Biophysical Study of the Mode of Action of the Tetracycline Antibiotics

Inhibition of Metalloflavoenzyme NADH Cytochrome Oxidoreductase

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A considerable amount of evidence indicates that the tetracyclines may produce their antibiotic action by inhibition of metalloflavoenzymes. In this investigation the inhibitory properties of a series of biologically active and inactive tetracycline analogs on the metalloflavoenzyme, NADH–cytochrome *c* oxidoreductase, were investigated. Those analogs capable of inhibiting the enzyme appeared to act, at least partially, by chelation of enzyme-bound metal. The site of chelation on the tetracycline molecule appeared to be the C-11 to C-12 chromophore, or the C-1, C-2, C-3 region involving the 2-carboxamide group of the tetracycline molecule. Both modification of the 2-carboxamide substituent and epimerization of the 4-dimethylamino group resulted in loss of inhibitory effect. This parallels known structure-antibiotic activity relationships for these compounds. Inactive isotetracycline, tetracycline methiodide, and dedimethylaminotetracycline were able to inhibit the enzyme at least as well as the parent tetracyclines. These compounds might lack antibiotic activity due to inability to enter bacterial cells.

In 1950, Loomis (1) observed that 7-chlorotetracycline[1] uncoupled mammalian aerobic phosphorylation, thus inhibiting the formation of ATP,[2] which is the primary source of energy for cellular functions. This observation suggested that tetracyclines might act by inhibiting the action of important components of the electron transport system and the process of oxidative phosphorylation. It was also found by several workers (2, 3) that divalent metal ions could reverse the tetracycline-induced inhibition of oxidative phosphorylation in mammalian mitochondria, and as a result of these findings, it was postulated (4) that the tetracyclines uncouple oxidative phosphorylation by interacting with metal bound to an enzyme without actually removing it.

In 1952, Miura *et al.* (5) showed that tetracyclines inhibit oxidative phosphorylation in intact bacteria at concentrations of approximately 10^{-6} *M*. In conjunction with this finding, it is of interest that metal ions can neutralize the antibiotic effects of tetracyclines in bacteria cultures (6, 7) and that this effect does not appear to be simply a result of removal of the drug by complex formation (4). Many of these findings correlated with Albert's view that metal chelation might well play a part in tetracycline action (8).

In 1953, Saz and Marmur (9) reported that the reduction of aromatic nitro groups by cell-free extracts of *E. coli* was inhibited by 7-chlorotetracycline. Saz and Slie (10) showed that this reduction of aromatic nitro compounds by a partially resolved bacterial nitro-reductase was inhibited by the tetracyclines, but that addition of manganese ion significantly reversed the inhibition. They suggested (11) that the antibiotic activity of the tetracyclines might be due to their ability to derange cellular metabolism by combining with sensitive cations of bacterial cells.

Saz and Martinez (12) isolated nitro-reductase from both 7-chlorotetracycline sensitive and 7-chlorotetracycline resistant *E. coli*. They showed that both the sensitive and resistant nitro-reductases are metalloflavoproteins, the conjugated flavin and metal of the enzyme derived from 7-chlorotetracycline sensitive bacteria being more readily dissociated from the protein. Saz and Martinez (12) also showed that the enzyme extracted from the 7-chlorotetracycline sensitive *E. coli* was inhibited by 7-chlorotetracycline, whereas that from the resistant *E. coli* was resistant to 7-chlorotetracycline inhibition. Saz and Martinez (13) later showed that nitro-reductase from both sensitive and resistant organisms each contain significant amounts of the total conjugated flavins found in the bacterial cells. They therefore concluded that the flavin associated with the enzyme represented a significant portion of the flavin involved in the electron transport

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[1] See Fig. 1 for numbering system of the tetracyclines.

[2] Abbreviations used in this paper are as follows: ATP, adenosine triphosphate; NAD^+, nicotinic acid adenine dinucleotide (oxidized form); NADH, nicotinic acid adenine dinucleotide (reduced form); K_m, the Michaelis constant; V, the maximum value of the observed velocity of an enzymatically catalyzed reaction, corresponding to saturation of the enzyme with the substrate.

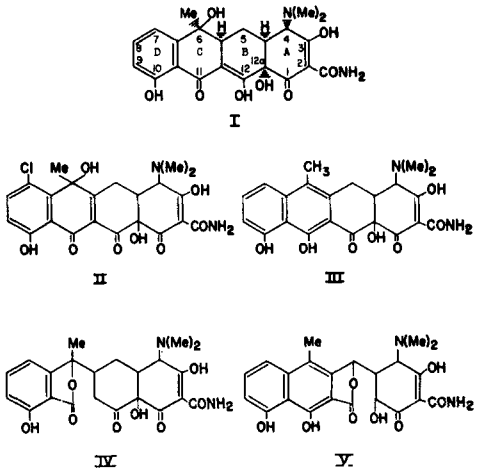

Fig. 1.—The structure of various tetracyclines. Key: tetracycline (I); 7-chloro-5a(11a)-dehydrotetracycline (II); 5a(6)-anhydrotetracycline (III); isotetracycline (IV); and apo-5-hydroxytetracycline (V).

mechanism of the intact cell. Inhibition of electron transport systems of bacterial cells could, of course, account for the findings of Miura *et al.* (5) that tetracyclines uncouple oxidative phosphorylation in bacteria.

Based on these findings, it appeared that a redox metalloflavoenzyme might provide a suitable model receptor site for study of the mode of action of the tetracycline antibiotics. The metalloflavoenzyme, NADH–cytochrome *c* oxidoreductase was selected to serve as the model enzyme for this study because it is available in relatively pure form and because a significant amount of knowledge concerning the mechanism of inhibition of this enzyme by chelating agents was available (14). The objective of this investigation was to study the inhibitory properties of biologically active and inactive tetracycline analogs on the NADH–cytochrome *c* oxidoreductase enzyme system in order to observe the effects of structural modifications of the tetracyclines on their inhibition of the model enzyme system, thus providing relationships between inhibitory properties, structure, and biological activity.

EXPERIMENTAL

Materials.—Samples of various tetracycline analogs and model compounds were supplied by the manufacturers.[3] Melting point determinations and ultraviolet and infrared spectra indicated that the compounds were of a high degree of purity. The structural formula for tetracycline, the parent compound of the tetracycline series, is shown as I in Fig. 1. Using the ring numbering system illustrated, tetracycline serves as a basis for the nomenclature of most of the other tetracycline analogs prepared in this study. Most of these derivatives have a tetracyclic nucleus identical with tetracycline and differ only in ring substituents. Several of the analogs employed have an altered nucleus, and these are indicated in Fig. 1. The natural stereochemistry of the tetracyclines is also depicted in I (15).

The enzyme used in this study was NADH–cytochrome *c* oxidoreductase[4] prepared from hog heart. Cytochrome *c*,[5] oxidized form, prepared from horse heart, served as the substrate of the enzymatically catalyzed reaction. In order to specify the cytochrome *c* substrate concentration in all experiments and to express the rate of the enzymatically catalyzed reaction in terms of molar concentration of cytochrome *c* reduced, it was necessary to assay each preparation of cytochrome *c* employed. The procedure used for the analyses of cytochrome *c* was essentially that described by Paul (16). The cofactor used for the enzyme studies was nicotinamide–adenine dinucleotide, reduced form, disodium salt (Na_2NADH).[6]

The water used in these experiments was prepared by passing distilled water through suitably charged Dowex 50W-X8 and Dowex 1-X4 resins. The ion content of the water prepared in this manner was determined using a Barnstead purity meter.[7] The ion content of the water corresponded to less than 0.1 p.p.m. as NaCl, which represents a specific conductance of less than 2.164×10^{-7} mho cm.$^{-1}$.

Analytical Measurements and Controls.—All spectral measurements were made using the Bausch & Lomb Spectronic 505 recording spectrophotometer, employing 1-cm. silica cells and reagent blanks, unless otherwise specified. Measurements made below 360 mμ were performed using the hydrogen lamp; for those above 360 mμ, a tungsten lamp was employed.

Temperature control of solutions during spectral measurements was maintained by means of a Bausch & Lomb constant-temperature cell holder by circulating water through it from a constant-temperature bath (Precision Scientific Co.) equipped with a Sargent mercurial thermoregulator. Temperature regulation by this system was found to be within $\pm 0.2°$. Control of the temperature of solutions other than during spectral measurements was maintained using a Bronwill constant-temperature circulator. Temperature control in this system was found to be within $\pm 0.1°$.

Measurements of pH were performed using a Beckman expanded scale pH meter equipped with a single combination glass–calomel electrode to facilitate measurements of the pH of solutions of volumes as small as 2 ml. Beckman standard buffers were used in the standardization of the pH meter. The standard buffer was always within 2 pH units of the pH measurement to be made.

[3] The authors are especially grateful to Dr. James H. Boothe, Lederle Laboratories, Pearl River, N. Y., and Dr. Charles R. Stephens, Charles Pfizer Laboratories, Groton, Conn., for their assistance in providing many of the tetracycline analogs used in this study. N^2-Pyrrolidinomethyltetracycline was the gift of E. R. Squibb and Sons, Inc.

[4] NADH–cytochrome *c* oxidoreductase was obtained from Mann Research Laboratories, New York, N. Y., and from Sigma Chemical Co., St. Louis, Mo.

[5] Cytochrome *c* was obtained from Sigma Chemical Co., St. Louis, Mo., from Nutritional Biochemicals Corp., Cleveland, Ohio, and from Dr. E. Margoliash, Abbott Laboratories, North Chicago, Ill.

[6] Na_2NADH, 98–100% pure, was obtained from Sigma Chemical Co., St. Louis, Mo.

[7] Barnstead Still and Sterilizer Co., Boston, Mass.

Reagent Solutions for Enzyme Assays.—The stock buffer solution used in the enzyme assay system was a 0.4 *M* solution of 2-amino-2-methyl-1,3-propanediol[8] (diol), pH 8.5. The buffer was prepared fresh weekly and kept refrigerated.

The cofactor stock solution was unbuffered 0.004 *M* Na_2NADH. This solution was kept refrigerated and used within 4 hr. from the time of its preparation.

Cytochrome *c* stock solution was prepared by dissolving sufficient oxidized cytochrome *c* preparation in sufficient water to provide a solution which was 0.667% in oxidized cytochrome *c*. This solution was used within 4 hr. from the time of its preparation.

The enzyme solution was prepared by dissolving a quantity of NADH–cytochrome *c* oxidoreductase in sufficient water to give a concentration of 0.600 mg./ml. of solution. This solution was then subdivided into several portions of approximately 1 ml. each. Each portion was immediately frozen in a dry ice chest until used. Such solutions were used within 4 hr.

Since the addition of unbuffered tetracycline analogs caused a marked change in the pH of the enzyme assay system to which they were added, it was necessary to buffer all of these inhibitor solutions to the same pH as the test system. This was especially true in light of the fact that the several different compounds tested for inhibitory effects differed considerably in acidity. The activity of NADH–cytochrome *c* oxidoreductase is maximal at pH 8.5, and even minor changes in pH on either side of the maximum result in a marked decrease in enzymatic activity (17). Hence, the inhibitor solutions were all buffered to pH 8.5 to produce uniform pH in all systems and to insure the validity of comparisons of inhibitory effects with various tetracycline analogs. The buffering effect was accomplished by incorporation of sufficient 2-amino-2-methyl-1,3-propanediol into the inhibitor solution in order to bring the pH to 8.5. The inhibitors were dissolved in sufficient 0.04 *M* diol and water to give solutions which were 0.006 *M* in inhibitor and of pH 8.5. The solutions were stored in an ice bath and were used within 30 min. from the time of their preparations to minimize the changes in the inhibitory potential of these compounds upon standing in solution.

Standard Assay Conditions for Measurement of Enzymatic Activity.—The procedure used for the measurement of enzymatic activity was that outlined in Colowick and Kaplan (18). It is based on the method developed by Edelhoch *et al.* (19) and modified by Mahler *et al.* (20).

In the standard assay system for measuring rates of reactions, the experimental cell contained the following reagent solutions: 0.1 ml. diol buffer, 0.4 *M*, pH 8.5; 0.1 ml. Na_2NADH, 0.004 *M*; 0.1 ml. cytochrome *c*, 0.667%; 0.1 ml. enzyme solution, 0.6 mg./ml.; and sufficient water to make a total volume of 2.00 ml. This provided a test solution which was 2×10^{-2} *M* in diol, 2×10^{-4} *M* in Na_2NADH, 2.69×10^{-5} *M* in cytochrome *c*, and 1×10^{-7} *M* in enzyme based on a reported purity of 35% and a molecular weight of 78,000 (17). The reference cell contained the same quantities of the same reagents as the experimental cell, except that it contained no enzyme (enzyme solution replaced with water).

[8] Nutritional Biochemicals Corp., Cleveland, Ohio.

The required volumes of water, diol buffer solution, NADH solution, cytochrome *c* solution, and inhibitor solution (if any), were added to the experimental cell and placed in the constant-temperature cell holder for approximately 3 min. This allowed more than sufficient time for the reaction mixture to come to an equilibrium temperature of 22°. During this time a portion of enzyme solution, freshly thawed from the frozen state, was brought to 22° by immersion of its container into a constant-temperature circulating bath. The required volume of enzyme solution was then added to the experimental cell. When delivery of enzyme solution from the pipet to the experimental cell was complete, a timer was immediately started (zero time), the reaction solution was mixed, the cell compartment was closed, and recording of the absorbance value was begun at 15 sec. The absorbance was automatically recorded by the spectrophotometer with the wavelength-drive gears disengaged, and the wavelength constant at 550 mμ. The increase in absorbance from 15 to 75 sec. (ΔA_{550} min.$^{-1}$) constituted the initial rate of the enzyme reaction (18). The initial rates in terms of absorbance (ΔA_{550} min.$^{-1}$) were converted to concentration units of cytochrome *c* reduced per minute (mole L.$^{-1}$ min.$^{-1}$).

Measurements of the pH of solutions before and after the enzymatic reaction showed no discernible pH change under the conditions used.

Procedure for Spectral Determination of Complexation of Tetracyclines with Ferrous Ion.—Ligand (tetracycline analog or model compound) solutions were all prepared to a concentration of 5×10^{-4} *M*. Certain of the less soluble compounds required small amounts of alcohol to effect their solution. Ferrous ammonium sulfate solution was prepared to provide a 5×10^{-4} *M* solution in ferrous ion. Buffers of various pH values were prepared using KCl–HCl, sodium acetate–acetic acid, and boric acid–sodium hydroxide buffer systems. All buffers were prepared at an ionic strength of 0.150 by addition of potassium chloride.

In determining the nature of the complexation (chelation) which occurs between the tetracyclines and ferrous ion, Job's method of continuous variation (21–23) was used. To prepare a Job plot, one obtains a value, $\bar{D}$, which is the difference between the observed absorbance of the chelate solution, and the absorbance which would have been obtained if no chelation had taken place. $\bar{D}$ is then plotted against a quantity, *R*, where

$$R = \frac{(\text{metal})}{(\text{metal}) + (\text{ligand})}$$

To prepare chelate solutions of desired *R* values, specified quantities of metal solution and ligand solution were added to buffer solutions of desired pH. The total metal plus ligand concentration was 5×10^{-5} *M*. Ligand blank solutions were also prepared containing the same reagents as the chelate solution, except that the metal was omitted.

It was found that the addition of ligand and metal in quantities used did not affect the pH of the solutions. The large excess of buffer in both chelate solutions and blanks kept all solutions at the same pH. All measurements for a given experiment were made at approximately the same time after the initial admixture of metal and ligand.

TABLE I.—COMPARISON OF VALUES FOR K_m AND V[a]

	Lineweaver-Burk	Eadie	Lit.
Km (mole L.$^{-1}$)	1.5×10^{-4}	1.4×10^{-4}	1.2×10^{-4}
V (mole L.$^{-1}$ min.$^{-1}$)	4.6×10^{-5}	4.3×10^{-5}	3.5×10^{-5}

[a] Standard assay for NADH–cytochrome c oxidoreductase activity. Enzyme, 1×10^{-7} M; NADH, 2×10^{-4} M; diol, pH 8.5, 2×10^{-2} M; temperature, 22°C.

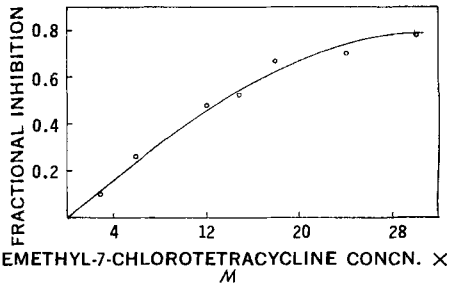

Fig. 2.—Fractional inhibition as a function of 6-demethyl-7-chlorotetracycline HCl concentration. Standard assay for NADH–cytochrome c oxidoreductase activity: enzyme, 1×10^{-7} M; NADH, 2×10^{-4} M; cytochrome c, 2.69×10^{-5} M; diol, pH 8.5, 2×10^{-2} M; temperature, 22° C.

RESULTS

Determination of K_m and V Under the Experimental Conditions Employed.—The effect of substrate (oxidized cytochrome c) concentration on reaction velocity was studied using standard assay conditions, but varying the concentration of substrate employed. K_m and V were determined according to the methods of Lineweaver-Burk (24) and of Eadie (25). The Lineweaver-Burk plot and Eadie plot were prepared using the method of least squares. The values for K_m and V obtained by both methods, together with literature values (17), appear in Table I.

Inhibitory Properties of the Tetracycline Analogs.—The inhibitory properties of the tetracycline analogs were determined using the standard assay procedure. Various quantities of buffered inhibitor solution (which had been prepared within the previous 30 min.) were added to the reaction mixture. For each inhibitor solution used, the fractional inhibition was determined using the expression

$$\text{fractional inhibition} = 1 - (v_i/v)$$

where v_i is the velocity (ΔA_{550} min.$^{-1}$) of the inhibited reaction, and v is the velocity (ΔA_{550} min.$^{-1}$) of the uninhibited reaction.

For each analog tested, the concentration of inhibitor was plotted against fractional inhibition. From these plots, the concentration required to produce a fractional inhibition of 0.5 (50% inhibition) was determined. An example of such a plot is shown in Fig. 2 for the case of 6-demethyl-7-chlorotetracycline HCl. Table II lists the molar concentration of each analog tested which was required to produce a fractional inhibition of 0.5.

Examination of Table II reveals that four of the tetracycline analogs tested produced no significant inhibition under the experimental conditions described here. It was observed, however, that if the buffered solutions of the 4-epi- compounds were tested for inhibitory properties near or beyond the 30-min. age limit imposed on the use of the other analog solutions, inhibition began to be observed, and the fractional inhibition produced was found to increase with the age of the 4-epi- solution being tested.

To study this phenomenon more carefully, buffered analog solution was prepared, and the time of dissolution of the analog was carefully recorded. Samples were withdrawn at definite time intervals from the time of dissolution and introduced into the standard assay system to provide a reaction mixture which was 1.5×10^{-3} M with respect to tetracycline analog. Fractional inhibition was then determined in the usual manner. The fractional inhibition values obtained with different ages of buffered 4-epi-7-chlorotetracycline solutions were found to increase as a function of the age of the buffered inhibitor solution. Since it appeared that this

TABLE II.—CONCENTRATION OF TETRACYCLINE ANALOGS REQUIRED TO PRODUCE A FRACTIONAL INHIBITION OF 0.5[a]

Tetracycline Analog[b]	Concn. × 10⁴, mole L.$^{-1}$
5a (6)-Anhydrotetracycline	17.1
α-Apo-5-hydroxytetracycline	1.18
7-Chloro-5a (11a)-dehydrotetracycline	10.5
7-Chloro-6-demethyltetracycline HCl	13.4
7-Chlorotetracycline HCl	16.8
2-Cyano-2-decarboxamidotetracycline	No inhibition
4-Dedimethylaminotetracycline	9.1
6-Demethyl-6-deoxy-9-aminotetracycline HCl	0.80
6-Demethyl-6-deoxy-9-nitrotetracycline	0.85
6-Demethyl-6-deoxytetracycline	4.35
12a-Deoxytetracycline	0.32
4-epi-7-Chlorotetracycline	No inhibition
4-epi-Tetracycline ammonium salt	No inhibition
Ethylenediaminetetraacetic acid[c]	22.0
8-Hydroxyquinoline-5-sulfonic acid[c]	23.7
2-Hydroxy-4-methyl-6-keto-cyclohexenylcarboxamide[d]	32.0
5-Hydroxytetracycline	17.9
Isotetracycline HCl	17.1
N^2-Pyrrolidinomethyltetracycline	No inhibition
Tetracycline HCl	18.0
Tetracycline methiodide	20.2

[a] Standard assay for NADH–cytochrome c oxidoreductase activity. Enzyme, 1×10^{-7} M; NADH, 2×10^{-4} M; cytochrome c, 2.69×10^{-5} M; diol, pH 8.5, 2×10^{-2} M; temperature, 22°C. [b] See Fig. 1 for clarification of tetracycline nomenclature. [c] These compounds are not tetracycline analogs but are known chelating agents which were used as inhibitors of the enzyme system by Mahler and Elowe (14). [d] This compound is a model of a portion of the tetracycline molecule. Its chemical structure is shown in Fig. 3.

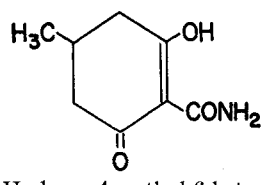

Fig. 3.—2-Hydroxy-4-methyl-6-keto-cyclohexenylcarboxamide.

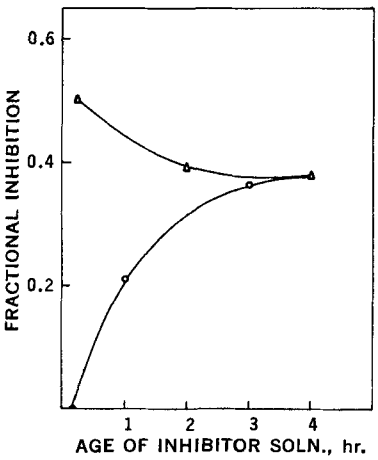

Fig. 4.—Fractional inhibition as a function of the age of buffered 7-chlorotetracycline HCl and buffered 4-epi-7-chlorotetracycline solutions. Standard assay for NADH–cytochrome *c* oxidoreductase activity: enzyme, 1×10^{-7} *M*; NADH, 2×10^{-4} *M*; cytochrome *c*, 2.69×10^{-5} *M*; diol, pH 8.5, 2×10^{-2} *M*; temperature, 22°C. Key: O, 4-epi-7-chlorotetracycline; △, 7-chlorotetracycline HCl.

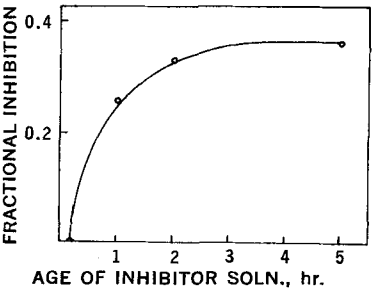

Fig. 5.—Fractional inhibition as a function of the age of buffered N^2-pyrrolidinomethyltetracycline solution. Standard assay for NADH–cytochrome *c* oxidoreductase activity: enzyme, 1×10^{-7} *M*; NADH, 2×10^{-4} *M*; cytochrome *c*, 2.69×10^{-5} *M*; diol, pH 8.5, 2×10^{-2} *M*; temperature, 22°C.

phenomenon, in the case of the 4-epi- compounds, might most probably be due to an epimerization of the 4-epi-compound to the biologically active epimer (26), it was decided to perform the same experiment with the so-called active epimer. If the observed increase in fractional inhibition as a function of age of buffered 4-epi-analog is due to an epimerization of this compound to the active epimer, then one would expect that a decrease in fractional inhibition would be observed with the active epimer. A plot of fractional inhibition as a function of the age of buffered inhibitor solution for both 4-epi-7-chlorotetracycline and 7-chlorotetracycline HCl appears in Fig. 4. The pattern of these two curves is reminiscent of the familiar pattern exhibited during the mutarotation of the α and β epimers of glucose (27).

It was realized that if the effect exhibited in Fig. 4 was, in fact, due to epimerization of the 4-dimethylamino group, then 4-dedimethylaminotetracycline should theoretically show no change in fractional inhibition as a function of the age of the inhibitor solution because there is no dimethylamino group in the four position which can epimerize. A study of fractional inhibition as a function of the age of buffered 4-dedimethylaminotetracycline solution was therefore undertaken. It was found that no significant change in the inhibitory potential of the buffered 4-dedimethylaminotetracycline solution occurred even over a 4-hr. period.

Boothe (28) has indicated that N^2-pyrrolidinomethyltetracycline is probably an active antibiotic by virtue of the fact that it readily hydrolyzes back to the parent antibiotic. This suggested that it would be of interest to see if the buffered solution of this compound might develop inhibitory properties upon standing (as did the 4-epi- compound). The experiment was performed in the same manner as was done with the 4-epi-7-chlorotetracycline. Figure 5 indicates that, as with the 4-epi- compound, the N^2-pyrrolidinomethyl analog can achieve ap-

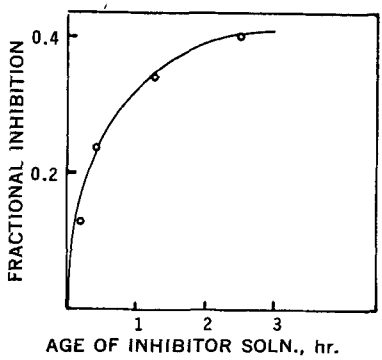

Fig. 6.—Fractional inhibition as a function of the age of buffered 2-cyano-2-decarboxamidotetracycline solution. Standard assay for NADH–cytochrome *c* oxidoreductase activity: enzyme, 1×10^{-7} *M*; NADH, 2×10^{-4} *M*; cytochrome *c*, 2.69×10^{-5} *M*; diol, pH 8.5, 2×10^{-2} *M*; temperature, 22°C.

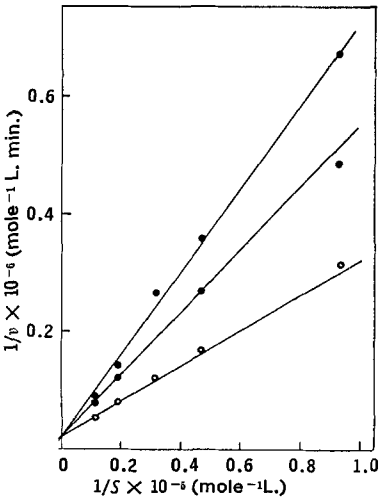

Fig. 7.—Competitive inhibition by 5-hydroxytetracycline HCl. Standard assay for NADH–cytochrome *c* oxidoreductase activity: enzyme, 1×10^{-7} *M*; NADH, 2×10^{-4} *M*; diol, pH 8.5, 2×10^{-2} *M*; temperature, 22° C. Key: O, no inhibitor; ◒, 1.2×10^{-3} *M* inhibitor; ●, 2.1×10^{-3} *M* inhibitor.

TABLE III.—SUMMARY OF K_i DETERMINATIONS[a]

Tetracycline Analog	K_i (mole L.$^{-1}$)
7-Chloro-6-demethyltetracycline HCl	1.0×10^{-3}
7-Chlorotetracycline HCl	1.6×10^{-3}
4-Dedimethylaminotetracycline	1.1×10^{-4}
6-Demethyl-6-deoxy-9-nitrotetracycline	1.6×10^{-4}
6-Demethyl-6-deoxytetracycline	7.3×10^{-4}
2-Hydroxy-4-methyl-6-ketocyclohexenylcarboxamide	1.6×10^{-3}
5-Hydroxytetracycline	2.0×10^{-3}
Isotetracycline HCl	2.6×10^{-3}

[a] Standard assay conditions for NADH–cytochrome *c* oxidoreductase activity: enzyme, 1×10^{-7} *M*; NADH, 2×10^{-4} *M*; diol, pH 8.5, 2×10^{-2} *M*; temperature, 22°C.

proximately the same inhibitory capacity as an equimolar solution of the parent compound.

Buffered 2-cyano-2-decarboxamidotetracycline solution was tested in the same manner as the N^2-pyrrolidinomethyl analog, and Fig. 6 indicates that it too can develop inhibitory capacity after standing in buffered solution.

Nature of the Tetracycline Inhibition.—The inhibitory effect of the tetracyclines on the NADH–cytochrome *c* oxidoreductase system was quantitatively characterized using the standard graphic kinetic procedures (29, 30). The standard assay procedures were used, and all Lineweaver-Burk plots were prepared employing the method of least squares. Velocity was determined at various substrate concentrations under three different conditions. These conditions were, first, no inhibitor present, then two different inhibitor concentrations present. Plots of reciprocal velocity *versus* reciprocal substrate concentration were prepared on the same graph, as illustrated in Fig. 7, for the case of 5-hydroxytetracycline HCl.

The fact that the three lines in Fig. 7 all intersect on the $1/v$ axis, indicates competitive inhibition (29, 30). This was found to be the case for all analogs tested, within the range of substrate concentrations in Fig. 7. At lower substrate concentrations deviations were observed. Mahler and Elowe (14) reported competitive inhibition when chelating agents were introduced into the NADH–cytochrome *c* oxidoreductase system. Table III provides a summary of each compound studied in this manner, together with the inhibitor constant (K_i) obtained for each.

Selection of the Optimum pH for Detecting Chelation by Spectral Measurements.—Using 5-hydroxytetracycline as the ligand, complexation with ferrous ion was studied at various pH values. It was found that no complexation with ferrous ion could be detected at pH 1.5. At pH 3.35, there were some slight spectral changes, but these were hardly enough evidence to permit precision of measurements. Complexation was detectable at pH 5.5, 7.4, and 8.4. At these pH values, complexation was observed at various wavelengths in both the ultraviolet and visible regions. The maximum absorbance changes were at pH 5.5.

The effect of chelation with ferrous ion at pH 5.5 on the spectrum of 5-hydroxytetracycline was as follows: between 230 and 270 mμ, chelation caused an increase in absorbance; between 270 and 380 mμ, chelation caused a decrease in absorbance; between 380 and 500 mμ, chelation caused an increase in absorbance. It is interesting to note that similar changes in absorbance were induced when ionization of 5-hydroxytetracycline beyond pH 5.5 occurred. When the pH of 5-hydroxytetracycline solution was raised from pH 5.5 to pH 7.4, absorbance was affected in the following manner: between 230 and 270 mμ, an increase in absorbance occurred; between 270 and 360 mμ, a decrease in absorbance occurred; between 360 and 500 mμ, an increase in absorbance occurred. Conover (31) has also noted these similarities between chelation and ionization effects on the spectral characteristics of the tetracyclines.

These observations probably explain why pH 5.5 has proved to be the most advantageous pH for detection of tetracycline chelation by the spectral means employed in this study. Below pH 5.5 (specifically at pH 3.35 and pH 1.5), the spectral shifts that occurred in the chelate solutions were too small for detection. This is probably due to the fact that at low pH the reaction between the most protonated form of tetracycline (AH_3^+) and metal (M^{2+})

$$AH_3^+ + M^{2+} \rightleftharpoons AH_2M^{2+} + H^+$$

is suppressed (in the forward direction) at high hydronium ion concentration.

Above pH 5.5 (specifically at pH's 7.4 and 8.4), spectral changes were observed, and there was evidence of chelation. However, the changes in the spectral properties of the ligand upon chelation were not so pronounced as those observed when chelation occurred at pH 5.5. This is probably due to the fact that the spectral changes induced by chelation are the same or very similar to those induced by ionization (increasing the pH). Hence, at those pH values at which the spectral patterns had already been more substantially shifted by the dissociation of proton, the spectral changes observed upon chelation were not so pronounced as at pH 5.5. Chelation does occur at the more alkaline pH values, but the spectral changes are not so pronounced at these pH values.

Spectral studies performed in this portion of the work indicated that 1:1 complexation was occurring with 5-hydroxytetracycline and 7-chlorotetracycline

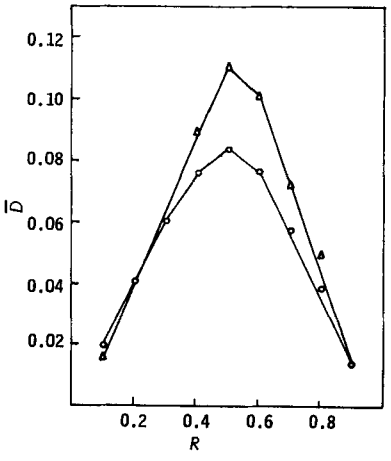

Fig. 8.—Job plot for complexation of 5-hydroxytetracycline HCl with ferrous ion at 400 and 235 mμ, pH 5.5, ionic strength 0.150, 25°C. Key: ○, 400 mμ; △, 235 mμ.

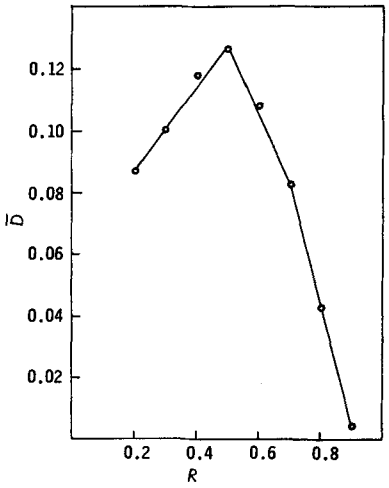

Fig. 9.—Job plot for complexation of 4-dedimethylaminotetracycline with ferrous ion at 420 mμ, pH 5.5, ionic strength 0.150, 25°C.

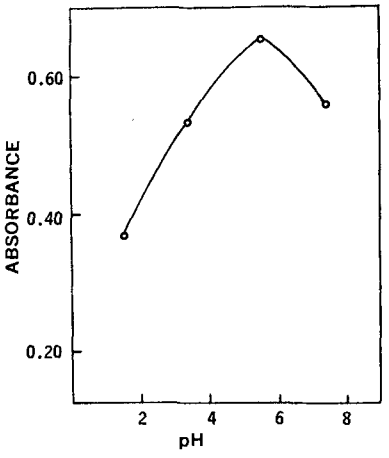

Fig. 10.—Absorbance of isotetracycline HCl at 289 mμ as a function of pH.

at all pH values at which complexation was detectable. This was observed in both the ultraviolet and visible regions of the spectrum.

Nature of Chelation for Some Tetracycline Analogs and Model Compounds.—The complexation of 5-hydroxytetracycline HCl with ferrous ion was studied at pH 5.5. Figure 8 shows the Job plot for complexation at both 235 and 400 mμ. Formation of a 1:1 complex is indicated in each case. The chelate solutions were markedly colored.

The complexation of 4-dedimethylaminotetracycline was studied in the same manner as 5-hydroxytetracycline HCl, and rather similar results were obtained. Figure 9 shows the Job plot for complexation in the visible region. Formation of a 1:1 complex is indicated.

In the iso analogs, the chromophoric β-diketone system is disrupted, depriving the analog of the characteristic tetracycline color. This chromophoric portion of the molecule (positions 9 through 11) is the sole contributor to spectral absorbance in the wavelength region beyond 340 mμ (26). Both the 9 through 11 chromophore *and* the ring A (positions 1 through 3) system contribute to spectral absorbance in the region 250 to 300 mμ (26). In accordance with this fact, it was found that at pH 5.5, isotetracycline did not give any absorbance beyond 320 mμ. When tested for evidence of chelation, it was found that a small but reproducible decrease in absorbance at 289 mμ did occur in the chelate solutions. This could indicate that there may be another binding site for metals on the tetracycline molecule other than on the position 11 to 12 chromophore. Other workers have shown that chelation does occur at the positions 11 to 12 diketone system, but possible chelation at additional sites on the tetracycline molecule has not been precluded (31, 32).

A study of the absorbance of isotetracycline HCl at 289 mμ, as a function of pH, revealed that there was an absorbance maximum in the vicinity of pH 5.5. Hence chelation, which has been shown to mimic ionization as far as its effects on the spectral properties of the tetracyclines are concerned, would be expected to lead to a decrease in absorbance at this pH. The effect of pH on the 289 mμ absorbance of 4.5×10^{-5} *M* isotetracycline HCl is shown in Fig. 10. The effect of ferrous ion complexation on the 289 mμ absorbance of isotetracycline HCl at pH 5.5 is shown in Fig. 11, which suggests a 1:1 complexation.

To study further the possibility of chelation at a site other than the 9 through 11 chromophore of the tetracycline molecule, the compound 2-hydroxy-4-methyl-6-ketocyclohexenylcarboxamide (Fig. 3) was employed. As its structure indicates, this compound can serve as a model for the ring A (positions 1 through 3) chromophore in the tetracyclines. If the chelation effect observed in isotetracycline is due to the ring A system, then some evidence of chelation should be observed with this model compound. It was found that when the compound was tested for complexation in the same manner as for isotetracycline, there *was* evidence of complexation at 257 mμ. As with isotetracycline, the chelation effect resulted in a decrease in absorbance. The decrease, as in the case of isotetracycline, was small, but reproducible. Investigation of the effect of pH on the absorbance

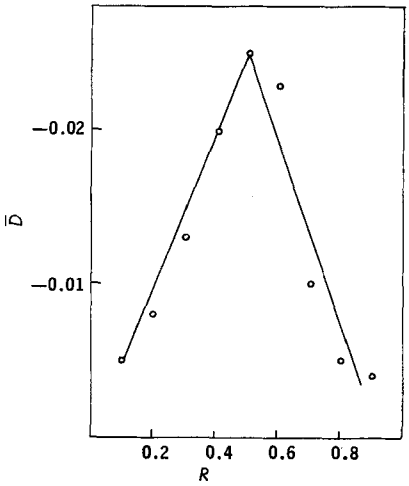

Fig. 11.—Job plot for complexation of isotetracycline HCl with ferrous ion at 289 mμ, pH 5.5, ionic strength 0.150, 25°C.

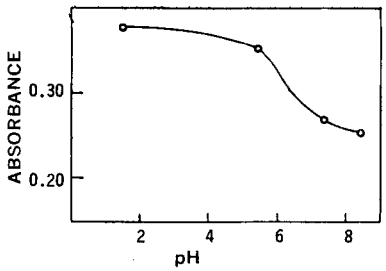

Fig. 12.—Absorbance of 2-hydroxy-4-methyl-6-keto-cyclohexenylcarboxamide at 257 mμ as a function of pH.

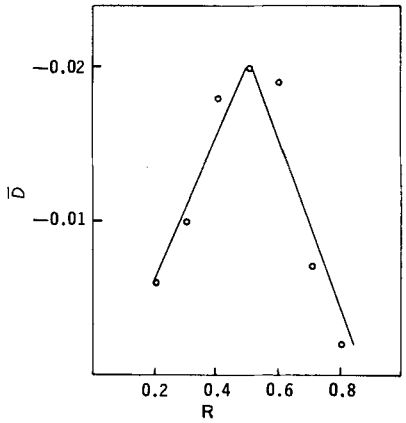

Fig. 13.—Job plot for complexation of 4-methyl-6-keto-cyclohexenyl carboxamide with ferrous ion at 257 mμ, pH 5.5, ionic strength 0.150, 25°C.

of this model compound at 257 mμ revealed that chelation at pH 5.5 produced the same effect as would ionization (*i.e.*, increase of pH beyond pH 5.5). The effect of pH on the 257 mμ absorbance of 2.25×10^{-5} *M* 2-hydroxy-4-methyl-6-ketocyclohexenylcarboxamide is shown in Fig. 12, and the effect of ferrous ion complexation on the 257 mμ absorbance of this compound is shown in Fig. 13, which suggests 1:1 complexation.

Ethyl - 5 - hydroxy - 10 - keto - 6,7,8,8*a*-tetrahydro - 9 - anthracenecarboxylate (Fig. 14) was tested for chelation properties. This compound was used as a model for the 11 to 12 chromophore of the tetracyclines which is responsible for the pronounced spectral changes in the visible region upon chelation of these compounds. When tested for chelation in the usual manner, it was found that a 2:1 (metal–ligand) complex was observed at 390 mμ. Figure 15 shows the Job plot for this compound. The chelate solution showed a definite color change.

DISCUSSION

Nature of the Enzymatic Inhibition.—Mahler and Elowe (14) have postulated an active site for NADH–cytochrome *c* oxidoreductase in which two of the iron's six coordination valences are involved in chelation of iron to the flavin prosthetic group. Two of the remaining coordination valences are involved in linkage of iron to enzyme protein, and two are involved in chelation of iron to cytochrome *c*. The metal provides a structural link between the flavoenzyme and the cytochrome *c* electron acceptor. The metal perhaps serves as a bridge over which electrons flow from reduced flavin to a cytochrome.

The results of the present investigation have revealed that the enzymatically catalyzed reduction of cytochrome *c* by the metalloflavoenzyme was subject to inhibition by the tetracycline antibiotics. Furthermore, it was shown that the inhibition was competitive with respect to cytochrome *c*. Based on the well-known ability of the tetracyclines to chelate metals (33) and the observation of Mahler and Elowe that chelating agents result in competitive inhibition of cytochrome *c* reduction, it is reasonable to assume that the tetracyclines exhibit their inhibitory effect on this enzyme system, at least in part, by chelating with the protein-bound iron.

Considering this model metalloflavoenzyme reactive site and recalling the hypothesis of Saz and Martinez (13) that the tetracyclines uncouple oxidative phosphorylation in bacteria by inhibition of metalloflavoenzymes, it is now possible to suggest that tetracyclines act by inhibiting metalloflavoenzymes due to chelation of enzymatically bound metal.

In the case of the model metalloflavoenzyme employed in this study, there are several specific ways in which chelation of iron by tetracyclines might occur. Tetracyclines may compete directly with cytochrome *c* for binding sites on iron. The com-

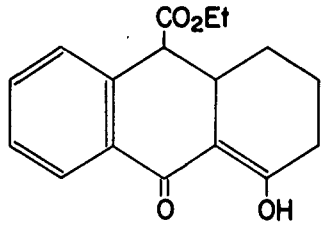

Fig. 14.—Ethyl - 5 - hydroxy - 10 - keto - 6,7,8,8*a* - tetrahydro-9-anthracenecarboxylate.

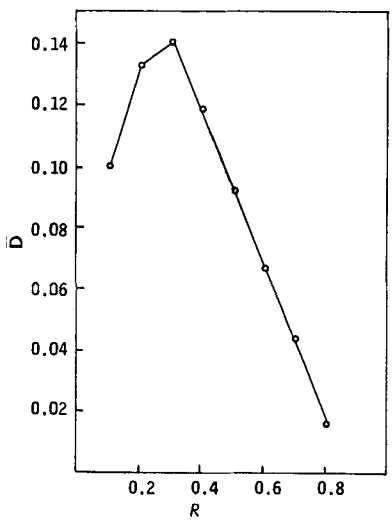

Fig. 15.—Job plot for complexation of ethyl-5-hydroxy-6,7,8,8*a* - tetrahydro - 9 - anthracenecarboxylate with ferrous ion at 390 mμ, pH 5.5, ionic strength 0.150, 25°C.

TABLE IV.—COMPARISONS BETWEEN THE RELATIVE ANTIBACTERIAL ACTIVITY OF 7-SUBSTITUTED 6-DEMETHYL-6-DEOXYTETRACYCLINES AND THE HAMMETT σ (*meta*) AND σ (*para*) FUNCTIONS

7-Substituent	σ (*meta*)[a]	σ (*para*)[a]	Relative Antibacterial Activity[b]
NO_2	0.710	0.778	640
Cl	0.373	0.227	300
Br	0.391	0.232	200
H	0	0	160
I	0.352	0.276	120
NH_2	−0.160	−0.66	40

[a] Values taken from Jaffe (38). [b] Based on value reported in the review article by Barrett (15).

petitive inhibition with respect to cytochrome *c*, which was demonstrated experimentally in this study, indicates that this is occurring, although other reactions could be simultaneously occurring to some extent. In metalloflavoenzymes of bacteria and lower organisms in general, the flavin prosthetic group is known to be readily dissociable (34). In such enzymes, tetracyclines could displace flavin by competing with it for iron binding sites. Chelating agents, such as the tetracyclines, could compete with enzyme protein itself for binding sites on iron, leading to dissociation of the metal component. It is also possible that tetracyclines could bind to substrates such as cytochrome *c*. This binding could possibly occur in the presence of exogenous metal, or it could be another type of complexation not involving metal. Such binding could block the attachment of substrate to enzyme.

Relationships Between Molecular Structure and Enzymatic Inhibitory Properties of the Tetracyclines.—The influence of the structural properties of the tetracyclines on their ability to inhibit the metalloflavoenzyme is illustrated in Table II. The results summarized there permit certain conclusions to be drawn about the structural relationships of tetracyclines in regard to the inhibition of the metalloflavoenzyme NADH–cytochrome *c* oxidoreductase.

Epimerization of the natural orientation of the 4-dimethylamino group results in an essentially complete loss of inhibitory effect on the enzyme system. However, removal of the dimethylamino group leads to no loss of inhibitory ability. In fact, inhibitory properties appear to be moderately enhanced by removal of this group. Based on the results of epimerization and on the effect of removal of the dimethylamino group, it appears that the dimethylamino group might influence inhibitory properties at the active site through steric effects. As a result of this hypothesis of steric influence for the dimethylamino group, one might ask what effect quaternization of this substituent with a methyl group has on inhibitory properties. At pH 8.5, according to the pKa assignments of Leeson *et al.* (35), the dimethylamino group would bear a positive charge regardless of whether it were quaternized. The only change then would be the somewhat bulkier nature of the quaternized compound. This should result in no great change in inhibitory capability. The fractional inhibition data (Table II) show, in fact, that the quaternized tetracycline methiodide is a little less active as an inhibitor than the normal parent tetracyclines. This small decrease in inhibitory activity could be due to steric interference from the small increase in the bulkiness of the dimethylamino group.

The effect of different substituents on the aromatic ring of the tetracycline molecule is difficult to account for in concrete terms. Saz and Martinez (13) noted that in the bacterial metalloflavoenzyme with which they worked, 7-chlorotetracycline was markedly more inhibitory than 5-hydroxytetracycline which both produced the same degree of inhibition in the bacterial enzyme. The results of our studies with mammalian NADH–cytochrome *c* oxidoreductase also indicate that 5-hydroxytetracycline and tetracycline are very similar in inhibitory activity and that 7-chlorotetracycline is a more potent inhibitor, although the difference in the case of 7-chlorotetracycline is not so striking as it was in the work of Saz and Martinez. Since the bacterial metalloflavoenzymes differ from those of mammalian origin in the extent of dissociability of the flavin coenzyme (34), it is not unreasonable to assume that the marked inhibition by 7-chlorotetracycline observed by Saz and Martinez is a reflection of the fact that the aromatic chloro substituent has an effect on the flavin, which tetracycline and 5-hydroxytetracycline do not have. It is altogether possible that the effect may be of the aromatic donor–acceptor complex type (36). It is possible that the aromatic ring of the tetracycline molecule, endowed with substituents providing suitable withdrawing power of electrons from the ring, could form aromatic molecular complexes with the isoalloxazine moiety of the flavin coenzyme. The isoalloxazine aromatic nucleus possesses two adjacent methyl substituents which are electron releasing in character, and thus push electronic charge into the aromatic ring (37). The chlorine substituent on the aromatic ring of 7-chlorotetracycline, having an opposite electronic effect from the methyl groups, could enhance the possibility of interaction between flavin and tetracycline and thus account for enhanced inhibitory effects.

In direct correlation with this discussion is the fact that there have been found to be some relationships between antibacterial activity and the electronic inductive effects of groups in the 7 position of tetracyclines. Table IV shows the relative antibacterial activity of a series of 7-substituted 6-demethyl-6-deoxytetracyclines. The authors have included in the table the Hammett σ (*meta*) and σ (*para*) functions for the substituents. Substituents with positive values for the Hammett functions are electron withdrawing with respect to hydrogen, and those with negative values are electron releasing. If one assumes that the large differences in antibacterial activity for the series of compounds in Table IV are possibly due to interaction between the aromatic portion of the isoalloxazine system of flavin enzymes and the aromatic nucleus of tetracyclines, then the data in the table show good correlation. The deviations observed between the antibacterial activity and the Hammett values for Cl, Br, and I may be due to the fact that in this series, steric effects are opposing electronic effects. In general, however, the data in the table support the idea that substituents strongly withdrawing electrons from the aromatic ring in tetracyclines might provide for a stronger interaction with the aromatic ring of the flavin system which has two adjacent electron releasing methyl groups.

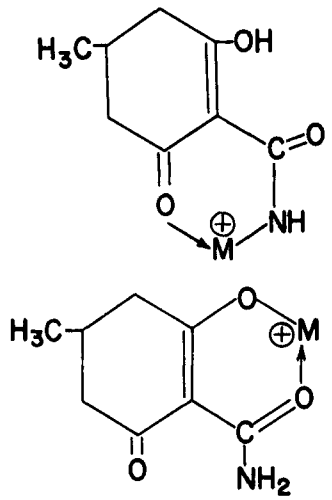

Fig. 16.—Possible metal chelates of 2-hydroxy-4-methyl-6-keto-cyclohexenylcarboxamide.

The formation of organic molecular complexes, as described here, does not involve chelation with metals. As will be shown later in this discussion, the portion of the tetracycline molecule responsible for chelation appears, from the results of this study, to be in the vicinity of the 2-carboxamido substituent and possibly at the chromophoric diketone region. Neither center of chelation is directly involved with the aromatic center. What is being suggested here, then, is that the aromatic ring accounts for an additional and different kind of interaction from chelation. Both kinds of interaction may be involved in tetracycline affinity to the receptor site and/or the antibacterial activity. The principles suggested here could be utilized to account for the marked effect on enzyme inhibition by 9-nitro and 9-amino substituted tetracyclines, which has been observed in this study. The 9-nitro analog was found to have a strikingly greater inhibitory activity than the normal parent tetracyclines. It would be difficult to account for this in terms of chelation effects at the 2-carboxamido group or the chromophoric diketone system. The possible formation of a molecular complex as an additional kind of drug–enzyme interaction might better account for this observation.

The position 11 to 12 chromophore of the tetracycline molecule has been shown to be one center capable of metal chelation (31, 32). This was confirmed by our spectral studies of the complexation of tetracyclines with ferrous ion. Isotetracyclines, in which the position 11 to 12 chromophore is destroyed, show no spectral evidence of chelation in the visible region. This is the wavelength region in which chelation with the 11 to 12 chromophore is manifested (26, 31, 32). The other normal tetracyclines and the model compound shown in Fig. 14, which possess the intact 11 to 12 chromophore, do show pronounced chelation in the visible region of the spectrum. The spectral studies with 2-hydroxy-4-methyl-6-keto-cyclohexenylcarboxamide (Fig. 3), both with regard to pH–absorbance profiles and spectral effects of chelation, indicate that this model compound and isotetracycline chelate *via* the same groups. The structure of the model compound suggests chelation involving the carboxamide substituent and either the keto group or the enolic hydroxyl group as shown in Fig. 16 (31, 39). Results of the spectral studies, as indicated, suggest a similar type of chelation for isotetracyclines.

On the basis of fractional inhibition studies and K_i determinations, isotetracycline shows inhibitory properties very similar to the parent antibiotics. It could be possible therefore, that the chelation effect considered to be important in tetracycline inhibition of NADH–cytochrome *c* oxidoreductase resides in the region of either the chromophoric diketone system *or* the carboxamido substituent, or both, and specifically in the substituents implicated in chelation as shown in Figs. 16 and 17. Isotetracycline exhibits the same inhibitory effect against the enzyme as parent tetracyclines, but does not have the 11 to 12 chromophoric system shown by the normal tetracyclines and the model compound shown in Fig. 14. The suggestion of the binding of the enzyme to a tetracycline through a metal bridge on the A ring is supported by the fact that 2-hydroxy-4-methyl - 6 - ketocyclohexenylcarboxamide exhibits inhibitory properties against the enzyme system. That the inhibitory effects of this model compound are not so strong as with isotetracycline could be due to the absence of a possible charge transfer binding site (as provided by the aromatic ring in isotetracycline).

The absence of inhibitory effects with N^2-pyrrolidinomethyltetracycline and with 2-cyano-2-decarboxamidotetracycline substantiates the hypothesis that the 2-carboxamido substituent might be essential in inhibiting the enzyme system either by chelation or by direct binding to the enzyme.

5*a*(6)-Anhydrotetracycline, apo-5-hydroxytetracycline, and 7-chloro-5*a*(11*a*)-dehydrotetracycline all have somewhat altered 11 to 12 chromophore systems, but all are at least as active enzyme inhibitors as the parent antibiotics. Removal of the 12*a*-hydroxyl group leads to greatly increased activity as an inhibitor of the enzyme. The reason for this is not apparent, although it is possible that the 12*a*-deoxy compound is readily converted to other more inhibitory forms (40).

Based on structure-activity relationships of the tetracyclines, it has been postulated (28) that groups at position 6 do not markedly effect activity, but that removal of the substituents at this position leads to an increase over the activity of the parent compounds. Studies with NADH–cytochrome *c* oxidoreductase indicate that removal of both the 6-methyl and 6-hydroxy group causes a substantial increase in inhibitory effect. These results seem to indicate that the nature of the effects at this position are steric in origin.

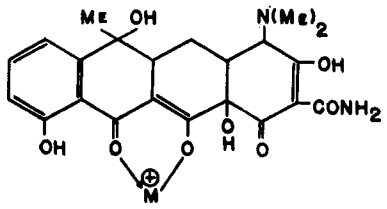

Fig. 17.—Chelation of the 11 to 12 chromophore of tetracycline.

Significance of the Results of This Study in the Interpretation of the Mode of Tetracycline Action.—It has been suggested that the tetracyclines exhibit their ultimate effect on bacteria by inhibiting protein synthesis (41). It has further been shown that the tetracyclines can inhibit oxidative phosphorylation in mammalian tissues (1) and in bacteria (5). The conclusion drawn from these observations (15) is that the tetracyclines probably exhibit their effect on bacteria by inhibiting oxidative phosphorylation. The work of Saz *et al.* (9–13) later showed that the inhibition of oxidative phosphorylation was due to the ability of the tetracycline to inhibit bacterial metalloflavoenzymes. Based on this knowledge, the authors selected NADH–cytochrome *c* oxidoreductase to serve as a model enzyme system to investigate further the mode of tetracycline action. The enzyme was selected because it is a metalloflavoenzyme available in relatively pure form and because it had been previously investigated to the extent that a proposed active site had been established (14). The authors have found that this enzyme seems to provide a reasonably good model of a proposed tetracycline receptor site.

It must be noted, however, that this enzyme is a mammalian enzyme rather than a bacterial one. Metalloflavoenzymes of bacterial origin differ from those of mammalian origin in that the bacterial enzymes have a more loosely bound flavin prosthetic group and possibly more loosely bound metal (34). Saz and Martinez (12), using bacterial nitro-reductase, showed that degree of dissociability of the coenzymes was the determining factor in bacterial resistance or sensitivity to the antibiotic. The tetracyclines inhibit oxidative phosphorylation in mammalian tissue, but only at higher concentrations than are required for inhibition in bacteria (3, 5). Thus, it appears that higher concentrations of tetracycline would be required to produce inhibitory effect on mammalian enzymes. This was borne out by the results of this study. The bacterial enzyme with which Saz *et al.* worked was inhibited 50% by 10^{-6} and 10^{-5} *M* concentrations of tetracyclines in the case of the sensitive and resistant extracts, respectively (13). In this study, 10^{-3} *M* concentrations of tetracycline were required to produce a similar degree of inhibition with the mammalian enyzme. Thus, based on the assumptions discussed here, it would appear that the mammalian enzyme has flavin significantly more tightly bound than even the resistant enzyme. This fact could, of course, serve as the basis for the selective toxicity of tetracyclines against microorganisms in animal hosts.

Another shortcoming of the model system of this study with respect to providing information about the action of the drug at the receptor site is a deficiency exhibited to some degree in all *in vitro* experiments on drug action. This deficiency lies in the impossibility of exactly reproducing all of the conditions and processes that the drug encounters or undergoes from the time it is administered to the time it elicits its ultimate response. The natural environment of the actual receptor site involves a particular pH and ionic strength, as well as definite concentrations of enzymes, substrates, and cofactors. Another serious drawback of most drug mechanism studies is their inability to distinguish between structural requirements at the site of action, and structural requirements important in drug transport. In considering this point with regard to the antibacterial activity of tetracyclines, the primary concern with respect to transport is the passage of antibiotic through the bacterial cell wall and the bacterial cell membrane. To accomplish this requires a certain balance between lipophilic and ionic character. At controlled pH, polyampholytes like the tetracyclines are no doubt dependent on their specific pKa values and the pH of the medium for passage through membranes. The relative proportion and arrangement of polar and nonpolar groups on the drug molecule is also of importance in this regard.

Bearing in mind the limitations of the model experiments performed in this study, it is now possible to attempt to provide a hypothesis concerning the mechanism of tetracycline action. The hypothesis is based on the assumption that tetracyclines inhibit bacterial growth by inhibiting metalloflavoenzymes responsible for oxidative phosphorylation within bacterial cells. The hypothesis must attempt to correlate known structure-activity relationships of the tetracyclines (15) with the experimental results of this and previous studies.

It appears that an essential requirement for antibiotic activity at the proposed metalloflavoenzyme receptor site is that the 4-dimethylamino group be in the same epimeric configuration found in the naturally occurring antibiotics. Epimerization from this configuration results in the inability of the compound to inhibit the enzyme receptor, and this accounts for the inactivation observed in bacterial inhibition studies with these compounds.

The results of this study indicate that the 2-carboxamido substituent is probably also essential for inhibition of the enzyme and that this may be due to its role in binding directly to the enzyme or through chelation of the enzyme bound metal. This fact would account for the inactive antibiotic properties of many of the 2-carboxamide modified tetracyclines.

Removal of the methyl and hydroxy substituents from position 6 was found to cause an increase in the ability of the tetracyclines to inhibit the model enzyme. These groups may offer steric hindrance when in place on the molecule. This finding could account for the fact that the 6-demethyl-6-deoxytetracyclines possess greater antibiotic activity than the normal tetracyclines.

Removal of the 4-dimethylamino group caused no loss in ability of the drug to inhibit the enzyme used in this study, even though dedimethylamino compounds are relatively inactive as antibiotics. This could well be explained by the fact that removal of this group significantly alters the acidic characteristics of the molecule and modifies its transport to the bacterial site of action. The pKa values for dedimethylaminotetracycline are 5.97 and 8.56 (35), as compared with 3.33, 7.68, and 9.69 for the parent compound. This alteration could lead to the inability of the molecule to traverse biological membranes. The fact that epimerization of the 4-dimethylamino group leads to greater loss of antibiotic activity than does its removal (42) might also indicate that the dimethylamino group is not required for inhibition at the receptor site, but that its loss decreases the concentration of drug that can get to the receptor site. Dedimethylaminotetracycline has been shown to exhibit 15% of the antibiotic activity of tetracycline (15).

Similar arguments might be invoked to explain the results obtained with isotetracycline. That is to say, these alterations may alter the pKa spectrum of these derivatives, adversely affecting their transport to the bacterial enzyme site.

REFERENCES

(1) Loomis, W. F., *Science*, **111**, 474(1950).
(2) Van Meter, J. C., *et al.*, *Proc. Soc. Exptl. Biol., N. Y.*, **81**, 215(1952).
(3) Brody, T. M., Hurawitz, R., and Bain, J. A., *Antibiot. Chemotherapy*, **4**, 864(1954).
(4) Hunter, F. E., and Lowry, O. H., *Pharmacol. Rev.*, **8**, 89(1956).
(5) Miura, Y., *et al.*, *Antibiot. Chemotherapy*, **2**, 152(1952).
(6) Jacob, F., and Monod, J., *J. Mol. Biol.*, **3**, 318(1961).
(7) Gale, E. F., and Folkes, J. P., *Biochem. J.*, **53**, 493 (1953).
(8) Albert, A., "Strategy of Chemotherapy," Cambridge University Press, London, England, 1958, pp. 112–138.
(9) Saz, A. K., and Marmur, J., *Proc. Soc. Exptl. Biol. Med.*, **82**, 783(1953).
(10) Saz, A. K., and Slie, R. B., *J. Am. Chem. Soc.*, **75**, 4626(1953).
(11) Saz, A. K., and Slie, R. B., *J. Biol. Chem.*, **210**, 407 (1954).
(12) Saz, A. K., and Martinez, L. M., *ibid.*, **223**, 285(1956).
(13) *Ibid.*, **233**, 1020(1958).
(14) Mahler, H. R., and Elowe, D. G., *ibid.*, **210**, 165 (1954).
(15) Barrett, G. C., *J. Pharm. Sci.*, **52**, 309(1963).
(16) Paul, K. G., "Methods in Enzymology," vol. II, Colowick, S. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1955, p. 749.
(17) Vernon, L. P., Mahler, H. R., and Sarkar, N. K., *J. Biol. Chem.*, **199**, 599(1952).
(18) Mahler, H. R., "Methods in Enzymology," vol. II, Colowick, L. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1955, pp. 688, 707.
(19) Edelhoch, H., Hayaishi, O., and Teply, L. J., *J. Biol. Chem.*, **197**, 97(1952).
(20) Mahler, H. R., *et al.*, *ibid.*, **199**, 585(1960).
(21) Job, P., *Ann. Chim.*, **9**, 113(1928).
(22) Martell, A. E., and Calvin, M., "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, pp. 28–34.
(23) Reilly, C. N., and Sawyer, D. T., "Experiments for Instrumental Methods," McGraw-Hill Book Co., Inc., New York, N. Y., 1961, p. 176.
(24) Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658(1934).
(25) Eadie, G. S., *Science*, **116**, 688(1952).
(26) McCormick, J. R. D., *et al.*, *J. Am. Chem. Soc.*, **79**, 2849(1957).
(27) Fruton, J. S., and Simmonds, S., "General Biochemistry," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1958, pp. 348–351.
(28) Boothe, J. H., "Antimicrobial Agents and Chemotherapy," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1962, p. 213.
(29) Dixon, M., and Webb, E. C., "Enzymes," Academic Press Inc., New York, N. Y., 1958, pp. 62–181.
(30) Neilands, J. B., and Stumpf, P. K., "Outlines of Enzyme Chemistry," John Wiley & Sons, Inc., New York, N. Y., 1958.
(31) Conover, L. H., "Oxytetracycline and Related Compounds," Symposium on Antibiotics and Mould Metabolites, Special Publication No. 5, The Chemical Society, London, England, 1956, pp. 48–81.
(32) Ishidate, M., and Sakaguchi, T., *Pharm. Bull. Japan*, **3**, 147(1955).
(33) Weinberg, E. D., "Metal Binding in Medicine," Seven, M. J., ed., J. B. Lippincott Co., Philadelphia, Pa., 1960, p. 329.
(34) Mahler, H. R., *Advan. Enzymol.*, **17**, 233(1956).
(35) Leeson, L. J., Krueger, J. E., and Nash, R. A., *Tetrahedron Letters*, (No. 18) **1963**, 1155.
(36) Andrews, L. J., *Chem. Rev.*, **54**, 713(1954).
(37) Morrison, R. T., and Boyd, R. N., "Organic Chemistry," Allyn and Bacon, Boston, Mass., 1959, p. 292.
(38) Jaffe, H. H., *Chem. Rev.*, **53**, 191(1953).
(39) Chenoweth, M. B., *Pharmacol. Rev.*, **8**, 57(1956).
(40) Burstall, M. L., *Mfg. Chemist*, **31**, 474(1960).
(41) Gale, E. F., *Pharmacol. Rev.*, **15**, 481(1963).
(42) Regna, P. P., "Antibiotics, Their Chemistry and Non-Medicinal Uses," Goldberg, H. S., ed., D. Van Nostrand Co., Inc., Princeton, N. J., 1959, p. 95.

Mechanism of Action of Phenolic Disinfectants VI

Effects on Glucose and Succinate Metabolism of *Escherichia coli*

By HENRY COMMAGER and JOSEPH JUDIS

A representative group of phenols was examined as to effects on aerobic and anaerobic utilization of glucose and aerobic utilization of sodium succinate. At low concentrations, *p*-chlorophenol, 2,4-dichlorophenol, *p*-chloro-*o*-cresol, and 2,4-dinitrophenol stimulated oxygen uptake with glucose as the substrate; phenol and *p*-chloro-*m*-xylenol were inhibitory, and *p-tert*-amylphenol had no measurable effect. At the higher concentrations used, all compounds except 2,4-dinitrophenol were inhibitory. Anaerobic metabolism of glucose was inhibited by concentrations of the compounds tested similar to those inhibiting glucose oxidation. Oxidation of sodium succinate was stimulated by low concentrations of *p*-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, *p*-chloro-*o*-cresol, dichloro-*m*-xylenol, and *p-tert*-amylphenol. All compounds were inhibitory at higher concentrations. In general, sodium succinate oxidation was the most sensitive to phenolics, and glucose oxidation the least sensitive. There was some correlation between phenol coefficients and inhibition of glucose and succinate metabolism. It is hypothesized that the lethal action of phenolic disinfectants is due to damage of permeability mechanisms, the repair of which is prevented by concomitant inhibition of energy-yielding metabolic reactions.

PREVIOUS WORK from this laboratory (1–3) strongly suggests that phenol disinfectants caused damage to permeability mechanisms in bacteria, though definitive correlation between such damage and the lethal effects of phenolic disinfectants must yet be established. Permeability damage would probably have to be rather extensive and irreversible for loss of macromolecules, such as proteins or nucleic acids, but the type of damage detected by β-galactosidase activity in cryptic (permeaseless) strains of

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