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Potentiometric and Spectral Investigations of Anhydrotetracycline and Its Metal-Ion Complexes

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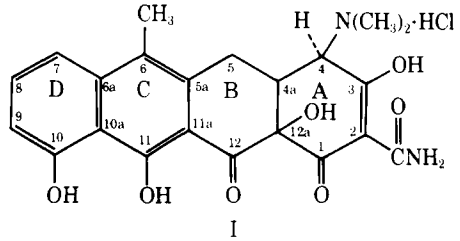
Abstract □ The interaction of various metal ions with anhydrotetracycline was investigated. A comparison of the UV–visible and fluorescence spectral characteristics of anhydrotetracycline and its metal-ion complexes with a number of modified anhydrotetracyclines in the presence of metal ions suggested that the C-11 oxygen was involved in metal-ion binding. Secondary binding was observed in the A-ring by circular dichroism when the primary site was blocked.

Keyphrases □ Anhydrotetracycline—and various metal-ion complexes, potentiometric and spectral investigation of binding properties □ Metal-ion complexes, various—with anhydrotetracycline, potentiometric and spectral investigation of binding properties □ Complexes, metal ion, various—with anhydrotetracycline, potentiometric and spectral investigation of binding properties □ Spectrometry, UV–visible, fluorescence, and circular dichroism—investigation of binding properties of anhydrotetracycline with various metal ions □ Potentiometry—investigation of complexation of anhydrotetracycline with various metal ions □ Antibiotics—anhydrotetracycline, complexation with various metal ions, potentiometric and spectral investigation

As a result of the extensive use of tetracyclines, several adverse side effects have been reported (1). Many of these side effects have been traced to impurities in the tetracycline preparation, as in the case of the "reversible Fanconi-type syndrome," which is thought to be induced by anhydrotetracycline (I) and 4-epianhydrotetracycline (1, 2). Tetracycline degradation in an acidic medium was investigated previously, and the principal degradation products were 5a,6-anhydrotetracycline and 4-epitetracycline (3).

Treatment of tetracycline with warm mineral acid results in the loss of a molecule of water from the C-5a and C-6 positions, forming anhydrotetracycline (4). X-ray diffraction studies revealed that the bond lengths of anhydrotetracycline agree with those of tetracycline, except for the C-ring and adjacent bonds. The anhydrotetracycline molecule was more planar in the C–D-ring region than its tetracycline parent (5). A slightly different conformation of the A-ring of anhydrotetracycline also was revealed. The nitrogen of the C-4 position was reported to be closer to the C-12a oxygen than to the C-3 oxygen in anhydrotetracycline (5). All active tetracyclines were reported to have the same conformation of the A-ring, with the C-4 nitrogen closer to the C-3 oxygen than to the C-12a oxygen (6, 7).



I

This investigation concerned the complexing properties of anhydrotetracycline, with particular emphasis on the determination of the complexation sites in this molecule. The functional groups proposed as binding sites in tetracycline remain in anhydrotetracycline and are considered possible binding sites of this compound. On the A-ring, these sites include a carbonyl and hydroxyl group at the C-1 or C-3 position, the amide group at the C-2 position, and the dimethylamine group at the C-4 position. The C-10–C-12 position carbonyl and hydroxyl groups of the B-, C-, and D-rings are also considered possible complexation sites.

While the UV region of both tetracycline and anhydrotetracycline contains multiple chromophore absorptions, the visible region of anhydrotetracycline exhibits a single absorption at 430 nm. This absorption is assigned to the $\pi \rightarrow \pi^*$ transition of the B–C–D-ring chromophore of anhydrotetracycline. In the presence of certain metal ions, this absorption band undergoes a bathochromic shift, strongly suggesting participation of this region of the molecule in complexation. However, the degree of intramolecular transfer of complexation effects is not known.

To establish the primary binding site(s) of metal ions to anhydrotetracycline, some modified anhydrotetracyclines were prepared. The effects of complexation within the A-ring were observed by comparison of the spectral properties of anhydrotetracycline with those of 4-dedimethylaminoanhydrotetracycline and 2-cyanoanhydrotetracycline. Possible complexation involving the C-10 hydroxyl was removed when 10-benzenesulfonyl-2-cyanoanhydrotetracycline was used; finally, any complexation through the C-11 hydroxyl was blocked using 4-dedimethylamino-11-methoxyanhydrotetracycline.

EXPERIMENTAL

Methods—The UV and visible spectra were obtained on a recording spectrophotometer[1] utilizing 1.0-cm quartz cells. Complete spectra of the compounds investigated were initially obtained in acetonitrile at concentrations of approximately 1.5×10^{-5} *M*. Samples for the visible studies were prepared at approximately 1×10^{-4} *M* in 50% chloroform in absolute ethanol. All studies of complexation with modified anhydrotetracyclines were carried out in the presence of equimolar amounts of triethylamine. The stock solutions of the various metal ions used were prepared from their chloride or nitrate salts in absolute ethanol.

Circular dichroism spectra were recorded on an optical rotatory dispersion–circular dichroism spectrometer[2]. Solutions of tetracycline and of anhydrotetracyclines were 1×10^{-4} *M* and 2.5×10^{-5} *M* in 90% methanol in water, respectively. The pH of all solutions was maintained at 7.4 with 0.1 *M* trimethylamine. A range of 0.1–0.2° full scale was used with a time constant of 3 sec.

The fluorescence spectra of the sample solutions were obtained using a spectrophotofluorometer[3]. Solutions were prepared in 50% chloroform in absolute ethanol at an approximate concentration of 1×10^{-4} *M*. Spectra were calibrated to the 450-nm fluorescence maximum of quinine sulfate.

Aqueous pKa values for the tetracycline hydrochloride salts were determined from potentiometric data by incorporation into a modified computer program (8); this program calculates pKa values from pH *versus* volume of titrant data utilizing the Noyes (9) method. Less soluble tetracyclines were titrated in 50% dimethylformamide in water, and apparent pH values were used to calculate the pKa values in these instances. Samples approximately 1.4×10^{-3} *M* were titrated with 0.5 ml of standardized 1.0 *M* sodium hydroxide in 0.01-ml increments using a micrometer syringe[4]. A constant ionic strength of 1.0 was maintained by the addition of potassium chloride. All pH values were taken on a servo-digital pH/volt meter[5] equipped with a glass electrode[6] and a calomel reference electrode.

Nonaqueous titrations in dimethylformamide of approximately 1.5×10^{-4} *M* samples were carried out using 20% tetra-*n*-butylammonium bromide as the supporting electrolyte. The dimethylformamide solvent was purified by vacuum distillation after standing for several days over barium oxide and stored over a type 4A (4–8-mesh) molecular sieve. A micrometer syringe[4] was used to deliver 0.01-ml aliquots of titrant accurately. A semimicro silver–silver chloride combination electrode[7] containing 2-propanol saturated with potassium chloride was used for all potential measurements made on a servo-digital pH/volt meter[5].

The analytical TLC method for tetracycline and related hydrochloride salts was described previously (2). Because the dedimethylaminotetracyclines remained on the solvent front in the previously described TLC system, these compounds were separated using silica gel H as the solid support and acetone–ethyl acetate–water (20:10:3) as the eluting solution.

Compounds—*Anhydrotetracycline Hydrochloride*—Tetracycline hydrochloride[8] (5.0 g) was added to a boiling solution of 2-propanol (42 ml), methanol (3 ml), and concentrated hydrochloric acid (15 ml). After refluxing for 20 min, the resulting precipitate was filtered, washed with 2-propanol, and dried under vacuum for 2 hr. The crude product was then boiled in 500 ml of benzene, removing excess water, methanol, and 2-propanol as azeotropes. The remaining precipitate was filtered and dried under vacuum for 18 hr to yield 3.9 g of product.

TLC yielded only one spot on a 5% edetate sodium[9]-treated Kieselguhr plate with R_f 0.88. An NMR peak was observed at 2.40 ppm for the C-6 methyl protons. The mass spectrum contained a parent ion at *m/e* 426. Titration in 50% dimethylformamide and water resulted in an equivalent weight of 446 ± 24 compared to a calculated value for the hydrochloride salt of 463. The calculated apparent pKa values were 4.6, 6.6, and 9.0.

2-Cyanotetracycline—The synthesis of 2-cyanotetracycline as outlined by Mani and Foltran (10) was followed. TLC using silica gel revealed a single spot at R_f 0.33. The decomposition temperature of the isolated yellow crystals was in excess of 280°. A mass spectrum of this compound could not be obtained because of its low volatility. Nonaqueous titration of the sample in dimethylformamide resulted in an equivalent weight of 427 ± 4 (calculated 426). The IR spectrum contained the nitrile absorption at 4.55 μm.

Anal.—Calc. for $C_{22}H_{22}N_2O_7$: C, 61.97; H, 5.20; N, 6.57. Found: C, 61.74; H, 5.12; N, 6.56.

2-Cyanoanhydrotetracycline—A sample of 2-cyanotetracycline was dissolved in a minimum volume of 50% anhydrous dimethylformamide in anhydrous acetone at 5°. Anhydrous hydrogen chloride was bubbled through the solution for 5 min. The resulting reaction mixture was maintained at 5° for 18 hr. The product was precipitated from the reaction mixture by treatment with anhydrous ether, purified by recrystallization from ethanol and water, and dried in a vacuum oven at 60° for 24 hr.

TLC of the sample on silica gel revealed one spot at R_f 0.25. Nonaqueous titration of the sample produced an equivalent weight of 406 ± 5 (calculated 408).

Anal.—Calc. for $C_{22}H_{20}N_2O_6$: C, 64.71; H, 4.90; N, 6.86. Found: C, 63.43; H, 5.01; N, 6.47.

10-Benzenesulfonyl-2-cyanotetracycline—The 10-benzenesulfonyl ester of tetracycline was prepared as outlined previously (4, 11). Nonaqueous titration of the dried product resulted in an equivalent weight of 546 ± 15 (calculated 566).

Anal.—Calc. for $C_{22}H_{26}N_2O_9S \cdot H_2O$: C, 57.53; H, 4.83; N, 4.79. Found: C, 57.37; H, 4.57; N, 4.81.

10-Benzenesulfonyl-2-cyanoanhydrotetracycline—A solution of 10-benzenesulfonyl-2-cyanotetracycline dissolved in anhydrous acetone was cooled to 5°. After the solution was treated for 5–10 min with anhydrous hydrogen chloride, the reaction mixture was allowed to stand for 18 hr. A 10-fold excess of distilled water was added, and the resulting precipitate was isolated and recrystallized from ethanol and water.

After drying in a vacuum oven at 60° for 24 hr, the sample revealed a single spot by TLC (silica gel) at R_f 0.57. Nonaqueous titration resulted in an equivalent weight of 265 ± 5 (calculated 274). The NMR spectrum in dimethyl sulfoxide-d_6 contained the typical anhydro C-6 methyl peak at 2.45 ppm.

4-Dedimethylaminotetracycline—The synthesis described by McCormick *et al.* (11) was used. The analytical data appeared previously (12).

4-Dedimethylaminoanhydrotetracycline and 4-Dedimethylamino-11-methoxyanhydrotetracycline—These compounds were prepared and identified as described previously (12).

DISCUSSION

Potentiometric Investigations—The titration of anhydrotetracycline hydrochloride in 50% dimethylformamide in water resulted in three pKa values: 4.6, 6.6, and 9.0. Tetracycline hydrochloride under similar conditions yielded pKa values of 4.3, 7.7, and 9.7. Comparison of these values indicates that the acidity of anhydrotetracycline is approximately the same as tetracycline, with the second and third pKa values being slightly more acidic. Doluisio and Martin (13) reported only two aqueous pKa values for anhydrochlortetracycline hydrochloride, 3.28 and 5.37, compared to 3.66, 7.40, and 9.27 for chlortetracycline hydrochloride, indicating a definite increase in the acidity of the anhydro analog.

The observed titration curves and calculated pKa values are the result of complex, multiple deprotonations of more than one acidic site within the molecule (14). The formation of anhydrotetracycline significantly changes the character of the C and D rings of tetracycline and is expected to alter significantly the acidity of any site(s) associated with this region of the molecule. The second and third dissociation values are strongly dependent on the microdissociation values (k_n) associated with the individual deprotonation sites. The theory and equations that apply to aqueous systems were developed previously (15). The equations that apply in this case are:

$$K_{a_1}K_{a_2}K_{a_3} = k_1k_{12}k_{123} = k_1k_{13}k_{132} \quad \text{(Eq. 1)}$$

$$1/K_{a_3} = 1/k_{123} + 1/k_{132} \quad \text{(Eq. 2)}$$

$$k_{12}k_{123} = k_{13}k_{132} \quad \text{(Eq. 3)}$$

[1] Cary model 14.
[2] Cary model 60.
[3] Aminco-Bowman.
[4] Agla, Burroughs Wellcome & Co.
[5] Heath model EU-302A.
[6] Sargent No. 5-30050-15C.
[7] Corning No. 476050.
[8] Pfizer, Inc., New York, N.Y.
[9] Versene.

Table I—Half-Neutralization Potentials (—Millivolts) of Various Tetracyclines and Model Compounds in Dimethylformamide

Compound	C-1 or C-3 Hydroxyl	C-4 Dimethylamine	C-10, C-11, or C-12 Hydroxyl
Tetracycline hydrochloride	474	788	976
Anhydrotetracycline hydrochloride	457	634	819 974
Tetracycline base	Zwitterionic	794	990
Tetracycline methiodide	622	Blocked	855 1240[a]
4-Dedimethylaminotetracycline	798	Not present	993
4-Dedimethylaminoanhydrotetracycline	635	Not present	893
4-Dedimethylamino-11-methoxyanhydrotetracycline	815	Not present	917[b]
2-Cyanotetracycline	Zwitterionic	649	914
2-Cyanoanhydrotetracycline	Zwitterionic	619	776
10-Benzenesulfonyl-2-cyanotetracycline	Zwitterionic	624	1038[c]
10-Benzenesulfonyl-2-cyanoanhydrotetracycline	Zwitterionic	564	652
2-Carbamoylcyclohexane-1,3-dione	932	—	—
1-Hydroxyanthraquinone	—	—	971
8,9,10-Trihydroxy-1-keto-1,2,3,4-tetrahydroanthracene	—	—	731
1,8-Dihydroxynaphthalene	—	—	735
1-Hydroxy-8-methoxynaphthalene	—	—	—

[a] Very small break in the titration curve. [b] C-11 blocked. [c] C-10 blocked.

$$K_{a_2} = k_{12} + k_{13} \quad \text{(Eq. 4)}$$

where K_{a_n}'s are the observed macroscopic dissociation constants, and k_n's are the dissociation constants of each individual site along a particular dissociation scheme. An increase in the acidity of the site assigned as the third deprotonation site (β-diketone system of C-10–C-12) results in an increase in k_{13} and k_{123} and is observed as an increase in the macroscopic dissociation values, K_{a_2} and K_{a_3}.

Because of the favorable solubility characteristics of the various modified tetracyclines and anhydrotetracyclines in dimethylformamide, all nonaqueous titrations were carried out in this solvent. The half-neutralization potential values for a number of tetracyclines and their anhydro analogs, as well as a number of tetracycline model compounds, are presented in Table I. Although the previously discussed equations do not apply directly to nonaqueous systems, the same trends were observed for the half-neutralization potentials of the tetracyclines and their anhydro analogs. The increase in the acidity of this region was also noted by the appearance of a fourth titratable proton from anhydrotetracycline, which was not observed with tetracycline under the same conditions.

As was the case with anhydro formation, the alteration of functional groups changed the acidity of the resulting compounds. The removal of the C-4 position dimethylamine group, for example, decreased the value of the first dissociation constant for both the tetracycline and the anhydrotetracycline derivatives (Table I). It is generally accepted that this dissociation should be assigned to the acidic proton of the A-ring tricarbonyl system, and a decrease in the acidity of this proton is expected as a result of removal of the electron-withdrawing dimethylamine group. A decrease in acidity was also observed for the proton dissociating from the B–C–D-ring system of these derivatives. Although the effect was not as great as that observed for the tricarbonyl proton, it does imply some intramolecular interaction between the A-ring and the remaining portion of the anhydrotetracycline molecule.

Conversion of the C-2 position amide to a nitrile would be expected to increase the acidity of the A-ring tricarbonyl proton; however, titration of the 2-cyano derivatives of anhydrotetracycline produced only two titratable protons, both weaker than predicted for this proton in these compounds (Table I). The 2-cyano derivatives may exist in zwitterionic form in solution with a weaker acidic site, probably the dimethylamine group of the C-4 position, acting as the proton receptor. The low vapor pressure and high melting points observed for these derivatives also were indicative of this ionic character. As observed for the 4-dedimethylamino derivatives, modification of the A-ring of both tetracycline and anhydrotetracycline to the 2-cyano derivative affected the acidity of the B–C–D-ring proton as well.

Conversion of the C-11 hydroxyl to the methyl ether decreased the hydrogen bonding of the C-10 hydroxyl proton, allowing this proton to be titrated. However, titration of the monomethyl ether of 1,8-dihydroxynaphthalene did not produce an observable break in the titration curve, indicating that the effect of hydrogen bonding alone was not sufficient to explain the observed increase in the acidity of this proton. The presence of the C-12 carbonyl was assumed to provide additional stabilization for the anion formed from 4-dedimethylamino-11-methoxyanhydrotetracycline. The increased carbonyl character of the C-12 position also resulted in an increase in the hydrogen bonding by the A-ring tricarbonyl proton on the C-1 oxygen. An accompanying decrease in acidity of this proton was observed, as indicated by the drop of the half-neutralization potential from −635 to −835 mv.

Spectral Characteristics—The UV–visible spectrum of anhydrotetracycline contained peaks of maximum absorption at 223 nm (ϵ = 36,900), 269 nm (ϵ = 58,370), and 430 nm (ϵ = 9820). The absorption maxima for model compounds of the B–C–D-chromophoric region and the A-ring of anhydrotetracycline are given in Table II. The bands at 430 and 269 nm were assigned to the $\pi \rightarrow \pi^*$ transitions of the B–C–D-chromophoric region. The absorption at 269 nm also contained a contribution from the A-ring chromophore. The 223-nm band was assigned to a $\pi \rightarrow \pi^*$ transition of the aromatic system of this compound, with accompanying benzenoid absorption bands observed in the 300-nm region of the spectrum.

The λ_{max} (ϵ) in the UV–visible spectra of the modified anhydrotetracyclines is presented in Table III. Comparison of the λ_{max} values of 4-dedimethylaminoanhydrotetracycline with anhydrotetracycline

Table II—UV-Visible Absorption Maxima of Model Compounds of Tetracycline and Anhydrotetracycline Chromophores

Compound	λ_{max}, nm (ϵ)	Reference
2-Carbamoylcyclohexane-1,3-dione	258 (17,800)	16
2-Methylcarbamoylcyclohexane-1,3-dione	260 (17,800)	16
2-Carbamoyl-4-dedimethylaminocyclohexane-1,3-dione	261 (15,500)	16
8-Hydroxytetralone	260 (9300), 335 (3600)	4
2-Acetyltetralone	235 (6800), 257 (5000), 300 (6400), 341 (13,000)	17
2-Acetyl-8-hydroxytetralone	236 (12,000), 267 (5500), 348 (12,000)	4
2-Acetyl-8-benzyloxytetralone	260 (4600), 343 (13,200)	17
2-Hydroxyacetophenone	252 (9300), 327 (3200)	18
2-Methoxyacetophenone	246 (11,500), 305 (3800)	18
2-Hydroxybenzaldehyde	255 (10,000), 325 (3000)	18
2-Methoxybenzaldehyde	253 (11,800), 319 (4300)	18
8,9,10-Trihydroxy-1-keto-1,2,3,4-tetrahydroanthracene	267 (35,500), 425 (6800)	4

Table III—UV-Visible Absorption Maxima, λ (ε), of Anhydrotetracyclines in Acetonitrile

Compound	λ_{max}, nm (ε)
Anhydrotetracycline hydrochloride	269 (58,400), 430 (9800)
2-Cyanoanhydrotetracycline	267 (45,000), 426 (6900)
4-Dedimethylaminoanhydrotetracycline	268 (51,400), 426 (9300)
10-Benzenesulfonyl-2-cyanoanhydrotetracycline	267 (26,500), 404 (41,00)
4-Dedimethylamino-11-methoxyanhydrotetracycline	266 (57,100), 392 (5900)

indicates that removal of the C-4 dimethylamine group only slightly affected the spectral characteristics of anhydrotetracycline. The λ_{max} values for 2-cyanoanhydrotetracycline were shifted slightly to shorter wavelengths, with a decrease in the intensity relative to anhydrotetracycline. The decrease in intensity (ε) was attributed to the change in the ionic character of the A-ring. The sulfonyl ester at the C-10 position produced a still further decrease in the intensity of the UV–visible absorptions of 10-benzenesulfonyl-2-cyanoanhydrotetracycline. The dramatic hypsochromic shift of the long wavelength maximum of 10-benzenesulfonyl-2-cyanoanhydrotetracycline and 4-dedimethylamino-11-methoxyanhydrotetracycline was the result of decreased intramolecular hydrogen bonding in these compounds. Conover (17) reported similar behavior in the spectra of *o*-hydroxy and *o*-alkoxy aldehydes and ketones.

Conversion to the anhydrotetracycline resulted in an enhancement of the fluorescent properties of the tetracycline. A single fluorescent maximum was observed for anhydrotetracycline at 520 nm, with an activation wavelength of 430 nm. Similar maxima were observed for the other modified anhydrotetracyclines (Table IV). The fluorescence intensity was observed to be pH dependent, decreasing with increasing pH. Fluorescence enhancement, as well as maxima shifts, was observed from solutions of anhydrotetracycline with some metal ions. Solutions containing the transition metal ions usually resulted in fluorescence quenching.

The results of visible and fluorescence investigations of metal-ion complexation with the modified anhydrotetracyclines are presented in Table IV. The metal ions chosen for this study all exhibited complexation with anhydrotetracycline, as indicated by either a bathochromic shift of the long wavelength $\pi \rightarrow \pi^*$ transition in the visible spectrum of metal ion–ligand solutions or a change in the fluorescence properties of these solutions. Metal-ion-containing solutions of both 4-dedimethylaminoanhydrotetracycline and 2-cyanoanhydrotetracycline produced the same spectral alterations observed for complexation with anhydrotetracycline. Any contribution to the visible absorption spectrum by complexation within the A-ring was apparently minimal. However, the bathochromic shift indicative of metal-ion complexation with anhydrotetracycline was not observed for metal-ion solutions of 4-dedimethylamino-11-methoxyanhydrotetracycline. It can be concluded that the C-11 hydroxyl is most likely involved as a binding site of these metal ions to anhydrotetracycline. A slight shoulder in the 460-nm region of the visible spectrum of the 11-methoxy derivative with Al^{+3} and Zr^{+4} suggests that possibly another site may become involved in the complexation of these metals. This contention is supported by an accompanying shift in the fluorescence maxima of these solutions.

The 10-benzenesulfonyl derivative also was not observed to form complexes with most of the metal ions investigated. Only the strong oxygen binding metals, antimony, aluminum, and zirconium, produced the spectral changes indicative of complexation with this derivative. Possible explanations for the inability of this derivative to form metal-ion complexes are direct participation of the C-10 oxygen in metal-ion chelation, steric interference of the bulky benzenesulfonyl ester, and complex formation too weak to be observed by the methods employed.

The formation of a metal-ion complex of anhydrotetracycline at the C-10 and C-11 hydroxyls would involve the loss of both protons of these functional groups or the rearrangement of the β-diketone system displacing a hydrogen to the C-12 oxygen. Potentiometric studies indicated that the displacement of a second proton from this region of the molecule is difficult. Although it was reported that strong oxygen binding metals such as zirconium and titanium do form such

Table IV—Visible Absorption Maxima and Fluorescence Emission Maxima of Metal-Ion Solutions of Anhydrotetracycline

Metal	Absorption λ_{max}, nm	Absorption ε	Fluorescence, λ_{max}, nm	Relative Intensity	Activation, λ_{max}, nm
Anhydrotetracycline					
None	433	7,300	520	1.0[a]	430
Antimony	442	7,800	545	1.0	440
Manganese	472	10,000	—	—[b]	—
Calcium	459	10,400	530	1.3	460
Magnesium	450	9,400	520	29.8	450
Tin	439	7,200	570	1.2	450
Zinc	441	9,600	530	11.0	450
Aluminum	480	8,500	550	25.0	475
Zirconium	475	8,000	557	13.5	470
4-Dedimethylaminoanhydrotetracycline					
None	432	8,200	523	1.0[a]	430
Antimony	430	8,300	536	1.0	435
Manganese	440	8,900	—	—[b]	445
Calcium	455	9,700	547	1.3	462
Magnesium	440	10,400	519	24.8	452
Tin	430	8,200	541	1.1	453
Zinc	448	10,200	532	10.5	451
Aluminum	478	11,000	556	24.4	475
Zirconium	490	8,507	577	10.5	488
2-Cyanoanhydrotetracycline					
None	430	8,300	538	1.0[a]	432
Antimony	437	9,000	560	1.8	456
Manganese	445	10,600	529	—[b]	430
Calcium	455	12,100	544	2.2	450
Magnesium	443	11,800	531	27.2	454
Tin	435	7,600	565	4.4	480
Zinc	450	10,500	531	16.1	454
Aluminum	475	10,100	559	18.3	475
Zirconium	497	9,300	578	14.5	485
10-Benzenesulfonyl-2-cyanoanhydrotetracycline					
None	405	5,100	500	1.0[a]	412
Antimony	412	5,100	505	0.8	415
Manganese	405	5,200	500	1.0	415
Calcium	406	5,000	505	1.1	412
Magnesium	405	4,900	500	1.0	415
Tin	405	5,300	505	1.0	415
Zinc	404	5,300	500	1.0	415
Aluminum	435	4,200	523	1.6	452
Zirconium	470	4,200	547	0.9	478
4-Dedimethylamino-11-methoxyanhydrotetracycline					
None	401	5,400	495	1.0[a]	400
Antimony	400	5,200	497	0.6	400
Manganese	398	5,200	500	0.5	400
Calcium	396	5,100	508	0.7	400
Magnesium	395	5,200	504	0.8	400
Tin	398	5,300	505	0.6	400
Zinc	397	5,300	503	0.8	400
Aluminum	396	5,100	521	0.7	400
Zirconium	400	5,200	534	0.8	425

[a] Intensity of anhydrotetracycline assigned as 1.0. [b] Fluorescence quenched.

double-proton displaced complexes with 1,8-dihydroxynaphthalene (19, 20), none of the other metals used in this investigation was reported to form complexes with this model compound of the C-10–C-11 chelating site. The data indicate that the C-11 oxygen participates as a chelating site in the interaction of metal ions with anhydrotetracycline. Much of the literature suggests that this is also the site of chelation in tetracycline along with the C-12 oxygen (17, 21). In the case of anhydrotetracycline, participation of the C-10 oxygen has not been eliminated by the evidence presented; however, it is highly unlikely that it would be involved in most cases. The similarities of the spectral changes induced by complexation of anhydrotetracycline with the various metal ions studied suggest that the same site is involved in all cases, namely the C-11–C-12 oxygens.

Positive evidence for additional involvement by other sites on the

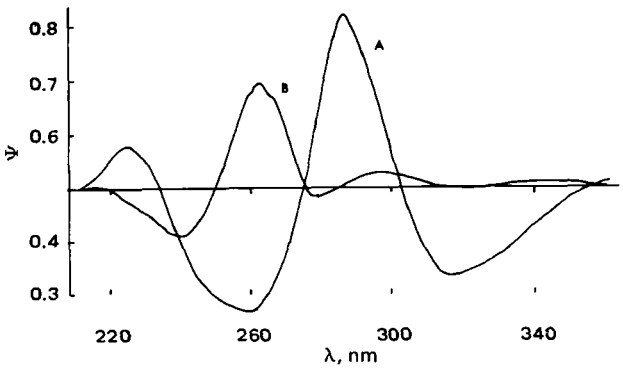


Figure 1—*Circular dichroism spectra of tetracycline hydrochloride and anhydrotetracycline hydrochloride.*

anhydrotetracycline molecules was observed only when the principal site investigated was blocked. This result is illustrated by the changes in the fluorescent properties of 4-dedimethylamino-11-methoxyanhydrotetracycline in the presence of Al^{+3} or Zr^{+4} (Table IV).

Circular Dichroism Investigations—The circular dichroism spectra of a number of tetracyclines were reported previously (6, 22). The stereochemistry at the ring junctures, C-12a, C-4a, and C-5a, are the principal factors contributing to the chirality of the B–C–D-ring chromophore of tetracycline. Therefore, the formation of anhydrotetracycline dramatically affects the circular dichroism spectra by removing the C-5a asymmetry. The circular dichroism spectra of tetracycline hydrochloride and anhydrotetracycline in methanol are shown in Fig. 1. While not all the absorptions observed have been assigned to specific chromophores, investigations of model compounds of the A-ring chromophore showed that a single absorption for this chromophore occurred in the 255–270-nm region (16).

The circular dichroism spectrum of 4-dedimethylamino-11-methoxyanhydrotetracycline in 90% methanol–water is presented in Fig. 2. At low pH, identical spectra were obtained for this compound in the presence of Ca^{+2} and Mg^{+2}, indicating no complex formation. At pH 7 and above, however, a 10-fold increase in the intensities of the principal absorptions for both Mg^{+2} and Ca^{+2} was observed.

The circular dichroism spectrum of the 11-methoxy derivative in the presence of Zr^{+4} is also presented in Fig. 2. A different type of complex is suggested by this spectrum. The definite shift observed in the 272-nm region is interpreted as participation of the A-ring chromophore in the complexation of this strong oxygen binding metal. Participation by the tricarbonyl system of the A ring has been generally disregarded because of the high acidity of this region. However, potentiometric studies indicated that the acidity of this region is greatly reduced in this derivative.

Table V presents the results of a study of the effect of complexation on the UV spectrum of a model A-ring compound, 2-carbamoylcyclohexane-1,3-dione (16). Complexation by Zr^{+4} with this compound resulted in a significant bathochromic shift, while no evidence of complexation was observed for Ca^{+2} until after the solution was treated with a base.

Because of the large shift observed for the Zr^{+4} complex of the model compound, it is postulated that similar complexation is observed in the circular dichroism spectrum of the 4-dedimethylamino-11-methoxyanhydrotetracycline–Zr^{+4} complex. No shifts were observed in the 270-nm region of the circular dichroism spectra for the Ca^{+2} and Mg^{+2} complexes; therefore, another site must be involved, possibly the C-1 and C-12 oxygens.

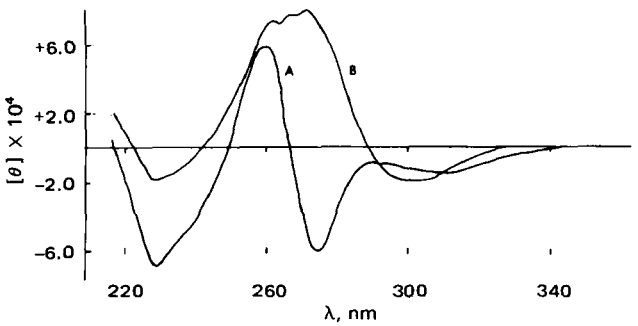


Figure 2—*Circular dichroism spectra of 4-dedimethylamino-11-methoxyanhydrotetracycline and its Zr^{+4} complex.*

Table V—Effect of Metal-Ion Complexation on the Absorption Maximum of an A-Ring Model Compound

	λ_{max}, nm (ϵ)
2-Carbamoylcyclohexane-1,3-dione[a]	257 (17,850)
Plus 1×10^{-3} *M* base	257 (18,300)
Plus 2×10^{-3} *M* Ca^{+2}	257 (17,500)
Plus 1×10^{3} *M* base and 2×10^{-3} *M* Ca^{+2}	264 (20,600)[b]
Plus 2×10^{-3} *M* Zr^{+4}	272 (18,100)
Plus 1×10^{3} *M* base and 2×10^{-3} *M* Zr^{+4}	272 (18,000)

[a] All solutions were 3×10^{5} *M* in absolute ethanol. [b] Additional shoulder at 245 nm.

It has been shown that secondary A-ring complexation can be observed in the circular dichroism spectra of modified anhydrotetracyclines. Mitscher *et al.* (22) observed such A-ring complexation of tetracycline; however, model compounds of the B–C–D-ring chromophore also yielded absorptions in the 260–270-nm region. Until the B–C–D-ring complexation site is blocked, it will remain extremely difficult to determine the effects of A-ring complexation from B–C–D-ring complexation in tetracycline.

SUMMARY

In conclusion, these studies indicate that anhydrotetracycline binds metals at more than one site. The visible spectra of anhydrotetracycline and A-ring-modified anhydrotetracycline exhibited a bathochromic shift of the 430-nm band in the presence of metal ions. The spectral properties, however, of the C-10 or C-11 blocked derivatives remained unchanged when metal ions were present. The fluorescent properties of the modified anhydrotetracyclines reflected the changes observed in the visible spectra in the presence of metal ions. Fluorescence evidence for A-ring complexation was noted when B–C–D-ring complexation was prevented. Circular dichroism investigations with the blocked C-11 position derivative confirmed metal-ion complexation within the A-ring. Variations in the circular dichroism spectra with metal ions indicated multiple binding possibilities within the A-ring.

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Lipid–Protein Interactions: Enhancement of Enzyme Activity of L-Glutamic Acid Dehydrogenase by Nonionic Detergents

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Abstract □ Five nonionic detergents enhanced the activity of L-glutamic acid dehydrogenase [L-glutamate:nicotinamide adenine dinucleotide phosphate oxidoreductase (deaminating) (EC 1.4.1.3)]. These detergents activated the enzyme toward α-ketoglutaric acid reduction, causing a decrease in the sensitivity of the enzyme to allosteric regulation by guanosine 5-triphosphate. There was also a diminution of the enhancing effect of the modifier adenosine 5-diphosphate on the enzyme's L-glutamic acid dehydrogenase activity. These detergents may cause a conformational change in the enzyme, and this change could lead to an increase in the binding of the substrates for the α-ketoglutaric acid reduction. Accompanied with this conformational change would be a decrease in the binding of the modifier guanosine 5′-triphosphate, with no concomitant change in the binding of the adenosine 5′-diphosphate modifier.

Keyphrases □ Lipid–protein interactions—L-glutamic acid dehydrogenase activity, effect of various nonionic detergents □ L-Glutamic acid dehydrogenase—effect of various nonionic detergents on activity □ Detergents, nonionic—effect on activity of L-glutamic acid dehydrogenase □ Enzyme activity—L-glutamic acid dehydrogenase, effect of various nonionic detergents

Nonionic detergents have been used for the isolation of membrane proteins (1, 2). However, the use of detergents can sometimes produce materials that possess altered activity. Thus, the cell surface antigen Thy-1.2 found on mouse thymocytes loses all activity when isolated with a polyoxyethylene ether nonionic detergent (3) whereas its activity is retained when it is solubilized by papain digestion (4, 5).

These changes in the biological activity of a protein in the presence of nonionic detergents prompted the investigation of the effects of such materials on a defined protein possessing an easily assayable biological activity. The enzyme L-glutamic acid dehydrogenase [L-glutamate:nicotinamide adenine dinucleotide phosphate oxidoreductase (deaminating) (EC 1.4.1.3)] was chosen as a model because it shows activity toward various substrates (6) and its activity is altered by allosteric modifiers (7).

EXPERIMENTAL

The concentration of L-glutamic acid dehydrogenase solutions was determined using an $E^{1\%}_{280}$ of 10.0 (8). Quantitation of the enzyme in detergent solutions and of ovalbumin was determined by fluorescamine (9).

L-Glutamic Acid Dehydrogenase Activity—This reaction was assayed in the direction of α-ketoglutaric acid reduction. A 6-μl aliquot of the enzyme, 0.4 mg/ml, was added to 3 ml of phosphate buffer (10^{-4} *M* ethylenediaminetetraacetic acid and 0.04 *M* sodium phosphate, pH 7.6) containing 3×10^{-4} mole of nicotinamide adenine dinucleotide phosphate reduced form, 1.5×10^{-3} mmole of α-ketoglutaric acid, and 3×10^{-1} mmole of ammonium chloride. The assays were monitored by recording the disappearance of the chromophore at 340 nm. An ϵ_{340} of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ was used. All initial rates were calculated between 15 and 30 sec after the start of the assay, during which time the reaction rate was linear.

L-Glutamic Acid Dehydrogenase Activity in Presence of Detergent[1]—This reaction was also assayed in the direction of α-ketoglutaric acid reduction. The L-glutamic acid dehydrogenase solutions in 25% (v/v) detergent were prepared by adding 0.5 ml of detergent to a 1.5-ml aliquot of a 0.53-mg/ml solution of the enzyme in phosphate buffer (1×10^{-4} *M* ethylenediaminetetraacetic acid and 0.04 *M* sodium phosphate, pH 7.4). A control was prepared by adding 0.5 ml of deionized water to a 1.5-ml aliquot of the same enzyme solution.

All samples were incubated at 37° for 15 min and then at 4° for 24 hr. Then 6-μl aliquots of this enzyme preparation were added to 3-ml aliquots of the phosphate buffer such that the detergent concentration in the assay mixture was 0.05%, and the assays were monitored as already described. The assays were performed immediately upon addition of the detergent solution of enzyme and at various time intervals over 42 hr.

A further control consisting of L-glutamic acid dehydrogenase solutions in 0.05% (v/v) detergent was prepared by adding 0.75 μl of detergent to a 1.5-ml aliquot of a 0.53-mg/ml solution of the enzyme in phosphate buffer (1×10^{-4} *M* ethylenediaminetetraacetic acid and 0.04 *M* sodium phosphate, pH 7.4). Then 6-μl aliquots of this enzyme mixture were added to 3-ml aliquots of the phosphate buffer con-

[1] The following nonionic detergents were purchased from Sigma Chemical Co.: Triton X-100, Triton X-102, Triton X-35, Triton X-45, Triton X-65, Triton CF-54, Triton CF-32, Triton N-101, Triton DF-12, Triton QS-15, Triton B-1956, Tween 20, Tween 40, Tween 60, Tween 80, Brij 96, and NP-40.