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

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

(1) J. T. Doluisio and A. N. Martin, *J. Med. Chem.*, **6**, 16 (1963).
(2) K. W. Kohn, *Nature*, **191**, 1156 (1961).
(3) R. C. Warner, *Trans. N. Y. Acad. Sci.*, **16**, 182 (1954).
(4) R. C. Warner and I. Weber, *J. Am. Chem. Soc.*, **75**, 5094 (1953).

or

$$K = K_{(HA)_2Cu}/K_{Con-Cu} \quad (3)$$

Therefore

$$\log K = \log K_{(HA)_2Cu} - \log K_{Con-Cu} \quad (4)$$

From previous work[1] it can be seen that log $K_{(HA)_2Cu}$ is approximately 13 for most of the 2:1 complexes of the tetracyclines and copper. Therefore

$$\log K = 13 - 16.5 = -3.5$$

Hence, this reaction is strongly favored to the left and, therefore, the removal of copper from conalbumin may be considered to be negligible when approximate equimolar quantities of conalbumin, tetracycline and cupric ions are present.

The stability constant for the reaction

$$\text{Con–Cu} + \text{HA} \rightleftharpoons \text{Con–Cu–HA} \quad (5)$$

may be evaluated. Curves of absorbance *vs.* initial concentration of tetracycline are prepared for various concentrations of the tetracycline when equilibrated with a length of dialysis tubing and for mixtures of the tetracycline, copper and conalbumin. The absorbance of the tetracycline when equilibrated with dialysis tubing corrects for any binding of the drug to the sack. The absorbancy of the free tetracycline is obtained directly from the second curve. Twice the vertical distance between the two curves yields the absorbancy of the tetracycline bound, that is, the absorbancy of the ternary complex. It is necessary to multiply by two since when the tetracycline is bound it can no longer leave the sack and, hence, is available to only one-half the total volume of the solution. The absorbances are converted to molar concentrations by use of the molar absorbancy index, a_M, for the tetracycline.

The concentration of conalbumin–copper is obtained by simply subtracting the concentration of the ternary complex from the initial concentration of conalbumin–copper.

The formation of the ternary complex, Con–Cu–HA, may be illustrated by the reactions

$$\text{Con} + \text{Cu} \rightleftharpoons \text{Con–Cu} \quad (6)$$

$$\text{Con–Cu} + \text{HA} \rightleftharpoons \text{Con–Cu–HA} \quad (5)$$

$$\text{Con} + \text{Cu} + \text{HA} \rightleftharpoons \text{Con–Cu–HA} \quad (7)$$

The over-all logarithmic stability constant for reaction 7 may be calculated by adding the logarithmic stability constant of reaction 6, 16.5, and the determined logarithmic stability constant of reaction 5.

Materials.—Tetracycline trihydrate, oxytetracycline dihydrate and 7-chloro-6-demethyltetracycline sesquihydrate were prepared from their respective hydrochlorides by Method A of Gans and Higuchi.[5] Melting point determinations indicated that the compounds were of a high degree of purity. 4-Epitetracycline monohydrate was prepared using the procedure of McCormick and associates.[6] Melting point determinations and ultraviolet absorption spectrum indicated that the compound was of a high degree of purity. The ultraviolet absorption spectrum in 0.01 *N* H_2SO_4 (aq) was similar to that reported by McCormick, *et al.*[6] Samples of anhydrochlorotetracycline and isochlorotetracycline monohydrate were obtained from Lederle Laboratories, Pearl River, New York. These compounds were supplied in relatively small amounts and, therefore, only melting point determinations were used to check their purity. The results indicated that the compounds were of a high degree of purity.

Metal-free conalbumin was used as obtained from Nutritional Biochemicals Corporation, Cleveland 28, Ohio. Visking No Jax Cellulose Dialysis Tubing (Visking Company, Chicago, Illinois), 2.38 cm., was utilized as the semipermeable membrane. It was found that this tubing allowed the equilibration of the tetracycline in less than 10 hr.

The buffer used in this investigation was prepared in the following manner. Approximately a 0.02 *M* solution of sodium biphosphate ($NaH_2PO_4 \cdot H_2O$) was prepared by dissolving 3.0 g. of the salt in 1 l. of deionized distilled water having a specific conductance of less than 2.5×10^{-6} ohm^{-1} cm.$^{-1}$. The resulting solution was adjusted to a pH of 7.4 by dropwise addition of 5.0 *N* sodium hydroxide solution.

Procedure.—A general outline of the procedure employed to determine the stability constant for the ternary complex conalbumin–copper–tetracycline is given. (a) The tetracycline (50.0 mg.) was dissolved in 500 ml. of pH 7.4 phosphate buffer. (b) Conalbumin (100 mg.) was dissolved in 100 ml. of pH 7.4 phosphate buffer to yield a 1.32×10^{-5} *M* solution or a 2.64×10^{-5} equiv. *M* solution. In the experiments which measure the binding in the presence of cupric ions, this conalbumin solution was made 2.64×10^{-5} *M* with respect to cupric ions. (c) Graded volumes of the tetracycline solution and sufficient buffer to make the total volume 20.0 ml. were equilibrated with 7.62 cm. strips of dialysis tubing in a constant temperature bath at 25° (±0.1°) for 16–20 hr. Aliquot portions of the solutions then were removed and analyzed for the tetracycline concentration using a Beckman Model B spectrophotometer. (d) Graded volumes of the tetracycline solution and sufficient buffer were added to wide-mouthed 30-ml. glass screw type bottles to make the total volume 10.0 ml. Conalbumin (10 ml.) or conalbumin–copper solution were added to a dialysis sack approximately 7.62 cm. in length. The tied sacks were placed into the bottles, the bottles were sealed and then agitated in a constant temperature bath at 25 (± 0.1°) for 16–20 hr. After equilibration, aliquot portions of the dialysate were removed and analyzed for the tetracycline concentration using a Beckman Model B spectrophotometer.

Treatment of Dialysis Data. a. Qualitative.—If no binding to conalbumin occurred, either in the absence or presence of cupric ions, the absorbance of the dialysate would be equal to the absorbance of the same concentration of the tetracycline when equilibrated with dialysis tubing alone. The expected absorbance, that is, the absorbance of the tetracycline when equilibrated with dialysis tubing, accounted for any binding of the drug to the sack, therefore a decrease from this value would indicate that binding was occurring to the conalbumin or conalbumin–copper, depending on which was present. The following example of anhydrochlorotetracycline illustrates the binding of tetracycline analogs to conalbumin.

Curve I, in Fig. 1, is a plot of the absorbance of various concentrations of anhydrochlorotetracycline equilibrated with dialysis tubing *vs.* the initial molar concentration in pH 7.4 buffer. Curves II and III, in Fig. 1, are plots of the absorbance of the dialysate *vs.* initial molar concentration of anhydrochlorotetracycline when equilibrated with conalbumin and conalbumin–copper, respectively. See Tables I and II for data.

For the most part Curve II coincides with Curve I, indicating that anhydrochlorotetracycline probably does not bind to conalbumin in the absence of copper. The downward displacement of Curve III indicates that binding occurred in the presence of cupric ions. From Fig. 1 it may be determined that approximately 1.9 moles of anhydrochlorotetracycline was bound per mole of conalbumin in the presence of cupric ions. This

(5) E. H. Gans and T. Higuchi, *J. Am. Pharm. Assoc.*, **46**, 458 (1957).

(6) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79**, 2849 (1957).

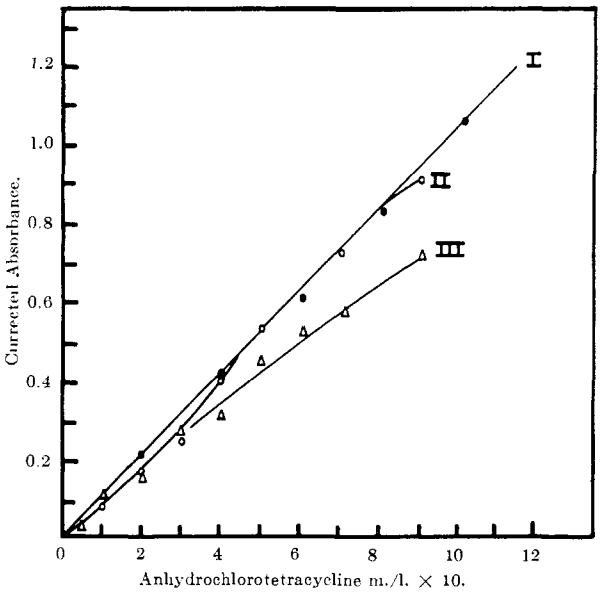

Fig. 1.—Dialysis of the binding of anhydrochlorotetracycline to conalbumin.

TABLE I

SPECTROPHOTOMETRIC DATA FOR ANHYDROCHLORTETRACYCLINE EQUILIBRATED WITH DIALYSIS TUBING

Anhydrochlorotetracycline Mg./20 ml.	Anhydrochlorotetracycline Init. *M* concn.	Absorbance, 440 mμ	Corrected absorbance $A_{440} - 0.020$
0.200	2.03×10^{-5}	0.230	0.210
0.400	4.07×10^{-5}	0.441	0.421
0.600	6.11×10^{-5}	0.629	0.609
0.800	8.14×10^{-5}	0.851	0.831
1.000	10.2×10^{-5}	1.08	1.06
Blanks			
0	0	0.023	
		Av.	0.020
0	0	0.017	

TABLE II

DIALYSIS DATA FOR ANHYDROCHLOROTETRACYCLINE AND CONALBUMIN IN THE ABSENCE AND PRESENCE OF CUPRIC IONS

	Anhydrochlorotetracycline Mg./20 ml.	Anhydrochlorotetracycline Init. *M* concn.	Absorbance 440 mμ	Corrected absorbance $A_{440} - 0.072$
Absence of cupric ions	0.100	1.02×10^{-5}	0.150	0.078
	.200	2.03×10^{-5}	.240	.168
	.300	3.05×10^{-5}	.311	.239
	.400	4.07×10^{-5}	.478	.406
	.500	5.09×10^{-5}	.603	.531
	.700	7.12×10^{-5}	.796	.724
	.900	9.16×10^{-5}	.980	.906
Presence of cupric ions	0.050	0.50×10^{-5}	0.095	0.023
	.100	1.02×10^{-5}	.175	.103
	.200	2.03×10^{-5}	.228	.156
	.300	3.05×10^{-5}	.356	.284
	.400	4.07×10^{-5}	.390	.318
	.500	5.09×10^{-5}	.525	.453
	.600	6.11×10^{-5}	.602	.530
	.700	7.12×10^{-5}	.653	.581
	.900	9.16×10^{-5}	.800	.728
Blanks	0	0	0.084	
				Av. 0.072
	0	0	0.060	

is the same as stating that approximately 1.0 mole was bound per *equivalent* mole of conalbumin. This value was determined at an initial concentration of anhydrochlorotetracycline of about 7.0×10^{-5} by dividing the molar concentration of the drug bound by the molar concentration of conalbumin, 1.32×10^{-5}. Since the *equivalent* molecular weight of conalbumin is one-half the true weight, the amount of drug bound per *equivalent* mole is one-half that bound per mole.

b. Calculation of Stability Constants.—The logarithmic stability constant, log K_2, for reaction 5 may be calculated in the manner previously described. Table III illustrates the calculation of this constant for the complex conalbumin-copper-anhydrochlorotetracycline.

For the conversion of absorbance to molar concentration it was necessary to determine the molar absorbancy index, a_M, of anhydrochlorotetracycline in pH 7.4 buffer. This value was found to be 1.04×10^4 l. mole^{-1} cm.$^{-1}$ at a wave length of 440 mμ by the usual quantitative procedures.

The over-all logarithmic stability constant for the formation of anhydrochlorotetracycline-copper-conalbumin is, therefore, approximately 21.0 at 25°. The term "approximately" is employed since the value of log K_1, 16.5, was determined at 30°.

$$\log K_3 = \log K_1 + \log K_2 = 16.5 + 4.5 = 21.0$$

TABLE III

THE BINDING OF ANHYDROCHLOROTETRACYCLINE TO CONALBUMIN IN THE PRESENCE OF CUPRIC IONS AND pH 7.4 PHOSPHATE BUFFER

Anhydrochlorotetracycline Init. *M* concn.	Free *m* concn.	Molar concentration of— Con-Cu-HA	Con-Cu	log K_2
2.03×10^{-5}	1.44×10^{-5}	1.06×10^{-5}	1.58×10^{-5}	4.67
4.07×10^{-5}	3.17×10^{-5}	1.35×10^{-5}	1.29×10^{-5}	4.52
5.09×10^{-5}	4.13×10^{-5}	1.38×10^{-5}	1.26×10^{-5}	4.42
6.11×10^{-5}	6.92×10^{-5}	1.83×10^{-5}	0.81×10^{-5}	4.51
			Av.	4.5

Results.—Samples of tetracycline trihydrate, oxytetracycline dihydrate, 7-chloro-6-demethyltetracycline sesquihydrate, anhydrochlorotetracycline, 4-epitetracycline monohydrate and isochlorotetracycline monohydrate were analyzed for binding with conalbumin in the absence and presence of cupric ions as outlined. The results have been summarized in Tables IV and V.

The only data available in the literature which resemble the above findings are those of the binding of tetracycline and oxytetracycline to albumin.[7] It was found that tetracycline bound to the extent of 0.06 and oxytetracycline to the extent of 0.2 mole per mole albumin in pH 7.4 phosphate buffer at 37°.

Discussion

The primary purpose of this research was to investigate the binding of various tetracycline analogs to conalbumin through the formation of ternary complexes and to correlate these findings with the antibacterial activities of the analogs.

It was found that the binding of therapeutically active tetracycline analogs,[1] tetracycline, oxytetracycline, 7-chloro-6-demethyltetracycline and anhydrochlorotetracycline, to the "model receptor," metal-free conalbumin, increased greatly in the presence of cupric ions, presumably through the formation of ternary complexes of the type shown in Fig. 2.

(7) J. M. Vandenbelt, Amer. Drug Manufact. Assoc., Research and Development Section, Atlantic City Meeting, Oct. 8, 1954.

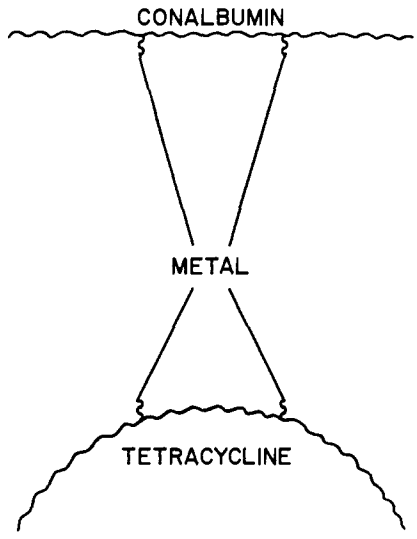

Fig. 2.

TABLE IV

APPROXIMATE VALUES FOR THE BINDING OF TETRACYCLINE ANALOGS TO CONALBUMIN IN THE ABSENCE AND PRESENCE OF CUPRIC IONS

Analog	A_{max} in pH 7.4 buffer, mμ	a_M	Moles analog bound per mole conalbumin: Without Cu^{++}	Moles analog bound per mole conalbumin: With Cu^{++}
Tetracycline trihydrate	364	1.39×10^4	0	1.8
Oxytetracycline dihydrate	364	1.43×10^4	0.4	1.7
7-Chloro-6-demethyltetracycline sesquihydrate	378	1.50×10^4	0	1.7
Anhydrochlorotetracycline	440	1.04×10^4	0	1.9
4-Epitetracycline monohydrate	365	1.40×10^4	0	0
Isochlorotetracycline monohydrate	345	7.00×10^3	0.5	0.5

since the maximum number of tetracycline molecules bound per molecule of conalbumin approached the number (2) of cupric ions bound per molecule. The values of the logarithmic stability constants of the ternary complexes ranged from 21.0 to 21.7. Chlorotetracycline could not be incorporated into this investigation because of the rapid decomposition of the free base.

No increase in the binding upon addition of cupric ions was evident with the inactive tetracycline analogs tested, 4-epitetracycline and isochlorotetracycline. Therefore, it may be assumed that ternary binding is not occurring.

TABLE V

APPROXIMATE STABILITY CONSTANTS FOR THE BINDING OF TETRACYCLINE ANALOGS TO CONALBUMIN IN THE PRESENCE OF CUPRIC IONS

Analog	log K_2	log K_s[a]
Tetracycline trihydrate	5.2	21.7
Oxytetracycline dihydrate	4.5	21.0
7-Chloro-6-demethyltetracycline sesquihydrate	4.8	21.3
Anhydrochlorotetracycline	4.5	21.0
4-Epitetracycline monohydrate	[b]	
Isochlorotetracycline monohydrate	[b]	

[a] Log K_1 was taken to be equal to 16.5. [b] No increase in binding was noted for these compounds in the presence of cupric ions; therefore, it was assumed that ternary complexation does not occur.

The inability of 4-epitetracycline to form a ternary complex may be explained by the alteration of the suggested binding site, *i.e.*, the epimerization of the C.4 dimethylamino group. The inability of isochlorotetracycline to form a ternary complex cannot be explained on this basis or from the data gathered thus far. However, these findings are consistent with previous results which have shown that isochlorotetracycline and the 4-epitetracyclines form only 1:1 chelates with cupric ions while the therapeutically active analogs form 2:1 chelates with cupric ions.

This evidence supports our previous observation[1] that a relationship exists between the antibacterial activity of the tetracyclines and their metal bonding properties. Furthermore, the results strongly suggest that ternary binding, a mechanism of action first suggested by Hunter and Lowry,[8] is involved in attachment of the drug to its site of action. However, this metal bridge is only one point of bonding between the tetracyclines and receptor site; undoubtedly others are also required in order for tetracyclines to exhibit their action.

By this investigation we do not wish to imply that conalbumin or, for that matter, cupric ions play an actual role in the antibacterial activity of the tetracyclines. Conalbumin was used because it serves as a readily available metal enzyme model. Cupric ion was employed in this study because its effect on conalbumin[3,4] and certain tetracyclines[1,9,10] already had been determined.

(8) F. E. Hunter and O. H. Lowry, *Pharmacol. Revs.*, **8**, 89 (1956).
(9) A. Albert and C. W. Rees, *Nature*, **177**, 433 (1956).
(10) A. Albert, *Nature*, **172**, 201 (1953).