L-Citrullyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine (1-Citrulline Bradykinin) (XX).—To a solution of the benzyloxycarbonyl nonapeptide methyl ester (XIX) (130 mg.) in methanol (1 ml.) 2 *N* sodium hydroxide (0.1 ml.) was added. After 1 hr. at room temperature water (2 ml.) was added and after another 15 min. the solution was acidified with *N* hydrochloric acid and kept in a refrigerator overnight. The supernatant was decanted and the semi-solid precipitate was dried *in vacuo* over sodium hydroxide. The solid residue thus obtained (88 mg.) was dissolved in a mixture of acetic acid-water (2:1) (12 ml.) and hydrogenated at atmospheric pressure for 48 hr. in the presence of 5% palladium on barium sulfate.[14] After removal of the catalyst the solution was freeze-dried. The residue was chromatographed on carboxymethylcellulose, using a gradually increasing concentration of ammonium acetate for the elution, as described for the purification of XI. The product was freeze-dried several times to remove residual ammonium acetate. The final product (30 mg., $[\alpha]^{20}D$ −91.2° (*c*, 1.0, *N* acetic acid)) was homogeneous on paper chromatograms (butanol–acetic acid–water 4:1:5, R_f 0.30) and by paper electrophoresis (pyridine acetate buffer pH 4.0 and ammonium acetate buffer pH 5.3) when developed with ninhydrin, Ehrlich (*p*-dimethylaminobenzaldehyde) and Sakaguchi reagents.

Amino Acid Analysis.—Gly:Ser:Pro:Phe:Arg:Cit-Orn-1:0.8:3:2:1.1:1.0.

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Metal Complexation of the Tetracycline Hydrochlorides

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Therapeutically active tetracycline analogs, tetracycline·HCl, chlorotetracycline·HCl, oxytetracycline·HCl, 7-chloro-6-demethyltetracycline·HCl, and anhydrochlorotetracycline·HCl, were found to complex with cupric, nickel and zinc ions to form 2:1 complexes with the same avidity. Inactive tetracycline analogs, anhydro-4-epitetracycline·HCl, 7-chloro-4-epitetracycline·HCl and isochlorotetracycline·HCl, were found to form only 1:1 complexes with cupric, nickel and zinc ions. By analysis of potentiometric data and examination of Stuart and Briegleb models, it is postulated that tetracycline chelation with these metal ions occurs through coördination with the C.4 dimethylamino group and either the C.3 or C.12a hydroxyl group.

Many possible modes of tetracycline action have been suggested, but one is of particular interest. Hunter and Lowry[1] have suggested that tetracyclines uncouple aerobic phosphorylation (that is, they inhibit the formation of ATP, which is a primary source of energy for cellular functions, without affecting oxygen consumption) by interaction with magnesium bound to an enzyme without actually removing it. In this investigation experiments were undertaken to determine whether there existed a correlation between the metal binding properties of the tetracyclines and effectiveness of antibacterial activity.

Albert[2,3] has calculated stability constants for the interaction of many metal ions with tetracycline·HCl, oxytetracycline·HCl and chlorotetracycline·HCl and has stated that chelation is likely to play a part in the mode of action "because substances with constants of such magnitude could not fail to compete with metals in the tissues."[4] He also points out that the action of the tetracyclines on bacteria is much slower than that of oxine and they are active in iron-depleted media.

Miura, *et al.*,[5] have shown that oxytetracycline and chlorotetracycline exhibit their activity by uncoupling aerobic phosphorylation. Recent work has indicated that manganese is essential for phosphorylation.[6] Saz and Slie[7,8] have presented evidence that manganese is essential for reduction of DPN^+ by malate in certain *Escherichia coli* extracts and suggest that the inhibition of nitroreductase by chlorotetracycline in such preparations is due to complexing with manganese and preventing the formation of DPNH which is essential for reduction. Brody, *et al.*,[9] have shown that the uncoupling of phosphorylation is prevented if excess magnesium is included in the medium; however, Burstall[10] suggests that small amounts of metal enhance, and may in some cases be necessary for, inhibition. Pancreatic lipase is inhibited by chlorotetracycline only in the presence of divalent ions.[11] Hamner[12] recently has reported the potentiation of demethylchlorotetracycline·HCl in the presence of zinc cations. This result was in contrast to effects obtained with nickel, magnesium, iron, copper and aluminum ions.

Goldman[13] has studied the inhibition of alanine dehydrogenase by oxytetracycline. He noted that crude

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(5) Y. Miura, Y. Nakamura, H. Matrudaira, and T. Komeiji, *Antibiotics and Chemotherapy*, **2**, 152 (1952).
(6) O. Linberg and L. Ernster, *Nature*, **173**, 1038 (1954).
(7) A. K. Saz and R. B. Slie, *Antibiotics Ann.*, 303 (1953).
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(13) D. S. Goldman, *J. Biol. Chem.*, **235**, 616 (1960).

preparations are inhibited by oxytetracycline while more purified preparations are not. When certain metal ions are added to purified preparations inhibition does occur with oxytetracycline. Goldman concluded from this evidence that a metal chelate of oxytetracycline is the actual inhibitor.

It is also interesting to note that certain enzyme systems inhibited by metals may be reactivated by the tetracyclines. For example, the beryllium-induced inhibition of rat plasma alkaline phosphatase is suppressed by the addition of chlorotetracycline or other metal-complexing agents.[14]

These studies suggest the importance of metals and, moreover, the importance of ternary binding in the mechanism of action of the tetracyclines; however, not all investigators favor this mechanism. Hahn,[15] in an article dealing with modes of antibiotic action, considers the hypothesis that tetracyclines inhibit bacterial growth by interfering with some metal requiring step in the energy metabolism but discounts this suggestion as a primary mode of action.

Experimental

Preliminary Considerations.—Albert[2,3] already has calculated the stability constants of tetracycline·HCl, chlorotetracycline·HCl and oxytetracycline·HCl with various metal ions. In his work he points out that only the functional group having an acidity constant of approximately 7 undergoes an ionization change when chelation takes place. This indicates that the complexing species of the tribasic tetracycline hydrochlorides, H_3A, is HA. Bjerrum[16] has shown that for the reaction

$$M(HA)_n + HA \rightleftarrows M(HA)_{n+1} \quad (1)$$

the general equation for the stepwise stability constants, K_i, is

$$\log K_i = p(HA) \quad \text{at } \bar{n} = i - 1/2 \quad (2)$$

where (HA) = molar concentration of the complexing species
i = integer indicating the particular stepwise reaction of interest
$\bar{n}$ = $\dfrac{\text{total concentration of ligand bound}}{\text{total concentration of metal}}$

For the special case where the maximum stoichiometric ratio is 2, it has been shown that the over-all stability constant, K_s, may be determined from either of the equations

$$\log K_s = \log K_1 + \log K_2 \quad (3)$$

$$\log K_s = 2p(HA) \quad \text{at } \bar{n} = 1 \quad (4)$$

From these equations it is evident that two quantities, $\bar{n}$ and p(HA), must be determined for the evaluation of the stability constants. The method of Platt and Martin[17] was used to calculate values of $\bar{n}$. The following derivation illustrates the calculation of p(HA) from potentiometric data.

The concentration of free and combined hydrogen ions, [XH], can be expressed in two ways:

$$[XH] = [H] + [HA] + 2[H_2A] + 3[H_3A] - [OH] \quad (5)$$

$$[XH] = 3C_A - [NaOH] \quad (6)$$

where C_A is the initial concentration of the tetracycline hydrochloride. Combining Equations 5 and 6, and substituting the appropriate acidity constants it may be shown that

$$[HA] + \frac{2[HA][H]}{k_2} + \frac{3[HA][H]^2}{k_1k_2} = 3C_A - [NaOH] - [H] + [OH] \quad (7)$$

or

$$[HA]\beta = 3C_A - [NaOH] - [H] + [OH] \quad (8)$$

where

$$\beta = 1 + \frac{2[H]}{k_2} + \frac{3[H]^2}{k_1k_2} \quad (9)$$

By taking logarithms of Equation 8 and rearranging, it can be seen that

$$p[HA] = \log \beta - \log [3C_A - [NaOH] - [H] + (OH)] \quad (10)$$

In the procedure described, two pH titration curves are required. The first is the potentiometric titration of the tetracycline hydrochloride in the absence of metals and the second is the potentiometric titration of the same concentration of the tetracycline hydrochloride reacted with half this concentration of divalent cation.

Materials.—Samples of tetracycline·HCl, chlorotetracycline·HCl, 7-chloro-6-demethyltetracycline·HCl, 7-chloro-4-epitetracycline·HCl, isochlorotetracycline·HCl and anhydrochlorotetracycline·HCl were donated by Lederle Laboratories. Oxytetracycline·HCl was donated by Pfizer Laboratories. Anhydro-4-epitetracycline·HCl was prepared by the procedure outlined by McCormick, *et al.*[18] Ultraviolet absorption spectra and/or melting point determinations indicated that the above compounds were of a high degree of purity. To prepare the water used in these experiments, distilled water was passed through Amberlite IR-4B (OH exchange) and Amberlite IR-120 (H exchange) resins and then boiled for 1 hr. to remove carbon dioxide. The resulting water had a specific conductance of 2.5×10^{-6} ohm^{-1} cm.$^{-1}$, or less, as determined by use of a Serfass conductivity bridge.

Procedure.—Thirty ml. of a carefully prepared 1.00×10^{-3} M solution of the tetracycline hydrochloride was diluted to 50 ml. with water and adjusted to an ionic strength of 0.01 with potassium chloride. The solution then was titrated potentiometrically, at 30° (± 0.2°) under nitrogen, with a standard solution of 0.01 – 0.02 N sodium hydroxide at 0.10 to 0.20 ml. increments.

To test for metal complexation, 15.0 ml. of a carefully prepared 1.00×10^{-3} M solution of divalent cation reacted with 30.0 ml. of the ligand solution. The resulting solution was diluted to 50.0 ml., adjusted to an ionic strength of 0.01 with potassium chloride and titrated with standard sodium hydroxide solution in the manner described.

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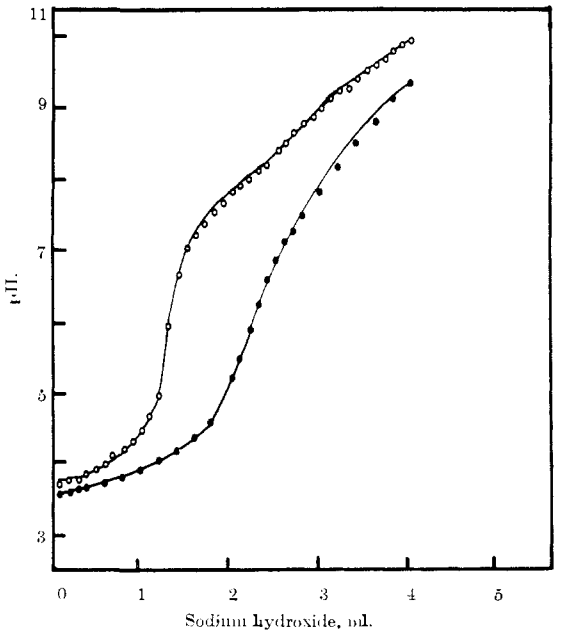

Fig. 1.—The potentiometric titration of tetracycline HCl in the absence and presence of cupric ions: ○, tetracycline·HCl; ●, tetracycline·HCl and cupric ions.

TABLE I

VALUES OF $\bar{n}$ FOR TETRACYCLINE AND CUPRIC IONS

Tetracycline·HCl, 5.64×10^{-4} mole/l.; cupric ions, 3.00×10^{-4} mole/l.; NaOH, 2.24×10^{-4} mole/l.; 30° (±0.2°).

pH	ml. NaOH per 50 ml. sample	Tetracycline complexed, moles/l. × 10^4	n
3.62	0.20	0.896	0.30
3.64	.25	1.12	.37
3.66	.30	1.34	.45
3.69	.35	1.57	.52
3.70	.40	1.79	.60
4.00	.50	2.24	.75
4.25	.60	2.69	.90
4.50	.64	2.86	.95
4.75	.70	3.04	1.04
5.00	.74	3.31	1.10
5.50	.82	3.67	1.22
6.00	.93	4.17	1.39
6.25	.97	4.35	1.45
6.50	1.00	4.48	1.49

TABLE II

VALUES OF p(HA) FOR TETRACYCLINE·HCl AND CUPRIC IONS

pH	NaOH, ml.	NaOH, moles/l. × 10^4	[H] × 10^4	β	p[HA]
3.62	0.20	0.896	2.40	5.66×10^4	7.62
3.64	.25	1.12	2.29	5.23×10^4	7.59
3.66	.30	1.34	2.19	4.87×10^4	7.56
3.69	.35	1.57	2.04	4.35×10^4	7.52
3.70	.40	1.79	2.00	4.20×10^4	7.50
4.00	1.12	5.02	1.00	1.48×10^4	7.13
4.25	1.45	6.50	0.562	6.78×10^3	6.84
4.50	1.70	7.61	.316	3.32×10^3	6.57
4.75	1.86	8.33	.178	1.72×10^3	6.32
5.00	1.94	8.69	.100	9.17×10^2	6.05
5.50	2.10	9.41	.0316	2.77×10^2	5.57
6.00	2.24	10.0	.0100	8.70×10	5.10
6.25	2.30	10.3	.00562	4.92×10	4.87
6.50	2.36	10.6	.00316	2.84×10	4.65
6.75	2.45	11.0	.00178	1.62×10	4.44

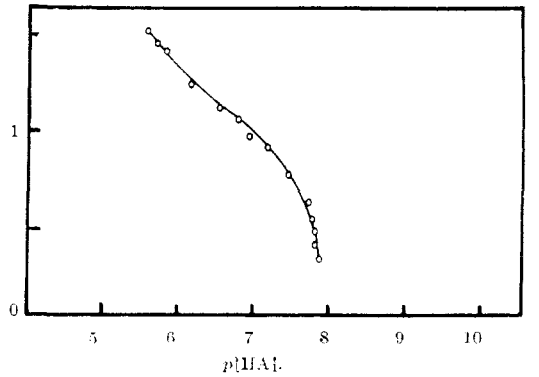

Fig. 2.—Formation curve for tetracycline·HCl and cupric ions.

Treatment of Potentiometric Data.—The acidity constants of the tetracycline hydrochlorides were determined using the procedure of Stephens, *et al.*[19] Calculated values of $\bar{n}$ and p(HA) for tetracycline·HCl and cupric ions are given in Tables I and II.

From the formation curve, Fig. 2, it seems that the values of $\bar{n}$ are approaching a value of 2 and, hence, the stoichiometric ratio of the complex is 2 tetracycline molecules per cupric ion. Approximate values of the stability constants also may be derived from this formation curve. At

$$\bar{n} = 0.5 \qquad \log K_1 = p[\mathrm{HA}] = 7.5$$

$$\bar{n} = 1.0 \qquad \log K_2 = 2p[\mathrm{HA}] = 2 \times 6.4 = 12.8$$

hence

$$\log K_2 = 12.8 - 7.5 = 5.3$$

Samples of various tetracycline hydrochlorides were analyzed for complexation with certain divalent cations as outlined. The results are summarized in Table III.

The acidity constants reported in reference 19 were determined at 25° and corrected to zero ionic strength. The logarithmic stability constants reported in references 2 and 3 were determined at 20° and an ionic strength of approximately 0.01. The experimentally determined values were obtained at 30° and an ionic strength of 0.01.

Discussion

Correlation of Antibacterial Activity with the Metal Binding Properties of Tetracycline Analogs.—Tetracycline, oxytetracycline, chlorotetracycline and 7-chloro-6-demethyltetracycline are antibiotics of known therapeutic value. The antibacterial activity of these compounds is approximately the same.[10,20] These compounds exhibited similar metal binding properties, all forming 2:1 complexes with cupric, nickel and zinc ions. The logarithmic stability constants range from 12.3 to 12.8 for the cupric complexes, from 10.4 to 11.3 for the nickel complexes and from 8.4 to 8.9 for the zinc complexes.

Anhydrochlorotetracycline possesses a different antibacterial spectrum from the above antibiotics. For example, it has only a fraction of the antibacterial activity of chlorotetracycline against *Staphylococcus aureus* but has a much greater activity than chloro-

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(20) J. R. D. McCormick, N. O. Sjolander, U. Hirsch, E. R. Jensen and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79**, 4561 (1957).

TABLE III
METAL COMPLEXATION OF THE TETRACYCLINE HYDROCHLORIDES

	Apparent acidity constants			Cation	log K_1	log K_2	log K_s	Ref.
				Tetracycline·HCl				
Found	3.69	7.63	9.24					
Lit. value	3.30	7.68	9.69					19
Found				Cu^{++}	7.5	5.3	12.8	
Lit. value				Cu^{++}	7.8	5.0	12.8	2
Found				Ni^{++}	6.1	5.1	11.2	
Lit. value				Ni^{++}	6.0	5.0	11.0	2
Found				Zn^{++}	5.1	3.8	8.9	
Lit. value				Zn^{++}	4.9	...	9[a]	2
				Chlorotetracycline·HCl				
Found	3.66	7.40	9.06					
Lit.	3.30	7.44	9.27					19
Found				Cu^{++}	7.5	4.9	12.4	
Lit.				Cu^{++}	7.6	5.0	12.6	3
Found				Ni^{++}	5.7	4.7	10.4	
Lit.				Ni^{++}	5.8	4.7	10.5	3
Found				Zn^{++}	4.8	4.1	8.9	
Lit.				Zn^{++}	4.5	...	8[a]	3
				Oxytetracycline·HCl				
Found	3.60	7.42	9.05					
Lit.	3.27	7.32	9.11					19
Found				Cu^{++}	7.6	5.1	12.7	
Lit.				Cu^{++}	7.2	5.0	12.2	3
Found				Ni^{++}	5.9	4.8	10.7	
Lit.				Ni^{++}	5.8	4.8	10.6	3
Found				Zn^{++}	4.7	3.7	8.4	
Lit.				Zn^{++}	4.6	...	8[a]	3
				7-Chloro-6-demethyltetracycline·HCl				
Found	3.85	7.31	9.23					
Found				Cu^{++}	6.9	5.4	12.3	
Found				Ni^{++}	6.4	4.9	11.3	
Found				Zn^{++}	5.3	3.6	8.9	
				Anhydrochlortetracycline·HCl				
Found	3.28	5.37						
Found				Cu^{++}	7.0	5.5	12.5	
Found				Ni^{++}	5.9	5.5	11.4	
Found				Zn^{++}	5.8	...	10[a]	
				Anhydro-4-epitetracycline·HCl				
Found	4.38	8.95						
Found				Cu^{++}	9.9	...	9.9[b]	
Found				Ni^{++}	9.3	...	9.3[b]	
Found				Zn^{++}	8.1	...	8.1[b]	
				7-Chloro-4-epitetracycline·HCl				
Found	4.07	7.56	9.26					
Found				Cu^{++}	7.6	...	7.6[b]	
Found				Ni^{++}	6.1	...	6.1[b]	
Found				Zn^{++}	4.6[a]	...	4.6[a,b]	
				Isochlorotetracycline·HCl				
Found	3.96	6.73	7.93					
Found				Cu^{++}	5.0	...	5.0[b]	
Found				Ni^{++}	3.9	...	3.9[b]	
Found				Zn^{++}	3.7[a]	...	3.7[a,b]	

[a] Notes approximate values. [b] In these cases only 1:1 complexes formed; hence, the over-all logarithmic stability constant, log K_s, is the same as log K_1.

tetracycline against actinomycetes.[21] Anhydrochlorotetracycline also forms 2:1 complexes with cupric, nickel and zinc ions. The logarithmic stability constants are approximately the same as those of tetracycline, oxytetracycline, chlorotetracycline and 7-chloro-6-demethyltetracycline.

The 4-epitetracycline analogs possess only about 5% of the antibacterial activity of their parent compounds.[18,20,22] Likewise, the metal binding properties of these compounds differ markedly from that of the parent compounds. For example, the 4-epi analogs appear to form only 1:1 complexes with cupric, nickel and zinc ions. The logarithmic stability constants varied from 7.6 to 9.9 for the cupric complexes, from 6.1 to 9.3 for the nickel complexes and from 4.6 to 8.1 for the zinc complexes.

Isochlorotetracycline has been reported to possess no known antibacterial activity.[10,23,24] This compound appears to form only 1:1 complexes with cupric, nickel and zinc ions. The logarithmic stability constants are 5.0, 3.9 and 3.7, respectively.

These data illustrate that the active and inactive tetracycline analogs tested differ markedly in their metal binding properties. This investigation has shown that therapeutically active analogs form 2:1 complexes with cupric, nickel and zinc ions while the inactive analogs tested appear to form only 1:1 complexes.

Site of Tetracycline Complexation.—From Table III it can be seen that epimerization of the C.4 position in the epitetracyclines and breaking of the "C" ring in isochlorotetracycline markedly changed the metal complexing properties with cupric, nickel and zinc ions. These compounds formed only 1:1 complexes while the other analogs of Table III formed 2:1 complexes. This observation brought up the question, "by which groups do the tetracyclines chelate?"

Albert[3,25] has stated that combination with metals requires that groups near pk_a7 be ionized but not those near 3 and 9. We have come to the same conclusion since, in Figure 1, it may be seen that the second dissociating hydrogen of tetracycline·HCl is the one displaced by the cupric ion. Stephens, *et al.*,[19] have demonstrated that the dimethylammonium cation is responsible for the acidity constant in the vicinity of 7. Therefore, it seems likely that this group is one of the coördinating groups of tetracycline.

By examination of Stuart and Briegleb models, it may be seen that, sterically, either a propanolamine type complex (between the dimethylamino group and the C.12a hydroxyl group) or an ethanolamine type complex (between the dimethylamino group and the C.3 hydroxyl group) can form between the tetracyclines and divalent cations. In the epitetracyclines, the Stuart and Briegleb models suggest that only the ethanolamine type complex may be formed.

It has been reported[10,19,26] that isochlorotetracycline does not bind with calcium. Therefore, calcium was investigated in our study and it was confirmed that this metal ion does not bind with isochlorotetracycline, but it was found that cupric, nickel and zinc ions did bind with this analog.

Conover,[26] by comparing the effect of metal ions on the absorption spectrum of oxytetracycline with the effect of such ions on the spectra of various model compounds, suggests that the ligand group is the enolized β-diketone system lying between C.11 and C.12. This coördinating site, indeed, seems likely for calcium complex formation. Calcium forms stable chelates with tetracycline, oxytetracycline and chlorotetracycline, and when the C.11–C.12 system is destroyed, as in isochlorotetracycline, no complexation occurs. The fact that a hydrogen is displaced by cupric, nickel and zinc in isochlorotetracycline supports our views

(21) J. J. Goodman, M. Matriskin and E. J. Barkus, *J. Bact.*, **69**, 70 (1955).

(22) M. A. Kaplan and F. H. Buckwalter, *Antibiotics Ann.*, 507 (1957).

(23) P. P. Regna, in "Antibiotics, Their Chemistry and Non-Medicinal Uses," H. S. Goldberg, ed., D. Van Nostrand Co., New York, N. Y., 1959, p. 95.

(24) C. W. Waller, B. L. Hutchings, R. W. Broschard, A. A. Goldman, W. J. Stein, C. F. Wolf and J. H. Williams, *J. Am. Chem. Soc.*, **74**, 4981 (1952).

(25) A. Albert, personal communication, 1961.

(26) L. H. Conover, Chem. Soc. Special Publication No. 5, 48 (1956).

that coördination occurs between the dimethylamino group and the C.12a or C.3 hydroxyl group since no hydrogen atoms are associated with the destroyed C 11–C.12 system in this analog.

The mention of hydrogen displacement brings up another point concerning our original views about the possibility of C.12a or C.3 hydroxyl coördination. As noted, the complexation of the tetracycline hydrochlorides causes the dissociation of one mole of hydrogen ions per mole of tetracycline. Both the ethanolamine and propanolamine type complexes would seem to cause the liberation of two moles of hydrogen, one from the dimethylammonium group and the other from the respective hydroxyl groups.

It has been stated previously that one mole of hydrogen ions is dissociated before actual metal complexation and one is dissociated during metal complexation. If the hydrogen being dissociated before actual complexation is that of the C.12a or C.3 hydroxyl group, our postulate would still be valid. Stephens, *et al.*,[19] have suggested that the dissociation of the first hydrogen is from the C.3 hydroxyl group. This would indicate that it is the ethanolamine type complex that is forming rather than the propanolamine type.

It should be pointed out that this discussion of the metal binding of the tetracyclines assumes that Stephens, *et al.*,[19] have correctly assigned the acidity constants of the tetracycline hydrochlorides.

The Binding of Tetracycline Analogs to Conalbumin in the Absence and Presence of Cupric Ions

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The binding of therapeutically active tetracycline analogs, namely tetracycline, oxytetracycline, 7-chloro-6-demethyltetracycline and anhydrochlorotetracycline, to the "model receptor," metal-free conalbumin, was found to increase greatly in the presence of cupric ions, presumably through the formation of ternary complexes. No increases in the binding were noted with the inactive tetracyclines, 4-epitetracycline and isochlorotetracycline. The evidence supports previous data[1] which suggested that a relationship exists between the antibacterial activity of the tetracyclines and their metal binding properties. This evidence also suggests that a metal bridge may be one point of bonding between the tetracyclines and their receptor site.

In a previous paper[1] we have described the relation of the antibacterial activity of tetracycline analogs and their metal complexing properties. It has been suggested by other workers that tetracycline inhibitions may be due to the formation of an active ternary complex of tetracycline, a metal and an enzyme. Kohn[2] has shown that certain metals may mediate the binding of tetracycline to macromolecules such as desoxyribonucleic acid (DNA) and serum albumin.

The general plan of this research was to investigate the binding of active and inactive tetracycline analogs to a model receptor, metal-free conalbumin, in the presence of cupric ions. Conalbumin resembles metal enzymes in that it has the ability to bind "specifically" certain metal ions through chelation.[3,4] Hence, conalbumin may serve as a metal enzyme in the laboratory, and we have used it for this purpose.

Experimental

Preliminary Consideration.—Warner and Weber[4] have shown that two cupric ions are bound per molecule of conalbumin and that these two sites are equivalent and non-interacting. For each cupric ion bound, two hydrogens are displaced. These workers have also calculated the logarithmic stability constant for conalbumin when coördinated with cupric ions to be 16.5 at 30°.

The molecular weight of conalbumin is approximately 76,000. In terms of metal binding, the *equivalent* molecular weight may be taken to 38,000. Hence, a molar solution of 1.32×10^{-5} may be taken to be 2.64×10^{-5} equiv. moles/l.

The binding of the tetracycline analogs to conalbumin in the absence of cupric ions in this investigation is undertaken so that an increase in binding in the presence of cupric ions, if occurring, may be noted. Hence, stability constants were not evaluated in the absence of cupric ions. Titration of conalbumin in the absence and presence of cupric ions showed that the dissociation of the metal-displaced hydrogen ions is complete at a pH of 5.7. Therefore, a pH 7.4 buffer was used to ensure the coördination of copper to conalbumin. The reaction

$$\text{Con–Cu} + 2\text{HA} \rightleftharpoons \text{Con} + (\text{HA})_2\text{Cu} \qquad (1)$$

where Con is Conalbumin and HA is complex forming species of the tetracycline, may be shown to be insignificant. For example, the logarithmic stability constant for the reaction may be determined

$$K = \frac{(\text{Con})((\text{HA})_2\text{Cu})}{(\text{Con–Cu})(\text{HA})^2} = \frac{(\text{Con})(\text{Cu})}{(\text{Con–Cu})} \times \frac{((\text{HA})_2\text{Cu})}{(\text{HA})^2(\text{Cu})} \qquad (2)$$

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