droquinone is oxidized to *p*-quinone. Then the differences in Cu, not extracted from the solution by the cation-exchange materials, would indicate a higher formation constant for Cu–*o*-quinone than for Cu–*p*-quinone.

The apparent effect of denaturation of the tyrosinase on the Cu^{++} complexed, as shown in Table II, is probably related to the amount of *o*-quinone formed rather than change in the enzyme. The apparent effect of time of addition of $Cu^{64}SO_4$ to the oxidizing solution on the amount of Cu^{++} complexed is probably due to the polymerization of the *o*-quinone and the decrease in active binding sites. That is, if the Cu^{++} was present at the time of formation of *o*-quinone, a greater amount was complexed than if the *o*-quinone was allowed to begin polymerization prior to addition of the $Cu^{64}SO_4$.

NEW ORLEANS, LOUISIANA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER & CO., INC., AND THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

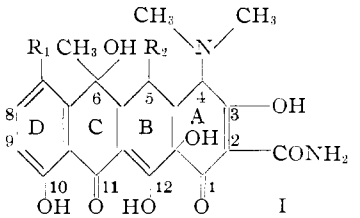
Acidity Constants of the Tetracycline Antibiotics

BY C. R. STEPHENS, K. MURAI, K. J. BRUNINGS AND R. B. WOODWARD

RECEIVED FEBRUARY 20, 1956

The three observed dissociation constants of the antibiotics oxytetracycline, chlorotetracycline and tetracycline have been assigned to specific acidic groupings. In each case, the first dissociation is due to the tricarbonyl system in ring A, the second to the dimethylammonium function[1] and the third to the phenolic β-diketone system (C.10, C.11, C.12). The findings reveal that in neutral solution these antibiotics exist largely as zwitterions.

The antibiotics oxytetracycline, tetracycline and chlorotetracycline may be illustrated by the general structural formula I.[2] It will be noted that the molecule I contains several acid groupings of a

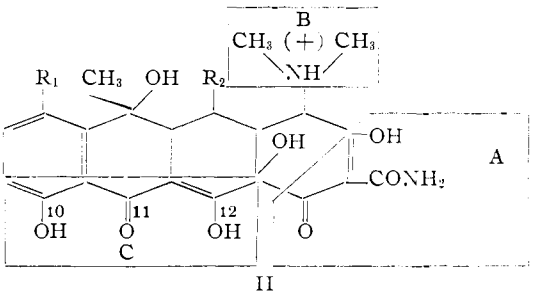


I

Ia, Oxytetracycline
$R_1 = H$, $R_2 = OH$
Ib, Chlorotetracycline
$R_1 = Cl$, $R_2 = H$
Ic, Tetracycline
$R_1 = R_2 = H$

rather unusual type. This communication is concerned with the assignment of the observed acidity constants of each of the three antibiotics to the specific functions responsible for the dissociation. From an examination of Table I, it is apparent that the three antibiotics are closely similar in acidity properties. This, of course, is consistent with their structural relationship. Thus, if we assign the pK_a's in any one of the antibiotics we will assume a similar relationship to apply in the other two.

The general formula I, written as an acid salt II, contains three distinct acid groups—the tricarbonylmethane system A, the ammonium cation B, and the



II

phenolic diketone system C.[3] The problem is thus to associate the 3, 7 and 9 pK_a values with the correct system (A, B or C).

TABLE I

pK_a VALUES[a] (OF HYDROCHLORIDES) IN AQUEOUS SOLUTION AT 25°

Oxytetracycline (Ia)	3.27	7.32	9.11
Chlortetracycline (Ib)	3.30	7.44	9.27
Tetracycline (Ic)	3.30	7.68	9.69

[a] These are carefully corrected thermodynamic pK_a's. They differ very slightly from previously reported values; *cf.* reference 2 and A. Albert, *Nature*, **172**, 201 (1953).

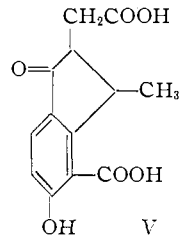
Our earliest data pertinent to the assignment of dissociation constants was derived through etherification studies on Terramycin.[2c] With diazomethane, a 10% yield of a dimethyl ether[2a] ($C_{24}H_{28}N_2O_9$) was obtained in addition to a larger amount of water-soluble, unstable, amorphous material. We have been able to prove that dimethyloxytetracycline contains one methoxyl at C.12 and another at either C.1 or C.3 (*cf.* I) in an otherwise unaltered molecule. Thus, the spectral properties and composition[2a] of the ether indicate that its basic Terramycin skeleton is still intact. Hydrolysis with

(1) ADDED IN PROOF.—Our more recent studies have placed the above assignments for pK_{a_1} and pK_{a_2} in serious jeopardy; see ref. 11.

(2) (a) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, THIS JOURNAL, **75**, 5455 (1953); (b) C. R. Stephens, L. H. Conover, R. Pasternack, F. A. Hochstein, W. T. Moreland, P. P. Regna, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **76**, 3568 (1954); (c) Terramycin is the registered trade-mark of Chas. Pfizer & Co., Inc., for the antibiotic oxytetracycline. Aureomycin is the registered trade-mark of the Lederle Laboratories Division of American Cyanamid Co. for chlorotetracycline. Tetracyn is the registered trade-mark of Chas. Pfizer & Co., Inc., for tetracycline; Achromycin is the trade-mark of American Cyanamid Co. for this antibiotic.

(3) In the case of system C we will make no effort to speculate as to which hydroxyl function (*i.e.*, the C.10 or C.12 —OH, etc.) actually loses its proton in this strongly chelated grouping of atoms.

dilute alkali gave terracinoic acid[4] (V), dimethylamine and ammonia—products readily obtainable

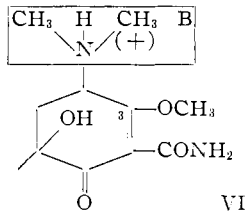


V

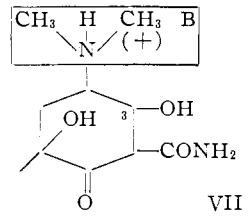
from Terramycin under similar conditions. Since the phenolic hydroxyl in terracinoic acid is the same[2a] as that originally present in Terramycin, it was evident that dimethyloxytetracycline contained no methoxyl at C.10. The presence of an *o*-hydroxyacetophenone system was indicated by the behavior of the ether in complexing with cupric chloride.[5] It was thus evident that one methoxyl group was attached at C.12 and the second either at C.1 or C.3. For the purpose of our argument, no closer definition of the structure is necessary.

Dimethyloxytetracycline, like the parent antibiotic, is amphoteric. It forms a hydrochloride with pK_a values 7.5 and 9.4. The amphoteric ether shows identical absorption peaks in neutral and in acidic solutions. However, a drastic absorption shift is observed in 0.1 *N* alkali. From these data we can conclude that the 7.5 pK_a value of the hydrochloride cannot be due to the phenolic hydroxyl[6] and must therefore be associated with the dimethylammonium cation B.

Comparison of one of the two possible structures of the A ring of dimethyloxytetracycline hydrochloride, *e.g.*, VI with the corresponding enolic expression for Terramycin hydrochloride (VII)

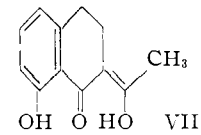


VI



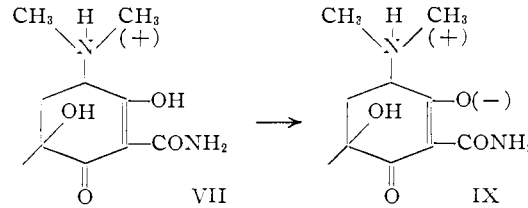
VII

reveals a relationship at C.1 and C.3 which is vinylogous to that of an ester and the corresponding carboxylic acid. "Esterification" (or etherification) might thus be expected to lead to an *increased* electronegative inductive effect on the ammonium cation B. Therefore, we felt certain that the acidity of this function could not *decrease* from pK_a 3.3 (pK_{a1} of Terramycin hydrochloride) to pK_a 7.5 in the ether hydrochloride. Thus, the ammonium cation B *could not be responsible for the first dissociation* ($pK_a \sim 3$ in water) of the antibiotics. That this first dissociation is not due to the phenolic β-diketone system C was apparent from titration of the model compound VIII.[2a] This substance showed pK'_a 8.3 (ethanol–water).



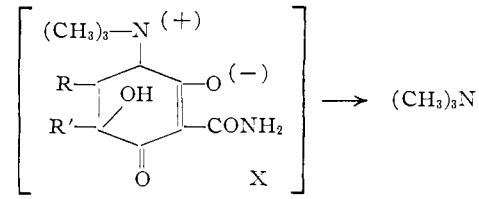
VIII

Thus, the first dissociation is due to system A[7] and can be represented by the expression VII → IX.



VII IX

From this conclusion and from the structural relationship of the three antibiotics, it is apparent that in the amphoteric form oxytetracycline, tetracycline and chlorotetracycline exist as zwitterions. The major product (*vide supra*) of diazomethane methylation of amphoteric Terramycin was consistent with a zwitterion concept. The water-soluble, unstable, amorphous material consisted largely of a quaternary salt—formulated as X—

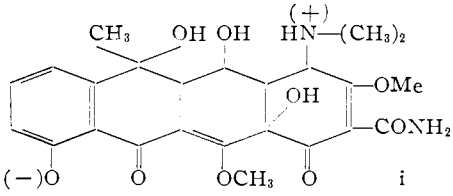


X

(4) R. Pasternack, L. H. Conover, A. Bavley, F. A. Hochstein, G. B. Hess and K. J. Brunings, THIS JOURNAL, **74**, 1928 (1952).

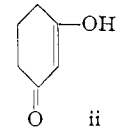
(5) This effect, observed by spectral changes, was similar in nature to that observed with 8-hydroxytetralone-1 (ref. 2a and 16).

(6) Such an assignment would require that amphoteric dimethyloxytetracycline exist as a zwitterion such as i, which would show a dissociated absorption curve in neutral medium.



i

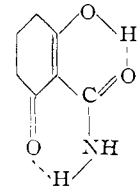
(7) The above assignment was postulated *ab initio* on the following grounds: if the effect of introduction of an ammonium cation into an α-position of dihydroresorcinol (ii) (pK_a 5.5) is assumed to be very roughly that (ΔpK_a 2.3) of a similar function



ii

α to a carboxyl group, the estimated value for the ionization VII → IX falls in the proper range for the first dissociation constant of Terramycin.

In this computation we neglected the acidifying effect of the C.2 carboxamide function, since we felt that this would be largely compensated by exceptionally strong hydrogen bonding stabilization of the non-dissociated species iii. The observed dissociation constant of desdimethylaminotetramycin (ref. 2a) confirms this belief. Thus, this material shows pK_{a1} 5.94. (In our earlier work (ref. 2a) apparent



iii

values in 50% dimethylformamide were given.) Ultraviolet data such as that recently recorded by Parke and Davis (*Anal. Chem.*, **26**, 642 (1954)) serve to graphically illustrate this assignment of pK_{a1}.

which decomposed in dilute alkali with evolution of trimethylamine.[2a] The formation of betaines is observed frequently in the reaction of zwitterions with diazomethane.[8]

The remaining problem was to allocate the pK_a's 7 and 9 between the ammonium cation B and the phenolic β-diketone C. Of great value in defining the acidity of system C was the complexing effect which the three antibiotics show with many polyvalent metallic ions. This effect is evidenced by a drastic change in apparent acidity constants.[9]

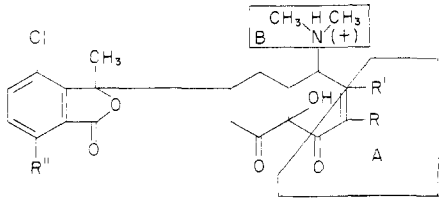
TABLE II[a]

Oxytetracycline·HCl (H_2O)	3.27	7.32	9.11
Oxytetracycline·HCl[b] (H_2O + 1 mole $CaCl_2$)	3.4[c]	5.5[c]	7.0[c]

[a] Ref. 9. [b] Chlorotetracycline and tetracycline show a substantially identical effect. [c] Uncorrected values taken from the half neutralization point in 4×10^{-3} M solution. The second apparent value—5.5 at this dilution, actually an acidity at half neutralization—varies drastically with concentration, since it is not controlled by an acid dissociation constant but is due to hydrogen ions liberated in the metal complexing reaction (*vide infra*).

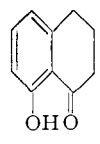
The apparent over-all effect (see Table II) is that both pK_2 and pK_3 change, *i.e.*, 7.3 → 5.5, 9.1 → 7.0. However, the same result would be obtained if pK_3 in water (9.1) changed to 5.5 in the presence of calcium chloride, and pK_2 (7.3) remained essentially constant (7.0). We felt that if we could associate a single acid grouping with this complexing effect and then measure the approximate magnitude of the acidity enhancement we might then be able to assign specific pK_a values with some rigor.

A very useful compound in this work proved to be isochlorotetracycline hydrochloride (XI).[10] It



XI, R″ = OH; R′ = OH; R = $CONH_2$

will be noticed that this substance contains the systems A and B intact and little changed in environment. It was thus most gratifying to note that the pK'_a's of this compound (3.1, 6.7, 8.3)[10] *were substantially unchanged by adding calcium chloride.* Thus, the complexing effect is unique to the system C and must furthermore involve the C.11–C.12 enol system, since both spectral and titration studies on dimethyloxytetracycline and the model compound 8-hydroxytetralone-1 (XII)[2a]



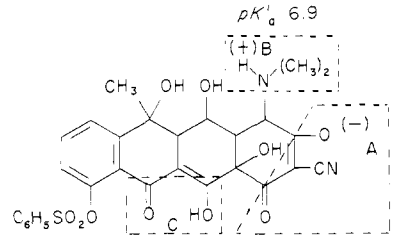
XII

(8) *Cf.* R. Kuhn and W. Brydowna, *Ber.*, **70**, 1333 (1937).

(9) (a) P. P. Regna, I. A. Solomons, K. Murai, A. E. Timreck, K. J. Brunings and W. A. Lazier, THIS JOURNAL, **73**, 4211 (1951); (b) A. Albert, *Nature*, **172**, 201 (1953).

(10) C. W. Waller, B. L. Hutchings, C. F. Wolf, A. A. Goldman, R. W. Broschard and J. H. Williams, THIS JOURNAL, **74**, 4981 (1952).

showed that calcium chloride had no acidifying effect on the C.10–C.11 system. Fortunately, a derivative of known structure was available which could be used to estimate the magnitude of the acidity enhancement effect of complex formation at the C.11–C.12 enol system. This compound, 10-benzenesulfonyloxytetracyclinonitrile[2a,11] (X-III), showed pK'_a's 6.9 and 11.5 in 50% aqueous dimethylformamide. In this case the acidity constant assignments indicated in expression XIII



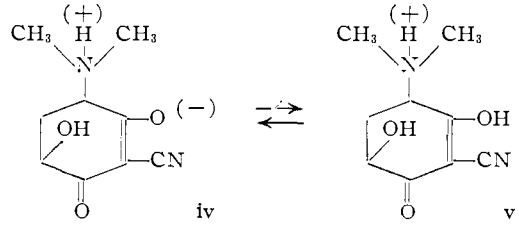
pK_a 11.5

XIII

could hardly be questioned.[12] When titrated in the presence of calcium chloride, compound XIII showed apparent pK_a's of 6.2 and 7.0[13] ($c \sim 4 \times 10^{-3}$ in 50% dimethylformamide–water). These acidity changes are quite comparable to those observed with the three antibiotics[14] and substantiate the interpretation that the third dissociation constant ($pK_a \sim 9$) is associated with system C in the parent antibiotics and give rise to the acidity enhancement effect noted in the presence of calcium chloride.

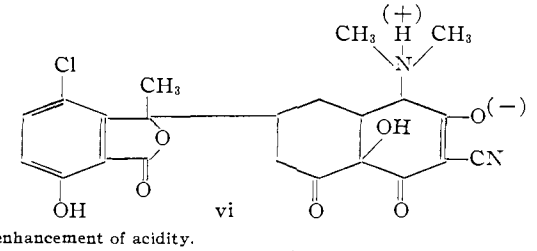
(11) C. R. Stephens and co-workers, to be published.

(12) The reverse situation (*i.e.*, reversal of B and C) would be inconceivable, since the introduction of the strongly electronegative nitrile function could only result in rendering the ammonium anion more acidic. The powerful acidifying influence of this nitrile group is dramatically illustrated by its effect on the enolic system A. In nitrile derivatives, this system (pK_a 3 in the parent antibiotics) becomes so acidic that the compounds are isolated only as zwitterions (iv) even from strongly acidic solutions. Efforts to prepare hydrochlorides (v) from nitrile analogs (*cf.* ref. 11) have thus been unsuccessful, since acidic conditions sufficiently rigorous to form the acid salt result in degradation of the compounds. The low solubility of XIII and related compounds in water required the use of the dimethylformamide–water solvent.



iv v

(13) We titrated isoaureomycinonitrile (vi, *cf.* ref. 11) in the presence of calcium chloride and noted that it, like isoaureomycin (XI), showed



vi

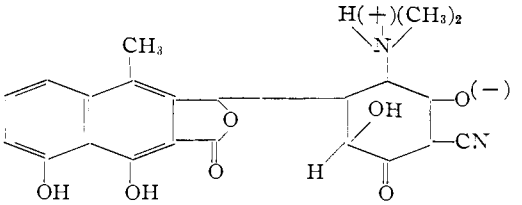
no enhancement of acidity.

(14) Amphoteric oxytetracycline, for example, shows pK'_a's of 5.8 and 7.3 in 50% dimethylformamide containing one mole of calcium chloride under similar concentration conditions.

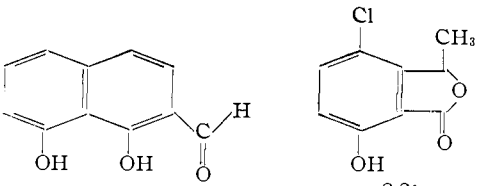
An equally definitive argument for the assignment of the pK_a 9 to the system C may be derived from a consideration of the series of nitrile analogs and model compounds listed in Table III. From

TABLE III

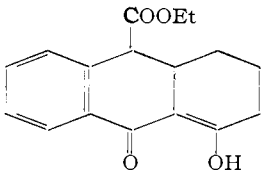
pK'_a's IN 50% DIMETHYLFORMAMIDE–H_2O



4.4, 7.1[a]



4.5[b] 8.2[c]



11.7[d]

XI (R″ = OH, R′ = O⁻, R = CN), 7.0, 8.4[a]
XI (R″ = $C_6H_5SO_2O$, R′ = O⁻, R = CN), 7.0[a]
XIII, 6.9, 11.5[b]

[a] Ref. 11. [b] Ref. 2a. [c] Ref. 2b and S. Kushner, J. Morton, II, J. H. Boothe and J. H. Williams, THIS JOURNAL, **75**, 1097 (1953). [d] L. H. Conover and co-workers, to be published.

the structures and acidities of these compounds, it is obvious that the pK'_a of the $(CH_3)_2NH^+$ function in nitrile analogs of this type remains very nearly 7.0 regardless of structural changes in the BCD system. Now if we consider the simple nitriles derived from these antibiotics (Table IV)

TABLE IV

Compound[a]	pK'_a's 50% dimethylformamide–water	
Tetracyclinonitrile[b] (XIVB)	7.2	10.1
Tetracycline (amphoteric) (XIVA)	8.3	10.2
Oxytetracyclinonitrile[b] (XIVB)	7.0	9.7
Oxytetracycline (amphoteric) (XIVA)	8.0	9.8

[a] Chlortetracyclinonitrile and chlortetracycline show the same relationship, although accurate values for the final constant are difficult to determine in this system due to rapid alkaline degradation; *cf.* ref. 2b. [b] Ref. 11.

we see that they exhibit a pK'_a 7 and also a second constant which has the same value as pK'_{a3} of the parent amides. (Comparison in 50% dimethylformamide is necessitated because of the low solubility of the nitriles.) In view of previous considerations (Table III) we know that the pK'_a 7 value must be associated with the dimethylammonium function in nitrile analogs. Thus, the final dissociation is due to the C.10–C.11–C.12 system in the nitriles. A similar relationship must exist in the parent antibiotics since changing the —$CONH_2$ function to —CN could hardly make either the pK 7 or the pK 9 function more basic.

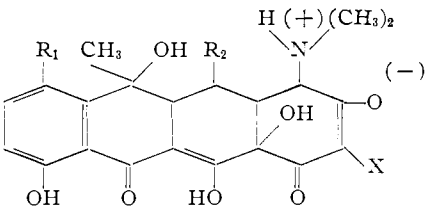
Thus, several independent lines of evidence demonstrate the pK_a assignments indicated in Table V for the antibiotics oxytetracycline,

TABLE V

pK_a assignments (*Cf.* Formula II)

	A	B	C
Oxytetracycline	3.27	7.32	9.11
Chlorotetracycline	3.30	7.44	9.27
Tetracycline	3.30	7.68	9.69

tetracycline and chlorotetracycline. These assignments require that the general formula I be written as the dipolar ion XIV A.



XIVA, X = —$CONH_2$
XIVB, X = CN

Experimental

The compounds cited in this theoretical argument have been described in other publications, references to which have been included.

Titrations were carried out under nitrogen by potentiometric methods similar to those previously described for Terramycin.[9a] The pK_a values listed in Table I,[15] as well as the constants for dimethyloxytetracycline hydrochloride, were computed by the procedure used previously[9a] and were in agreement with values obtained by the rigorous classical calculation based on data obtained by titration in 0.1 and 0.01 *M* potassium chloride solutions with activities computed using Debye–Hückel approximations. The pK_a' values cited are apparent values at the point of 50% neutralization, ($c \sim 5 \times 10^{-3}$ mole).

Alkali degradation studies on dimethyloxytetracycline were performed in a manner similar to that previously described for Terramycin.[4] The degradation products were identified by comparison (infrared absorption, etc.) with authentic samples.

The complexing behavior of dimethyloxytetracycline was observed by measurement of the ultraviolet absorption curve of the compound in the presence and in the absence of cupric chloride (methanol solvent). The observed shifts in absorption peaks were similar to those found with 8-hydroxytetralone-1.[16]

Acknowledgments.—We are indebted to Glenn Hess and his associates for the titration data in non-aqueous media. We would also like to express our gratitude to P. N. Gordon, L. H. Conover, F. A. Hochstein and F. J. Pilgrim for many stimulating discussions on this problem.

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(15) The values listed for chlorotetracycline (Table I) were first reported by A. Albert (reference 9b) and have been duplicated in our laboratories. Our pK_a values for oxytetracycline differ slightly from those reported by Albert.

(16) We are indebted to P. N. Gordon for this observation.