Table IV—Aziridinylbenzoquinone Activity Based on Selected Criteria [a]

Compound	Ependymoblastoma	Intracerebral L-1210	P-388	Intraperitoneal L-1210	B16
II			X	X	
III	X		X		
IV	X		X		
VI	X	X	X		
VII	X		X		
XII			X		X
XIII		X	X		X
XIV	X	X	X	X	X
XV	X	X			
XVI			NT[b]		X
XVII	X		X		
XVIII			X	X	X
XX			X	X	
XXI			X	X	X
XXII	X	X	X		
XXIII			X	X	
XXVII	X				

[a] An X indicates a compound that meets the following criterion: ependymoblastoma, multiple long-term survivors in both experiments; intracerebral L-1210, confirmed T/C >150%; P-388, confirmed T/C >200%; intraperitoneal L-1210, confirmed T/C >200%; and B16, confirmed T/C >140%. [b] Not tested.

XIV was inactive against subcutaneous and intracerebral B16 melanocarcinoma as well as subcutaneous and intravenous Lewis lung carcinoma. It had confirmed activity against nine murine models: the five models in Table IV and the four shown in Table V.

Table V—Additional Antitumor Data for XIV [a]

Tumor	O.D.	Treatment Schedule	T/C	Activity Criterion (T/C)
CD mammary (mouse)[b]	12.5	Q7D × 5	23[c]	≤42
Intracerebral P-388[d]	1.5	QD 1–9	151	≥125
C38 colon[b]	25	Q7D × 3	32[c]	≤42
C26 colon[e]	3.1	Q4D × 3	240 (4)	≥130

[a] Highest reproduced T/C; see notes of Table III for definitions. [b] Subcutaneous tumor implantation. [c] Based on mean tumor weights estimated from tumor diameter. [d] Intracerebral tumor implantation. [e] Intraperitoneal tumor implantation.

Pharmacological studies are currently underway with XIV. After development of an acceptable formulation, preclinical toxicological studies will be initiated.

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Solubility of Doxycycline in Aqueous Solution

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Abstract □ The solubility of doxycycline monohydrate and doxycycline hydrochloride dihydrate was investigated in aqueous solution. The hydrochloride dihydrate salt was isolated and identified from solutions initially containing doxycycline hyclate in water. The pKa′ = 3.09 (μ = 0.1 and 25°) for protonation of doxycycline was determined spectrophotometrically. The pH–solubility profiles were determined for doxycycline monohydrate in water and in 1.0 *M* $NaNO_3$–HNO_3 and NaCl–HCl. The pH–solubility profile at 25° for doxycycline in aqueous hydrochloric acid without added salt reached a sharp maximum of 50 mg/ml at pH 2.16. Added chloride ion strongly suppressed the solubility of the hydrochloride dihydrate salt. The apparent solubility product was not constant but decreased as the concentration of added salt increased. A theoretical model was developed involving dimerization of doxycycline and applied to the experimental data. The dimerization constant, K_d = 24 M^{-1}, and true solubility product, $K^0_{sp} = 1.8 \times 10^{-3} M^2$, were calculated. The effect of concentration on NMR and visible spectra indicated that dimerization resulted from intermolecular hydrogen bonding of the phenolic β-diketone portion of the molecule.

Keyphrases □ Doxycycline—monohydrate and hydrochloride dihydrate salts, solubility in aqueous solution, effect of pH □ Solubility—doxycycline monohydrate and hydrochloride dihydrate in aqueous solution, effect of pH □ Antibacterials—doxycycline monohydrate and hydrochloride dihydrate, solubility in aqueous solution, effect of pH

Knowledge of quantitative solubility relationships in purely aqueous solutions is highly important to pharmaceutical research and product development. In water, solute activity is the summation of numerous associative interactions of the drug with other drug molecules and the solvent. These interactions often cause solutions of phar-

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maceutical interest to deviate from thermodynamic ideality (1).

The complex nature of liquid water and its self-association *via* hydrogen bonding are major complicating factors in the study of aqueous solubility. Another problem is that concentration is usually measured rather than thermodynamic activity (2), except in the case of hydrogen ions. Specific solute–solute and solute–solvent interactions may also be present, such as self-association of drug, hydrogen bonding, and ion pairing.

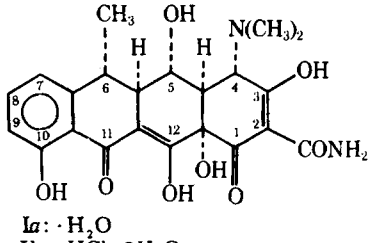
The effects of pH, drug ionization, salts, solvent (in mixed aqueous systems), and temperature on the solubility of several drugs were studied (1–3). The observed pH–solubility relationships in most cases followed basic mathematical expressions derived from the law of mass action.

An unusual characteristic of aqueous systems leading to nonideal behavior is the existence of common ion effects. Although the concept of solubility product equilibrium originated in inorganic chemistry, this phenomenon also has been observed for organic compounds of pharmaceutical interest. Triamterene exhibited common ion equilibria with hydrochloric, nitric, sulfuric, and phosphoric acids (3). pH–solubility profiles containing maxima at pH 2–3 were reported for chlortetracycline, demeclocycline, and methacycline hydrochlorides in sodium acetate–hydrochloric acid buffers (4). The decrease in solubility at lower pH values was attributed to the common ion effect of chloride on the solubility product equilibrium of the hydrochloride salts. The apparent dissolution rates and solubilities of these hydrochloride salts were less than those of the respective free base forms in chloride-containing media. Tetracycline hydrochloride, however, exhibited a low sensitivity to the chloride ion.

The present paper reports a quantitative study of the solubility properties of doxycycline monohydrate (I*a*) and hydrochloride dihydrate (I*b*) crystal forms. Common ion effects due to chloride and nonideal solubility behavior were investigated.

EXPERIMENTAL

Materials—Doxycycline monohydrate[1] was used as received. Doxycycline hydrochloride dihydrate was prepared from doxycycline hyclate[2] (Ic) by aqueous recrystallization. Doxycycline hyclate was dissolved in warm (40°) water to make a 30% solution. The solution was purged with nitrogen and refrigerated for 4 days. The resulting bright-yellow crystals were isolated and air dried at room temperature. Based on elemental analyses (C, H, N, Cl^-), water content (Karl Fischer method), and neutralization equivalent, the product was identified as the hydrochloride dihydrate salt of doxycycline. Paper chromatography and UV spectrophotometry confirmed that degradation had not occurred.



Ia: $\cdot H_2O$
Ib: $\cdot HCl \cdot 2H_2O$
Ic: $\cdot HCl \cdot 0.5H_2O \cdot 0.5C_2H_5OH$

All other chemicals were reagent grade. Water was deionized and double distilled.

Solubility Determination—Equilibration at 25 ± 0.2° was achieved with a vibratory mixer[3] and constant-temperature water bath. Excess amounts of drug were added to amber ampuls containing 5 ml of solution. The sealed ampuls were attached to a plastic holder assembly and vibrated for 18 hr. Preliminary experiments established that this time was sufficient for equilibration. The suspensions at equilibrium were filtered[4], and the first 2 ml of filtrate was discarded.

Apparent binding of doxycycline to the membrane filter caused the concentration in the initial volumes of filtrate to be lower than in later aliquots. This technical artifact has been observed for several other compounds (5). The pH was determined[5] after filtration. Solutions were diluted in 0.1 *N* HCl and assayed spectrophotometrically at 345 nm[6]. Solutions followed Beer's law under these conditions.

pKa′ Determination—The apparent ionization constant, pKa′, for formation of monoprotonated doxycycline was determined spectrophotometrically in citrate buffers at μ = 0.1 (NaCl) and 25°. The absorption spectra of the ionized and nonionized forms indicated that 262 nm was a suitable analytical wavelength. The absorbance of doxycycline was measured at several pH values, and the pKa′ was calculated by the method of Albert and Serjeant (6).

Spectral Studies—NMR spectra were obtained using a 100-MHz spectrometer[7]. Visible absorption measurements were made on a double-beam spectrophotometer[8]. Linearity of detector response over the absorbance range used was verified by constructing a Beer's law plot for FD&C Yellow No. 6 dye in water.

THEORY

Basic pH–Solubility Relationships—Mathematical expressions for the solubility of acid–base forms of weak electrolytes were reported previously (1–3). The terminology of Kramer and Flynn (1) is used here. For weak bases in the pH region where the solubility of the protonated form is limiting, the total solubility is:

$$S_{T,pH<pH_{max}} = [BH^+]_s + [B] = [BH^+]_s(1 + K'_a/[H^+]) \quad \text{(Eq. 1)}$$

where BH^+ is the protonated or salt form, B is the free base, K'_a is the apparent ionization constant, and subscript *s* denotes saturation. The pH_{max} is defined as the pH at which both base and salt species are simultaneously saturated. In a similar manner, the total solubility in the pH region where the free base solubility is limiting may be expressed as:

$$S_{T,pH>pH_{max}} = [BH^+] + [B]_s = [B]_s(1 + [H^+]/K'_a) \quad \text{(Eq. 2)}$$

The present treatment differs from that in Ref. 1 in the theoretical development of pH_{max}. The solubility at this pH is given by:

$$S_{T,pH=pH_{max}} = [BH^+]_s + [B]_s \quad \text{(Eq. 3)}$$

Therefore, Eqs. 1–3 should be sufficient to explain the solubility of a weak base throughout the pH region where pKa′ is applicable. The pH_{max} is defined as:

$$pH_{max} = pKa' + \log \frac{[B]_s}{[BH^+]_s} \quad \text{(Eq. 4)}$$

Application of the Gibbs phase rule to a system at pH_{max} indicates that it is thermodynamically invariant. Therefore, addition of small amounts of acid or base cannot change the equilibrium pH *or* solubility. For example, addition of the conjugate acid, *e.g.*, hydrochloric acid for a hydrochloride salt, temporarily upsets the equilibrium by forming protonated species from base species in solution. Since the concentration of the salt form is then above saturation, precipitation occurs to reestablish its solubility equilibrium. Solid base form also must dissolve to replace the amount "removed" from solution by protonation.

During these events, the pH decreases initially upon addition of acid but then returns to pH_{max} at equilibrium. The net result is that the solid base form is converted to the solid salt form *via* the solution phase. This transformation may proceed until the supply of the solid base form is

[1] Lot 42376, Pfizer.
[2] Lot 55028, Pfizer.
[3] Vibro-Mixer E1, 60 Hz, Chemapec, Woodbury, N.Y.
[4] Millipore, 0.45 μm.
[5] PHM 62, Radiometer, Copenhagen, Denmark.
[6] Model 240, Gilford.
[7] Varian XL-100.
[8] Beckman Acta III.

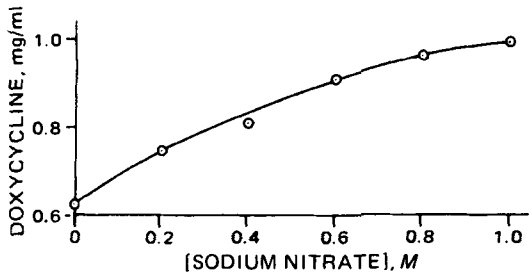


Figure 1—*Solubility of Ia as a function of sodium nitrate concentration in water at 25°. Concentration is expressed as free base equivalent.*

exhausted. The system would then be controlled by Eq. 1 in the normal manner.

A similiar explanation is applicable for addition of hydroxide to convert a salt of a weak base into its free base form. This treatment cannot account for salt effects such as would arise from the generation of sodium chloride from a hydrochloride salt reacting with sodium hydroxide.

The *in situ* salt to base conversion, or its reverse, is often experimentally expedient since both forms of a particular compound are often not available in the pure state. The by-product salt generated must not be neglected in interpretation of the data, however, since the solubility of many compounds is quite dependent on the presence of an electrolyte. This problem may be avoided in some cases by addition of a background electrolyte to maintain ionic strength essentially constant.

Common Ion Effects—The equilibrium for the solubility of a hydrochloride salt of a weak base in water is described by Scheme I and Eq. 5:

$$(BH^+Cl^-)_{solid} \overset{K}{\rightleftharpoons} [BH^+]_s + [Cl^-]$$

Scheme I

$$K = \frac{[BH^+]_s[Cl^-]}{(BH^+Cl^-)_{solid}} \qquad \text{(Eq. 5)}$$

Concentration and activity must be assumed to be equivalent since direct measurement of activity is often not possible for the species of interest other than the hydrogen ion.

If it is assumed that the activity of the solid material is constant, Eq. 5 may be converted to the familiar expression for the solubility product constant:

$$K^0_{sp} = [BH^+]_s[Cl^-] = K(BH^+Cl^-)_{solid} \qquad \text{(Eq. 6)}$$

The apparent solubility product calculated from experimental data is defined by:

$$K'_{sp} = S_T[Cl^-] \qquad \text{(Eq. 7)}$$

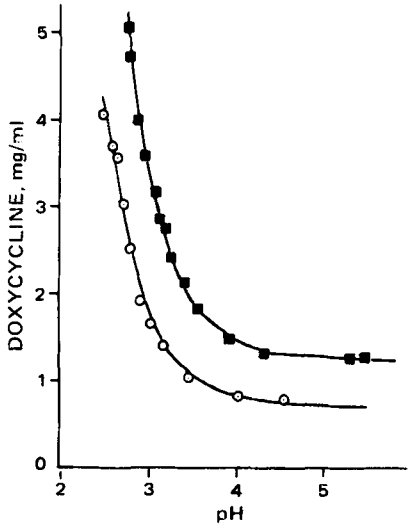


Figure 2—*The pH-solubility profiles for Ia in 1.0 M $NaNO_3$-HNO_3 (■) and 1.0 M NaCl-HCl (⊙) at 25°. The solid lines were calculated according to Eq. 2 using the following constants: $NaNO_3$-HNO_3, pKa' = 3.25, $[B]_s$ = 1.25 mg/ml; and NaCl-HCl, pKa' = 3.20, $[B]_s$ = 0.72 mg/ml. Concentration is expressed as free base equivalent.*

Table I—Effect of Ammonium Chloride and Sodium Chloride on the Solubility of Ib in Water at 25°

[Salt], *M*	S_T, *M*	pH	$[Cl^-]$, *M*[a]	K^0_{sp}, $M^2 \times 10^{-3}$ [b]
0	0.121	2.16	0.121	13.1
Ammonium chloride				
0.02	0.0913	2.16	0.111	9.1
0.05	0.0631	2.26	0.113	6.2
0.1	0.0336	2.44	0.134	3.7
0.2	0.0193	2.57	0.219	3.2
0.4	0.0091	2.76	0.409	2.5
0.8	0.0049	2.89	0.805	2.4
1.0	0.0039	2.93	1.004	2.3
Sodium chloride				
0.02	0.0905	2.16	0.111	8.9
0.05	0.0648	2.25	0.115	6.5
0.1	0.0385	2.40	0.139	4.4
0.2	0.0180	2.58	0.218	3.0
0.4	0.0079	2.75	0.408	2.2
0.8	0.0040	2.89	0.804	2.0
1.0	0.0031	2.95	1.003	1.8

[a] Calculated from Eq. 9. [b] Calculated from Eq. 8 using pKa' = 3.09.

Substitution of Eqs. 1 and 7 into Eq. 6 yields an expression for K^0_{sp} as a function of pH:

$$K^0_{sp} = \frac{K'_{sp}}{(1 + K'_a/[H^+])} \qquad \text{(Eq. 8)}$$

The $[Cl^-]$ in Scheme I and Eqs. 5–8 must be the total chloride ion present in the system. This value is obtained by summation of $[M^+Cl^-]$, where $M^+ = BH^+, H^+, Na^+$, *etc.*, according to:

$$[Cl^-] = \sum_i [M^+Cl^-]_i \qquad \text{(Eq. 9)}$$

When the system is composed of monovalent cations and chloride is the only anion, $[Cl^-]$ is equal to the ionic strength.

RESULTS AND DISCUSSION

Ionization Constant—The pKa' of doxycycline was determined spectrophotometrically. A value of 3.09 ± 0.09 (17 measurements) was calculated at μ = 0.1 and 25°. This pKa' is in agreement with literature values for doxycycline, pKa = 3.4, as well as the pKa = 3.3 reported for oxytetracycline, chlortetracycline, and tetracycline (7, 8).

The microscopic ionization scheme for tetracycline was studied by ^{1}H-NMR (9) and ^{13}C-NMR (10) methods. These studies concluded that protonation of the C-1–C-3 tricarbonylmethane portion of the tetracycline molecule occurred in the pH 1–6 region, forming the corresponding acid salt. The conjugate base species exists predominantly as a zwitterion involving the C-1–C-3 oxyanion and the protonated C-4 dimethylammonium group. Two additional microspecies of the base form, the nonionized neutral molecule and a zwitterion between the C-10–C-12 phenolic diketone anion and the C-4 dimethylammonium group, are present but exist in much lower concentration.

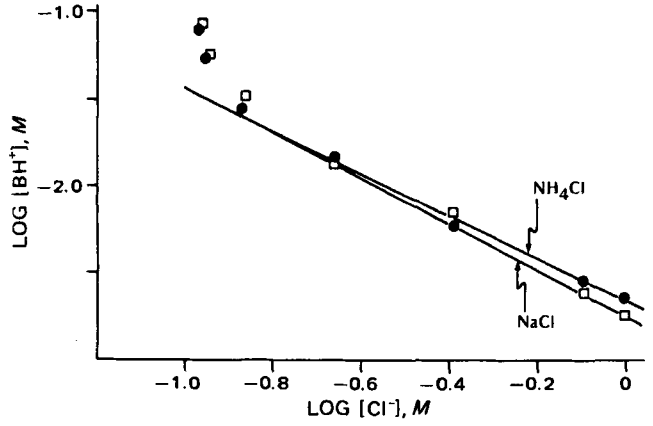


Figure 3—*Logarithmic plot of the relationship between $[BH^+]_s$ calculated from Eq. 1 using pKa' = 3.09 and $[Cl^-]$. The solid lines represent regression slopes of −1.2 (●, NH_4Cl) and −1.3 (□, NaCl).*

Table II—Solubility of I*b* in Aqueous Sodium Chloride and Hydrochloric Acid Solutions at 25°

[HCl], *M*	[NaCl], *M*	pH	S_T, *M*	[Cl⁻], *M*[a]	K'_{sp}, $M^2 \times 10^{-3}$ [b]
0.05	0	1.42	0.0530	0.103	5.3
0.05	0.05	1.41	0.0285	0.128	3.6
0.05	0.15	1.42	0.0128	0.213	2.7
0.05	0.35	1.37	0.0053	0.405	2.1
0.05	0.55	1.35	0.0035	0.603	2.1
0.05	0.75	1.32	0.0026	0.803	2.1
0.05	0.95	1.29	0.0019	1.002	1.9
0.2	0	0.86	0.0128	0.213	2.7
0.2	0.1	0.84	0.0080	0.308	2.5
0.2	0.2	0.82	0.0055	0.407	2.2
0.2	0.3	0.80	0.0044	0.504	2.2
0.2	0.4	0.77	0.0035	0.604	2.1
0.2	0.6	0.75	0.0025	0.803	2.0
0.2	0.8	0.71	0.0020	1.002	2.0
0.01	—	1.92	0.0917	0.102	8.8
0.02	—	1.72	0.0763	0.096	7.0
0.05	—	1.42	0.0530	0.103	5.3
0.10	—	1.11	0.0223	0.122	2.7
0.20	—	0.89	0.0118	0.212	2.5
0.40	—	0.61	0.0056	0.406	2.3
1.0	—	0.21	0.00245	1.002	2.5

[a] Calculated from Eq. 9. [b] Calculated from Eq. 8 using pKa′ = 3.09. $K'_{sp} = K^0_{sp}$ since $K'_a/[H^+] \ll 1$.

Since doxycycline and other tetracyclines are structurally identical in the ionizing functional groups as well as in solution conformation (11), an identical ionization scheme is assumed for these compounds. The chromophore of tetracyclines in the region of 262 nm has been attributed to the $\pi \rightarrow \pi^*$ transition of the C-1–C-3 tricarbonylmethane function (11, 12). The observation that this wavelength exhibited the maximum UV absorptivity change for doxycycline is additional evidence for the assignment of protonation at the C-1–C-3 tricarbonylmethane portion of the molecule.

Salt and pH Effects on Solubility of I*a*.—The effect of sodium nitrate on the solubility of I*a* is shown in Fig. 1. A 65% increase in solubility was found in 1.0 *M* sodium nitrate compared to water. This strong positive salt effect is related to the zwitterionic nature of doxycycline in aqueous solution, which is characteristic of the tetracyclines (13). As a dipolar species, it should be stabilized in an increasingly polar environment. In contrast, uncharged organic species are usually salted out by the addition of an electrolyte.

The pH-solubility profiles for I*a* in the presence of nitrate and chloride ions at μ = 1.0 are shown in Fig. 2. Although the solubility of I*a* was enhanced by nitrate compared to water, it was only slightly affected by chloride ions. Solubility increased uniformly according to Eq. 2 due to protonation of doxycycline. The pKa′ values obtained by curve fitting of these data at μ = 1.0 were in good agreement with those measured spectrophotometrically at μ = 0.1.

The experimental data in Fig. 2 cannot be extended to lower pH values or higher solubility by addition of small increments of acid. Therefore, the systems have reached pH_{max}. By definition, pure base form $[B]_s$ and salt form $[BH^+]_s$ must both be present for the system to be invariant. The maximum solubilities in Fig. 2 are ~10-fold lower than can be attained in purely aqueous solution. This difference is due to the effect of these anions on the solubility product equilibrium of the corresponding acid salt of doxycycline. The existence of doxycycline nitrate was confirmed by dissolving I*a* in water with nitric acid and allowing the product to crystallize. The anhydrous nitrate salt was isolated and identified.

Solubility Product Constant for I*b*.—The stable crystalline form of doxycycline in aqueous hydrochloric acid is I*b*. This salt was isolated from solutions originally prepared with I*c*. Solutions of I*c* can be easily supersaturated by severalfold with respect to the solubility of I*b*. Nucleation and/or crystal growth occur slowly for the interconversion of I*c* → I*b* in water.

The effect of added chloride ion on the solubility of I*b* in water was investigated to determine K^0_{sp}. Ammonium and sodium chlorides were used in the 0.02–1.0 *M* concentration range. Table I summarizes the data from these experiments. The solubility, S_T, decreased strongly with an increasing concentration of either salt. Since the data for the two salts are similar, specific effects due to the cation are considered negligible. The pH increased with an increasing salt concentration due to the lower concentration of I*b*. Since external buffers were not present, the pH of the system was controlled by the concentration of I*b*.

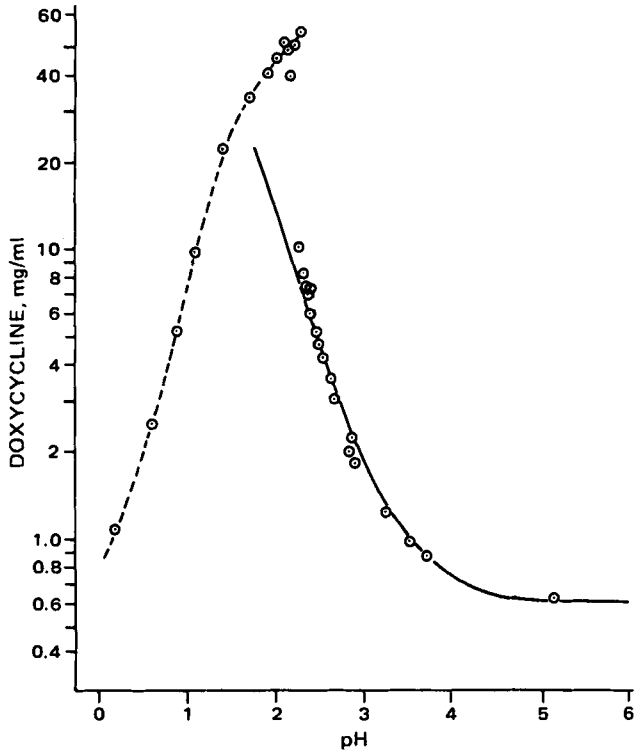


Figure 4—*The pH-solubility profile for I in aqueous hydrochloric acid at 25°. At pH_{max} = 2.16, both I*a *and I*b *were in equilibrium with the solution. Below pH_{max}, I*b *was the solid phase (dashed line). Above this pH, I*a *was in equilibrium with the solution. The solid line is theoretical according to Eq. 2 using $[B]_s$ = 0.625 mg/ml and pKa′ = 3.30. Concentration is expressed as free base equivalent.*

The solubility product, K^0_{sp}, decreased more than fivefold over the concentration range studied. The apparent molecular order of interaction between $[BH^+]$ and $[Cl^-]$ is plotted logarithmically in Fig. 3. A slightly greater than first-order inverse relationship is apparent with strong positive deviation from linearity at low added salt concentrations. A small amount of added salt (0.02–0.05 *M*) caused a much greater than first-order decrease in solubility. The total ionic strength, indicated by $[Cl^-]$ in Table I, was essentially constant at 0.11–0.13 *M* in the region where K^0_{sp} was most sensitive to added salt. In the μ = 0.2–1.0 *M* range, the K^0_{sp} became practically constant.

Since the pH change in these experiments was a complicating factor in data interpretation, the solubility product measurement was repeated under the conditions shown in Table II. Since $K'_a/[H^+]$ was much less than one under these conditions, $K'_{sp} = K^0_{sp}$. Mixtures of sodium chloride and hydrochloric acid, as well as varying concentrations of hydrochloric acid, were used up to 1.0 *M* total added chloride-ion concentration. The downward trend in K'_{sp} with increasing $[Cl^-]$ is again apparent.

The nature of the cation, Na^+ or H^+, had little influence on K'_{sp}. For example, added chloride ion of 0.4 *M* composed of 0.05 *M* HCl + 0.35 *M* NaCl, 0.2 *M* HCl + 0.2 *M* NaCl, or 0.4 *M* HCl resulted in K'_{sp} values of 2.1, 2.2, and 2.3 × 10^{-3} M^2, respectively. These differences were well within experimental error in the value of K'_{sp}. When plotted logarithmically, as in Fig. 3, the data of Table II exhibit linear regression slopes of −1.1 and −1.2.

The K'_{sp} value calculated from a suspension of I*b* in water, *i.e.*, S_T^2, is the most deviant value in the series. Addition of 0.02 *M* NaCl or NH_4Cl (Table I) caused the doxycycline concentration to decrease by 0.03 *M*. This result is impossible according to the principles of common ion equilibria. The solubility cannot decrease by an amount *greater* than the amount of common ion added. Clearly, nonideal behavior was occurring under these conditions. The observed variation in K'_{sp} was not due to changing solution ionic strength. On the contrary, ionic strength (indicated as $[Cl^-]$ in Tables I and II) had little effect on the equilibrium. As already discussed, K'_{sp} varies most under conditions where μ is constant. When μ is changing, K'_{sp} is relatively constant.

pH-Solubility Profile for I*a* and I*b* in Aqueous Hydrochloric Acid—Figure 4 is the pH-solubility profile for I*a* and I*b* in aqueous hydrochloric acid without added salt. Maximum solubility was observed

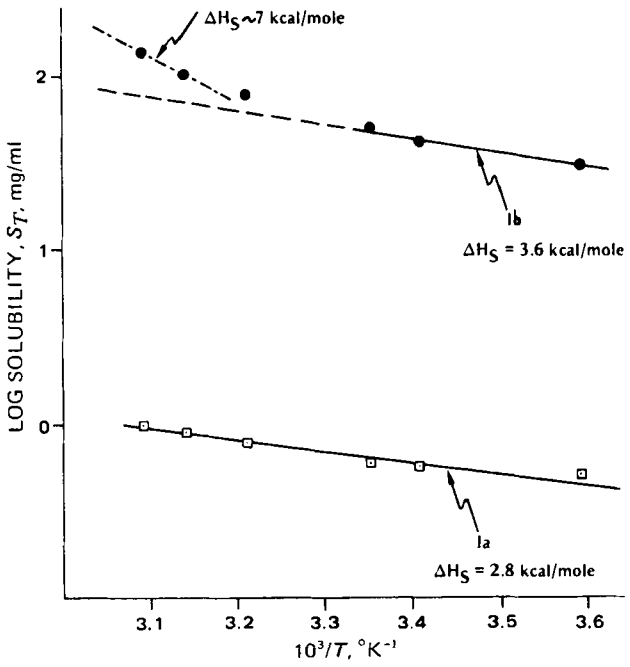


Figure 5—*A van't Hoff-type plot for the solubility of Ia (lower line, ⊡) and Ib (upper line, ●) in water.*

at pH 2.16, which was the result of simultaneous equilibration of crystal forms I*a* and I*b* in water. The ionic strength was not kept constant because of interference of added ions with the solubility equilibria.

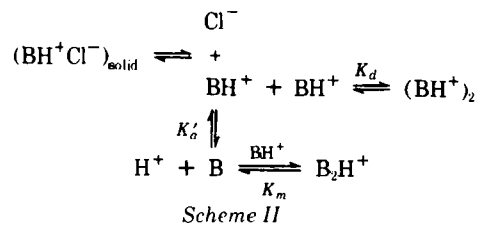
A remarkable feature of these data is the approximately fivefold increase in solubility from pH 2.3 to 2.2. Therefore, a much greater than first-order dependence on hydrogen ion would be applicable in this region. Positive deviations of the experimental points from the theoretical line based on Eq. 2 were apparent below pH 2.5. The decrease in solubility below pH_{max} was due to the previously discussed common ion effect of chloride on the solubility product equilibrium.

Solubility-Temperature Relationship—The temperature dependence for solubility of I*a* and I*b* in the 5–50° range is shown graphically in Fig. 5 as van't Hoff-type plots. At the lower temperatures, I*a* and I*b* had similar heats of solution of 2.8 and 3.6 kcal/mole, respectively. At higher temperatures, the slope for I*b* showed upward curvature whereas the slope for I*a* remained linear. In the 45–50° range, ΔH_s for I*b* increased to approximately 7 kcal/mole.

Mechanism of Nonideal Behavior—The data in Figs. 3–5 and Tables I and II clearly show that doxycycline solubility does not follow the expected theoretical relationships given in Scheme I and Eqs. 1–8. All data are consistent with a mechanism involving self-association of doxycycline to form dimeric species such as $(BH^+)_2$, B_2H^+, and, possibly, higher order self-associated forms. The presence of associated species in solution would cause the apparent solubility and, therefore, K'_{sp} to be higher than the values predicted from basic theoretical relationships.

It was previously shown that ionic strength, pH, and specific ion effects (H^+, Na^+, and NH_4^+) were not responsible for the observed changing of K'_{sp}. The absence of crystal transformation during equilibration was verified microscopically and is consistent with the fact that I*a* and I*b* were originally isolated from water. The instability of doxycycline during equilibration could not be a factor affecting solubility since the compound has a half-life of ~100 hr at pH 5 and 50° and shows better stability at lower pH values and temperatures (14).

Scheme II describes the equilibria that would be present if dimerization were to occur forming $(BH^+)_2$ or B_2H^+ species. In this scheme, the solubility of BH^+ is assumed to be limiting.



Scheme II

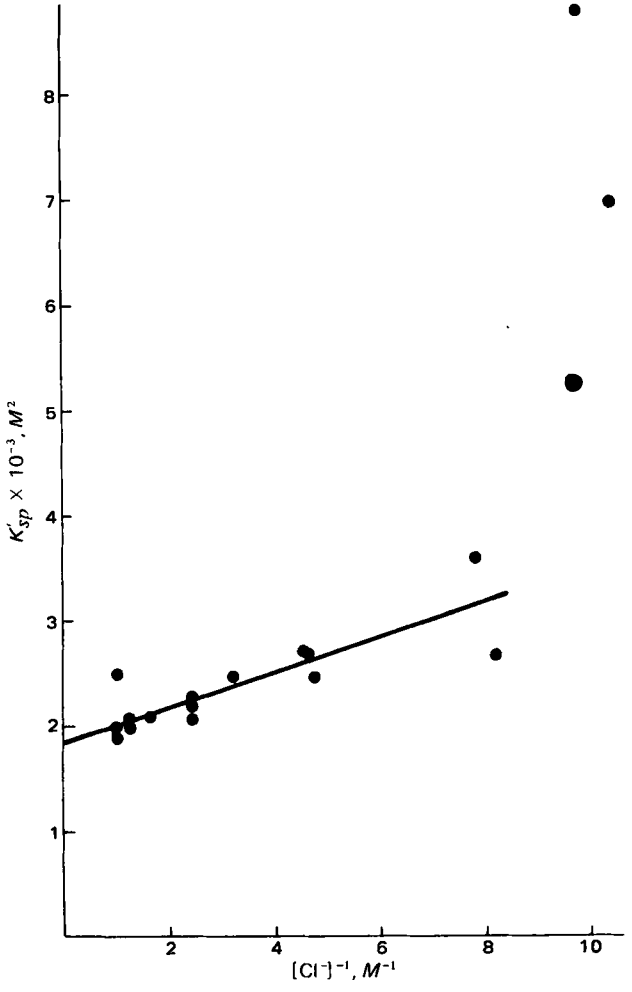


Figure 6—*Apparent solubility product for Ib as a function of the inverse chloride concentration plotted according to Eq. 16.*

The dimerization constants may be defined as:

$$K_d = \frac{[(BH^+)_2]}{[BH^+]^2} \qquad \text{(Eq. 10)}$$

$$K_m = \frac{[B_2H^+]}{[B][BH^+]} \qquad \text{(Eq. 11)}$$

Total solubility is expressed as the sum of the species, taking into account the dimer stoichiometry:

$$S_T = [BH^+]_s(1 + K'_a/[H^+]) + 2[BH^+]_s^2(K_d + K_mK'_a/[H^+]) \qquad \text{(Eq. 12)}$$

Substitution of Eqs. 6 and 12 into Eq. 7 yields an expression for the apparent solubility product:

$$K'_{sp} = K^0_{sp}(1 + K'_a/[H^+]) + \frac{2(K^0_{sp})^2(K_d + K_mK'_a/[H^+])}{[Cl^-]} \qquad \text{(Eq. 13)}$$

The expression analogous to Eq. 12 when the solubility of B is limiting can be shown to be:

$$S_T = [B]_s(1 + [H^+]/K'_a) + 2[B]_s^2(K_d[H^+]^2/K'^2_a + K_m[H^+]/K'_a) \qquad \text{(Eq. 14)}$$

Equation 13 predicts that, at constant pH, K'_{sp} should be an inverse function of the chloride concentration, which is in agreement with the experimental data in Table II. Equation 14 predicts that S_T should first follow Eq. 2 when $[H^+]/K'_a$ is small and then deviate from first-order behavior at lower pH values; these predictions are identical to the solubility data in Fig. 4.

At low pH values, *i.e.*, $K'_a/[H^+] \ll 1$, Eqs. 12 and 13 may be simplified to Eqs. 15 and 16, respectively:

$$S_T = [BH^+]_s(1 + 2K_d[BH^+]_s) \qquad \text{(Eq. 15)}$$

$$K'_{sp} = K^0_{sp} + \frac{2(K^0_{sp})^2K_d}{[Cl^-]} \qquad \text{(Eq. 16)}$$

Table III—Concentration Dependence of ^{1}H-NMR Signals for C-7, C-8, C-9, and C-4 Dimethylamino Protons of I*b* in Deuterium Oxide[a]

[Doxycycline], *M*	Chemical Shift, δ, ppm			
	C-9 H[b]	C-7 H[b]	C-8 H[c]	C-4 $N(CH_3)_2$
0.10	6.67	6.86	7.44	3.04
0.05	6.78	6.99	7.54	3.06
0.017	6.90	7.08	7.64	3.06

[a] Ambient temperature. [b] Doublet, J = 8 Hz. [c] Triplet, J = 8 Hz.

According to Eq. 16, a plot of K'_{sp} *versus* $[Cl^-]^{-1}$ should be linear with slope = $2(K^0_{sp})^2K_d$ and intercept = K^0_{sp}. Figure 6 shows the data of Table II plotted in this manner. Values of K_d = 24 M^{-1} and K^0_{sp} = 1.8×10^{-3} M^2 were calculated from the slope and intercept. Deviation from linear behavior as solubility approaches that of pure I*b* may be due to incomplete equilibrium or existence of higher than second-order association complexes.

It is not possible to prove the existence of the mixed dimer, B_2H^+, with the present data. Under conditions where K_m would be determinable, $(BH^+)_2$ and B_2H^+ species would coexist and separation of the stability constants would be difficult. Attempts to solve Eq. 13 or 14 for K_m did not give reproducible results because of the large error involved in taking the difference between numbers of similar magnitude. Since doxycycline is a large molecule and the site of self-association is remote from the ionizing group, B_2H^+ should have a stability constant at least as large as K_d. The reduced electrostatic interaction in this species indicates that it should be more stable than the dication, $(BH^+)_2$.

The concentration dependence of NMR chemical shifts for certain hydrogens of I*b* in deuterium oxide is shown in Table III. Proton assignments were adapted from those reported for tetracycline (15). An upfield shift in the C-7, C-8, and C-9 aromatic protons of ~0.2 ppm occurred as the concentration was increased from 0.017 to 0.10 *M*. The C-4 dimethylamino protons, however, were essentially unchanged, as were other protons not listed in Table III. Rigler *et al.* (9) found that the chemical shift of the C-8 proton moved 0.28 ppm upfield during ionization of the phenolic β-diketone system (macro pKa = 7.7 and 9.7). The similarity of the concentration-dependent and ionization-induced change in chemical shift of these protons indicates that the phenolic β-diketone moiety is the site of self-association.

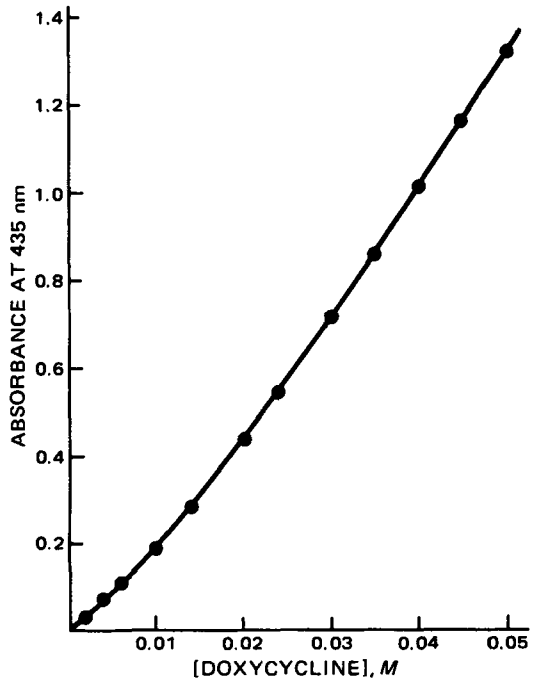


Figure 7—*Absorbance of Ic as a function of concentration in 0.05* M *phosphoric acid buffer (pH 2.0) at 25°. Concentration is expressed as free base equivalent. Points are experimental, and the line is theoretical according to Eq. 18. The theoretical line was constructed using the following constants:* a = *1.0 cm,* E_m = *12* M^{-1} cm^{-1}, E_d = *76* M^{-1} cm^{-1}, *and* K_d = *24* M^{-1}.

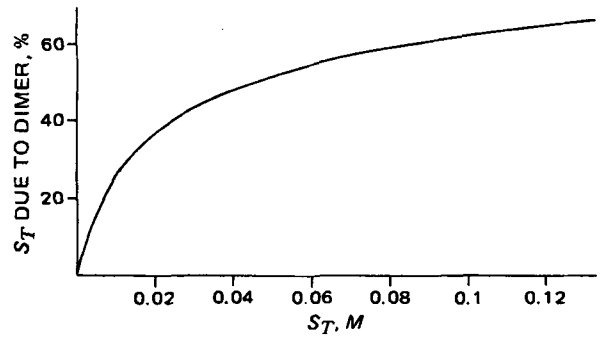


Figure 8—*Theoretical curve of the percent of total solubility contributed by dimer species* $(BH^+)_2$ *as a function of total solubility. Dimerization constant,* K_d = *24* M^{-1}, *was used to calculate the curve.*

Planarity of the aromatic ring with the C-11–C-12 β-diketone groups (12, 16) would allow dimerization to occur *via* multiple intermolecular hydrogen bonds. The C-10 phenol and C-11–C-12 enol–keto groups of one molecule could associate with those of the other molecule, forming an edge-to-edge oriented dimer in solution. In this configuration, the environment of the C-4 methyl protons would not be affected, which is consistent with the data.

Self-association of doxycycline also was observed by visible spectrophotometry (Fig. 7). Absorbance at 435 nm was not linear with concentration but showed an apparent parabolic relationship. The 0–0.05 *M* concentration range was chosen because the solubility product data indicated that dimerization was predominant in this region. The chromophore at this wavelength is a shoulder of the ~350-nm maximum of doxycycline, which is generally attributed to the phenolic β-diketone moiety of tetracyclines (12).

The total concentration, C_T, can be expressed as the sum of the monomer, C_m, and dimer according to:

$$C_T = C_m + 2K_dC_m^2 \qquad \text{(Eq. 17)}$$

The total absorbance, A_T, is defined by:

$$A_T = a(E_mC_m + K_dE_dC_m^2) \qquad \text{(Eq. 18)}$$

where a is the cell path length, E_m is the molar absorptivity of the monomer, and E_d is the molar absorptivity of the dimer.

The theoretical line in Fig. 7 was determined as follows. The value of C_m was calculated from Eq. 17 at various values of C_T, assuming K_d = 24 M^{-1} (determined from the solubility product data). An initial estimate of E_m was taken from the initial slope in Fig. 7, and then E_m and E_d were adjusted to give the best fit of the data according to Eq. 18. The curve was sensitive to changes in constants E_m and E_d, which primarily affected the lower and upper parts of the curve, respectively. The value of the dimerization constant cannot be verified by this procedure, however, because other values of K_d can be fitted to the data as well. For example, assumption of a larger value of K_d will cause the C_m value calculated by Eq. 17 to become smaller. In Eq. 18, however, this decrease can be compensated by adjustment of E_m and E_d to yield an equally good fit of the data.

The molar absorptivity of the dimer species required to fit the data is approximately sixfold larger than that of the monomer. Simple aggregation or hydrophobic association would be expected to yield a ratio nearer twofold. Perturbation of the aromatic system by hydrogen bonding is a possible explanation for the high absorptivity of the dimer.

Figure 8 is a theoretical plot showing the strong effect of dimerization on the apparent solubility of doxycycline. If it is assumed that only the monomer and dimer are present, more than 50% of the total solubility would be contributed by the dimer species at S_T >0.04 *M*. Self-association in solution is probably common to tetracyclines in general because of the similarity of the interacting functional groups.

At concentrations near saturation, all compounds must begin to self-associate. When pushed to slightly higher concentrations, the association complexes become active nuclei for the crystallization process. The lack of suitable analytical methods for the determination of free solute concentrations has limited the investigation of solute properties in saturated or nearly saturated solutions. Analysis of solubility product equilibria was a sensitive indicator of the concentration of unassociated doxycycline cation. This method should be a powerful tool for the study of solution equilibria in general.

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Synthesis and Therapeutic Testing of Mono- and Dialkyl Esters of Pentetic (Diethylenetriaminepentaacetic) Acid for Decorporation of Polymeric Plutonium

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Abstract □ The synthesis, characterization, and therapeutic evaluation of a series of partially esterified derivatives of pentetic (diethylenetriaminepentaacetic) acid are reported. These compounds were prepared in an attempt to promote increased decorporation of insoluble colloidal forms of plutonium, which are not removed by pentetic acid alone. The dimethyl, diethyl, dibutyl, dioctyl, and monoethyl esters were synthesized by reaction of the appropriate alcohol with the dianhydride of pentetic acid. These esters were injected intravenously into mice as their calcium chelates in saline. None of the esters was effective in removing plutonium from the liver. All esters removed approximately 20% of the plutonium in the skeleton. However, when the esters were given together with pentetic acid, only the dioctyl ester showed enhanced removal of plutonium compared to pentetic acid alone. The small increase in effectiveness and the increased acute toxicity make these esters of limited practical interest in plutonium decorporation therapy.

Keyphrases □ Pentetate esters, various—synthesized, evaluated for ability to decorporate polymeric plutonium in mice □ Plutonium, polymeric—decorporation in mice, various pentetate esters evaluated □ Structure-activity relationships—various pentetate esters evaluated for ability to decorporate polymeric plutonium in mice □ Chelating agents—various pentetate esters synthesized, evaluated for ability to decorporate polymeric plutonium in mice

The disposition kinetics of the easily hydrolyzable toxic radiometal plutonium following accidental exposure depend on the physicochemical form to which an individual is exposed as well as the route of exposure. Systemically deposited plutonium (*i.e.*, plutonium that has reached the circulation and is subsequently deposited in the tissues) is generally considered to be soluble ("monomeric") plutonium. However, aggregated insoluble forms of plutonium have been observed at later times in the liver and spleen (1, 2). As a model for insoluble systemically deposited plutonium, a colloidal ("polymeric") plutonium preparation is injected intravenously in animals. A large fraction of this plutonium is rapidly deposited in organs rich in reticuloendothelial elements, particularly the liver (3).

BACKGROUND

Pentetic acid (diethylenetriaminepentaacetic acid or DTPA), *N,N*-bis[2-[bis(carboxymethyl)amino]ethyl]glycine, administered intravenously as the calcium chelate trisodium salt (I), presently is considered the treatment of choice for accidental exposure to certain multivalent lanthanide and actinide radioelements such as plutonium. This drug has also been used for the treatment of lead poisoning, acute iron intoxication, and iron storage disease (4). Although I has been effective in removing soluble plutonium from the liver and, to a lesser extent, from the skeleton (5, 6), this chelating agent has not been successful in decorporating the more insoluble polymeric plutonium (7). Since hepatically deposited polymeric plutonium is primarily associated with lysosomes (8) and I is predominantly distributed in the extracellular space (9) and is rapidly excreted in the urine (10), it was reasoned that increased intracellular uptake of I might place a sufficient concentration of ligand at the site of plutonium deposition to promote increased plutonium decorporation.

Previously, two different approaches were attempted to increase the uptake of I into cells. Markley (11), using the pentaethyl ester of pentetic acid, found that this more lipid-soluble form removed additional plutonium from the mouse liver beyond that removed with I alone. When I and its pentaethyl ester were administered together, their effects in the liver were approximately additive, suggesting that each form was acting on a separate and distinct fraction of hepatic plutonium. However, the pentaethyl ester was much more toxic than I. In the second approach, I encapsulated in phospholipid liposomes was injected intravenously. The encapsulated I deposited intracellularly in the liver (12) to a large

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