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Acid-Base Equilibria in Aqueous Solutions of Tetracycline Hydrochloride

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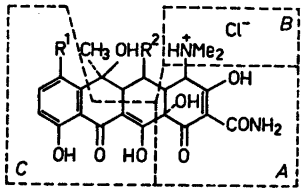
An ultraviolet spectral examination of tetracycline hydrochloride in aqueous solution indicated the existence of monoprotonated, neutral, anionic, dianionic and trianionic species across the pH 1 – 14 range. These findings were supported by molecular orbital, polarographic and biological absorption studies, and polarography was further used to study the nature of the complex formed between tetracycline, calcium ions and barbitone.



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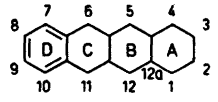


1a

Although many authors [1–5] agree that tetracycline hydrochloride (*1*: $R^1 = R^2 = H$) exhibits three acid-base equilibria in the 1 – 10 pH range, there is lack of agreement as to the sites with which the phenomena are associated. Stephens *et al.*,[3] for example, used a potentiometric method to determine pK_a values of $pK_1 = 3.30$, $pK_2 = 7.68$ and $pK_3 = 9.69$, and subsequently assigned the equilibria to activity at three non-interacting proton-active sites (*A*, *B* and *C*, respectively) within the molecule. This group also suggested the existence of a zwitterionic form of tetracycline which was thought to be the predominant species present in solutions of $pK_1 < pH < pK_2$. Leeson *et al.*,[4] however, have proposed a reversal in assignments for pK_2 and pK_3 based upon a comparison with values obtained for the quaternary ammonium salt. These conclusions were supported by Garrett [5] following an investigation of the relationship between the dielectric constant and the apparent pK_a values of tetracycline in dimethylformamide – water solvent mixtures. Rigler,[6] on the other hand, has exploited NMR chemical shift information to determine a microscopic dissociation scheme for tetracycline in which it was assumed that the shifts observed for the dimethylamino and D-ring protons (*cf.* ring nomenclature in *1a* were due to protonations occurring solely at sites *B* and *C*. These findings tended to bear out the conclusions of Stephens *et al.*[3] and, in addition, a further pK_a value (10.67) was reported for the methiodide derivative of tetracycline.

In general, most treatments of this subject involve a comparison of the behaviour of tetracycline with that of analogues containing the positively-charged 4-dimethylammonium moiety.

In order to gain further insight into the nature of these acid-base equilibria, particularly those

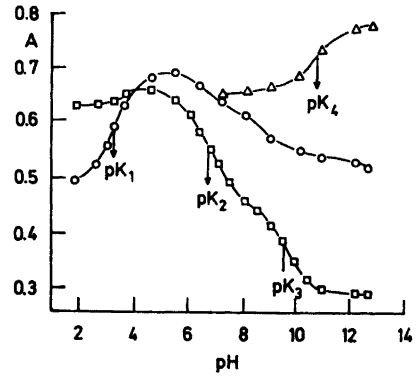
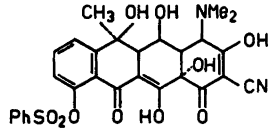


Fig. 1. Plots of absorbance *vs.* pH for tetracycline hydrochloride in aqueous solution at selected wavelengths (○, $\lambda = 300$ nm; □, $\lambda = 325$ nm; △, $\lambda = 240$ nm).

involving the A-ring, an appropriate molecular orbital computer programme was used to explain the changes observed in the UV spectrum of tetracycline at acid pH. Polarography has also been used to study the acid-base properties of this compound in aqueous media [7] and, in particular, the complex which tetracycline forms with Ca^{2+} ions and barbitone.[8]

RESULTS AND DISCUSSION

UV spectral behaviour and the pK_a *values of tetracycline hydrochloride.* Tetracycline hydrochloride exhibited four distinct changes in ultraviolet spectral behaviour in aqueous solutions of pH 1 – 14. The spectral characteristics of each species and the pK_a values, as determined from plots of absorbance *vs.* pH at selected wavelengths (Fig. 1), are collected in Table 1. The pK_1 and pK_3 values obtained are within 0.1 pK unit of the potentiometrically-determined values,[3] whilst the value determined for pK_2 is lower by *ca.* 0.8 pK unit. The spectral results also provided evidence for the existence of a fourth pK_a (10.9) which is in qualitative agreement with both the 10.67 value determined for the tetracycline methiodide derivative [6] and the 11.5 value assigned to deprotonation of the 12-OH group in 10-benzenesulfonyloxy-tetracyclinonitrile (*2*).[3]



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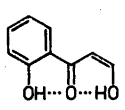
It has been shown [3] for oxytetracycline (*1*: $R^1 = H$, $R^2 = OH$) that the chromophore involving the *C* part of the molecule is alone responsible for UV absorption at wavelengths > 330 nm. This would appear to be consistent with both the aromatic nature of the D-ring and (hence) the intense $\pi - \pi^*$ transitions evident from Table 1. Further examination of the spectral information in Table 1 reveals a substantial shift to longer wavelength (369→380 nm), with no change in molar extinction coefficient, ε, once pK_3 is exceeded. Coupling this observation with the facts that (*i*) sulfonylation of the 10-OH group results in the disappearance of pK_3, and (*ii*) *para*-substitution by chlorine in chlortetracycline (*1*: $R^1 = Cl$, $R^2 = H$) produces a decrease [3] in

Table 1. Spectral characteristics and pK_a values determined for tetracycline hydrochloride.

Species	λ_{max}/nm (10^{-4} ε/dm^3 mol^{-1} cm^{-1})	pK_a values (wavelength at which determined/nm)
H_4A^+	220 (1.25); 270 (1.9); 358 (1.5)	$pK_1 = 3.3$ (300)
H_3A	248 (1.2); 276 (1.6); 360 – 365 (1.7)	$pK_2 = 6.9$ (325)
H_2A^-	248 (1.3); 272 (1.6); 287*sh.* (1.5); 369 (1.7)	$pK_3 = 9.6$ (325)
HA^{2-}	247 (1.3); 269 (1.4); 288*sh.* (1.2); 380 (1.7)	$pK_4 \simeq 10.9$ (240)
A^{3-}	238 (1.35); 245 (1.4); 267 (1.4); 288*sh.* (1.2); 380 (1.7)	

pK_3 from 9.69 to 9.27, it is possible to unambiguously assign this equilibrium to deprotonation of the phenolic OH group at C-10.

The hydroxyl group at C-12, however, is situated in a less activating environment than that at C-10 and is probably extensively stabilized by hydrogen-bonding to the two adjacent carbonyl groups; the hydroxyl group at C-10, on the other hand, can bond to only one carbonyl. These observations together suggest that 12-OH ionization will be markedly more difficult than that of the 10-OH group; pK_4 is thus ascribed to deprotonation at this site. Irreversible decomposition is detectable at pH≫12, suggesting either skeletal rearrangement or ring-opening of tetracycline in strongly alkaline media.



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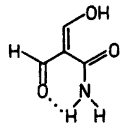

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CNDO/2 molecular orbital calculations. MO calculations [9] were carried out on what were assumed, using the following assumptions, to be two simplified, non-interacting, UV-active tetracycline fragments: *3* and *4*. (*a*) The 4-dimethylamino group in tetracycline (*1*: $R^1 = R^2 = H$) is isolated from fragment *4* by an sp^3-hybridized carbon, thus preventing π-delocalization through this centre; (*b*) the two fragments, *3* and *4*, are similarly separated by C-12a, and (*c*) the hydroxyl moieties at C-6 and C-12a are likely to behave as simple alcohol groups, and hence will not be protonated or deprotonated in the pH range under consideration.

Preliminary MO calculations on fragment *3* and the anions generated by deprotonating the 10- and 12-OH groups revealed the latter process to be considerably more difficult (in energy terms) than deprotonation of the phenol function, providing justification for our pK_3 and pK_4 assignments.

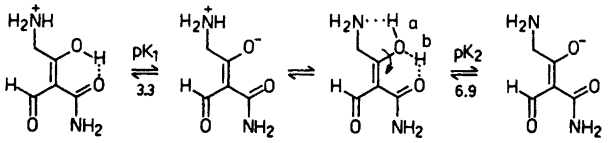
MO calculations on fragment *4* and derived cations and anion, whilst indicating deprotonation of the conjugated 3-OH group to be energetically more favourable than protonation of either the ketone of carboxamide functions, failed, however, to provide a reasonable correlation between the computed and observed 210 – 330 nm spectral behaviours. It is inferred, therefore, that the 4-dimethylamino moiety does in fact interact with the A-ring chromophore, and that MO calculations of this nature require reintroduction of this function. Calculations were subsequently executed for each of the structures *5* – *8* and the computed results are given in Table 2.

Examination of the information in Table 2 reveals a fair correlation between the equivalent wavelengths computed for structures *5* and *8* and the experimental 210 – 330 nm λ_{max} values witnessed for tetracycline species H_4A^+ and H_2A^- (*cf.* Table 1).*

Rather poor agreement is evident (Table 2) for the zwitterion *6* and the neutral H_3A form of tetracycline, in contrast with the excellent λ_{max} values calculated for the alternative unionized structures, *7a* and *7b*. These results lead us to infer that a hydrogen-bonded H_3A species resembling *7* is more likely to represent the neutral solution form of tetracycline than the hitherto postulated zwitterion.[3] Species *7a*, in fact, provided the best λ_{max} correlation and lowest calculated total energy of the three neutral fragments studied. It is therefore suggested that the first two acid-base equilibria of tetracycline hydrochloride ($H_4A^+ \rightleftharpoons H_3A + H^+ \rightleftharpoons H_2A^- + 2H^+$) can be represented by structures analogous to $5 \rightleftharpoons 7 \rightleftharpoons 8$, as shown.

Polarographic behaviour. The polarographic behaviour of tetracycline has been studied in

* It should be pointed out, however, that the transition moment probabilities remain to be determined for the structures examined.



5 6 7a,b 8

Table 2. CNDO/2 molecular orbital energies and equivalent electronic transition wavelengths [a] computed for tetracycline fragments *5* – *8*.

Species	Computed energies/Hartrees [b] Total energy	Orbital energies [c]	Calculated λ_{max}/nm
5	−120.328	−0.6469, −0.6075, −0.1314, −0.0527, *0.0242, 0.0607, 0.1483, etc.*	216.5
6	−119.790	−0.5058, −0.4380, −0.3552, *0.0986, 0.1248, 0.2221, 0.2663, etc.*	207, 220, 226, 237, 243, 255, 285, 301
7a	−119.957	−0.6480, −0.5001, −0.4468, *0.0497, 0.1747, etc.*	248.2, 275.7
7b	−119.842	−0.6402, −0.5038, −0.4502, *0.0493, 0.1708, etc.*	247.0, 273.8
8	−119.121	−0.4982, −0.4801, −0.4700, −0.3355, −0.2880, −0.1810, *0.2975, 0.3687, 0.4572, etc.*	214, 216, 233, 248, 285

[a] Values computed for the 210 – 330 nm spectral region only. [b] 1 Hartree ≡ 2.626 MJ mol^{-1}. [c] Italicized values denote *unoccupied* molecular orbitals.

great detail by Caplis [7] in both aqueous and aprotic media. He has shown that the first wave obtained in acid media (comprised of two segments, E_1 and E_2) is due to reduction of the protonated tertiary amine function and has the characteristics of a surface reaction. This is in agreement with the observations that dedimethylaminotetracycline and the free tetracycline base *do not* give this reduction wave in aprotic media, in contrast to the behaviour of tetracycline hydrochloride in similar media (*e.g.*, in DMF, $E_{\frac{1}{2}}$(tetracycline.HCl) = −1.08, −1.35 V; $E_{\frac{1}{2}}$(tetracycline) = −1.4 V; $E_{\frac{1}{2}}$(dedimethylaminotetracycline) = −1.36 V).

At pH > pK_2, the segment E_1 disappears and E_2 becomes independent of pH, illustrating that at pK_1 < pH < pK_2, where a species analogous to *6* (*or 7a*) is predominant, reduction of the protonated *tert*-amine function still occurs *via* surface protonation.

These results refute the reversal in assignment of the pK_2 and pK_3 values put forward by Leeson *et al.*[4] and Garrett [5] since deprotonation at site *C* would be unlikely to affect the polarographic reduction occurring in ring A.

Although Caplis did not identify the nature of the second wave (E_3), it is reasonable to assume that it corresponds to the reduction(s) of α,β-unsaturated ketone(s) in the molecule. This was further substantiated by a differential pulse polarographic study of the mixed complex formed with barbitone and Ca^{2+} ions, as previously described by Kohn.[8]

The results of this are shown in Fig. 2, where it can be seen that whereas E_1 and E_2 are shifted to more negative values with increasing Ca^{2+} ion concentration, E_3 completely disappears and is replaced by a new wave at a more positive potential. Kohn [8] has suggested that complex-formation involves the 1-substituent (in its enolized form) and also depends to a large degree upon the presence of dimethylamino and 12a-OH substituents. This would explain the shift of the first two waves to more negative potentials since the protonated *tert*-amine should be more difficult to reduce. The disappearance of the third wave (E_3) and the emergence of a wave (E_4) with a peak current approximately half that of the original wave suggests that Ca^{2+} does indeed bind to tetracycline at the 1-position (thus leaving it

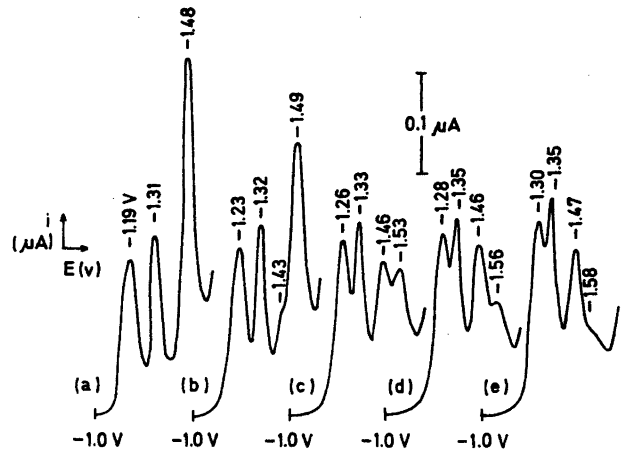


Fig. 2. Effect of calcium ions upon the polarographic behaviour of tetracycline hydrochloride (6.8 μg cm^{-3}) in barbitone buffer (0.1 M barbitone, 0.1 M LiCl in 40 % CH_3OH), pH 4.7: (*a*) tetracycline hydrochloride (TC.HCl); (*b*) TC.HCl + 0.1 μg cm^{-3} $CaCO_3$; (*c*) TC.HCl + 0.3 μg cm^{-3} $CaCO_3$; (*d*) TC.HCl + 0.5 μg cm^{-3} $CaCO_3$; (*e*) TC.HCl + 0.7 μg cm^{-3} $CaCO_3$.

polarographically inactive), leaving the keto-group in the 11-position, whose reduction accounts for the reduction wave E_4.

Absorption studies using biological membranes. The work of Pindell *et al.*[10] has established that the absorption of tetracycline throughout the gastrointestinal tract is pH-dependent (Table 3), and that the rate of absorption is greatest at $pK_1 < pH < pK_2$. These results cannot, however, be taken as absolute since the various sites of absorption have different physiological characteristics (membrane structure, surface area, *etc.*) which might account for the differences observed. It was decided, therefore, to conduct absorption studies on the same biological membrane throughout a controlled region of pH. Two models were chosen: *in vivo* studies with buccal mucosa, and *in vitro* studies with rat colon. The pH range of these experiments was restricted to 4.5 – 7.5 since solutions of lower pH were both unpleasant to hold in the mouth and led to increased saliva production (to such an extent that it interfered with the assay), and more alkaline solutions tended to degenerate the tissue.

The results of these experiments are shown in Fig. 3, where it can be seen that there is an

Table 3. In vivo absorption of tetracycline from various parts of the gastrointestinal tract of dogs.[a]

Site of absorption	Approximate pH	Serum level attained[b] /μg cm^{-3}
Stomach	1.0 – 1.5	0.8
Duodenum	4.5 – 5.5	3.2
Ileum	6.0 – 7.0	2.0
Colon	7.0 – 7.5	0.2

[a] Taken from the work of Pindell *et al.*, Ref. 10.
[b] Determined 1 h after a 20 mg kg^{-1} dose.

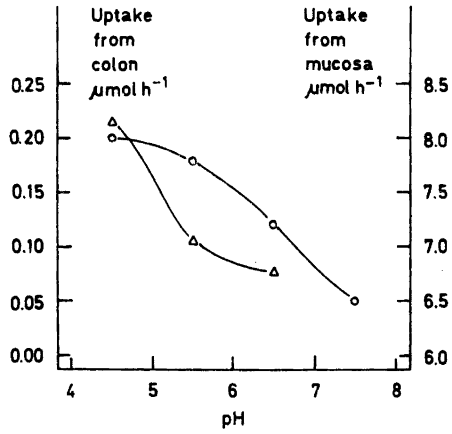


Fig. 3. Plot of rate of absorption of tetracycline hydrochloride (μmol h^{-1}) *vs.* pH using buccal mucosa (△) and rat colon (○) membranes.

increase in the rate of absorption through these membranes with decreasing pH. These results, particularly with the rat colon, are consistent with tetracycline being present as a neutral species in solutions of $pK_1 < pH < pK_2$, and agree with the results of Colaizzi and Klink [11] on the partition of tetracycline between octanol and aqueous phases of varying pH.

METHODS AND EXPERIMENTAL

Ultraviolet absorption spectra were recorded using a Perkin-Elmer Model 137 spectrophotometer with matched 10 mm silica cells. Readings of pH were made on an EIL, Model 23 A, direct-reading pH meter.

Solutions were prepared for spectrophotometry by diluting the appropriate amount of stock tetracycline hydrochloride solution with the appropriate Britton-Robinson (B.R.) buffer (prepared from a stock solution, 0.04 M in orthophosphoric, glacial acetic and boric acids, by addition of 0.2 M NaOH) to give solutions in the pH range 2 – 12 and 5.56×10^{-5} in the drug. To extend the pH range studied at either end of the scale, 1 M HCl, 0.1 M HCl, 0.1 M NaOH and 1 M NaOH were used. The range from pH 0 – 14 was scanned for the drug in increments of 1 pH unit to determine the approximate position of each pK_a value by observation of the spectral changes over the whole range. The region around each pK_a value was then studied in more detail with buffers differing by increments of *ca.* 0.3 pH unit. From the spectra obtained, pK_a values were evaluated using the Henderson equation. The wavelength range scanned was 200 – 390 nm. A slow scan speed (8 min for the range) was used, and the instrument reference beam contained a blank of buffer solution.

Molecular orbital (CNDO/2) calculations were carried out at the University of London Computer Centre (CDC 6600). For the application of the particular computer programme,[9] data words were prepared for each atom in the molecule stating the atomic number and the spatial coordinates relative to a set of mutually orthogonal X, Y and Z planes. Atomic coordinates were calculated geometrically using literature interatomic bond lengths and dihedral angles. Programme 92 requires further information concerning (*i*) the number of atoms composing the species of interest, (*ii*) the overall charge on the species, and (*iii*) the spin multiplicity of the system. Spin multiplicities were in all cases taken as unity since the various species were assumed to be in their lowest energy singlet ground-states with all electrons paired.

For the polarographic studies, the PAR 174A Polarographic Analyser was operated in the differential pulse mode. A scan speed of 10 mV s^{-1} and a controlled drop time of 1 s were used. The tetracycline hydrochloride concentration was held constant at 6.8 $\mu g\ cm^{-3}$ in a supporting electrolyte of pH 4.7 (0.1 M barbitone, 0.1 M LiCl in 40 % methanol), and increasing amounts of calcium (0 – 0.7 $\mu g\ cm^{-3}\ CaCO_3$) were added. The differential pulse polarographic behaviours of these mixtures were then examined.

For the absorption studies involving biological membranes, 10^{-3} M solutions of tetracycline hydrochloride were prepared in the modified form of the Krebs-Ringer buffer. This buffer has been devised to exclude Ca^{2+} (known to complex with tetracycline) and Cl^- ions, and employs isotonic mixtures of disodium orthophosphate and citric acid to give solutions of pH 4.5 – 7.5.

Absorption studies on human buccal mucosa were based on the method of Beckett and Triggs,[12] and the buccal washes were assayed for tetracycline using the spectrophotometric method of Chatten and Krause.[13] Rat colon everted sacs were prepared by the method of Parsons and Paterson.[14] Aliquots of 0.2 cm^3 of the serosal and mucosal fluids and the original solution were added to 1.8 cm^3 of 5 % (*w/v*) trichloroacetic acid and centrifuged at 3000 rpm for 10 min; 0.5 cm^3 aliquots of the supernatants were then taken and assayed as for the buccal washes.

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