Measurements of Ionization Constants and Partition Coefficients of Guanazole Prodrugs

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Abstract ☐ A series of guanazole prodrugs, which are less water soluble than the parent compound and have relatively higher molecular weights, was recently synthesized, and their antineoplastic activities were measured *in vitro.* In the present work, the ionization constants and partition coefficients of these compounds were measured for the first time. In contrast to guanazole, which is a weak base, guanazole prodrugs have been shown to be weak to moderate acids due to the electronic effects of the acyl group and the heterocyclic ring. Possible tautomeric and resonance structures are presented to account for the pKa values observed. Both the inductive and resonance effects of the substituent are important in determining the values of the ionization constant. The preparation of the prodrugs not only altered the lipophilicity but also drastically changed the acid–base property of the parent compound. The observed true partition coefficient values in most guanazole prodrugs studied were higher than those calculated from the π-constants. Under highly ionized conditions, the small amount of water in the octanol layer has a significant effect in trapping a substantial amount of the ionized species in the octanol layer and gives rise to a higher log P value than expected. Under nonionized conditions, intramolecular hydrogen bonding plays an important factor in electron delocalization and reduction of hydrogen bonding with water molecules, causing the nonionized species of the guanazole prodrugs to be more lipophilic than expected.

Keyphrases ☐ Guanazole-prodrugs, ionization constants and partition coefficients ☐ Ionization constants—guanazole prodrugs ☐ Partition coefficients—guanazole prodrugs ☐ Prodrugs—guanazole, ionization constants and partition coefficients ☐ Antitumor agents—guanazole prodrugs

Guanazole, a known ribonucleotide reductase inhibitor was shown to have antitumor activity against the murine leukemias L-1210 and K-1964, carcinoma, and reticulum cell sarcoma A-RCS (1). Brockman *et al.* found that guanazole acts by inhibiting the incorporation of adenine, hypoxanthine, and uridine into DNA to a much greater extent than into RNA (2).

BACKGROUND

Guanazole has been shown to be effective clinically in patients with acute and chronic myelogenous leukemias (3). In addition to the side effects observed in therapy with guanazole, such as hematological toxicity and some myelosuppression (4, 5), guanazole has some biopharmaceutical disadvantages. Pharmacokinetic studies on guanazole have shown that 95% of the drug was excreted in the urine of mice within 3 hr and most of it was unchanged (6). It also has a very short half-life: 30 min in mice (7), 68 min in rats (8), and 5 hr in humans (8, 9). Thus, a high dose or continuous administration is necessary to obtain the required therapeutic effect. The short half-life of guanazole can be attributed to its high polarity and its low molecular weight.

To circumvent these problems, a series of new guanazole prodrugs was prepared by acylation of guanazole by various organic anhydrides and acid chlorides (10). The antineoplastic activity *in vitro* against leukemia L-1210 has already been studied. The results showed that four of the prodrugs had comparable or greater *in vitro* activity than guanazole itself. (*In vivo* testing is presently being conducted by the National Cancer Institute.)

To carry out structure–activity relationship studies to serve as guidelines for further molecular modification, it is necessary to have pertinent physicochemical parameters for the guanazole prodrugs. However, these properties of the new compounds are not available in the literature. This need prompted the investigation of the ionization constants (pKa) and partition coefficients (log P) since these are the most important physicochemical constants used in structure–activity relationship studies (11).

Guanazole prodrugs have a triazole structure with two ionization sites, deprotonation and protonation. However, under the present experimental conditions, pKa values represent only the deprotonation process. Since the optical properties of the undissociated forms differ from those of the dissociated forms, UV spectroscopy was utilized to measure the pKa values of the prodrugs.

The partition coefficient (log P) may be measured at a pH in which the drug is partially ionized and partially nonionized (12, 13). However, the ionized form of a molecule does not partition into octanol (13). The true partition coefficient may be calculated from the observed value, the buffer pH, and the pKa of the compound. Therefore, the apparent partition coefficient is pH dependent (14–16) and should be converted to the "true" (corrected) partition coefficient, which is pH independent as indicated by the following:

$$P_{corr} = \frac{C_o}{C_a(1 - \alpha)} = \frac{P_{app}}{(1 - \alpha)} \qquad (Eq. 1)$$

where P_{corr} is the corrected partition coefficient, P_{app} is the apparent partition coefficient, C_o is the concentration of the drug in the organic phase, C_a is the concentration of the drug in the aqueous phase, and α is the degree of ionization calculated from the Henderson–Hasselbalch equation (17) as follows. For acids:

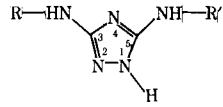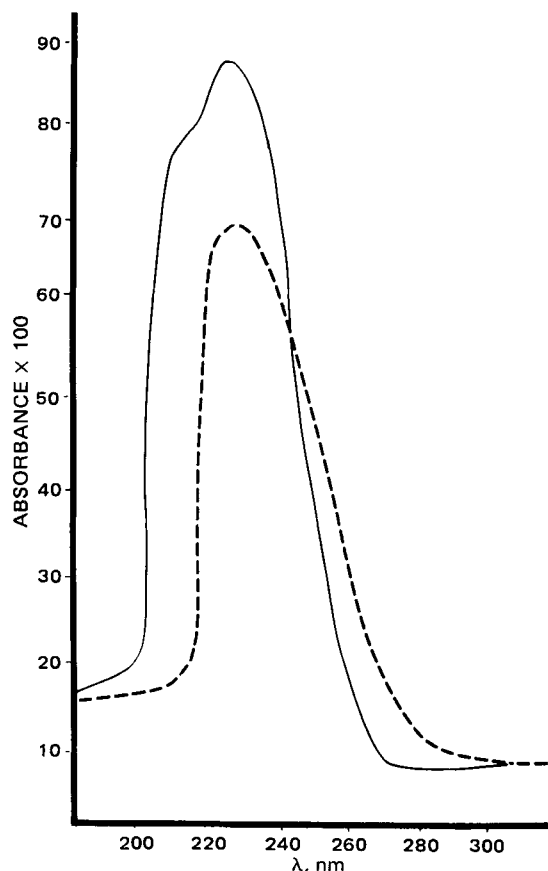$$\alpha = \frac{1}{1 + antilog\ (pKa - pH)} \qquad (Eq. 2)$$

Figure 1—*UV spectrum of 3,5-diacetamido-1,2,4-triazole. Key:* —, *pH 4.50; and* - - -, *pH 12.50.*

Table I—Structural Formulas of Guanazole Prodrugs

R—HN, N, NH—R'

	Chemical Name	R	R'
I	3,5-Diacetamido-1,2,4-triazole	$COCH_3$	$COCH_3$
II	3,5-Dipropionamido-1,2,4-triazole	$COCH_2CH_3$	$COCH_2CH_3$
III	3,5-Dibutyramido-1,2,4-triazole	$COCH_2CH_2$-CH_3	$COCH_2CH_2$-CH_3
IV	3,5-Di(trifluoroacetamido)-1,2,4-triazole	$COCF_3$	$COCF_3$
V	3-Amino,5-heptafluorobutyr-amido-1,2,4-triazole	H	COC_3F_7
VI	3,5-Dibenzamido-1,2,4-triazole	COC_6H_5	COC_6H_5
VII	3,5-Di(p-nitrobenzamido)-1,2,4-tri-azole	$COC_6H_4NO_2$	$COC_6H_4NO_2$

For bases:

$$\alpha = \frac{1}{1 + \text{antilog} (pH - pKa)} \quad \text{(Eq. 3)}$$

An aqueous phase with a suitable buffering system is required to enhance the dissolution and ionization or to maintain a particular pH with an organic solvent. However, the effects of buffer species on the apparent partition coefficient were not studied until recently (12). It was found that phosphate buffer is the best system for measuring the true partition coefficient for quantitative structure–activity relationship work for both acidic and neutral drugs whenever the drug is soluble in this system. For many drugs, it is necessary to correct for the discrepancies when different buffer systems are used.

Due to the limited solubility, the phosphate buffer is not suitable for some of the prodrugs studied. It was also observed that the degree of ionization of some prodrugs is very high with most of the buffer systems. Accordingly, the small amount of water molecules in the octanol layers may trap a reasonable amount of the ionized species, giving rise to a higher log P value than predicted (12, 18).

EXPERIMENTAL

Materials—A UV spectrometer[1] equipped with a digital recorder, a pH meter[2], and an automatic electrobalance[3] were used. Buffer solutions such as hydrochloric acid–potassium chloride (pH 1.0–2.5), acetate (pH 3.6–5.9), phosphate (pH 6.0–8.0), tromethamine (pH 7.2–9.0), borate (pH 9.2–10.7), and bicarbonate (pH 9.28–10.1) were prepared as reported (19). They were then stored as 0.1 N solutions and diluted to the proper ionic strength (17, 20). The pH values were then adjusted by using 0.1 N HCl or 0.1 N NaOH.

Compounds—Guanazole prodrugs (Table I) were synthesized previously (10). Guanazole (3,5-diamino-1,2,4-triazole) was obtained commercially[4]. The melting point (158°) of salicylic acid[5] was determined to check for purity.

Purification of Octanol—Octanol[6] was purified by shaking with 1 N sodium hydroxide, 1 N sulfuric acid, and 5% sodium bicarbonate and then drying with excess anhydrous magnesium sulfate and fractional distillation (21, 22). The purified solvent did not show any UV-absorbing impurities (200–800 nm) for the boiling range of 192–195°.

Determination of Ionization Constants—A previously described procedure (17) was used for the determination of ionization constants. Its accuracy was checked with an authentic sample of salicylic acid (at 25°), and the result (2.94) was in close agreement with that reported (2.97) (23). The concentration of the buffers utilized for compounds I–VII was 0.05 M.

Determination of Apparent and True (or Corrected) Partition Coefficients—An accurate weight of the drug under investigation was dissolved in a suitable buffer solution to make up the stock solution (100 ml). The problem of low solubility was circumvented by the use of 2% C_2H_5OH, 1% $(CH_3)_2SO$, or 0.05 N NaOH (pH 12.4). Aliquots of the stock solution were partitioned between the 1-octanol–aqueous system (each

Table II—Ionization Constants and the Apparent and Corrected Partition Coefficients of Guanazole Prodrugs

Compound	pKa	Log P Calc.	$1 - \alpha$	Observed Log P_{app}	Log P_{corr}
I	9.35	−2.70	0.989637	−1.02	−1.01
II	9.34	−1.48	0.991776	−0.06	−0.07
III	9.34[a]	−0.54	0.990040	0.78	0.79
IV	5.90	−1.56	0.034265	−0.86	0.62
V	6.10	0.96	0.047727	0.23	1.54
VI	10.21	0.50	0.006415	−0.95	1.25
VII	8.64	0.44	0.953309	0.53	0.55

[a] The pKa of the dipropionated form was used.

phase being presaturated with the other) in 50-ml glass-stoppered bottles. These two phases were vigorously shaken by hand to ensure rapid distribution. The bottles were then placed in a horizontal bottle shaker for 3–4 hr at room temperature (25 ± 1°), followed by centrifugation of 10 ml of the aqueous layer for 20 min. Alternatively, the partitioned phases were centrifuged for 1 hr at 2000 rpm after 2 min of vigorous shaking.

Standard curves were run from duplicate samples using two separate weighings of the drug. In most cases, a 1:1 (v/v) ratio of 1-octanol to buffer (25 ml:25 ml) was used. The drug concentration in the aqueous phase after centrifugation was measured from UV absorbance and deduced from the standard curve. The apparent partition coefficient was calculated from the equation:
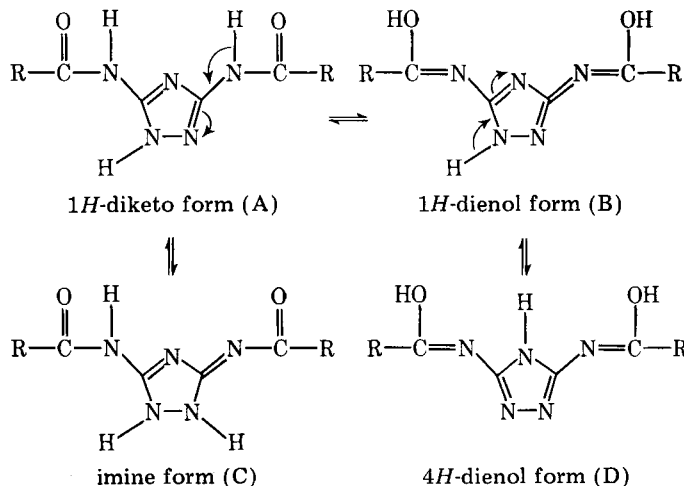
$$P_{app} = C_{octanol}/C_{aqueous} \quad \text{(Eq. 4)}$$

where $C_{octanol}$ and $C_{aqueous}$ are the prodrug concentrations in the octanol and aqueous phases, respectively. Based on the apparent partition coefficient and its corresponding pH and pKa values, the true or corrected partition coefficient value was calculated from Eq. 1.

RESULTS AND DISCUSSION

The ionization constants and both the apparent and true (corrected) partition coefficients of Compounds I–VII were measured for the first time in this study and are listed in Table II.

Dedichen (24) first measured the basicity constants of 1,2,4-triazole and some of its 2-amino-3,5-dialkyl substituted products. The applicability of Hammett's equation to the triazole ring was shown previously (25, 26). In addition, it was found (27) that basicity increases in the presence of an electron-donating group but decreases in the presence of an electron-withdrawing group. By employing the Hückel approach in conjunction with the linear combination of atomic orbitals–molecular orbital (LCAO–MO) method, the protonation center in 1H-1,2,4-triazole was shown to be the N-4 ring nitrogen atom (28). The basicity constants of a series of 5-(3)-substituted 3-(5)-amino-1,2,4-triazoles was determined by potentiometric titration (29). A high degree of quantitative correlation between the pKa values and the Hammett constants was found. Furthermore, the pKa from 3,5-diamino-1,2,4-triazole was 4.43, which was attributed to the conjugate acid of N-4 of the triazole ring (29). On the other hand, the ionization constants of the deprotonation of the hetero-imino group of a series of 1,2,4-triazole derivatives were measured previously (30) and were in the range of 6.10–11.08. Consequently, it appears

R—C—N ... N—C—R   R—C=N ... N=C—R

1H-diketo form (A)   **1H-dienol form (B)**

R—C—N ... N—C—R   R—C=N ... N=C—R

imine form (C)   **4H-dienol form (D)**

Scheme I

[1] Perkin-Elmer double-beam Coleman 124.
[2] Beckman model 3500.
[3] CAHN 25.
[4] Aldrich Chemical Co.
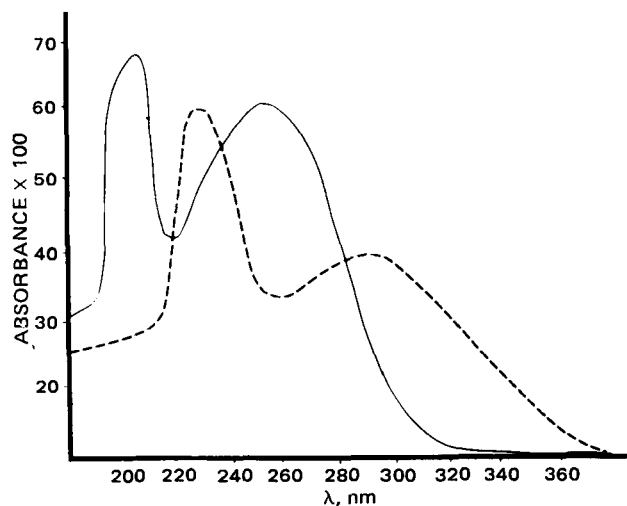[5] Mallinckrodt Chemical Works.
[6] Fisher Chemicals.

Figure 2—*UV spectrum of 3,5-dibenzamido-1,2,4-triazole. Key:* —, *pH 5.00; and* - - -, *pH 12.50.*

that the pKa values determined in this study (Table II) belong to the deprotonation process of the weakly acidic amido —NHCO— group or the 1,2,4-triazole ring rather than to the protonation of any basic nitrogen.

This finding was utilized in determining the percent ionization at the pH values used to measure the ionization constants of the compounds.

Some work has been published on the tautomerism of 1,2,4-triazole derivatives (26, 31, 32); however, nothing has yet been reported concerning the tautomeric isomers of guanazole derivatives. Nevertheless, a theoretical postulate can be drawn for the possible tautomeric isomers of guanazole derivatives as shown in Scheme I.

It has been shown (33) by IR spectral comparison of amidine analogs that alkyl groups (Y) tend to prefer attachment to the amino system 2A rather than to 2B but that the reverse is true for aryl groups.

Chua et al. (34) quantified the energy differences between the two possible tautomers by applying the basicity method (35), which depends on the formation of the cation for both the tautomeric forms A and B, and comparing their basicities to fixed models of A and B. From their findings, it appears likely that the tautomeric structure A is the predominant structure for I–III. On the other hand, the tautomeric structures B and C are more likely for IV–VII.

Apparently, there are two different kinds of tautomerism: the imino and keto-enol types. Furthermore, there are four possibilities for dissociation. A comparison of the UV spectra of the neutral and the anion species is important in determining the dissociation site. It was found (27) that the splitting off of a proton attached to the heteronitrogen atom of 3-nitro-1,2,4-triazole-5-carboxylic acid causes a marked (50 nm) bathochromic shift in the UV spectrum while dissociation at the carboxyl group of 1-methyl-5-nitro-1,2,4-triazole-3-carboxylic acid gives a very small (5 nm) bathochromic shift.

The UV spectrum of I (Fig. 1) at various pH values shows a negligible bathochromic shift (4 nm) of the B band of the triazole ring. Therefore, the dissociation process and the possible canonical forms of I–III can be represented by Scheme II.

Likewise, the UV spectra of the neutral and the anionic species of VI is shown in Fig. 2. For the neutral form, there are two absorption maxima, one at 260 nm, which is characteristic of the aromatic ring and is analogous to the K-band (36), and one at 215 nm for the B-band, which is characteristic of the heteroaromatic ring. The splitting off of the proton causes a very strong bathochromic shift of 20 and 40 nm for the B- and K-bands, respectively. Therefore, the removal of the proton causes a delocalization of electrons in both rings, which indicates a possible pathway for the deprotonation process and the resulting resonance structures (Scheme III).

The UV spectrum of VII is shown in Fig. 3. There is a red shift (14 nm) for the K-band as compared to VI, which is attributable to the electron-withdrawing activity of the nitro group. However, the B-band remains
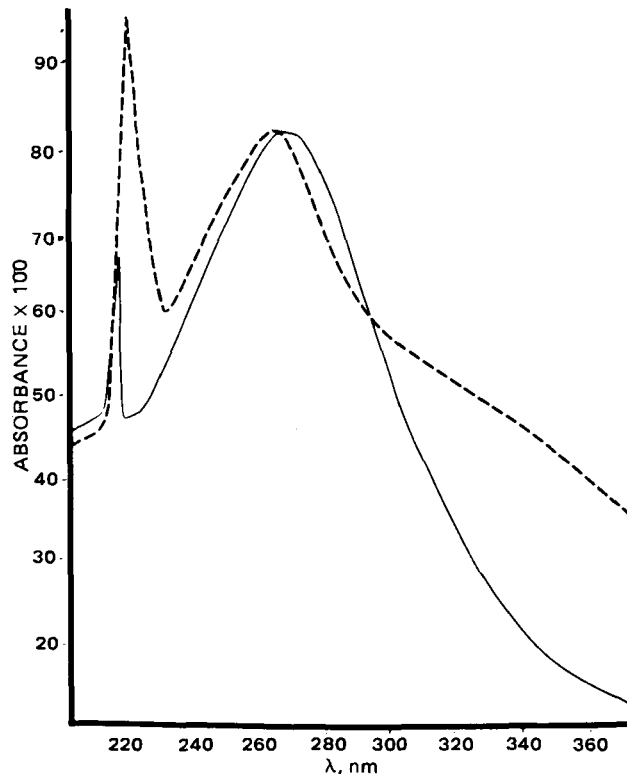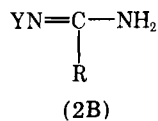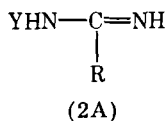

Figure 3—*UV spectrum of 3,5-di(p-nitrobenzamido)-1,2,4-triazole. Key:* —, *pH 4.50; and* - - -, *pH 12.50.*

the same as that of VI. Deprotonation causes the solution to change from colorless to yellowish, but a negligible change is observed for both the K- and B-bands. The contributing structures for the visible absorption may be illustrated as shown in Scheme IV.

From Table II, it appears that there is a substantial dependence of the ionization constants on the electronic character of the substituent. The inductive and resonance effects both contribute to the ionization constant in a collective manner.

It was shown (11) that changing the electronic properties of the substituent groups may affect the prototropic equilibria and result in a more active tautomeric form of a particular drug. This investigation found that, in contrast to guanazole which is a weak base, guanazole prodrugs range from weak to moderate acids. Preparation of the prodrugs not only altered the lipophilicity but also drastically changed the acid–base property of the parent compound.

Table II shows the measured and calculated values of the partition coefficients of the prodrugs examined. It was shown (37) that log P is not entirely additive. It has been suggested (38) that the additivity of π-values will break down when there is mutual interaction between substituents (intramolecular hydrogen bonding, steric, and field effects) or when a substituent cannot be dissolved to its maximal potential (39).

The nature of the interaction between guanazole derivatives and both octanol and water can be illustrated by Scheme V.

The preferred interaction of guanazole with the aqueous or organic layers probably would be determined in part by the electronic properties of the X substituent. Electron-withdrawing groups would inhibit hydrogen bonding of the type shown on the right side of Scheme V due to the delocalization of the lone-pair electrons but enhance hydrogen bonding of the type shown on the left side of Scheme V. Octanol, being more nucleophilic than water (40), would compete more effectively in binding with the hydrogen of the amino nitrogen. The differences between the observed and calculated values of log P in Table II can be rationalized in part from the substituent effect on the availability of the lone-pair electrons.

Although the true partition coefficient takes into account the ionization of the drug at a given pH, the assumption that the ionized species of an ionizable drug is insoluble in the nonaqueous layer may be incorrect. First, the small amount of water in the octanol layer is expected to accommodate some concentration of the ionized species (12, 18). Thus, the observed log P would be higher than the calculated value. The partition coefficients of ring-substituted aspirin were measured at different pH values (18). It was found that the discrepancies between the observed and

$$\text{YHN}-\underset{\underset{\text{R}}{|}}{\text{C}}=\text{NH} \qquad \text{YN}=\underset{\underset{\text{R}}{|}}{\text{C}}-\text{NH}_2$$

(2A)   (2B)
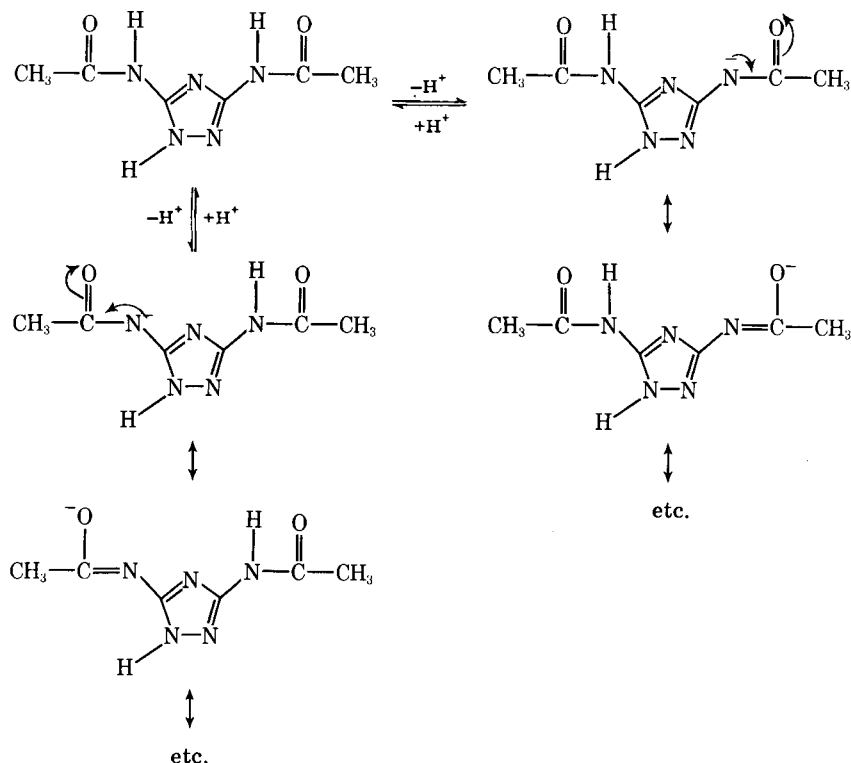
Scheme II

calculated values correlated well with the calculated lipophilicities, which was attributed to ion-pair formation with buffer counterions (41).

Second, the ionized species may have a different conformation than that of the nonionized form. The partition coefficient (log P = 4.33) of salicylic acid in a 1-octanol–bicarbonate buffer (pH 9.20) system has been shown to be very different from that in a 1-octanol–acetate (pH 4.00) system (log P = 2.87). Wang and Lien (12) considered the first factor as well as the effect of the ionic strength of the buffer to rationalize such a deviation. The difference in the intramolecular hydrogen bonding between the ionized and the nonionized forms may also play a significant role. At a pH of 9.2, the deprotonated form of salicylic acid is predominant (Scheme VI).

Scheme III

Therefore, the ionized form is more capable of intramolecular hydrogen bonding than the nonionized form and gives rise to a higher log P. However, the ionized form also has a higher preference to a hydrogen bond with water molecules than the nonionized form. This decreases the log P. The net effect on log P depends on the buffer species used as well as the pH value.

Compared to the calculated values, the higher values of the observed log P for I–III can be attributed to both the inductive effect of the substituent and the intramolecular hydrogen bonding between the carbonyl group and the triazole ring. Compounds IV–VI, with a high degree of ionization (low 1 − α values) at the given pH values, may be trapped by the small amount of water molecules in the octanol layer, giving a higher log P value. Compound VII has an observed log P value only slightly higher than the expected value.

Compound VII can be considered a nitrobenzene derivative substituted at position 4. It can be considered similar to a dinitro-benzene derivative where each nitro group can nullify the effect of the other, resulting in a smaller difference between the observed and calculated log P. Furthermore, at pH 7.40, the degree of ionization of VII is relatively small (5%). Accordingly, the amount of ionized drug trapped by the small amount of water in the octanol layer is negligible. The observed value of log P did not deviate significantly from the calculated value.
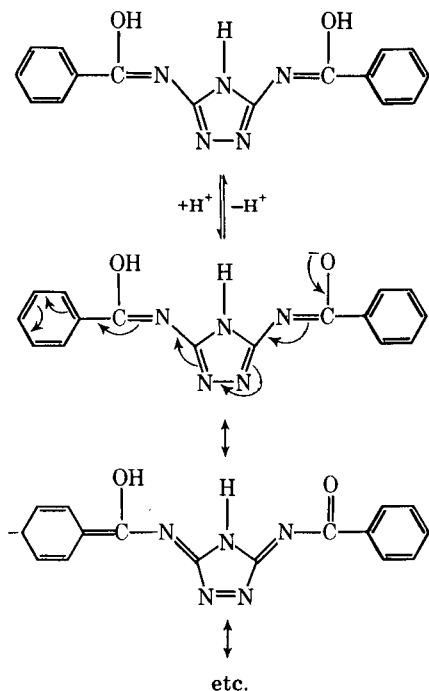
The partition coefficient of salicylic acid was measured utilizing two different procedures:

1. After 2 min of vigorous shaking, the two layers were partitioned for 3–4 hr at 25° in a bottle shaker, and the aqueous layer was centrifuged for 20 min.

2. After 2 min of vigorous shaking, the two layers were centrifuged together for 30–45 min in a centrifuge tube.

The apparent partition coefficients from both methods were identical. However, the pH values, after partitioning from the first and second methods, were 8.90 and 9.20, respectively. Therefore, the true partition coefficient was lower in the first method (4.33 *versus* 4.63). The difference can be attributed to the efficiency of the first method over that of the second in getting clear separation of the aqueous from octanol layer or to the temperature effect that might happen under the longer period of centrifugation in the second method.

Selection of the proper method depends on the compounds being investigated and the volume ratio (42). The first method should be more desirable for a compound that needs a long period of time to reach equilibrium, *i.e.*, phenol (43). For highly ionized compounds, it is advisable to have an efficient separation to minimize any emulsification or clouding of the phases.
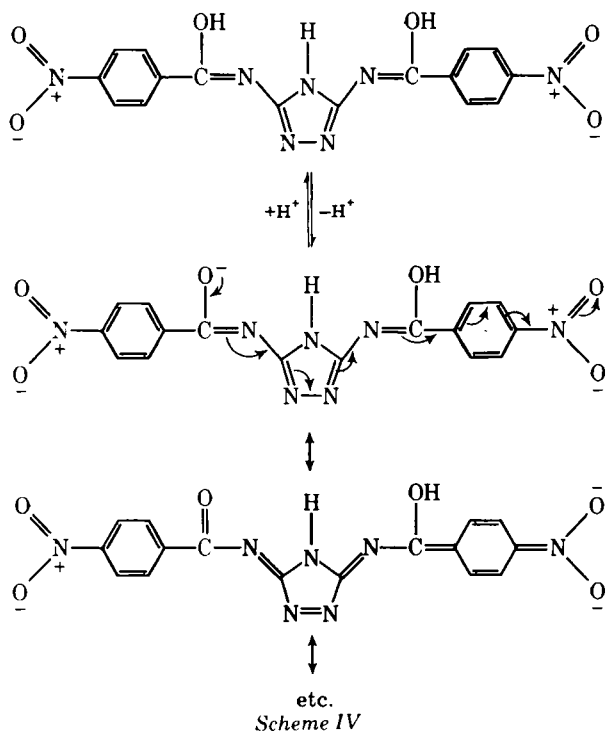
In this study, the separation of the two layers with phosphate buffer

etc.

Scheme IV

was more efficient than other systems used under the same experimental conditions. This finding is in agreement with earlier data (12).
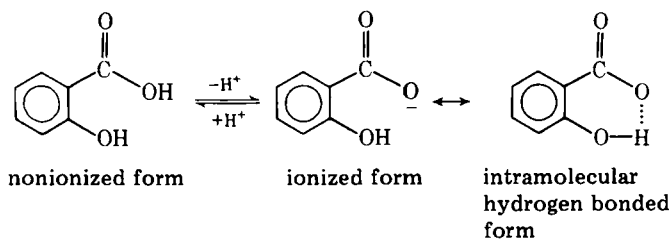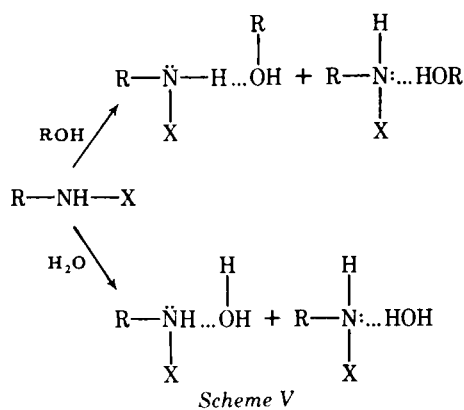
Distribution coefficients (D) of ionizable compounds have been used in structure–activity correlations (43). From the mathematical formula for D, it can be shown that D is the same as the apparent partition coefficient:

$$D = AH_l/(AH_w + A^-)$$

$$(P_{app} = C_0/C_w = AH_l/(AH_w + A^-)$$

$$D/(1 - \alpha) = AH_l/(AH_w + A^-)(1 - \alpha) = AH_l/AH_w = P_{corr}$$

where α = degree of ionization = $A^-/(AH_w + A^-)$. AH_l and AH_w and the free acid concentration in the lipid and the water layer, respectively, and A^- is the concentration of the anion in the water layer.

Scheme V

nonionized form   ionized form   intramolecular hydrogen bonded form

Scheme VI

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Heterogeneity of Biochemical Actions among Vasodilators

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Abstract □ Thirty-four vasodilators were screened in several *in vitro* biochemical assays related to smooth muscle excitation–contraction coupling, binding to β_1^-, β_2^-, and α-adrenergic receptors, inhibition of phosphodiesterase activity, and antagonism of calcium accumulation. Isoproterenol and perhexiline only exhibited binding to β-adrenergic sites. Ergocryptine, tolazoline, and amotriphene only bound to α-adrenergic receptors. Leniquinsin, papaverine, proquazone, dioxyline, hoquizil, quazodine, and theophylline were active only as phosphodiesterase inhibitors. Isoxsuprine, nylidrin, and bencyclane bound to α- and β-receptors. Pentoxifylline bound to β_1-sites and inhibited phosphodiesterase. Cyclandelate bound to β_2-sites and blocked calcium accumulation. Cinnarizine and flunarizine antagonized calcium accumulation and bound to α-sites. Prazosin bound to α-sites and inhibited phosphodiesterase. Ethaverine and dipyridamole were inhibitors of phosphodiesterase and calcium accumulation. Nafronyl bound to β_2- and α-sites and antagonized calcium accumulation. Mebeverine bound to β_2- and α-receptors and inhibited phosphodiesterase activity and calcium accumulation. Verapamil bound to α-sites, and blocked phosphodiesterase and calcium accumulation. Quinazosin bound to β_2- and α-receptors and antagonized both phosphodiesterase activity and calcium accumulation. Vasodilators that were inactive in all assays included niacin, nicotinyl alcohol, inositol nicotinate, amyl nitrite, sodium nitroprusside, diazoxide, hydralazine, and protoveratrine. Vasodilators should not be considered as a single drug class since they act on various mechanisms related to coupling of neuronal excitation to muscular contractility.

Keyphrases □ Vasodilators—biochemical mechanisms □ Adrenergic receptors—α, β_1, and β_2, effect of various vasodilators □ Calcium—effect of various vasodilators on intracellular accumulation, red blood cells □ Phosphodiesterase—inhibition by various vasodilators

The treatment of cardiovascular diseases such as hypertension (1, 2), angina pectoris, cardiac failure (3, 4), and certain peripheral vascular diseases (1, 5, 6) utilizes drugs that produce vasodilation. Vasodilation results from an increase in the internal diameter of blood vessels, presumably through relaxation of vascular smooth muscle. Significant progress has been made in elucidating the biochemical mechanisms controlling smooth muscle contractility. Molecular events in that process include the interaction of catecholamines with specific adrenergic receptors of the smooth muscle cell membrane, the activities of enzymes that regulate cellular levels of cyclic AMP, the control of cellular calcium-ion transport and storage, and the interaction of calcium with the contractile proteins of the smooth muscle cell (7–9).

By using this concept of excitation–contraction coupling in smooth muscle as a working hypothesis, the mechanism of action of various vasodilators has been studied. Thirty-four vasodilators (1, 2, 5, 10, 11) were tested in three types of *in vitro* screening models: (*a*) binding to α- and β-adrenergetic receptor sites, (*b*) inhibition of cyclic AMP phosphodiesterase, and (*c*) inhibition of intracellular calcium accumulation. The results suggest that vasodilator drugs interact with a variety of distinct events in the excitation–contraction coupling mechanisms of vascular smooth muscle.

EXPERIMENTAL

Adrenergic Radioreceptor Assays—β-*Adrenergic Receptor Binding Assays*—Sarcolemma-enriched membranes from guinea pig hearts were prepared as described previously (12). Guinea pigs were killed by cervical dislocation, and the hearts were immediately placed in ice-cold physiological saline. Hearts (20–25 g) were minced in 10 volumes of 2.5 mM imidazole buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (I) and briefly homogenized with a tissue homogenizer. The homogenate was centrifuged for 1 min at 22×g.

The supernatant fraction was rehomogenized and recentrifuged five times. The pooled supernatant fractions were passed through three layers of cheesecloth and centrifuged at 1600×g for 20 min. The resulting pellet was resuspended in 200 ml of 10 mM [tris(hydroxymethyl)aminomethane hydrochloride] buffer (II), pH 7.5, containing 0.1 M sucrose and homogenized using a conical polytef[1]/glass tissue grinder. The homogenate was centrifuged at 1600×g for 20 min.

These resuspension, homogenization, and centrifugation steps were then repeated. The pellet was resuspended in 50 ml of 0.4 M sucrose in 10 mM II (pH 7.5) and osmotically shocked by slow dilution into 1 liter of 0.25 mM I in 5 mM II (pH 7.0) buffer. The membranes were collected by centrifugation at 1600×g for 30 min, and the pellet was suspended in 20 ml of 0.4 M sucrose in 10 mM II (pH 7.5). The suspension was diluted with three volumes of a 13 mM II buffer (pH 7.0) containing 1.3 M KCl, 2.6 mM I, and 0.4 M sucrose. After stirring for ~16 hr at 4°, the extract was diluted with an equal volume of buffer [1 M KCl–2 mM I–10 mM II (pH 7.0)]. After centrifugation at 1600×g for 30 min, the pellet was washed three times in 100 ml of 0.1 M sucrose in 10 mM II (pH 7.0) buffer. The final pellet was suspended in 0.1 M sucrose and stored in liquid nitrogen.

Bronchial tree membranes were prepared from either fresh guinea pig lungs or lungs stored at −100°. Bronchial trees were exposed by combing through the lungs several times with a steel animal hairbrush to strip them of alveolar tissue. Extraneous tissue was removed by scraping with a scalpel. The bronchial trees were placed in a small volume of ice-cold II (pH 7.4) containing 1 mM MgCl₂ and 0.25 M sucrose. Then they were finely minced and homogenized by four 10-sec bursts with a tissue homogenizer.

The homogenate was filtered through a single layer of cheesecloth, and the filtrate was centrifuged for 10 min at 400×g. The pellet was discarded, and the supernate was centrifuged for 10 min at 20,000×g. The resulting pellet was washed in 50 mM II (pH 7.5) containing 10 mM MgCl₂ and centrifuged at 28,000×g for 10 min. The pellet was resuspended in 75 mM

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[1] Teflon.