. E., and Soine, T. O., THIS JOURNAL, 53, 275(1964).

[1] The collection of these roots was made by Dr. T. G. Call, California State Polytechnic College, San Luis Obispo, Calif., on June 19, 1956, in the neighborhood of Missoula, Mont., at an elevation of 3400 ft. We are indebted to Dr. Call for the original identification and to Dr. G. B. Ownbey, Botany Department, University of Minnesota, Minneapolis, for confirmation of the identity. It may be noted that the modern nomenclature for this plant is *Lomatium dissectum* var. *multifidum* (Nutt.) Math. and Const. A specimen has been deposited in the herbarium of the Botany Department, University of Minnesota, Minneapolis.

diluted with water. To this solution was added enough concentrated hydrochloric acid to make it approximately 10% in HCl; it was then refluxed for 30 minutes and filtered immediately before cooling. The filtrate, on cooling, deposited fine brownish needles which were recrystallized several times from boiling water to yield 1.6 Gm. of white needles, m.p. 161–163°. A mixed melting point with authentic columbianetin[2] failed to depress the melting point.

The acetate of the compound was made by refluxing it (200 mg.) with pyridine (5 ml.) and acetic anhydride (5 ml.) for 6 hours, after which the solution was poured into ice cold water (50 ml.). The dark crystalline product was recrystallized several times from boiling water to yield 90 mg. of white needles, m.p. 133.5–135°. A mixed melting point with authentic columbianetin acetate[3] was 128–131°. On allowing the melt to cool and again taking the melting point, the melting point was 133–135°.

The respective specific rotations, ultraviolet spectra, and infrared spectra of the lactone and columbianetin were determined and were virtually identical.

Isolation of Sucrose and Glycoside-Sucrose Complex.—A fresh quantity (500 Gm.) of the powdered root was extracted successively with Skellysolve B, ether, chloroform, and methanol, respectively, in a Soxhlet apparatus; the marc was air-dried between each extraction. The methanol extract[4] became turbid after 2 hours of extraction, at which time it was replaced by fresh solvent. After another 8 hours, the again turbid methanol extract was replaced by fresh methanol which extracted the marc in 24 hours. On cooling, a white crystalline compound, m.p. 165–190° dec., was deposited in the first extract and a brown inorganic material in the second and third extracts. The white crystalline material was identified as sucrose by conversion to the octaacetate, m.p. 84–88°, which when mixed with authentic sucrose octaacetate (m.p. 84–88°)[5] failed to depress the melting point. The filtrate from the first extract was diluted with methanol and allowed to concentrate at room temperature. After a few days, a yellowish-white amorphous substance separated which was filtered off and washed with cold methanol. The crude material, m.p. 186–225°, gave a positive Molisch test and reduced Fehling's solution after hydrolysis with 10% hydrochloric acid. Recrystallization of the crude material from methanol did not improve the melting point. Chromatography through silica gel and elution with water likewise failed to improve the melting point, although the color was removed. It was observed that an aqueous solution, when spotted on filter paper and examined in the dark under ultraviolet light, gave a brilliant blue fluorescence.

A portion (500 mg.) of the substance was boiled with 10% sulfuric acid for 20 minutes and filtered before cooling. On cooling, the filtrate deposited a crystalline material (70 mg.), which was recrystallized from boiling water to give white crystals, m.p. 160–163°. A mixed melting point with authentic columbianetin failed to depress the melting point.

The above glycosidic compound (1 Gm.) was refluxed with acetic anhydride (20 ml.) and sodium acetate (1 Gm.) for 3 hours. The reaction mixture was diluted with glacial acetic acid (10 ml.) and allowed to stand in the refrigerator overnight. A white crystalline residue (150 mg.) was deposited and was recrystallized from boiling water, m.p. 217–222°. A mixed melting point with authentic columbianin acetate[6] showed no depression of the melting point. The acidic mother liquor from the above white crystalline residue was concentrated under reduced pressure until all volatile material had been removed; then the residue was extracted with benzene. The benzene extract was dried over anhydrous sodium sulfate and, upon removal of the solvent, yielded a semisolid mass. This was dissolved in ethanol and diluted with water to a slight turbidity. On standing, a finely crystalline material (600 mg.) separated, m.p. 84–88°. A mixed melting point of this material with authentic sucrose octaacetate showed no depression.

A comparison of the ultraviolet spectrum of the original white glycosidic material (m.p. 186–225°) to that of authentic columbianin showed identical maxima and minima but with a greatly reduced intensity. The intensity of absorption was 15% that of pure columbianin.

A mixture of 15% columbianin and 85% sucrose showed an identical melting point and ultraviolet spectral behavior after crystallization from methanol.

Isolation of the Glycoside.—After removing inorganic material from the second and third methanolic extracts, they were mixed together and allowed to concentrate slowly at room temperature. After several days, an amorphous white material was separated which was purified by elution with water through a silica gel column to give a white crystalline material, m.p. 273–275.5°. A mixed melting point determination with authentic columbianin[7] showed no depression of the melting point.

A small quantity of the white crystalline material, on hydrolysis with boiling 10% sulfuric acid, gave a compound identical to columbianetin in all respects.

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[2] Previously isolated and characterized in these laboratories (3).
[3] The melting point reported by Willette and Soine (3) for columbianetin acetate was 127.5–128.5°, and that reported by Lloyd and Jenkins for the acetate of their lactone was 126–127° (1). An authentic sample of the former was melted and allowed to resolidify. On remelting, this sample showed a melting point change to 133–135°.
[4] Work on the other extracts is in progress.
[5] Prepared by acetylation of authentic sucrose with acetic anhydride and sodium acetate in the conventional manner.
[6] Previously prepared in these laboratories (3), m.p. 219–221°.
[7] Previously isolated in these laboratories, m.p. 274–275°.

Potential Antiradiation Compounds II. Extension of the Nitro-Thiolacetate Route

By K. VENKATRAMANA BHAT and WALTER C. MCCARTHY

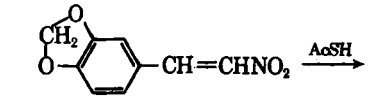
Thiolacetic acid was added to 3,4-methylenedioxy-ω-nitrostyrene to give 1-(3′,4′-methylenedioxyphenyl)-1-acetthio-2-nitroethane which was reduced with lithium aluminum hydride to produce 1-(3′,4′-methylenedioxyphenyl)-1-mercapto-2-aminoethane. Other substituted nitrostyrenes containing the 3,4-dimethoxy- and 3-methoxy-4-acetoxy groups also reacted with thiolacetic acid to give the corresponding addition products, but 3-acetoxy-4-methoxy-ω-nitrostyrene underwent an anomalous reaction with the formation of elemental sulfur. 1-Phenyl-2-nitro-1-propene added thiolacetic acid to give 1-phenyl-1-acetthio-2-nitropropane, but the latter compound was cleaved by lithium aluminum hydride to benzyl mercaptan and ethylamine.

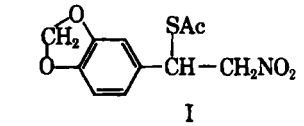
CONTINUED INTEREST in compounds containing a mercapto group and an amino group on adjacent carbon atoms has prompted us to investigate the possible extension to substituted analogs of the synthetic route previously reported (1) by means of addition of thiolacetic acid to ω-nitrostyrene, followed by reduction with lithium aluminum hydride.

The initial reaction of the sequence—addition of thiolacetic acid across the double bond—proceeded smoothly with the next higher side chain homolog, 1-phenyl-2-nitro-1-propene, to give 1-phenyl-1-acetthio-2-nitropropane (IV).[1] Ring-substituted ω-nitrostyrenes were also investigated, and those with the 3,4-methylene-dioxy, 3,4-dimethoxy, and 3-methoxy-4-acetoxy groups all gave the expected thiolacetate addition product. 3-Acetoxy-4-methoxy-ω-nitrostyrene underwent an anomalous reaction with thiolacetic acid to give a gum which could not be crystallized. Isolation of elemental sulfur from this reaction mixture indicated that an oxidation reduction reaction had taken place.

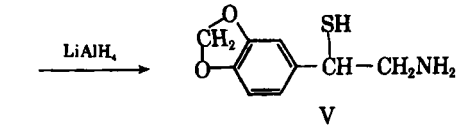
In the second reaction of the sequence, a typical ring-substituted member of the series, 1-(3′,4′-methylenedioxyphenyl) - 1 - acetthio - 2 - nitroethane (I), was reduced with lithium aluminum hydride to the mercapto-amine, 1-(3′,4′-methylenedioxyphenyl)-1-mercapto-2-aminoethane (V). Because such mercaptans are readily oxidized to disulfide during the process of isolation, the oxidation equivalent was determined with iodine to prove that the isolated product was, in fact, the mercaptan. The yield in this reduction was low (11%), but this could undoubtedly be improved by further workup of the residues from the reduction.

Reduction of the side chain homolog, 1-phenyl-1-acetthio-2-nitropropane (IV), gave cleavage of the carbon chain; none of the expected mercapto-amine



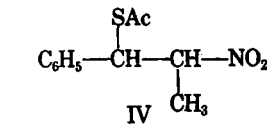


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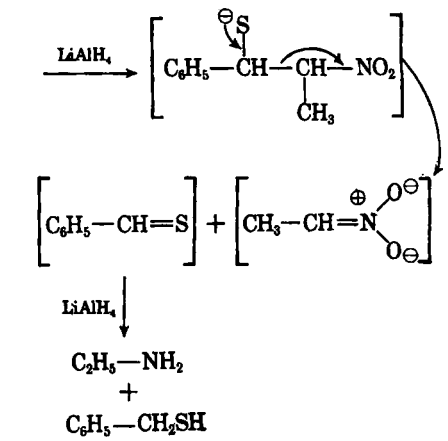


V

was isolated. The reduction products actually isolated were benzyl disulfide and ethylamine hydrochloride. It would appear that the initial reduction took place at the thiolacetate group to give an intermediate salt of a mercaptan which then underwent reverse aldol condensation. The initial cleavage products, thiobenzaldehyde and the salt of nitroethane, were then reduced to the lithium aluminum complexes of benzyl mercaptan and ethylamine, respectively. During the course of hydrolysis and isolation, the benzyl mercaptan was apparently air-



IV



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Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.

[1] Through the courtesy of Dr. Thomas R. Sweeney, Department of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, D. C., 1-phenyl-1-acetthio-2-nitropropane was reported to show "some activity" in protection of mice against an LD_{100} dose of radiation at a dosage level in the range 151 to 350 mg./Kg. The next lower homolog, 1-phenyl-1-acetthio-2-nitroethane, synthesis of which has been described previously (1), was reported to show the same level of protection. For comparison, it is of interest that the mercaptoamine derived from the latter compound, 2-mercapto-2-phenylethylamine hydrochloride (1) was reported to show no protective activity when administered at a dosage level in the range 51 to 150 mg./Kg. The test procedure and evaluation scale used are described more fully by Field, L., *et al.*, *J. Med. Chem.*, 7, 39(1964).

TABLE I.—SUBSTITUTED 1-PHENYL-1-ACETTHIO-2-NITROETHANES

No.	Substituent	M.p., °C.	Yield, %	Formula	C, %		H, %		N, %		O, %	S, %	
					Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Calcd.	Found
I	3',4'-Methylenedioxy	96–97	75	$C_{11}H_{11}NO_5S$	49.06	49.30	4.12	4.30	5.20	5.09	29.71	11.91	11.73
II	3',4'-Dimethoxy	115–116	66	$C_{12}H_{15}NO_5S$	50.51	50.24	5.30	5.31	4.90	5.00	28.10	11.24	11.06
III	3'-Methoxy-4'-acetoxy	98–99.5	50	$C_{13}H_{15}NO_6S$	49.83	49.88	4.83	4.93	4.47	4.58	30.64	10.23	10.10
IV	2-Methyl	83–84.5	52	$C_{11}H_{13}NO_3S$	55.23	55.32	5.47	5.41	5.85	5.70	...	13.40	13.34

oxidized to the disulfide. Leonard and co-workers (2) observed a similar carbon-carbon cleavage of part of the product during the lithium aluminum hydride reduction of 1-anilino-2-nitro-1-phenylethane. Their isolation of *N*-benzylaniline could be explained by a similar mechanism.

Apparently the cleavage must occur prior to or during the reduction of the nitro group, as derivatives of structurally similar mercapto-amines do not show this cleavage on reduction with lithium aluminum hydride. For instance, 3,4-dimethyl-5-phenylthiazolidine-2-thione is readily reduced in good yield with lithium aluminum hydride to 1-phenyl-1-mercapto-2-(dimethylamino)propane (3).

EXPERIMENTAL

1 - (3',4' - Methylenedioxyphenyl) - 1 - acetthio-2-nitroethane (I).—Thiolacetic acid (1.6 ml., 0.02 mole) and then 2 drops of tri-*n*-butylamine were added to a suspension of 3.9 Gm. (0.02 mole) of 3,4-methylenedioxy-*β*-nitrostyrene (4) in 20 ml. of benzene. The mixture became warm, and the solid slowly dissolved. The product crystallized overnight, as the reaction mixture was allowed to stand at room temperature. After two recrystallizations from ethanol, the yield was 4 Gm. (75%), m.p. 96–97°.

Analyses and data on analogous compounds are shown in Table I.

Reaction of Thiolacetic Acid with 3-Acetoxy-4-methoxy-*β*-nitrostyrene.—Thiolacetic acid (0.08 ml., 0.001 mole) and then 2 drops of tri-*n*-butylamine were added to a suspension of 0.237 Gm. (0.001 mole) of 3-acetoxy-4-methoxy-*β*-nitrostyrene (4) in 1.5 ml. of benzene. The solid went into solution with the evolution of heat. A brownish-red gum separated overnight, as the reaction mixture was allowed to stand at room temperature. Addition of ethanol to the gum dissolved most of it, leaving a small amount of yellow crystals (0.024 Gm.), m.p. 113–114.5°, identified as elemental sulfur. The alcohol-soluble gum could not be crystallized.

1 - (3',4' - Methylenedioxyphenyl) - 1 - mercapto-2-aminoethane (V).—A solution of 13.46 Gm. (0.05 mole) of 1-(3',4'-methylenedioxyphenyl)-1-acetthio-2-nitroethane in 750 ml. of anhydrous ether was added dropwise to a solution of 9.5 Gm. (0.25 mole) of lithium aluminum hydride in 1 L. of anhydrous ether. The mixture was refluxed with stirring for 10 days. The reaction complex was hydrolyzed by the dropwise addition of 18 ml. of water, followed by 24.3 ml. of concentrated hydrochloric acid. The filtered ether solution was evaporated to dryness, and the white residue was recrystallized from alcohol to give a yield of 1.1 Gm. (11%), m.p. 110.5–112°.

Anal.—Calcd. for $C_9H_{11}NO_2S$: C, 54.80; H, 5.62; N, 7.10; O, 16.22; S, 16.26; mol. wt., 197.3. Found: C, 54.78; H, 5.76; N, 7.20; O, 16.47; S, 16.17; neut. equiv., 194 and 196 (by semimicro nonaqueous titration with acetous perchloric acid); oxidation equivalent, 194 (by oxidation of mercaptan to disulfide with excess iodine and back-titration with thiosulfate).

Reduction of 1-Phenyl-1-acetthio-2-nitropropane.—A solution of 24 Gm. (0.1 mole) of 1-phenyl-1-acetthio-2-nitropropane in 300 ml. of anhydrous ether was added dropwise to a solution of 15.2 Gm. (0.4 mole) of lithium aluminum hydride in 300 ml. of ether. The mixture was heated under reflux with

stirring for 80 hours. The reaction complex was hydrolyzed by dropwise addition with vigorous stirring of 28.8 ml. of water, followed by 39.5 ml. of concentrated hydrochloric acid. The ether filtrate was evaporated to give 10.5 Gm. of an oily semisolid. Repeated recrystallization of this residue from mixtures of benzene and petroleum ether gave 2 Gm. of white crystals, m.p. 69–70°. Chemical analysis indicated that this compound was benzyl disulfide. Comparison to an authentic specimen showed identical infrared spectrum and no depression of melting point.

In a subsequent identical run, during the evaporation of the ether solution to dryness, the escaping gases were passed through a trap containing hydrochloric acid. Evaporation of the solution from this trap left a residue of 4.5 Gm., m.p. 95–105°. Recrystallization from ethanol-ether mixtures gave 2.5 Gm. (28%) of ethylamine hydrochloride, m.p. 108–109°. This is in agreement with the known melting point of 109° (5). Conversion to the benzamide derivative gave a product which melted at 68–69°, in agreement with the previously reported melting point for *N*-ethylbenzamide of 69° (6).

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Use of Hammett Graphs in Stability Programs

By J. THURØ CARSTENSEN, E. G. SERENSON, and J. J. VANCE

HAMMETT GRAPHS have been used for many years by organic and physical chemists, primarily for elucidation of reaction mechanisms.

Research programs involving the screening of a host of related compounds may benefit from this sort of approach. By establishing a few pKa values and rate constants, rapid estimates of degradation rates of new compounds can be made simply by a pKa measurement. The advantage of this in pharmaceutical formulation is quite obvious.

The condition that a linear relationship holds (1, 8) is that the entropy of reaction is either zero or is the same for all the hydrolyses (or whatever reaction is pertinent) or that ΔH and ΔS be linearly related.

In general, the substituent parameter is referred to as σ, and the reaction parameter as ρ; the latter in particular is useful in deducing the actual reaction mechanism. Substituent σ values and reaction ρ values are abundantly reported in the literature (1).

The authors have exemplified the utility of this sort of approach by a study of a series of allylbarbituric acids.

The materials used in this study were obtained by suitable extraction from commercially available dosage forms in the case of talbutal and secobarbital. 5-Allyl-5(2-cyclopenten-1-yl) barbituric acid,[1] itobarbital,[2] and allobarbital[3] were obtained from suppliers. Unsubstituted allylbarbituric acid was synthesized according to the method of Arnold *et al.* (2) and Johnson and Hill (3).

Experimentally, samples were prepared by dissolving 250 mg. of barbiturate in form of the acid in 50 ml. of 95% ethanol. This was then diluted 1:50 with a borate buffer of the following composition: 0.97 Gm. of boric acid, 1.15 Gm. of potassium chloride, 344 ml. of 0.1 *N* sodium hydroxide and adjusted to 1000 ml. with distilled water, yielding a final pH of 11.8.

Ampuls of these solutions were stored at 85° and assayed at six storage times. The decrease in barbiturate was determined by a decrease in absorbance at the peak wavelength (240–242 mμ) after suitable dilution with the described borate buffer. Pseudo first-order rate constants (hours^{-1}) are denoted k in Fig. 1 and Table I.

The pKa's of the barbiturates were determined spectrophotometrically. pKa° and k_o in the following refer to the unsubstituted allylbarbituric acid.

In a more formal treatment (4, 5, 7, 9, 10), the reaction constant is calculated from substituent constants based on dissociation constants in water. The σ-constants as defined here hence refer to the dissociation constants relative to the dissociation of unsubsituted allylbarbituric acid. Statistical im-

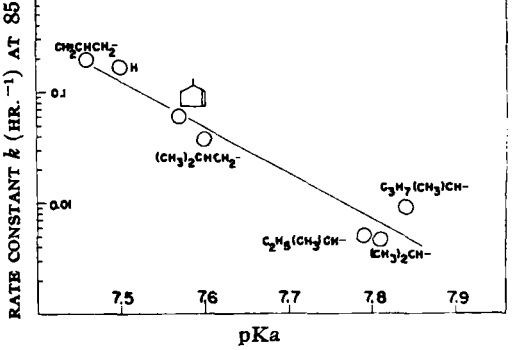


Fig. 1.—Form in which rate constants can most conveniently be calculated for an unknown substance with known pKa with previous knowledge of rate constant and pKa of compounds related to the substance in question. [Brønsted plot (11).]

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Accepted for publication August 20, 1964.
[1] Obtained from Gane's Chemical Works, Carlstad, N. J.
[2] Itobarbital was kindly supplied by Mr. S. Weinstein, Sandoz Pharmaceuticals, Hanover, N. J.
[3] Allobarbital was kindly supplied by Mr. J. Cooper, Ciba Pharmaceutical Co., Summit, N. J.

stirring for 80 hours. The reaction complex was hydrolyzed by dropwise addition with vigorous stirring of 28.8 ml. of water, followed by 39.5 ml. of concentrated hydrochloric acid. The ether filtrate was evaporated to give 10.5 Gm. of an oily semisolid. Repeated recrystallization of this residue from mixtures of benzene and petroleum ether gave 2 Gm. of white crystals, m.p. 69–70°. Chemical analysis indicated that this compound was benzyl disulfide. Comparison to an authentic specimen showed identical infrared spectrum and no depression of melting point.

In a subsequent identical run, during the evaporation of the ether solution to dryness, the escaping gases were passed through a trap containing hydrochloric acid. Evaporation of the solution from this trap left a residue of 4.5 Gm., m.p. 95–105°. Recrystallization from ethanol-ether mixtures gave 2.5 Gm. (28%) of ethylamine hydrochloride, m.p. 108–109°. This is in agreement with the known melting point of 109° (5). Conversion to the benzamide derivative gave a product which melted at 68–69°, in agreement with the previously reported melting point for *N*-ethylbenzamide of 69° (6).

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Use of Hammett Graphs in Stability Programs

By J. THURØ CARSTENSEN, E. G. SERENSON, and J. J. VANCE

HAMMETT GRAPHS have been used for many years by organic and physical chemists, primarily for elucidation of reaction mechanisms.

Research programs involving the screening of a host of related compounds may benefit from this sort of approach. By establishing a few pKa values and rate constants, rapid estimates of degradation rates of new compounds can be made simply by a pKa measurement. The advantage of this in pharmaceutical formulation is quite obvious.

The condition that a linear relationship holds (1, 8) is that the entropy of reaction is either zero or is the same for all the hydrolyses (or whatever reaction is pertinent) or that ΔH and ΔS be linearly related.

In general, the substituent parameter is referred to as σ, and the reaction parameter as ρ; the latter in particular is useful in deducing the actual reaction mechanism. Substituent σ values and reaction ρ values are abundantly reported in the literature (1).

The authors have exemplified the utility of this sort of approach by a study of a series of allylbarbituric acids.

The materials used in this study were obtained by suitable extraction from commercially available dosage forms in the case of talbutal and secobarbital. 5-Allyl-5(2-cyclopenten-1-yl) barbituric acid,[1] itobarbital,[2] and allobarbital[3] were obtained from suppliers. Unsubstituted allylbarbituric acid was synthesized according to the method of Arnold *et al.* (2) and Johnson and Hill (3).

Experimentally, samples were prepared by dissolving 250 mg. of barbiturate in form of the acid in 50 ml. of 95% ethanol. This was then diluted 1:50 with a borate buffer of the following composition: 0.97 Gm. of boric acid, 1.15 Gm. of potassium chloride, 344 ml. of 0.1 *N* sodium hydroxide and adjusted to 1000 ml. with distilled water, yielding a final pH of 11.8.

Ampuls of these solutions were stored at 85° and assayed at six storage times. The decrease in barbiturate was determined by a decrease in absorbance at the peak wavelength (240–242 mμ) after suitable dilution with the described borate buffer. Pseudo first-order rate constants (hours^{-1}) are denoted k in Fig. 1 and Table I.

The pKa's of the barbiturates were determined spectrophotometrically. pKa° and k_o in the following refer to the unsubstituted allylbarbituric acid.

In a more formal treatment (4, 5, 7, 9, 10), the reaction constant is calculated from substituent constants based on dissociation constants in water. The σ-constants as defined here hence refer to the dissociation constants relative to the dissociation of unsubsituted allylbarbituric acid. Statistical im-

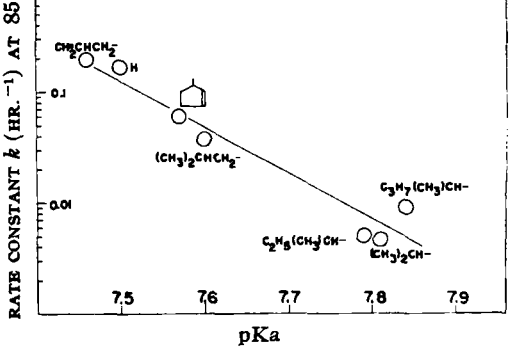
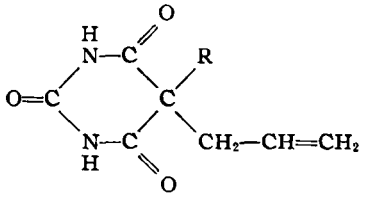


Fig. 1.—Form in which rate constants can most conveniently be calculated for an unknown substance with known pKa with previous knowledge of rate constant and pKa of compounds related to the substance in question. [Brønsted plot (11).]

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[1] Obtained from Gane's Chemical Works, Carlstad, N. J.
[2] Itobarbital was kindly supplied by Mr. S. Weinstein, Sandoz Pharmaceuticals, Hanover, N. J.
[3] Allobarbital was kindly supplied by Mr. J. Cooper, Ciba Pharmaceutical Co., Summit, N. J.

TABLE I.—HYDROLYSIS DATA AT 85° C.



R	5-Substituent	Generic Name	Hydrolysis Rate Constant at 85°C. k (Hr.$^{-1}$)	k/k_o	pKa	pKa° − pKa
H			0.168		7.50	
C_5H_7−	Cyclopentenyl	None[a]	0.10	0.595	7.57	−0.07
$C_2H_5CH(CH_3)$—	*sec*-Butyl	Talbutal	0.0050	0.030	7.79	−0.29
$C_3H_7CH(CH_3)$—	1-Methylbutyl	Secobarbital	0.0092	0.054	7.84	−0.34
$(CH_3)_2CH$—	Isopropyl	Aprobarbital	0.0046	0.028	7.81	−0.31
CH_2=$CHCH_2$−	Allyl	Allobarbital	0.198	1.180	7.46	+0.04
$(CH_3)_2CHCH_2$—	Isobutyl	Itobarbital	0.037	0.220	7.60	−0.10

[a] Chemical name: 5-allyl-5(2-cyclopenten-l-yl) barbituric acid.

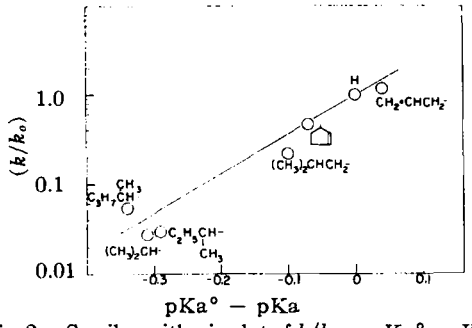


Fig. 2.—Semilogarithmic plot of k/k_o *vs.* pKa° − pKa. (See text for notation.)

TABLE II.—SUBSTITUENT SIGMA VALUES

Substituent	σ_B		Ref.
$(CH_3)_2CH$—	−0.35	$\sigma_+ = -0.151$	(7)
		$\sigma_p = -0.280$	(6)
C_5H_7—	−0.05		
CH_2=CH—			
CH_2—	+0.02		
$(CH_3)_2CHCH_2$—	−0.15	$\sigma = -0.115$	(6)
$C_3H_7CH(CH_3)$—	−0.28		
$C_2H_5CH(CH_3)$—	−0.34	$\sigma = -0.123$	(6)
		$\sigma = -0.983$	(10)

provement (7) over this is accomplished by the (confined) least-square relationship

$$\log k/k_o = 4.47 \log K_a/K_a°$$

derived from Fig. 2 and then obtaining individual σ's from $\log k/k_o = \sigma \cdot \rho = 4.47\ \sigma$. Values calculated in this manner are listed as σ_B in Table II. Substituent values have been reported in the previous literature for only three of the six substituents tested here. Previously published σ values are predominantly for substituents in aryl (10) and alkyl (10, 11) compounds in a variety of reactions, and some values are listed in Table II (with references) for comparison purposes.

Actual reaction mechanisms of the barbiturate hydrolysis have not been dealt with here; the primary scope of this report has been stability prediction.

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Opium Alkaloids—Separation and Identification by Gas, Thin-Layer, and Paper Chromatography

By E. BROCHMANN-HANSSEN and T. FURUYA*

A large number of opium alkaloids has been chromatographed by gas, thin-layer, and paper chromatography for the purpose of identification. Gas chromatography is particularly valuable because of its speed, simplicity, and reproducibility. The two other methods are useful for certain alkaloids which either do not lend themselves to gas chromatography or give unsatisfactory separation.

FEW NATURAL products have been studied so extensively as opium, and none has presented problems of such magnitude from chemical, biological, and social viewpoints. The principal alkaloids of opium were discovered during the first half of the 19th century. During the next 50 years, many minor alkaloid constituents were isolated, and the list has continued to grow even during the 20th century. Today perhaps 20 are recognized as genuine alkaloids in opium and the opium poppy. In addition, several heterocyclic nitrogen compounds have been isolated which probably do not occur in the fresh latex but are formed as a result of oxidation, hydrolysis, or racemization.

Most of the opium alkaloids which have been isolated as pure compounds are now reasonably well known and, except for a few, their structures have been established. Nevertheless, it is not an easy task to identify the minor alkaloids of opium because they are often difficult to purify, and the identity tests which have been described are not specific. Thus, Fulton (1) has reported that he has isolated several phenolic opium alkaloids which he has been unable to identify. Recently, Pfeifer and Teige (2) have described a new opium alkaloid whose structure has not yet been established. More alkaloids will undoubtedly be discovered as modern methods of isolation and characterization are developed. In connection with such work, it is essential to be able to identify microquantities of the alkaloids which have already been isolated. For this purpose, the chromatographic behavior of a large number of opium alkaloids was studied by gas, thin-layer, and paper chromatography.

EXPERIMENTAL

Gas Chromatography.—The instrument used for this work was a Barber-Colman model 15 gas chromatograph equipped with an argon ionization detector and U-shaped glass columns 6 ft. long and $^1/_8$ in. in inside diameter. The solid support was Gas-Chrom A, washed with acid and base and siliconized with hexamethyldisilazane (3). It was inactivated further with thin coats of polyethylene glycol 4000 and nonylphenoxypolyethyleneoxyethanol, each coat representive of about 0.05% of the weight of the support. This material was coated rapidly with 2% of silicone rubber SE-30 by a filtration method (4). The alkaloid samples were dissolved in acetone and applied with a microsyringe as 1 μl. containing 5 to 10 mcg. of each alkaloid. The results are given in Table I.

Thin-Layer Chromatography.—Silica gel G was used as the adsorbant, and the chromatograms were developed with methanol–chloroform (1:9) and ethanol–benzene (1:4), as described by Mary and Brochmann-Hanssen (5). After drying, the plates were observed under ultraviolet light and sprayed with potassium iodoplatinate reagent. In the case of porphyroxine, the plate was sprayed with hydrochloric acid and dried; a characteristic red spot was produced. Occasionally, spraying with Gibbs' reagent (2,6-dichloroquinone chlorimide) was used to develop the spots of laudanine, laudanidine, and reticuline. The R_f values in thin-layer chromatography greatly depend on the brand of silica gel and the activation of the thin-layer plates. The results in Table II are average values based on a large number of runs.

Paper Chromatography.—Of the many solvent systems used for paper chromatography of alkaloids, a modification of the procedure described by Miram and Pfeifer (7) appeared to be most satisfactory for the opium alkaloids. Sheets of Whatman No. 1 filter paper were cut in the machine direction into strips 46 cm. long and 20 cm. wide. With a soft lead pencil, lines were drawn across the papers 6.5 cm. and 32.0 cm. from one end; the first line marked the starting point. The papers were sprayed with

TABLE I.—RELATIVE RETENTION TIMES OF OPIUM ALKALOIDS

Alkaloid	
Codeine	0.38
Neopine	0.41
Morphine	0.48
Thebaine	0.59
10-Hydroxycodeine	0.66
Laudanosine	1.00
Laudanine	1.12
Laudanidine	1.12
(±)-Reticuline	1.18
Papaverine	1.65
Protopine	1.95
Cryptopine	2.41
Narcotine (noscapine)	4.40
Carrier gas	Argon
Inlet pressure, p.s.i., lb.	19
Column dimensions	6 ft. × $^1/_8$ in.
Liquid phase	2% SE-30
Column temp.	207°
Injection port temp.	302°
Cell bath temp.	230°
Laudanosine time, min.	16.4

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The authors are indebted to Mallinckrodt Chemical Works and Merck A. G. (Darmstadt) and to Dr. A. Baerheim Svendsen, Dr. V. Deulofeu, Dr. E. Hardegger, Dr. S. Pfeifer, and Dr. M. Tomita for making available most of the rare alkaloids used in this study.

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TABLE II.—R_f VALUES OF THIN-LAYER CHROMATOGRAMS

Alkaloid	Solvent System MeOH:CHCl₃ (1:9)	Solvent System EtOH:C₆H₆ (1:4)
Narceine	0.09	0.03
Morphine	0.12	0.08
10-Hydroxycodeine	0.17	0.15
(±)-Reticuline[a]	0.28	0.18
Codeine	0.35	0.15
Neopine	0.38	0.12
Protopine	0.46	0.42
Laudanine	0.47	0.28
Laudanidine	0.47	0.28
Cryptopine	0.48	0.40
Thebaine	0.65	0.38
Laudanosine	0.74	0.36
Narcotoline	0.88	0.71
Porphyroxine[b]	0.93	0.65
Papaverine	0.97	0.77
Narcotine (noscapine)	0.97	0.87

[a] Isolated from opium (6). [b] Isolated from opium as described by Pfeifer and Teige (2).

McIlvain buffers, as illustrated in Fig. 1. The excess of buffer solution was removed between clean filter papers, and the paper strips were allowed to air-dry for 2 hours. Spots of the alkaloid solutions (in chloroform or methanol) were placed on the starting line 3 cm. apart; after a few minutes, the papers were placed in chromatographic jars, the atmosphere of which was saturated with the solvent system to be used. After 1 hour of equilibration, the chromatograms were developed with water-saturated *n*-butanol or water-saturated ether, respectively. The results are illustrated in Fig. 1, a composite picture based on a number of chromatograms.

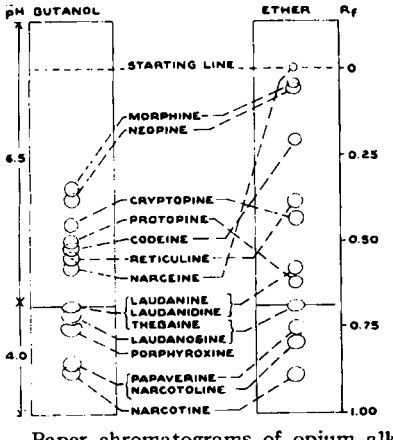


Fig. 1.—Paper chromatograms of opium alkaloids developed by descending chromatography.

DISCUSSION

The gas chromatographic method was most generally applicable and permitted separation of the largest number of alkaloids. Even when no clear-cut separation of mixtures could be obtained, as in the case of codeine and neopine or laudanine and reticuline, there was difference enough in the retention times to make possible the identification of the pure alkaloids. The isomeric alkaloids, laudanidine and laudanine (*dl*-laudanidine), could not be separated by the three methods. The peak shift technique described by Anders and Mannering (8) was very useful for the identification of phenolic alkaloids.

Thin-layer chromatography is also a good method for identification of many opium alkaloids, but the retention data are not nearly so reproducible as for gas chromatography. Although several alkaloids have R_f values too close for separation, it is often possible to identify the spots by observing them under ultraviolet light and by using selective spray reagents. The relative differences in R_f values for the two solvent systems are also useful as means of identification.

The paper chromatographic method showed considerable selectivity in some instances where the other two methods were unsatisfactory. It was the only method which gave a clear separation of codeine and neopine.

It would seem that the three methods when considered together should permit identification of practically all known opium alkaloids. When extracts of opium were subjected to these procedures, the alkaloids chromatographed as the pure compounds could readily be identified. It also became apparent that opium contains several alkaloidal substances in addition to those described in this paper. An investigation is being conducted to isolate and characterize these unknown alkaloids.

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Determination of Anhydrotetracycline and 4-Epianhydrotetracycline in a Tetracycline Mixture

By R. G. KELLY

A method of analysis for anhydrotetracycline and 4-epianhydrotetracycline in the presence of large quantities of tetracycline (about 95 per cent) has been developed. The method involves separation of the components by column chromatography, followed by spectral determination of column eluates. In the presence of 50 mg. of tetracycline, 0.9 mg. of added anhydrotetracycline was determined with the analytical recovery of 100.5 per cent, and 0.9 mg. of added 4-epianhydrotetracycline was determined with the analytical recovery of 92.5 per cent.

RECENTLY, reports have appeared in the literature describing a Fanconi-type syndrome, usually reversible, induced in patients who have ingested outdated or degraded tetracycline (1–5). The qualitative analyses of degraded capsular material by paper chromatographic methods (6) have revealed the presence of three degradation products (Scheme I) typical of what might be expected if tetracycline hydrochloride (I) were exposed to conditions of high temperature and moisture (7, 8). These degradation products are 4-epitetracycline (II), anhydrotetracycline (III), and 4-epianhydrotetracycline (IV). Of these, 4-epianhydrotetracycline in relatively large doses was the causative agent of renal tubular damage in the rat and the dog producing urinary findings suggestive of a Fanconi-type syndrome (9).

Therefore, it was necessary to develop an analytical procedure capable of determining the anhydrotetracyclines in the presence of large quantities of tetracycline.

EXPERIMENTAL

Materials and Methods

Reagents.—Acid-washed diatomaceous earth,[1] 0.1 *M* ethylenediaminetetraacetic acid (EDTA) disodium salt brought to pH 7.8 with ammonium hydroxide, reagent grade chloroform, 0.1 *M* EDTA in 0.2 *M* NH_4OH, and 0.1 *N* HCl were employed.

Procedure.—Equilibrate equal volumes of pH 7.8 EDTA solution and chloroform. To 10 Gm. dry Celite, add 5 ml. of the upper phase in a small conical flask, and shake until the Celite is moistened evenly.

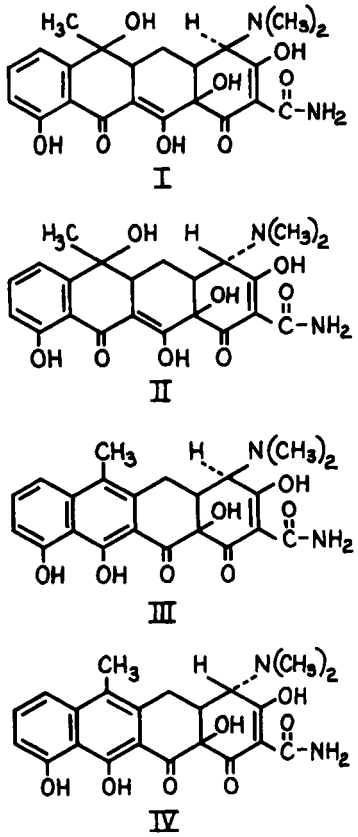
Prepare a column 15 × 1.8 cm. (an ordinary 6-in. test tube with a stopcock) of the moist Celite, using a small circle of filter paper (about 1 cm. diameter) as a plug, tightly packing about 80% of the Celite (10–11 cm.). Dilute an amount of the sample equivalent to 50 mg. of tetracycline to 1 ml. with 0.1 *N* HCl, and mix, effecting as much solution as possible of the sample.

Add 0.25 ml. of the solution (or fine suspension) to 1 Gm. of dry Celite, and mix thoroughly with a small glass stirring rod; add 0.25 ml. of 0.1 *M* EDTA in 0.2 *M* NH_4OH, and mix thoroughly again. Pack the sample Celite mixture on the column. Pack an additional 1 cm. of the EDTA-moistened Celite on top of the sample, and cover with a layer of sand.

Elute with the lower phase of the equilibrated solvent mixture; more rapid elution may be effected by applying a positive pressure to the top of the column after the solvent has passed the tetracycline charge. Collect 5-ml. fractions, and read absorbances at 430 mμ, using lower phase solvent as a blank. Anhydrotetracycline is in the first eluted tube and epianhydrotetracycline in tubes 2–7. (This will vary slightly, according to the precise pH conditions of the column.)

RESULTS AND DISCUSSION

The solubility differences between the anhydro derivatives of tetracycline and tetracycline itself suggested that a method for the determination of these derivatives in the presence of tetracycline



Scheme I

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[1] Marketed as Celite 545 by the Johns Manville Co.

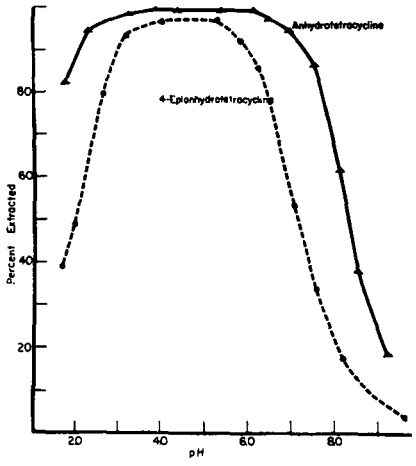


Fig. 1.—Percentage of anhydrotetracycline and 4-epianhydrotetracycline extracted from water into chloroform at various pH's.

might be evolved from a preliminary separation of the various components, followed by a spectroscopic analysis of the purified compounds. In the course of work involving the development of an analytical method for the determination of tetracycline in rat bone (10), the extraction of anhydrotetracycline from an aqueous medium into chloroform was maximal at pH 4.5. Work done subsequently indicated that the same pH maximum for extraction occurred with epianhydrotetracycline. However, in the construction of pH *versus* distribution coefficient curves, at certain pH values differences in the curves derived from the two anhydro compounds were apparent (Fig. 1). These differences occur in the pH ranges 1.5–2.5 and 6.5–8.5. On the basis of this information, column chromatography, using a pH 7.8 chloroform–water solvent system on an inert carrier, was attempted for the separation of anhydrotetracycline from epianhydrotetracycline.

Figure 2 shows the results obtained on a column run on a mixture of 50 mg. of tetracycline hydrochloride, 910 mcg. of anhydrotetracycline hydrochloride, and 894 mcg. of 4-epianhydrotetracycline hydrochloride. The column eluates were read in a Beckman model B spectrophotometer at 430 mμ. For the anhydrotetracycline hydrochloride, an absorptivity of 1.755×10^{-2} was used for calculation of concentration; for 4-epianhydrotetracycline, an absorptivity of 1.62×10^{-2} was used. The absorptivity is defined as absorbance per microgram per milliliter of eluate using a 1-cm. light path. These absorptivities were determined by dissolving (or suspending) an accurately weighed amount of the compound under consideration in a 10-ml. volume of the solvent equilibrated aqueous phase of the pH 7.8, 0.1 *M* EDTA solution and extracting with successive small volumes of the chloroform phase until all of the yellow color was in the chloroform extracts. These were pooled and diluted to a specific volume, then read in the spectrophotometer at 430 mμ.

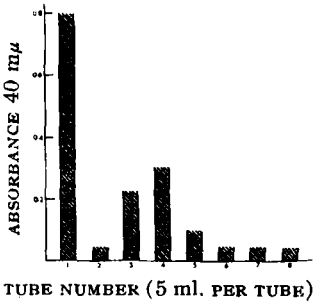


Fig. 2.—Column chromatography of a mixture of tetracycline, anhydrotetracycline, and 4-epianhydrotetracycline.

In the column described by Fig. 2, anhydrotetracycline was in tube 1, and epianhydrotetracycline was in tubes 2–5. The slight absorption in tubes beyond the fifth tube was shown by reading absorbances at 360 mμ to be due to tetracycline, which has an absorptivity of approximately 1.6×10^{-4} at 430 mμ.[2]

Although anhydrotetracycline is in the first eluted fraction in spite of variations in pH over the range 7.5–8.0, epianhydrotetracycline and tetracycline elutions are somewhat affected by such variations. In agreement with the curve in Fig. 1, at a pH more acid than 7.8 the epianhydrotetracycline will be eluted more rapidly than desirable; conversely, at pH's higher than that recommended, the elution of this compound will be slower and extend over a larger number of fractions. Rather than to impose a precise control of pH on the methodology, it is simpler to permit a variable elution rate. The fractions to be estimated as epianhydrotetracycline are easily selected on the basis of their yellow color.

For the column shown in Fig. 2, the tubes 2–5 were chosen in this manner. The recovery of added epianhydrotetracycline was determined as 92.5%; the recovery of added anhydrotetracycline, all of which was in tube 1, was 100.5%. In the original mixture, each of these components represented less than 2% of the total tetracycline content.

In two other experiments performed, recoveries of 95.5 and 102% were obtained on added anhydrotetracycline, and recoveries of 86.0 and 101.0% were derived for epianhydrotetracycline in mixtures of the two compounds. These columns were run without added tetracycline. In one, epianhydrotetracycline was eluted in tubes 2–10 and in the other in tubes 2–7.

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Infrared Absorption Ratio Method for Determination of Triethanolamine Salicylate in Ointment

By H. ORVILLE HEISEY*

A rapid and convenient infrared method was developed for the assay of triethanolamine salicylate in an ointment base. The method involves drying the ointment, scanning the infrared spectrum from 5.5 to 8.7 μ, and calculating the ratio of the absorption peaks at 6.3 and 6.85 μ. Advantages of the method are that no chemical isolation or treatment of the drug is necessary and no weight or volume measurement of sample is required.

A METHOD was sought for determining triethanolamine salicylate (TEAS) salt complex (2, 2′, 2″-nitrilotriethanol salicylate) in ointment. A literature search turned up only three direct references to the compound (1–3); its analytical determination was not mentioned. It was desirable to have a method specific for the TEAS complex and not dependent on an analytical reaction of any of its components. Preliminary studies on colorimetric and titrimetric methods did not give promising results. Investigation of the infrared spectra of the complex and of the ointment base revealed that absorption by the ionized carboxyl group along with the absorption of the methylene groups in the system could be used as the basis for an assay method. Like most ointment bases, the one used in this work consists largely of high molecular weight aliphatic compounds, so the strongest absorption is due to methylene groups. Since the $pKa^{25°}$ for salicylic acid is 3.00, the contribution to the quantity of ionized carboxyl from any free salicylic acid present would be negligible. Hence the absorption by the ionized carboxyl group of the salicylic acid moiety of the TEAS complex is taken as specific for the TEAS content; the absorption peak occurs at about 6.3 μ in this system. (Commercial grades of triethanolamine contain varying amounts of diethanolamine with traces of monoethanolamine. Any salicylate complexes involving these amines would also contribute to this absorption band.) The CH_2 group absorbs at about 6.85 μ in this ointment system. The ester absorption band appears at about 5.8 μ. Under normal conditions (*i.e.*, temperatures less than about 65°), only a negligible amount, if any, of the ester would be present. At any rate, the ester would not contribute to the absorption at 6.3 μ by the ionized carboxyl of the TEAS salt; hence, this latter absorption would indicate correctly the content of salicylate complex.

Since the ratio of TEAS to CH_2 groups will always be the same in a given ointment sample or batch (assuming homogeneity) regardless of the quantity of ointment taken, the ratio of the absorbances of these two chemical entities is a measure of the relative amount of TEAS present. This eliminates the need for weight, volume, or thickness measurements of sample taken. For the assaying of ointments, this is a very desirable feature. The ratio of interest is the ratio of the absorbance at 6.3 μ to the absorbance at 6.85 μ.

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EXPERIMENTAL

Apparatus.—An infrared recording spectrophotometer with NaCl optics, two or three NaCl plates and holders or a demountable cell, an infrared heating lamp, a Wig-L-Bug (or similar vibrator-mixer) and stainless steel capsule and pellet, and a thermometer, microspatula, ringstand, and microscope slide were employed.

Preparation of Standard Curve.—A series of synthetic ointments was prepared containing these percentages of TEAS in ointment base (w/w): 5, 7, 8, 9, 9.5, 10, 10.5, 11, 12, 13, 15. Samples of these standards were prepared as outlined under *Procedure*, and the infrared spectrum of each was obtained from 5.5 to 8.7 μ on a Beckman IR 5 spectrophotometer. The absorbance ratio was calculated, and the plot of absorbance ratio *versus* per cent TEAS followed Beer's law over the region of interest.

Procedure.—Attach the heating lamp to a ringstand so that the lens is about 1 ft. above the base of the stand. Arrange a thermometer horizontally, so that it registers the temperature at the base; turn on the lamp and allow the temperature to reach about 60° before drying the sample. The temperature should not be allowed to rise above about 70°.

Mix the ointment thoroughly; with a microspatula, spread a layer of sample about $^1/_{16}$ in. or less in thickness on a glass slide. Place the slide directly under the lamp and next to the thermometer bulb. Note the time and temperature at the beginning and end of the heating period, which should be 10 minutes for an ointment containing about 60% water. Remove the slide from the heat and mix the melted sample with the spatula; when it has solidified after 1 minute or 2, scrape off the sample as much as possible into the stainless steel capsule with the pellet and vibrate it in the Wig-L-Bug for 20 seconds. This assures thorough mixing of the crystals of TEAS with the dehydrated ointment base, which is essential for obtaining an accurate assay result.

Prepare two NaCl salt plates as windows, warming them slightly under the lamp. Apply a thin film of the dried mixed ointment to the larger plate, then press a smaller plate over it to form a continuous film of an area appropriate to the cross section of the sample beam of the spectrophotometer. Adjust the sample thickness so that the absorbance at 6.85 μ is approximately 0.53 (or 30% T) and obtain the infrared spectrum from 5.5 to 8.7 μ. It is desirable to reduce the background absorption by placing a single salt plate in the reference beam.

Draw a base line joining the transmission maxima at 5.6 and 8.55 μ. Determine the appropriate absorbance values and calculate the 6.3/6.85 ratio from

$$\text{ratio} = \frac{(A_{6.3}{}^{p}) - (A_{6.3}{}^{bl})}{(A_{6.85}{}^{p}) - (A_{6.85}{}^{bl})}$$

where

$A_{6.3}{}^{p}$, $_{6.85}$ = absorbance of peak at 6.3 or 6.85 μ

$A_{6.3}{}^{bl}$, $_{6.85}$ = absorbance of base line at 6.3 or 6.85 μ

DISCUSSION

It is important in the sample treatment to ascertain that all water is driven out. There are water absorption bands in the region of interest, and the presence of water in the sample being scanned will give variable results for the absorbance ratio. On the other hand, care must be taken that the temperature does not rise high enough to cause ester formation, which would obviously result in a lowered salicylate salt content. There should be no problem if the procedure outlined above is followed. Other drying methods are available, but this was the method of choice in terms of convenience and simplicity.

Replicates of four spectra for each of 10 lots of ointment gave an average deviation of ± 0.006 in the absorbance ratio, which represents an error of approximately 0.2% in TEAS content. This precision is adequate for routine control of commercial samples.

The time required for the assay is about 30 minutes and includes drying and preparing the sample, running four spectra, and calculating results.

SUMMARY

A convenient, rapid, and accurate absorbance ratio method for the assay of triethanolamine salicylate in ointment base is proposed. The method is based on the spectrophotometric measurement of the infrared absorption of the salicylate ionized carboxyl and the system methylene groups. No measurement of amount of sample taken is necessary, and no standard needs to be run after the standard curve has been established.

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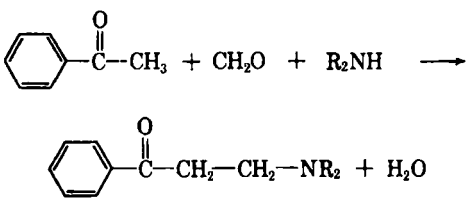
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Mannich Bases and Alcohols from Hexamethylenimine

By W. LEWIS NOBLES and B. BLACKBURN THOMPSON*

The synthesis of a group of Mannich bases and secondary and tertiary γ-aminoalcohols derived from hexamethylenimine are described. These compounds are to be screened for possible pharmacological action.

THE MANNICH reaction has been extensively reviewed (1–4) and consists of the condensation of ammonia or a primary or secondary amine, usually as the hydrochloride salt, an aldehyde, and a compound capable of supplying one or more replaceable hydrogen atoms. A typical condensation with acetophenone as the active hydrogen compound may be illustrated as



The mechanism of the Mannich reaction has been investigated by others (5–9). Hellmann and Opitz (5) and Cummings and Shelton (6) proposed that the reaction is initiated by a condensation between the amine and formaldehyde to yield an aminomethanol. The subsequent steps may be visualized as follows: attack of a proton on the oxygen atom of the aminomethanol, followed by expulsion of water, leads to the formation of a resonance-stabilized carbonium-immonium ion. This carbonium ion then reacts with the carbanion, which results from ionization of the active hydrogen-containing compound.

A large number of β-aminoketones (Mannich bases) have been prepared and tested as antispasmodics, local anesthetics, analgesics, and antibacterial agents (10–18). Secondary γ-aminoalcohols, prepared from Mannich bases by reduction with sodium borohydride (15, 19, 20), and tertiary γ-aminoalcohols, obtained by reacting Grignard reagents with Mannich bases (21), have been prepared and tested for similar pharmacological activity (15, 22, 23).

Reduction of piperidine Mannich bases to yield the corresponding secondary alcohols or reaction with Grignard reagents to yield the appropriate tertiary alcohols gave agents of greater antispasmodic activity (10, 22). Mannich bases derived from propiophenone appear to have enhanced analgesic activity (16, 21) and are better local anesthetics (12, 13) and antifungal agents (16) than acetophenone derivatives. Certain β-aminoketones with complex amine moieties have shown an unexpected order of antibacterial activity (24).

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* Present address: School of Pharmacy, University of Georgia, Athens.

Draw a base line joining the transmission maxima at 5.6 and 8.55 μ. Determine the appropriate absorbance values and calculate the 6.3/6.85 ratio from

$$\text{ratio} = \frac{(A_{6.3}{}^{p}) - (A_{6.3}{}^{bl})}{(A_{6.85}{}^{p}) - (A_{6.85}{}^{bl})}$$

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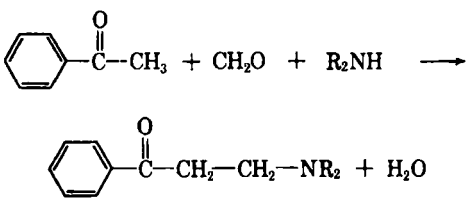
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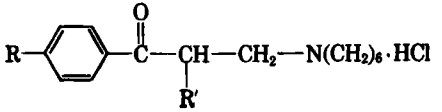
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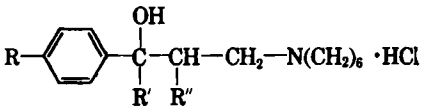
TABLE I.—MANNICH BASES



No.	R	R′	Formula	M.p., °C.[a]	Yield, %	Recryst. Solvent[b]	Microanalytical Data[c] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
1	H	H	$C_{15}H_{22}ClNO$	167.0–168.0[d]	45.0	A	67.27	67.01	8.28	8.18	...	...
2	H	CH_3	$C_{16}H_{24}ClNO$	183.5–184.5[e]	63.5	A	68.19	68.19	8.58	8.45	...	...
3	CH_3	H	$C_{16}H_{24}ClNO$	151.0–152.5[f]	35.0	A	68.19	68.34	8.58	8.58	4.97	4.98

[a] Melting points are uncorrected. [b] Recrystallization solvents are as follows: A, ethanol–acetone (1:4 by volume); B, 2-propanol–petroleum ether (60–110° boiling range) mixture (1:4 by volume); C, 2-propanol. [c] Microanalyses were performed by Weiler and Strauss, Oxford, England; A. Bernhardt, Mülheim (Ruhr), West Germany; and H. Galbraith, Knoxville, Tenn. [d] Reported to melt at 165–166°C. (26). [e] Kost *et al.* (26) reported m.p. 198–200°C. [f] Literature melting point was 171–173°C. (26).

TABLE II.—γ-AMINOALCOHOLS



No.	R	R′	R″	Formula	M.p., °C.[a]	Yield, %	Recryst. Solvent[b]	Microanalytical Data[c] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
1	H	H	H	$C_{15}H_{24}ClNO$	115.5–117.5	82.2	B	66.77	66.63	8.97	8.86	5.19	5.32
2	H	H	CH_3	$C_{16}H_{26}ClNO$	231.5–232.5	87.5	B	67.70	67.92	9.23	9.23	4.94	4.88
3	CH_3	H	H	$C_{16}H_{26}ClNO$	118.5–120.5	29.0	B	67.70	67.51	9.23	9.40	4.94	4.76
4	H	CH_3	H	$C_{16}H_{26}ClNO$	188.0–190.0	6.2	B	67.70	67.49	9.23	9.31	4.94	5.06
5	H	CH_3	CH_3	$C_{17}H_{28}ClNO$	187.5–189.0	45.5	A	68.55	68.43	9.47	9.42	4.70	4.51
6	CH_3	CH_3	H	$C_{17}H_{28}ClNO$	209.5–210.0	7.9	C	68.55	68.51	9.47	9.49	4.70	4.55
7	H	C_2H_5	H	$C_{17}H_{28}ClNO$	167.5–168.5	7.5	B	68.55	68.39	9.47	9.44	4.70	4.77
8	H	C_2H_5	CH_3	$C_{18}H_{30}ClNO$	197.0–198.5	50.1	C	69.31	69.21	9.70	9.76	4.49	4.46
9	CH_3	C_2H_5	H	$C_{18}H_{30}ClNO$	197.0–197.5	7.5	B	69.31	69.20	9.70	9.53	4.49	4.66
10	H	C_6H_5	H	$C_{21}H_{28}ClNO$	229.0–231.0	8.2	B	72.91	72.84	8.16	8.02	4.05	4.21
11	H	C_6H_5	CH_3	$C_{22}H_{30}ClNO$	208.5–211.0	39.2	C	73.41	73.52	8.40	8.50	3.89	3.86
12	CH_3	C_6H_5	H	$C_{22}H_{30}ClNO$	223.0–225.0	10.1	C	73.41	73.18	8.40	8.56	3.89	3.95

[a] Melting points are corrected. [b] Recrystallization solvents are as follows: A, ethanol–acetone (1:4 by volume); B, 2-propanol–petroleum ether (60–110° boiling range) mixture (1:4 by volume); C, 2-propanol. [c] Microanalyses were performed by Weiler and Strauss, Oxford, England; A. Bernhardt, Mülheim (Ruhr), West Germany; and H. Galbraith, Knoxville, Tenn.

Luts and Nobles (25) reported anti-inflammatory activity, anticonvulsant activity, and analgesic activity in members of a series of β-aminoketones and secondary and tertiary γ-aminoalcohols derived from heptamethylenimine. β-Aminoketones derived from hexamethylenimine were reported to possess CNS tranquilizing ability (26) and local anesthetic activity (26, 27). Most of the β-aminoketones were also observed to exhibit febrile activity (26, 27) and vasodilator activity (27). Extension of the series of hexamethylenimine derivatives to include the secondary and tertiary γ-aminoalcohols seemed desirable. The preparation and chemical properties of β-aminoketones and secondary and tertiary γ-aminoalcohols derived from hexamethylenimine are described in this manuscript. The results of the pharmacological evaluation will be published at a later date.

EXPERIMENTAL

Mannich Bases (β-Aminoketones).—The procedure of Mannich and Lammering (12), as applied by Nobles *et al.* (14, 15), was employed for the preparation of the Mannich bases. Pertinent data are provided in Table I.

Secondary γ-Aminoalcohols (Table II, No. 1–3).—The preceding Mannich bases were reduced with sodium borohydride in aqueous methanol according to the procedure of Chaikin and Brown (19), as employed by Nobles (20) and by Rogers and Nobles (15). The data for these compounds are listed in Table II.

Tertiary γ-Aminoalcohols (Table II, No. 4–12).—The procedure of Pohland and Sullivan (21) was employed for the preparation of these compounds. Relevant physical and chemical properties are given in Table II.

DISCUSSION OF RESULTS

Hexamethylenimine (azepane), a homolog of piperidine, was converted to the hydrochloride salt and condensed with acetophenone, propiophenone, or *p*-methylacetophenone and paraformaldehyde in ethanol according to the procedure of Mannich and Lammering (12). *sym*-Trioxane, an acetal, failed to function as a source of formaldehyde in the reaction. Evidently acetals are unable to depolymerize in the relatively dry ethanol employed in the reaction, while hemiacetals, such as paraformaldehyde, are readily converted to free aldehyde. Pertinent data are given in Table I.

The Mannich bases were reduced with sodium borohydride according to the procedure of Chaikin and Brown (19), as applied by Nobles (20) and by Rogers and Nobles (15). The products of the reduction, secondary γ-aminoalcohols, were isolated from dry ether as the hydrochlorides. Physical and chemical properties are listed in Table II.

In a similar manner the procedure of Pohland and Sullivan (21) was employed for the preparation of tertiary γ-aminoalcohols. Methyl-, ethyl-, and phenylmagnesium bromide were reacted with Mannich bases of acetophenone, propiophenone, and *p*-methylacetophenone; the resultant aminoalcohols were isolated as the hydrochlorides. Pertinent data are provided in Table II.

In the isolation of all the secondary and tertiary γ-aminoalcohols it was desirable to chill the dry ethereal solution before treatment with anhydrous hydrogen chloride. Oil formation, and possibly side reactions, appear to be retarded by this measure. Prolonged treatment of the solution with anhydrous hydrogen chloride gave some evidence of decomposition.

The yields of Mannich bases, secondary, and tertiary γ-aminoalcohols are uniformly low for *p*-methylacetophenone and consistently high for propiophenone. In general, acetophenone derivatives were obtained in intermediate yields. These data may reflect solubilities in the recrystallization solvents more closely than they reflect reaction yields since members of the propiophenone series were all rapidly precipitated on recrystallization, while members of the *p*-methylacetophenone series were obtained in crystalline form only after refrigeration for several days at sub-zero temperatures.

The order of addition of Grignard reagent and Mannich base has been reported to be important in obtaining high yields of the tertiary γ-aminoalcohols (28). No definite conclusions could be drawn from the present studies. Preliminary data, while not conclusive, indicated that the best order of addition depends more on the nature of the reactants than on any inherent advantage of the order of addition *per se*. Further studies are planned.

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Identification of 3′,4′,7-Trihydroxyflavone in Ladino Clover

By A. L. LIVINGSTON and E. M. BICKOFF

A yellow crystalline flavone, $C_{15}H_{10}O_5$, previously isolated from ladino clover (*Trifolium repens*), has now been identified as 3′,4′,7-trihydroxyflavone. This structure was confirmed by comparing the ultraviolet and infrared spectra and melting point of the unknown to those of an authentic synthetic sample.

PHENOLIC COMPOUNDS of forages are under investigation at this laboratory. To date, the estrogenic compound, coumestrol (1); the related coumestan, trifoliol (2); the estrogenic isoflavones, genistein, daidzein, biochanin A, and formononetin (3); the dicoumaryl ether, daphnoretin (4); and the flavone, tricin (5), have been isolated and identified.

Recently, the isolation of 17 phenolic compounds from ladino was reported (6). One of these, compound XVII, having the empirical formula $C_{15}H_{10}O_5$, has now been identified. The formation of a triacetate indicated the presence of three phenolic hydroxyl groups. The similar ultraviolet spectra of compound XVII triacetate ($\lambda_{max.}$ 299, 253) and of the unsubstituted compound, flavone itself ($\lambda_{max.}$ 297, 250), suggested that the unknown was also a flavone (7). Production of orange-red color, upon treatment of an alcoholic solution of the parent compound with magnesium and hydrochloric acid, further confirmed its flavone structure (8).

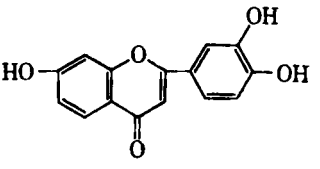
A marked shift in the ultraviolet spectrum of the parent compound in the presence of boric acid–sodium acetate solution indicated an *o*-dihydroxyl substitution (9). A comparison of the melting points of the phenolic compound and its triacetate to those reported in the literature for known trihydroxyflavones indicated that it might be 3′,4′,7-trihydroxyflavone.

This structure was unequivocally confirmed by comparing the spectra and mixed melting points to those of authentic samples. Although the synthesis of this compound has been described (10), this is the first report (to our knowledge) of its occurrence in a natural plant product.

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I

EXPERIMENTAL

A paper giving the details of the isolation of this yellow crystalline compound (compound XVII) by countercurrent distribution from an acetone extract of ladino clover was recently prepared (6). The flavone was recrystallized from methanol to yield the analytical sample, m.p. 318–322° dec.; no depression in melting point with a synthetic sample, m.p. 318–322° dec. $\lambda_{max.}^{EtOH}$ 342 (log ϵ, 4.33), 313 (sh) (log ϵ, 4.18), 236.5 (log ϵ 4.31) mμ. (Spectrum identical to that of synthetic 3′,4′,7-trihydroxyflavone.) The infrared spectra of the isolated and synthetic flavones were also identical.

Anal.—Calcd. for $C_{15}H_{10}O_5$: C, 66.7; H, 3.70. Found: C, 66.8; H, 3.65.

Thirty milligrams of the parent phenol was treated in the usual way with acetic anhydride and fused sodium acetate to form the triacetate. The triacetate was recrystallized from acetone to give 28 mg. of white needles, m.p. 209–210°; no depression in melting point with a synthetic sample, m.p. 209–210°. $\lambda_{max.}^{EtOH}$ 299 (log ϵ, 4.43), 253 (log ϵ, 4.25) mμ. The ultraviolet and the infrared spectra of the natural and synthetic acetates were identical.

Anal.—Calcd. for $C_{21}H_{16}O_8$: C, 63.8; H, 4.04; CH_3CO, 32.6. Found: C, 63.6; H, 4.414; CH_3CO, 32.8.

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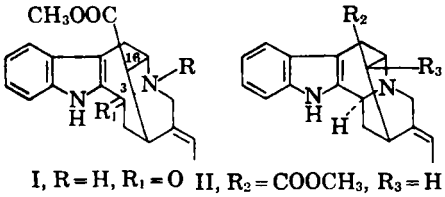
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Pericyclivine, a New *Catharanthus* Alkaloid

Sir:

In the course of the structure elucidation of perivine (I) (1), a major alkaloid of *Catharanthus roseus* and *C. lanceus* (2), the authors had occasion to convert I to the hitherto unknown pentacyclic deoxy compound II. The isolation of an alkaloid, pericyclivine, identical to substance II from the leaves of *C. lanceus*, is now reported.

Of the six classified *Catharanthus* species, only the two mentioned above have been phytochemically enumerated to date (3). Several identical alkaloids appear to be present in both species, *i.e.*, perivine and leurosine (2). Perivine (I) is a 2-acyl indole-containing compound; as such, it is capable in its reduced form of readily forming dimeric substances through condensation with activated aromatic rings (1). The alkaloid voacamine (III) is an example of such a condensation product (4). No natural dimer of this type has been found in the genus *Catharanthus* as yet.



I, R = H, R_1 = O II, R_2 = $COOCH_3$, R_3 = H

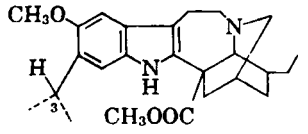
IV, R_2 = H, R_3 = $COOCH_3$

V, R_2 = $COOCH_3$, R_3 = CH_2OH

VI, R_2 = CH_2OH, R_3 = $COOCH_3$

VII, R_2 = H, R_3 = CH_2OH

III, R = CH_3, R_1 =



Following the procedure utilized for *C. roseus* (5), the *A* fraction from *C. lanceus* leaf (100 Gm.) was chromatographed on alumina (partially deactivated, Alcoa F-20). After elution of perivine, leurosine, and yohimbine, crude pericyclivine was obtained from the chloroform–benzene(2:1) eluates. Two crystallizations from ethanol afforded 0.225 Gm. of pericyclivine ($C_{20}H_{22}N_2O_2$), m.p. 232–233°, monoclinic prisms with elongation parallel to the *b* axis, pK'a 6.75 (33% DMF), $[\alpha]_D^{26}$ + 5.2 (*c* = 1.0, $CHCl_3$), which was shown to be identical to II, prepared from perivine (1), by comparison of I.R. and NMR spectra and mixed melting points (1). An examination of the NMR spectrum of pericyclivine indicated that the ester function (CH_3 singlet 3.05 δ) at C-16 was oriented toward the indole moiety as shown in II. Treatment of the base with sodium methoxide in methanol resulted in epimerization of the ester-methyl (3.78 δ) to yield the known dehydroxymethyl akuammidine[1] (IV) (6).

A number of indole alkaloids containing the pericyclivine ring system are known (7). Of particular interest is the occurrence of both ester-carbinol isomers in akuammidine (V) and polyneuridine (VI) and also the 16-carbinol, normacusine-B (VII). This represents the first example of an alkaloid in this series containing only an ester function at C-16. The finding of this alkaloid, an obvious progeny of perivine, indicates that perivinol or its biosynthetic equivalent must exist in *C. lanceus*. It will be interesting to see if dimeric compounds containing perivine will eventually be discovered in this plant.

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[1] The logical name of pericyclivine should be dehydroxymethyl akuammidine since the ester function in akuammidine (V) is oriented as shown for the former compound (II). However, treatment of akuammidine with potassium *tert*-butoxide yields compound IV isomerized at position 16. Therefore, to avoid confusion we have chosen the trivial name "pericyclivine."