Mechanism of Fluorometric Analysis of Tetracycline Involving Metal Complexation

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Abstract □ Tetracycline complexation with calcium and organic ligands was studied using fluorescence, circular dichroism, and solvent extraction methods. The results were used to interpret the mechanism of the commonly used fluorometric methods for the analysis of tetracycline in biological fluids. Tetracycline formed ternary calcium complexes with barbital sodium and L-tryptophan in alkaline solutions. The circular dichroism studies indicate that the calcium ion in these complexes is bound to the C-4 dimethylamino and the C-12a hydroxyl groups of tetracycline. These ternary complexes are strongly fluorescent and can be extracted easily into 1-butanol or ethyl acetate. Based on the characteristics of the ternary complexes and of the tetracycline degradation products, it is concluded that only the active form of tetracycline can be complexed and extracted for fluorescence analysis.

Keyphrases □ Tetracycline—complexation with calcium and organic ligands, mechanism of fluorometric analysis □ Complexation—tetracycline with calcium and organic ligands, mechanism of fluorometric analysis □ Fluorometry—mechanism of analysis, tetracycline complexation with calcium and organic ligands □ Antibacterials—tetracycline, complexation with calcium and organic ligands, mechanism of fluorometric analysis

Tetracycline is amphoteric, forming salts with either acids or bases. The drug is commonly marketed as the hydrochloride salt (I), in which the tetracycline molecule is protonated at the C-4 dimethylamino group.

Tetracycline hydrochloride exhibits three pKa values (1): pK_1 at 3.3 is associated with the tricarbonyl methane system at the C-1, C-2, and C-3 positions; pK_2 at 7.8 is associated with the C-11–C-12 β-diketone system; and pK_3 at 9.5 indicates the deprotonation of the C-4 dimethylammonium ion.

BACKGROUND

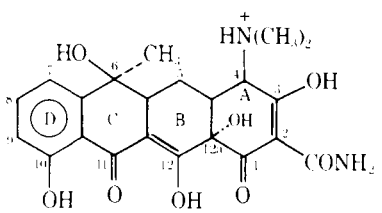
The pH of the medium has a great effect on the stability of tetracycline and its interaction with other molecules. Most tetracyclines are unstable in basic solutions. Kelly *et al.* (2) reported that chlortetracycline converts rapidly into isochlortetracycline at pH 10, and similar reactions for other tetracyclines were observed (3). Tetracycline undergoes oxidation with atmospheric oxygen (4), which may be catalyzed by riboflavin under light (5). Epimerization occurs at the C-4 position and leads to the formation of anhydrotetracycline, a biologically inactive compound often found in aged tetracycline products (6, 7). Dehydration and aromatization of the C-ring lead to the formation of anhydro derivatives, a process occurring predominantly at low pH.

Several methods of tetracycline analysis were developed recently. Tetracycline in biological fluids has been assayed primarily by microbiological (8, 9) and fluorometric (10–14) techniques. The disadvantages inherent in the microbiological methods are that the results may be subjected to large errors (±15%) (15) and that the assay is of limited specificity since several tetracycline-like compounds have similar biological activity (16). The serum tetracycline concentrations determined by these methods are consistently lower than those determined by chemical analysis, presumably because of protein binding, which may inhibit the diffusion necessary in the microbiological procedures. Furthermore, the microbiological assay requires sample incubation at pH 4.5 for 3–4 hr, conditions conducive for epimerization to the biologically inactive epimer.

Fluorometric techniques have become popular because of their high sensitivity and reduced variability. The analysis is based on the fluorescence quantitation of tetracycline extracted from biological fluids. Since tetracycline is nearly nonfluorescent, the assays must provide complete extraction as well as large enhancement of the tetracycline fluorescence. Tetracycline forms complexes with many divalent and trivalent cations, and some of these complexes are strongly fluorescent when compared to nonchelated tetracycline. However, these complexes are fairly water soluble and difficult to extract with organic solvents at physiological concentrations. For these reasons, some early fluorometric procedures (10, 11) involved primarily the extraction of the lipid-soluble degradation products of tetracycline followed by treatment with alcoholic calcium or aluminum chloride to enhance the fluorescence.

A method combining both the extraction and fluorescence enhancement was first reported by Kohn (12); tetracycline was extracted with ethyl acetate from solutions containing 0.01 *M* calcium chloride and 0.3 *M* barbital sodium at pH 9. Similar success was reported (13, 14) using magnesium acetate buffer and different solvents. The function of barbital sodium in Kohn's method was to enhance both extraction and fluorescence by forming a neutral ternary complex with calcium and tetracycline. A structure of this complex (II) was proposed by Kohn based on his solvent distribution data. However, the structure and the role of barbital in fluorescence enhancement have been questioned (13, 14, 17).

Poiger and Schlatter (17) suggested that barbital sodium only enhances the fluorescence but does not facilitate tetracycline extraction, whereas others (13, 14) suggested that barbital sodium has no effect on tetracycline fluorescence. The contradictions may be partly due to the use of different analogs of tetracycline in different studies; nevertheless, the study of the ternary complex of calcium, tetracycline, and barbital is important for several reasons. While most fluorometric procedures are adequate for *in vivo* studies of tetracycline, information has not been presented to indicate whether the complex is extracted selectively from the active form of tetracycline. The drug is known to form nonactive degradation products under various conditions. The analytical results could be misleading if some of the results are due to the contribution of the nonactive products.



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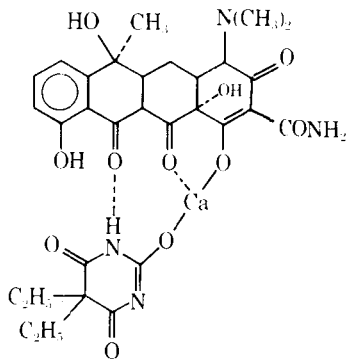

II

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Studies on the ternary complex will aid in understanding the extracted species. From the pharmacological point of view, metal binding of tetracycline has long been known to affect the drug's absorption in biological systems. Studies of the binary and ternary complexes of metal–tetracycline and other ligands should provide useful information on the action of the tetracycline antibiotics.

Under these guidelines, studies of the complexation involving calcium, tetracycline, and several other ligands were carried out using solvent extraction, fluorescence, and circular dichroism techniques. Emphasis was placed on the nature of these complexes and of their importance in the chemical analysis of tetracycline.

EXPERIMENTAL

Materials—Tetracycline hydrochloride[1], barbital sodium[2], and L-tryptophan[3] were obtained commercially. 1-Butanol and other solvents were spectroscopic grade. All other chemicals were analytical grade.

Instruments—Fluorescence measurements were made with a spectrophotofluorometer[4] equipped with a 150-w xenon lamp, a 1P21 photomultiplier tube, and an x–y spectral recorder[5]. Absorption studies were carried out with a spectrophotometer[6] using a matched set of UV cells. Circular dichroism measurements were made with a spectrophotometer[7] equipped with constant nitrogen flush.

Fluorescence Measurements—The fluorescence of tetracycline and its complexes was determined at the emission maximum of 512 nm with the excitation wavelength at 390 nm. A primary fluorescence standard (12) was prepared by mixing a 2-ml aliquot of 5×10^{-5} M tetracycline hydrochloride in water with 0.4 ml of 0.9 M barbital sodium in water. The mixture was then diluted to 50 ml with a solution of 3×10^{-4} M calcium chloride and 8×10^{-3} M barbital prepared in methanol.

After allowing the instrument to warm up for 1 hr, the standard was used to adjust the instrument to give a fluorescence reading of 90 on a fixed sensitivity setting. This adjustment was done periodically during the fluorescence measurements. All sample measurements were carried out at 25° using a matched set of fluorescence cells. Dilution of samples for fluorescence measurement, when necessary, was made with a microsyringe.

Fluorescence–pH Titrations—A 100-ml volume of aqueous solution containing 0.01 M calcium chloride and an appropriate ligand was adjusted to the desired pH with dilute sodium hydroxide or sulfuric acid. A 2-ml aliquot was pipetted into the cell, and to it was added 4 μl of 1×10^{-3} M tetracycline in methanol. The sample fluorescence was then measured. For titrations using only calcium and tetracycline, 20 μl of 1×10^{-2} M tetracycline in methanol was added to 100 ml of aqueous calcium chloride, and pH adjustments were made as described.

All solutions for the pH studies were prepared using double-distilled water. Argon gas was constantly used to prevent pH errors due to carbon dioxide interference in nonbuffered solutions prior to and during titrations.

Distribution Studies—Solvent extraction studies were carried out according to the following procedure. A 10-ml aliquot of tetracycline in a 0.01 M tris(hydroxymethyl)aminomethane–0.1 M hydrochloric acid buffer was equilibrated with 4 ml of the organic solvent (1-butanol or ethyl acetate) by hand shaking for 2 min, followed by centrifuging for 4 min at 2500 rpm; pH adjustments were made prior to extraction. The concentration of the extracted tetracycline was determined spectrophotometrically (Table I). The extracted tetracycline in the complexed forms showed an absorption at 385 nm in 1-butanol or ethyl acetate, whereas the uncomplexed tetracycline absorbed at 360 nm. The molar absorptivities of the complexed tetracycline in 1-butanol and ethyl acetate were determined to be 2.53×10^4 and 1.75×10^4 M^{-1} cm^{-1}, respectively.

Data Treatment—The solvent distribution data were used to determine the stability constants of the binary and ternary complexes involving calcium, tetracycline, and other ligands. In the calcium–tetracycline binary system, the stability constant, K, can be determined from:

$$K = [MT_n]/[M][T]^n \qquad \text{(Eq. 1)}$$

[1] Lot 1110/337, Mylan Laboratories, Morgantown, W.Va.
[2] Fisher Scientific, Pittsburgh, Pa.
[3] Nutritional Biochemical Corp.
[4] Aminco-Bowman, American Instruments, Silver Spring, Md.
[5] Omnigraphic model 2000, Houston Instruments, Houston, Tex.
[6] Cary 118, model C, Varian Instruments, Palo Alto, Calif.
[7] Jasco ORD/CD model 5.

Table I—Percent Extraction of Tetracycline Solutions with 1-Butanol

Solution[a]	pH	Extraction, %
—	7.5	2
—	9.0	43
0.3 M Barbital sodium	9.0	88
0.1 M Acetic acid	9.0	51
0.025 M L-Tryptophan	9.0	89

[a] All solutions contained 0.01 M calcium chloride and 1×10^{-6} M tetracycline (see *Experimental* for extraction procedure).

where $[MT_n]$ is the concentration of the tetracycline complex, $[M]$ is the free concentration of the calcium ion, $[T]$ is the unbound concentration of tetracycline, and n is the number of tetracycline molecules bound to one calcium ion.

In this study, $[MT_n]$ was determined in terms of the bound tetracycline concentration, $[T_b]$. The latter was taken to be the extracted concentration of tetracycline in the organic layer because: (*a*) no extraction can be made without the presence of calcium chloride; and (*b*) the measured absorption of the extracted species is at 385 nm, which is clearly different from that of the unbound tetracycline at 360 nm. Since the concentration of calcium chloride (0.01 M) used is much greater than that of tetracycline (in the range of 10^{-6} M), the initial concentration of calcium chloride, C_m, is essentially taken to be the free concentration, $[M]$. Substitution of C_m for $[M]$ and $[T_b]/n$ for $[MT_n]$ in Eq. 1 leads to:

$$K = [T_b]/n[T]^n C_m \qquad \text{(Eq. 2)}$$

and:

$$-\log[T] = (\log nKC_m)/n - \log[T_b]/n \qquad \text{(Eq. 3)}$$

The plot of $-\log[T]$ *versus* $-\log[T_b]$ should give a straight line with the intercept and slope equal to $(\log nKC_m)/n$ and $1/n$, respectively. From these results, K and n can be easily calculated.

In the ternary system, the overall stability constant for the reaction $M + T + A = MTA$ can be expressed as:

$$K = [MTA]/[M][T][A] \qquad \text{(Eq. 4)}$$

where $[MTA]$ is the concentration of the ternary complex, which is equal to $[T_b]$, and $[A]$ is the free concentration of the second ligand. Equation 4 is based on the assumption that only one molecule of T or A is involved in the complex. The validity of this assumption will be discussed under *Results*. In a condition such that the tetracycline concentration is much smaller than the concentrations of the calcium ion and the ligand A, $K[M][A]$ is essentially a constant. Thus, the plot of $-\log[T]$ *versus* $-\log[MTA]$ should yield a straight line. From the intercept of the plot, the stability constant can be calculated with known values of $[M]$ and $[A]$.

In the present study, the ligand A represents barbital, L-tryptophan, and acetate. These compounds show very weak binding with calcium; *i.e.*, the free concentrations of the ligand and the calcium ion, $[A]$ and $[M]$, may be approximated to be their initial concentrations. With this approximation, the stability constants for these complexes were then calculated. The constants calculated in this manner are slightly lower than the actual values; nevertheless, these results are adequate for comparison.

RESULTS

The pH profile of tetracycline fluorescence in solutions containing calcium chloride and barbital sodium is shown in Fig. 1. Tetracycline is only weakly fluorescent in a basic medium. Calcium chloride addition, however, results in a large enhancement of the tetracycline fluorescence, with a maximum at pH 7.5. Calcium chloride can cause a shift of the pK_2 of tetracycline to a more acidic pH (18), so that the deprotonation of the BCD-ring β-diketone system is nearly complete at pH 7.5. Therefore, the strong fluorescence of the calcium–tetracycline complex at pH 7.5 is due to the chelation of calcium at the β-diketone system of tetracycline. Model studies of the complex indeed show that the calcium ion forces the tetracycline molecule to maintain a planar, rigid conformation, which is optimal for fluorescence.

At pH 9 and above, the calcium–tetracycline solution shows a marked decrease in fluorescence. This result indicates that the calcium ion may have changed its binding site on the tetracycline molecule. At this pH, the C-4 dimethylammonium group of tetracycline undergoes proton dissociation and conformational change. Mitscher *et al.* (19, 20) found

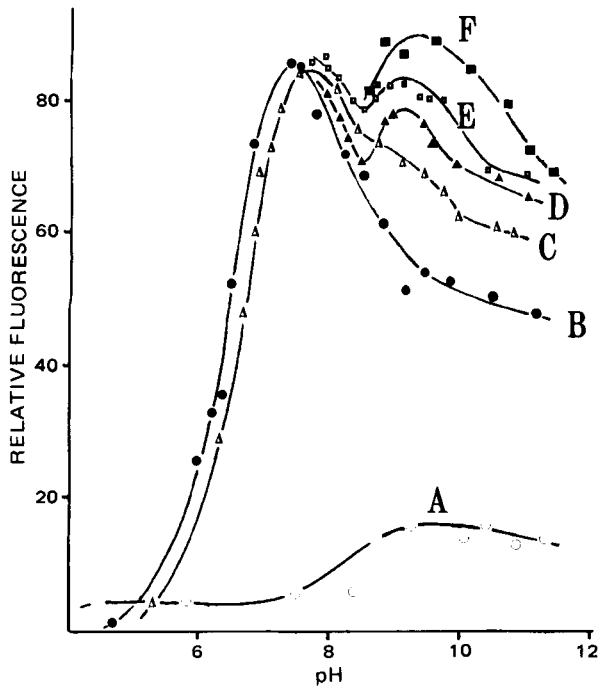

Figure 1—*The pH profile of tetracycline fluorescence (excitation, 390 nm; emission, 512 nm; and concentration of tetracycline, 2×10^{-6} M) in various solutions. Key: A, tetracycline alone; B, with 0.01 M calcium chloride; C, with 0.01 M calcium chloride and 0.025 M barbital sodium; D, with 0.01 M calcium chloride and 0.06 M barbital sodium; E, with 0.01 M calcium chloride and 0.08 M barbital sodium; and F, with 0.01 M calcium chloride and 0.1 M barbital sodium.*

that this conformational change may be enhanced by the presence of calcium and suggested that the calcium is bound to the A-ring system. The present study supports the A-ring binding theory, because the fluorescence of the solution should have been as strong as that in pH 7.5 if the calcium were bound predominantly at the β-diketone system.

In the presence of barbital sodium, the fluorescence of the solution at pH 7.5 remains unchanged; strong enhancement of the fluorescence, however, is observed at pH 9. As shown in Fig. 1, the peak becomes more prominent at higher barbital concentrations. The results indeed suggest the formation of a ternary calcium–tetracycline–barbiturate complex since, in the absence of calcium, barbital sodium has no effect on the tetracycline fluorescence. Furthermore, the mixture of calcium chloride and barbital sodium does not exhibit fluorescence at the wavelength measured.

The effect of barbital sodium on the fluorescence of the calcium–tetracycline solution at pH 9.5 can be used to determine the number of

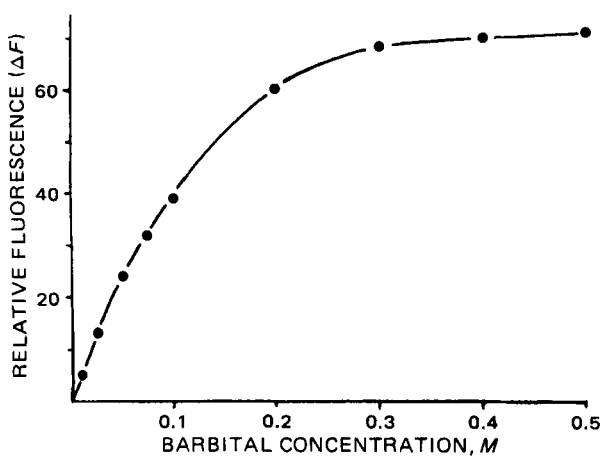

Figure 2—*Plot of the fluorescence enhancement, ΔF, as a function of barbital sodium concentration for solutions containing 0.01 M calcium chloride, 2×10^{-6} M tetracycline, and varying concentrations of barbital sodium at pH 9.5.*

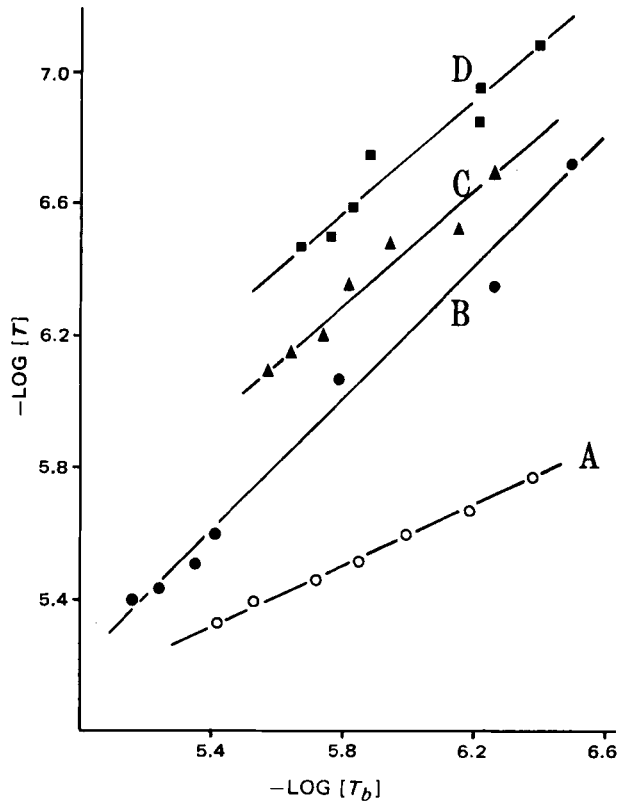

Figure 3—*Tetracycline distribution between pH 9 buffer and 1-butanol (expressed by the plot of $-log[T]$ versus $-log[T_b]$) for solutions containing 0.01 M calcium chloride (A), 0.01 M calcium chloride and 0.2 M acetic acid (B), 0.01 M calcium chloride and 0.025 M L-tryptophan (C), and 0.01 M calcium chloride and 0.1 M barbital sodium (D).*

barbiturate molecules involved in the ternary complex. Figure 2 shows the plot of the fluorescence enhancement, ΔF, induced by barbital sodium *versus* the barbital sodium concentrations, where ΔF is the difference in fluorescence between the ternary and binary systems. If it is assumed that the ternary complex is formed by the reaction $M + T + pA = MTA_p$, then $K = [MTA_p]/[M][T][A]^p$. Here K is the equilibrium constant, p is the number of barbiturate molecules, $[M]$ and $[A]$ are the respective concentrations of calcium and barbital sodium, and $[MTA_p]$ and $[T]$ are the concentrations of the bound and free tetracycline, respectively.

The plot of $\log[MTA_p]/[T]$ *versus* $\log[A]$ should yield a straight line with the slope equal to p. The values of $\log[MTA_p]/[T]$ may be taken to be the values of $\log \Delta F/(\Delta F_0 - \Delta F)$, where ΔF_0 is the maximum fluorescence enhancement, which may be seen in Fig. 2 as a plateau in the region of high barbiturate concentrations. From the data of Fig. 2, a slope of 1.1 was obtained from the plot. This result indicates that only one barbiturate molecule is involved in the ternary complex.

Table I shows the distribution data of the extraction of 1×10^{-6} *M* tetracycline with 1-butanol from solutions containing 0.01 *M* calcium chloride and various ligands. Acetic acid is used as a ligand because it was reported (13) to replace barbital sodium in a fluorometric assay of tetracycline. The results show that nearly complete extraction can be achieved by the presence of 0.3 *M* barbital sodium or 0.025 *M* L-tryptophan, whereas acetic acid shows only little or no effect on the extraction.

Figure 3 shows the log–log plots of the tetracycline distribution between water and 1-butanol in the absence (line A) and presence (lines B, C, and D) of acetic acid, L-tryptophan, and barbital sodium. In the absence of ligands, the plot gives a straight line with $n = 2$, indicating two tetracycline molecules in the extracted complex. In the presence of ligands, however, the plots give straight lines with a slope of 1. This result indicates that one tetracycline molecule in the binary complex is replaced by these ligands to form a ternary complex.

The equilibrium data of the binary and ternary complexes are shown in Table II. In the binary system, the log K values, determined from Eq. 3, were 5.90 at pH 7.5 and 7.04 at pH 9. These results are quite reliable, since similar studies (21) reported log $K = 3.0$ for a 1:1 calcium–oxytetracycline complex at pH 7.4. The equilibrium constant measured at pH

Table II—Equilibrium Data of Tetracycline Complexes

Complex[a]	pH	n	log K
A	7.5	2.1	5.90
B	9.0	2.0	7.04
C	9.0	1.2	5.07
D	9.0	1.2	4.92
E	9.0	1.0	2.90

[a] A = calcium–tetracycline; B = calcium–tetracycline, C = calcium–tetracycline–L-tryptophan, D = calcium–tetracycline–barbital, and E = calcium–tetracycline–acetate.

9 is about 14-fold greater than that at pH 7.5. The large difference between the two values also suggests that calcium forms two different complexes at these pH values. In the ternary system, the equilibrium constants for the barbiturate and the L-tryptophan complexes are about 100-fold greater than the constant of the acetate, indicating that the acetate is a poor agent for tetracycline extraction. Successful extraction of tetracycline in physiological concentrations using 1-butanol can be made with barbital sodium and L-tryptophan. The results show a linear relationship between fluorescence and the tetracycline concentration. L-Tryptophan appears to be more effective in inducing extraction but is less effective in enhancing fluorescence than barbital sodium. In contrast to some earlier reports (13, 14), this study shows that barbital sodium markedly affects the tetracycline fluorescence.

The circular dichroism spectra of tetracycline and of its calcium complexes at pH 7.5 and 9 are shown in Fig. 4. The BCD-ring chromophore is responsible for the bands at 360, 320, 285, and 225 nm; the A-ring chromophore contributes the 262-nm band (19). The circular dichroism spectrum is extremely sensitive to configuration change at the C-4 position of the tetracycline molecule (22). At pH 7.5, only the bands associated with the BCD-ring chromophore are affected by calcium ion (Fig. 4). At pH 9, however, binding of calcium to the A-ring is evident from the striking change of the 262-nm band.

Spectral differences between the two calcium complexes are also seen in the 350–400-nm region. At pH 7.5, the spectrum (C) shows a weak band at 370 nm and a trough at 400 nm; at pH 9, strong positive and negative bands (spectrum D) are observed at 360 and 400 nm, respectively. This result may indicate that the A-ring binding also produces a conformational change of the BCD-ring chromophore. Similar studies, reported by Newman and Frank (23), suggest that at pH 7.4 the calcium is bound to the BCD-ring and that the C-4 dimethylamino group of tetracycline is not involved. The A-ring binding occurs only in high pH solutions, with the deprotonation of the C-4 dimethylammonium group (pK_3 = 9.5) being critical.

To study the solvent effect on the complexation, the calcium–tetracycline complex formed in pH 9 buffer was extracted into 1-butanol and ethyl acetate. The circular dichroism spectra were then taken (Fig. 5, spectra B and C). For comparison, the spectrum obtained in pH 9 buffer (spectrum A) is also included in the figure. Besides the A-ring binding as indicated by the 262-nm band in all three spectra, a remarkable inversion of the peaks at 360 and 400 nm is observed as the complex is extracted into nonpolar solvents. This phenomenon resembles the reciprocal relationship found in the circular dichroism studies of adenosine 5-mononicotinate (24). Adenosine has an absorption at 259 nm, whereas nicotinate absorbs at 264 nm. In adenosine 5-mononicotinate, stacking of the two aromatic moieties results in a reciprocal relationship where the 259-nm band becomes more negative as the stacking becomes more prominent. In the calcium–tetracycline complex, the inversion of the 360- and 400-nm bands may indicate a certain interaction between the two tetracycline molecules in nonpolar solvents. In aqueous solution, this interaction becomes less likely because of the interaction of water with the complex.

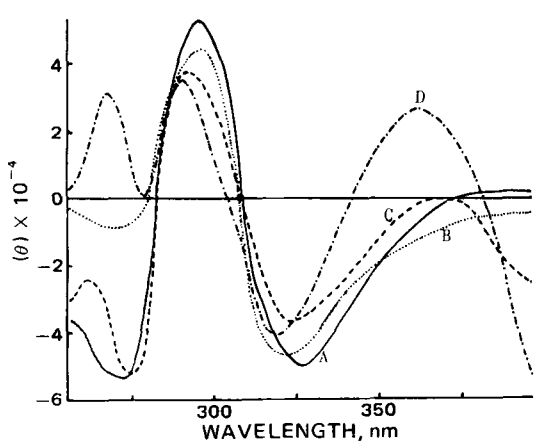

Figure 4—*Circular dichroism spectra of 1 × 10⁻⁴ M tetracycline in pH 7.5 buffer (A), 1 × 10⁻⁴ M tetracycline in pH 9 buffer (B), 1 × 10⁻⁴ M tetracycline and 0.01 M calcium chloride in pH 7.5 buffer (C), and 1 × 10⁻⁴ M tetracycline and 0.01 M calcium chloride in pH 9 buffer (D).*

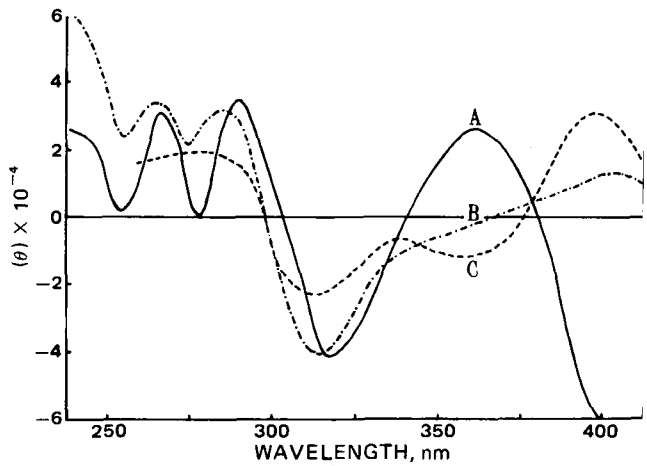

Figure 5—*Circular dichroism spectra of 0.01 M calcium chloride and 1 × 10⁻⁴ M tetracycline in pH 9 buffer (A), 1-butanol (B), and ethyl acetate (C).*

The effect of barbital sodium on the circular dichroism spectrum of the calcium–tetracycline complex in ethyl acetate is shown in Fig. 6. The positive band at 400 nm of the binary complex is diminished and becomes negative in the presence of barbital sodium. However, the 360-nm band increases with increasing barbiturate concentration. These results are consistent with the data shown in Fig. 5 since, in this case, the interaction between the tetracycline molecules is no longer possible as one tetracycline molecule is replaced by the barbiturate.

Figure 7 shows the circular dichroism spectrum of the ternary barbital complex in 1-butanol. The sharp peaks at 260 and 270 nm strongly indicate that, in the ternary complex, the calcium ion is bound to tetracycline at the A-ring binding site. A similar spectrum also was obtained for the ternary complex involving acetate as a ligand. However, the spectrum

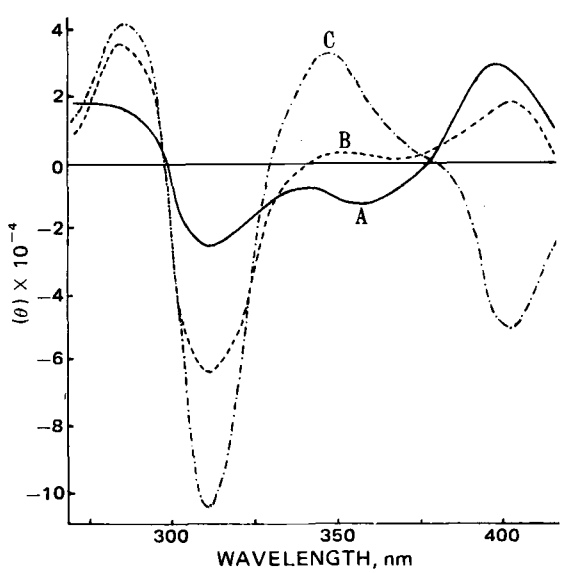

Figure 6—*Effect of barbital sodium on the circular dichroism spectrum of 0.01 M calcium chloride and 1 × 10⁻⁴ M tetracycline in ethyl acetate. Key (concentration of barbital sodium): A, 0; B, 0.01 M; and C, 0.05 M.*

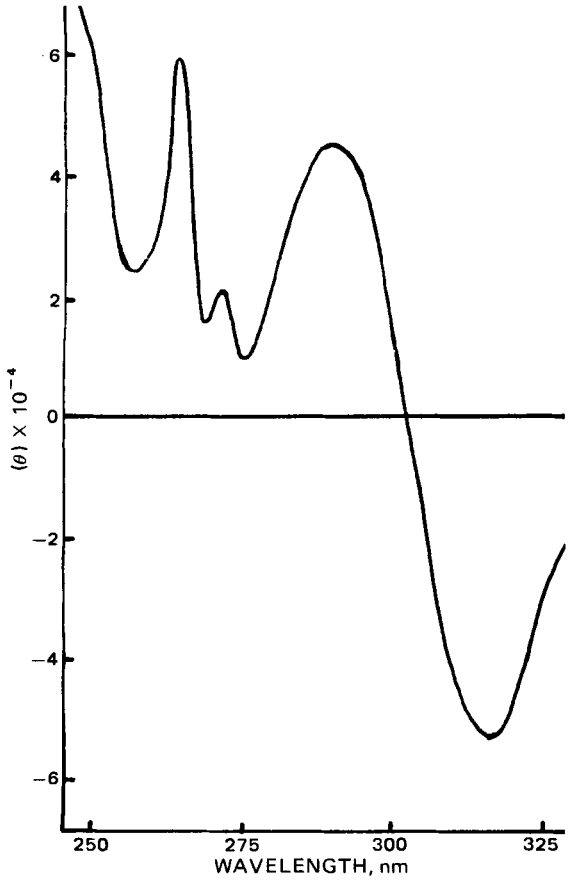

Figure 7—*Circular dichroism spectrum of the ternary calcium–tetracycline–barbiturate complex in 1-butanol (concentration of tetracycline was 1 × 10⁻⁴ M).*

for the ternary complex involving tryptophan cannot be obtained, because L-tryptophan exhibits strong absorptions in the low wavelength region.

DISCUSSION

Fluorometric analysis of tetracycline analogs, a method widely used in many laboratories, involves essentially the extraction of a highly fluorescent tetracycline complex. For tetracycline to be strongly fluorescent, the molecule has to maintain a planar structure in its BCD-ring-conjugated system. This structure may be achieved by rigid binding at the β-diketone system of the tetracycline molecule, as experimentally demonstrated by pH studies of calcium–tetracycline binding. As shown in Fig. 1, at pH 7.5, where the calcium ion is predominantly bound to the β-diketone groups, the complex exhibits the highest fluorescence. At pH 9 or above, the calcium–tetracycline complex is noticeably different from that of pH 7.5, as indicated by the fluorescence, solvent extraction, and circular dichroism studies. The circular dichroism studies, in particular, suggest that the binding occurs at the A-ring binding site. These results are in agreement with other studies (19, 20, 22, 25).

The calcium–tetracycline complex formed in basic solution produces lipid-soluble ternary complexes with barbital sodium and L-tryptophan. These compounds are very effective for tetracycline extraction. A structure of the ternary barbiturate complex was proposed by Kohn (12) (Structure II), but this arrangement is rather unfavorable considering the geometry of the tetracycline molecule. X-ray studies (26) on the structure of 4-hydroxytetracycloxide have shown that the A-ring is nonplanar with respect to the BCD-ring and that chelation at the C-1 and C-12 groups is quite impossible because of their different orientations. In tetracycline, under basic conditions, the C-4 dimethylamino and the C-12a hydroxyl groups rotate toward each other and force the A-ring away from the BCD-plane. This structural change not only results in a nonplanar relationship among the C-11, C-12, and C-1 groups but also creates an unfavorable situation at the C-1 and C-12 carbonyl groups.

In the present study, the calcium ion in the ternary complex is proposed to chelate to the C-4 dimethylamino and the C-12a hydroxyl functions because:

1. While barbital sodium induced the tetracycline extraction at pH 9, it showed no effect at pH 7.5 on either the fluorescence or extraction of the calcium–tetracycline complex, which indicates that barbital sodium does not affect the BCD-ring binding between calcium and tetracycline.
2. The circular dichroism spectrum for the ternary barbital complex strongly suggests the A-ring binding.
3. Circular dichroism studies (25) indicated that the calcium ion, even in large excess, does not exhibit A-ring binding with 4-dedimethylaminotetracycline at pH 9.
4. Kohn (12) reported that the use of barbital sodium is practically ineffective in the attempt to extract 12a-deoxytetracycline, 4-dedimethylaminotetracycline, and 4-epitetracycline.

The binding of calcium to the A-ring does not preclude the BCD-ring binding. Simultaneous binding of the calcium ion to the two binding sites can maintain the planar condition of the BCD-ring and, therefore, result in strong fluorescence. This reaction may explain the strong fluorescence of the barbiturate complex in basic solution. The tryptophan complex is equally effective in extraction but less fluorescent than the barbiturate complex, presumably because of the steric effect of the rather large tryptophan molecule, which may hinder the chance of the BCD-ring binding.

Tetracycline is known to have remarkable resistance toward metabolic changes (2). The chemical degradation, however, may present potential problems for the assay of tetracycline in biological fluids. The principal degradation products of tetracycline are anhydrotetracycline, 4-epitetracycline, and some oxidation and basic degradation products. Under basic conditions, chlortetracycline rapidly converts to isochlortetracycline, in which the C-6 hydroxyl group interacts with the β-diketone system to form a five-member ring including the C-6–O–C-11 linkage. The reaction is also known to result in a shift of the fluorescence maximum from 520 to 425 nm (27). A similar reaction, although less rapid, has been reported for other tetracyclines having C-5 or C-6 hydroxyl groups. For example, oxytetracycline is known to form the C-5–O–C-12 linkage under basic conditions (3).

In the present study, certain oxidation products were observed, although not yet identified, during the experiments. The degradation, which occurs rapidly under light, give a purple-colored water-soluble product. Based on the examination of the characteristics of these degradation products and of the calcium–tetracycline–barbiturate complex, it appears that Kohn's (12) method is quite specific for the assay of the active form of tetracycline. Serious structural changes in the tetracycline molecule, such as the chlortetracycline–isochlortetracycline reaction, affect the fluorescence spectrum greatly. Yet the fluorescence of the ternary complex is measured at about 510 nm, the fluorescence maximum common to all active tetracyclines. Structural change may also change the A-ring conformation, thus preventing the formation of the ternary complex. This reaction may explain why Kohn's method is not effective for the analysis of oxytetracycline, which undergoes similar degradation under basic conditions. In the case of epimerization, the ternary complex cannot be formed because of the unfavorable configuration of the C-4 dimethylamino group.

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Degradative Behavior of a New Bronchodilator, Carbuterol, in Aqueous Solution

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Abstract □ The degradation kinetics of carbuterol in aqueous solution were investigated at 85° and constant ionic strength over the pH 0.25–13.3 range under anaerobic conditions. The results demonstrated a complex kinetic pattern involving specific acid and specific base catalyses at the pH extremes. Degradation resulted primarily from intramolecular catalysis and indicated that both the protonated and unprotonated phenolic groups participated in the reaction. High-pressure liquid chromatography was used to isolate carbuterol and its degradation product. Mass spectrometric examination showed that the degradation product was a cyclized derivative formed by intramolecular attack of the phenoxy group on the ureido carbonyl with ammonia expulsion. The apparent activation energy for carbuterol at pH 4.0 and 10.0 was 22.3 and 11.7 kcal/mole, respectively. The agreement between the calculated theoretical pH–rate profile and the experimental points supports the hypothesis presented concerning the reactions involved in carbuterol degradation.

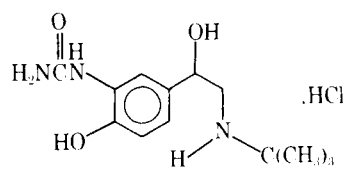
Keyphrases □ Carbuterol—degradation kinetics in aqueous solution, pH effect □ Degradation kinetics—carbuterol in aqueous solution, pH effect □ Kinetics—carbuterol degradation in aqueous solution, pH effect □ Bronchodilators—carbuterol, degradation kinetics in aqueous solution, pH effect

Carbuterol, [5-[2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxyphenyl]urea hydrochloride (I), is a new potent bronchodilator. It is stable in solid admixtures, capsules, and tablets. Like other bronchodilators of similar structure, carbuterol oxidizes readily in aqueous solution (1, 2). Unlike other similar bronchodilators, carbuterol exhibits significant solution degradation under anaerobic conditions in the presence of antioxidants. This study investigated the unusual degradative behavior of carbuterol in aqueous solution.

EXPERIMENTAL

Materials—All chemicals were analytical reagent grade.

Buffers—Hydrochloric acid–potassium chloride (pH 0.25–2.0), citrate buffer (pH 3.0–5.5), succinate buffer (pH 6.0), phosphate buffer (pH 6.5–8.0), borate buffer (pH 8.5–9.0), carbonate buffer (pH 9.35–11.0), trisodium phosphate buffer (pH 12.0), and sodium hydroxide–potassium chloride (pH 13.0–13.3) were used. The buffers were freshly prepared by dissolving hydrochloric acid, citric acid, succinic acid, monobasic sodium phosphate, boric acid, sodium bicarbonate, tribasic sodium phosphate, or sodium hydroxide together with potassium chloride in distilled water and adding concentrated sodium hydroxide to achieve the desired pH. The buffers were 0.1 *M* with respect to hydrochloride, citrate, succinate, phosphate, borate, carbonate, and hydroxide ions (except when buffer effects were investigated) and were adjusted to an ionic strength of 0.5 with potassium chloride (except when the primary salt effect was investigated).



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High-Pressure Liquid Chromatographic (HPLC) Isolation of Degradation Product—The liquid chromatograph[1] was fitted with a precision photometer at 254 nm. The column, 2 mm i.d. × 1 mm, was packed with strong cationic exchange[2] resin and was operated at ambient temperature, a pressure of 900 psig, and a flow rate of 1 ml/min.

The mobile phase was 0.01 *M* borate buffer containing 0.1 *M* anhydrous sodium sulfate adjusted to pH 10.0 with sodium hydroxide. The concentration was normal at about 2.5 mg/ml; the injection size was 5 μl in aqueous solution. The attenuation was 64×10^{-2}, and the chart speed was 2.54 cm/5 min.

Several injections of degraded solution were placed on the column, and the decomposition product was collected as it was eluted. The solution was evaporated to dryness, and the residue was washed several times with aliquots of methanol. The methanol subsequently was evaporated to dryness, and the residue was submitted for analysis by mass spectrometry.

[1] Dupont 820.
[2] Dupont 820960002.

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