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Note added in proof: The authors of a recent Communication in *J. Pharm. Sci.* [**65**, 1262(1976)], accepted after this research article, reported the formation of a heptafluorobutyramide derivative of pilocarpine. The chemical structure of pilocarpine would seem to preclude formation of an amide. The evidence presented in this research article indicates that either the 2′- or 4′-position of the imidazole ring is acylated with heptafluorobutyric anhydride when the reaction is catalyzed with triethylamine. Since the authors of the Communication employed reaction conditions similar to those presented in this article, it is likely that they formed the same acylated derivative of pilocarpine.

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Circular Dichroism Spectra of Tetracycline Complexes with Mg^{+2} and Ca^{+2}

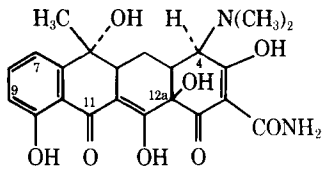
E. C. NEWMAN [*] and C. W. FRANK [x]

Abstract □ The study of Ca^{+2} and Mg^{+2} complexes of tetracycline in buffered solution was undertaken to determine their stoichiometry and the chelation sites. Circular dichroism was used to follow complex formation. Modified tetracyclines, in which potential complexation sites were blocked, were used to determine the participation of particular sites in complexation. Calcium formed a 2:1 metal-ion to ligand complex, while the magnesium complex formed at a 1:1 ratio. Formation of the calcium complex involved addition of one metal ion to the C-10, C-11 site with subsequent addition of a second metal ion at the C-12, C-1 site. The magnesium chelate occurred at the C-11, C-12 β-diketone site.

Keyphrases □ Tetracycline—complexes with Ca^{+2} and Mg^{+2}, circular dichroism spectral investigation of binding properties □ Metal-ion complexes—Ca^{+2} and Mg^{+2} with tetracycline, circular dichroism spectral investigation of binding properties □ Complexes, metal ion—Ca^{+2} and Mg^{+2} with tetracycline, circular dichroism spectral investigation of binding properties □ Spectrometry, circular dichroism—investigation of binding properties of tetracycline complexes with Ca^{+2} and Mg^{+2} □ Antibiotics—tetracycline, complexes with Ca^{+2} and Mg^{+2}, circular dichroism spectral investigation of binding properties

The mode of action of the tetracycline (I) antibiotics is dependent upon the presence of certain metal ions, and the metal-ion complexation of these drugs has been the subject of numerous investigations. The complex chemistry of the tetracyclines and the large number of potential binding sites have led to much confusion as to the location of these sites. A major contributing factor in this problem has been the failure of the commonly used spectroscopic techniques to pinpoint the complexation centers. Circular dichroism, however, seems to be capable of filling this void, as indicated by recent work (1–4).

From an antimicrobial standpoint, the tetracycline complexes with Mg^{+2} and Ca^{+2} are probably the most important, because the concentrations of these cations



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and the large formation constants of their complexes with tetracycline dictate that *in vivo* tetracycline would exist as one of these two complexes.

The tetracycline complexes of Mg^{+2} and Ca^{+2} were studied in pH 7.4 buffered 90% methanol, and differences found in the circular dichroism (as well as fluorescence and UV–visible) spectra were attributed to different conformations and thus different binding sites for the two complexes (1). A four-coordinate binding involving positions C-11, C-12, C-2, and C-3 was proposed for the Ca^{+2} complex; the Mg^{+2} complex was thought to be at a different, unspecified site.

Mitscher *et al.* (2) concluded that, in aqueous solutions of pH 7.4 or below, Ca^{+2} and Mg^{+2} both bind somewhere at the B, C, or D complexation site (C-10, C-11; C-11, C-12; or C-12, C-1) of tetracycline. Above pH 7.5, Ca^{+2} binds between the C-4 dimethylamine group and the C-12a hydroxyl group, but Mg^{+2} cannot.

EXPERIMENTAL

Circular Dichroism Spectra—All circular dichroism spectra were recorded on an optical rotatory dispersion–circular dichroism instrument[1]. Concentrations of tetracyclines were always 1×10^{-4} *M*.

[1] Cary 60.

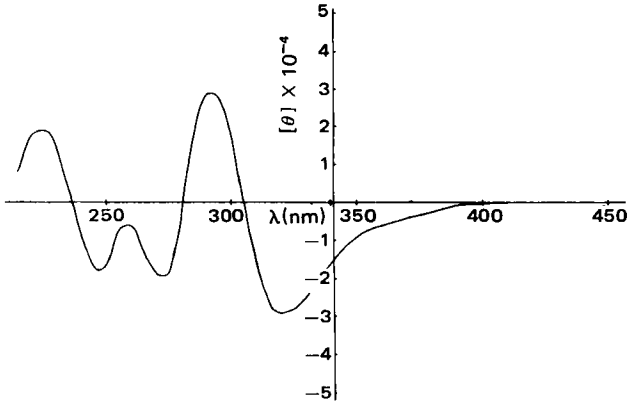

Figure 1—*Circular dichroism spectrum of tetracycline in methanol–water (90:10).*

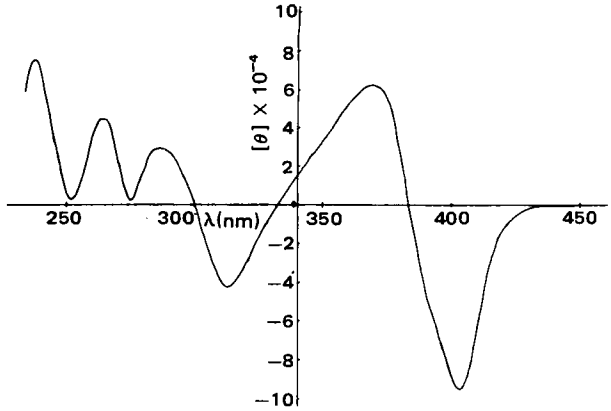

Figure 2—*Circular dichroism spectrum of the calcium complex of tetracycline in methanol–water (50:50).*

The buffer was 0.1 *M* tromethamine, adjusted to pH 7.4 with concentrated hydrochloric acid. The circular dichroism spectra of solutions in this buffer were identical to those of solutions in 0.1 *M* KCl adjusted to pH 7.4, indicating no buffer interference. Solutions were prepared by adding 1 ml of 2.5×10^{-3} *M* tetracycline and an aliquot of 1.25×10^{-2} *M* metal ion to give the desired metal-ion to ligand ratio, and these solutions were diluted to 25 ml with the buffer.

Modified Tetracyclines—Dedimethylaminotetracycline was prepared according to the method of Boother *et al.* (5). The 2-cyanotetracycline was prepared according to the method of Soder and Siedel (6).

RESULTS AND DISCUSSION

The circular dichroism spectrum of tetracycline in 90% methanol contained up to seven clearly defined Cotton effect peaks (as compared to two UV–visible peaks). Since there were also seven potential binding sites, it is theoretically possible to assign each peak to a binding site. Unfortunately, most Cotton effects are not as yet assigned. Some Cotton effects, however, can be assigned to particular portions of the tetracycline molecule on the basis of model compounds. Those Cotton effects that are not assigned are at least useful for fingerprint comparisons.

The circular dichroism spectrum of tetracycline (Fig. 1) in pH 7.4 buffer in 90% methanol contained positive Cotton effects at 225 and 291 nm and negative peaks at 247, 273, and 321 nm with a weak shoulder in the 360-nm region. The two negative peaks at 247 and 273 nm were assigned to the combination of two Cotton effects centered at about 260 nm, one positive and the other negative. One of these two peaks resulted from the β-diketone system of the A-ring, while the other resulted from the phenolic β-diketone system of the B-, C-, and D-rings.

A comparison of the circular dichroism spectra of the complexes of tetracycline and dedimethylaminotetracycline serves to establish the possible involvement of the C-4 dimethylamino group in complexation. Similarly, the use of 2-cyanotetracycline can be used to determine the involvement of the C-2 amide group.

Ca^{+2} Complex of Tetracycline—As Ca^{+2} was added to a buffered solution of tetracycline, some striking spectral changes were noted (Fig. 2). The positive Cotton effect at 225 nm underwent a bathochromic shift to 236 nm and was greatly increased in intensity. These effects were complete once the Ca^{+2} concentration gave a 1:1 metal-ion to ligand ratio. The positive peak at 260 nm was shifted to 264 nm and the intensity was enhanced; the 247- and 273-nm peaks were slightly shifted to 251 and 274 nm, respectively, and decreased in intensity. These effects were complete when a metal-ion to ligand ratio of 1:1 was achieved.

The intensity of the positive Cotton effect at 291 nm was not affected by complexation; however, the peak was shifted to 286 nm. This shift did not begin until the metal-ion to ligand ratio was 1:1 and was complete at a 2:1 ratio. The negative Cotton effect at 321 nm was slightly enhanced and shifted to 313 nm at a metal-ion to ligand ratio of 1:1, after which there was no change. Two new Cotton effects, a positive absorption at 370 nm and a negative absorption at 403 nm, developed from the shoulder near 360 nm and became very intense. These Cotton effects remained unchanged after the metal-ion to ligand ratio reached 2:1 (Fig. 3).

These changes are consistent with the stepwise formation of a 2:1 Ca^{+2}–tetracycline complex. The first Ca^{+2} is added to a site that affects the positive peak at 225 nm, the positive peak at 260 nm, and, to a lesser extent, the positive peak at 291 nm.

The circular dichroism spectrum of dedimethylaminotetracycline is presented in Fig. 4. Its similarity to that of tetracycline indicates that the removal of the dedimethylamino group does not produce major conformational changes in the molecule. There were positive Cotton effects at 225, 256, and 284 nm and negative peaks at 246, 264, and 319 nm.

The addition of Ca^{+2} to a solution of dedimethylaminotetracycline buffered to pH 7.4 produced effects in its circular dichroism spectrum similar to those observed with tetracycline (Fig. 5). The positive Cotton effect at 225 nm was shifted to 236 nm, and its intensity was enhanced up to a metal-ion to ligand ratio of 1:1. This behavior was identical to that observed with tetracycline. The positive peak at 256 nm was also enhanced and shifted to 261 nm, while the intensity of the negative peaks at 246 and 264 nm decreased and shifted to 250 and 273 nm, respectively. These changes were essentially complete at a metal-ion to ligand ratio of 1:1. As with tetracycline, the positive Cotton effect at 284 nm was unchanged in intensity while the negative Cotton effect at 319 nm was slightly enhanced and shifted to 307 nm. These changes were complete up to a metal-ion to ligand ratio of 1:1. Two new Cotton effects, a positive absorption at 366 nm and a nega-

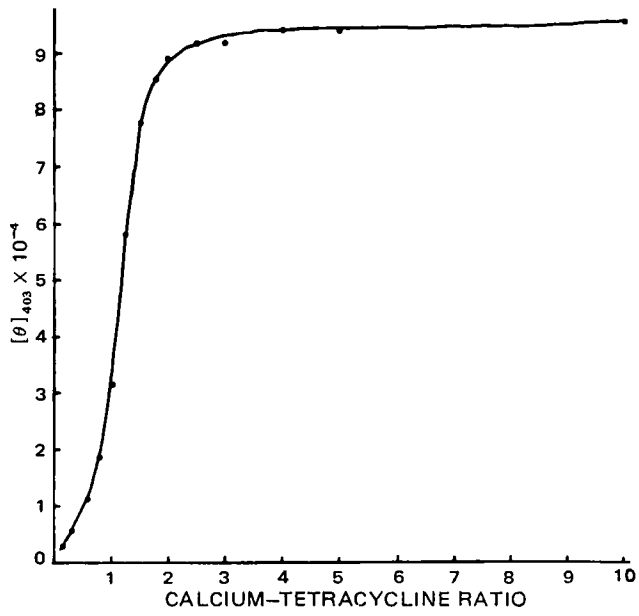

Figure 3—*Mole ratio plot for the calcium–tetracycline complex at 403 nm in methanol–water (50:50).*

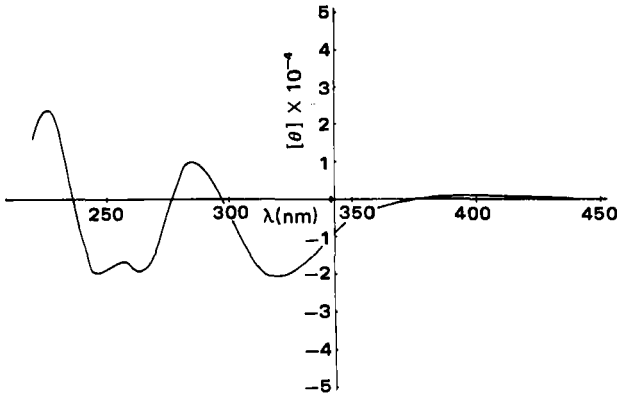

Figure 4—*Circular dichroism spectrum of dedimethylaminotetracycline in methanol–water (50:50).*

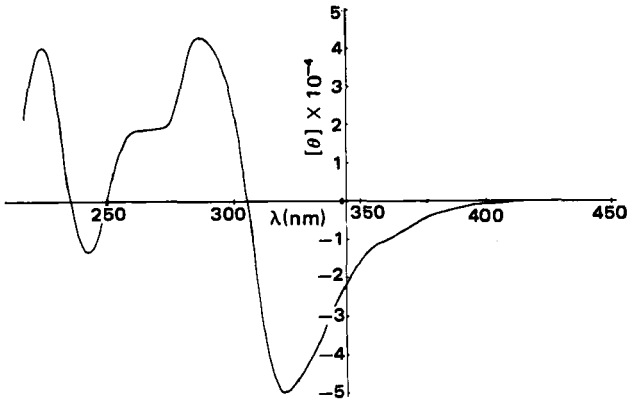

Figure 6—*Circular dichroism spectrum of 2-cyanotetracycline in methanol–water (50:50).*

tive absorption at 403 nm, were produced and became very intense at a metal-ion to ligand ratio of 2:1.

The spectra of the calcium complexes of tetracycline and dedimethylaminotetracycline were strikingly similar at the various stages of the development of the complexes. Both ligands formed 2:1 metal-ion to ligand complexes with Ca^{+2}. It is concluded that tetracycline and dedimethylaminotetracycline complex with Ca^{+2} at the same sites. Furthermore, neither of these sites involves the C-4 dimethylamino group, since this group is absent in dedimethylaminotetracycline.

The circular dichroism spectrum of 2-cyanotetracycline (Fig. 6) was slightly different from the spectra of tetracycline and dedimethylaminotetracycline in that the negative peak at 273 nm was absent. Thus, the positive 260-nm peak appeared as a shoulder on the larger, positive peak at 289 nm. The conversion of the amide group to a nitrile would be expected to change the contribution of the A-ring chromophores to the circular dichroism spectrum in the 260-nm region. The other Cotton effects, the positive peaks at 225, 260, and 289 nm, and the negative peaks at 244 and 320 nm were all present. These peaks were similar in shape and intensity to the corresponding peaks in the tetracycline spectrum. The circular dichroism spectrum was similar to that of tetracycline, indicating that no major conformational changes took place in the conversion of the amide to a nitrile.

The addition of Ca^{+2} to a pH 7.4 buffered solution of 2-cyanotetracycline changed the circular dichroism spectrum (Fig. 7) to one similar to spectra of tetracycline and dedimethylaminotetracycline. The positive Cotton effect at 225 nm was shifted to 236 nm and increased in intensity. This effect was complete when the metal-ion to ligand ratio reached 1:1. In addition, the positive peak at 260 nm was enhanced, the negative peak at 273 nm observed in tetracycline reappeared, and the negative peak at 244 nm was decreased in intensity and shifted to 249 nm. These three changes were complete at a metal-ion to ligand ratio of 1:1 and resulted in a spectrum that was similar to that of tetracycline in the 260-nm region. The 289-nm positive chromophore was only slightly enhanced, as was the 320-nm negative peak. Changes of these two Cotton effects were complete at a metal-ion to ligand ratio of 1:1. As with tetracycline and dedimethylaminotetracycline, two new peaks, a positive absorption at 356 nm and a negative absorption at 393 nm, were produced. These peaks were approximately 10 nm lower than the corresponding peaks in tetracycline and changed only slightly after the metal-ion to ligand ratio reached 1:1.

The circular dichroism spectrum of the calcium chelate of 2-cyanotetracycline was similar to spectra of tetracycline and dedimethylaminotetracycline in the region below 340 nm but differed significantly at longer wavelengths. This finding indicates that the first Ca^{+2} chelates at the same site as the first Ca^{+2} in tetracycline or dedimethylaminotetracycline but that complexation at the other site is incomplete.

The failure of 2-cyanotetracycline to bind the second calcium ion is explained by identifying one A-ring site, or an adjacent site, as the region of interaction for this second cation. The absorptions affected by chelation of the second Ca^{+2} to tetracycline are at 370 and 403 nm, which are assigned to the B-, C-, and D-rings of the tetracycline molecule. Neither of the A-ring sites gives rise to these spectral changes; therefore, assignment of the binding of Ca^{+2} to the C-12, C-1 site accounts for both the long wavelength absorptions and the failure of the 2-cyano derivatives to add the second calcium ion.

The first Ca^{+2} to complex with tetracycline and dedimethylaminotetracycline also complexes with 2-cyanotetracycline. Because the derivative without the C-4 dimethylamino group forms this chelate, sites involving this position (C-4, C-12 and C-3, C-4) are removed as possibilities for the first binding site. Sites involving the C-2 amide (C-1, C-2 and C-2, C-3) are removed as sites because this same complex is formed by 2-cyanotetracycline.

Thus, only three sites (C-10, C-11; C-11, C-12; and C-12, C-1) remain as viable possibilities. Since the second Ca^{+2} chelates at the C-12 position and the calcium ions are chelated by tetracycline at two of three sites, the first Ca^{+2} must bind in the C-10, C-11 site. The C-12, C-1 site must be assigned to the second Ca^{+2} to account for the only partial complexation of this second cation with 2-cyanotetracycline.

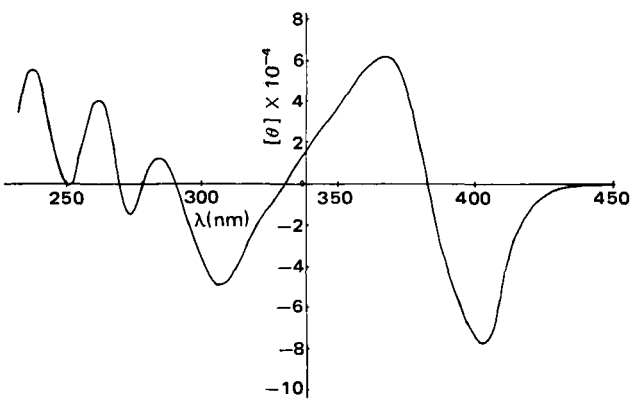

Figure 5—*Circular dichroism spectrum of the calcium complex of dedimethylaminotetracycline in methanol–water (50:50).*

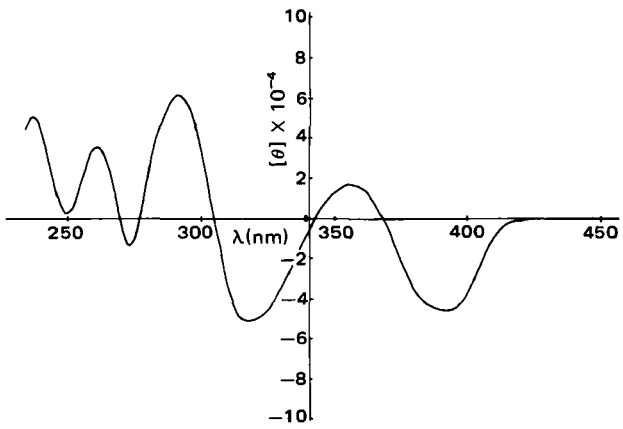

Figure 7—*Circular dichroism spectrum of the calcium complex of 2-cyanotetracycline in methanol–water (50:50).*

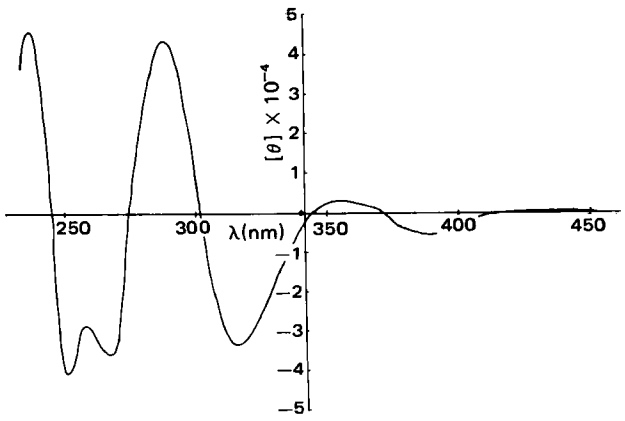

Figure 8—*Circular dichroism spectrum of the magnesium complex of tetracycline in methanol–water (90:10).*

This order of complexation also explains the effect on the longer wavelength region of the circular dichroism spectrum of this molecule.

Mg^{+2} Complex of Tetracycline—The addition of Mg^{+2} to a solution of tetracycline buffered to pH 7.4 produced changes in its circular dichroism spectrum (Fig. 8) that differed from those observed for the Ca^{+2} complex of tetracycline. The 225-nm positive Cotton effect was shifted to 237 nm and was enhanced in intensity, an effect similar to that with Ca^{+2}. The two negative peaks at 247 and 273 nm were also increased in intensity at the expense of the positive 260-nm peak. The opposite effect was noted for the calcium chelate as the positive 260-nm Cotton effect was increased. The positive Cotton effect at 291 nm, which was essentially unchanged by the addition of Ca^{+2}, was greatly increased in magnitude by the addition of Mg^{+2}. The negative peak at 320 nm was only slightly affected by the addition of Mg^{+2}. Two new peaks, a positive absorption at 355 nm and a negative absorption at 390 nm (similar to those produced in the circular dichroism spectrum of the Ca^{+2} complex of tetracycline), formed only to a minor extent.

All changes in the circular dichroism spectrum of the tetracycline complex with Mg^{+2} were complete once the metal-ion to ligand ratio was 1:1 (Fig. 9). Because the spectral changes accompanying the formation of this complex were different from those observed with

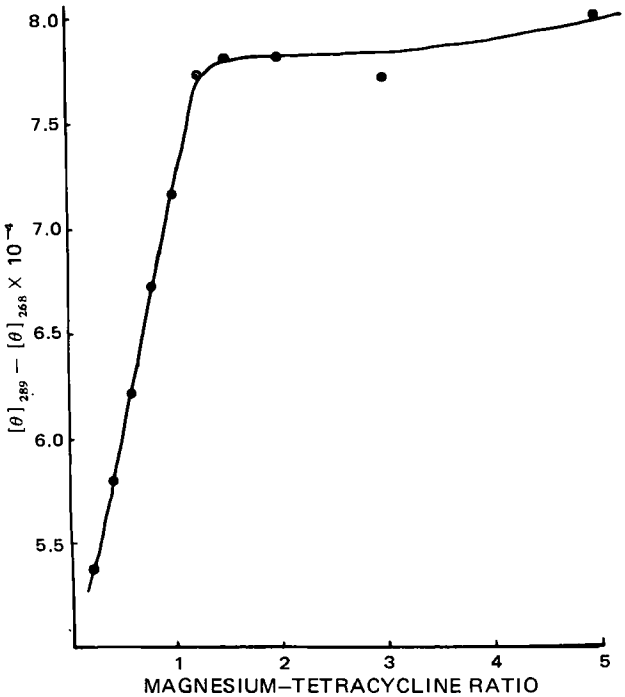

Figure 9—*Mole ratio plot for the magnesium complex of tetracycline in methanol–water (90:10).*

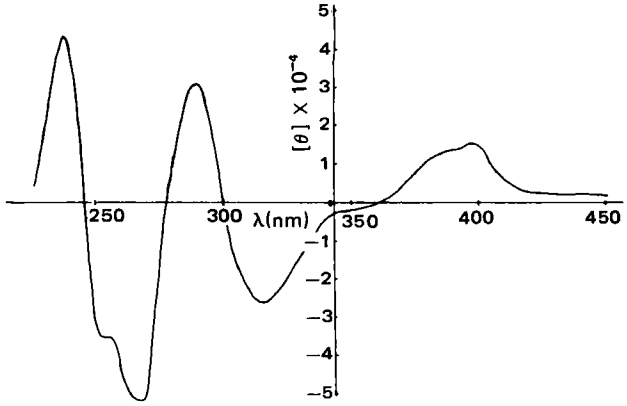

Figure 10—*Circular dichroism spectrum of the magnesium complex of dedimethylaminotetracycline in methanol–water (90:10).*

the addition of metal ion to either site proposed for the tetracycline complex with calcium, a third complexation site is indicated.

When Mg^{+2} was added to a solution of dedimethylaminotetracycline in 90% methanol buffered to an effective pH of 7.4, its circular dichroism spectrum (Fig. 10) was affected in a manner similar to the spectrum of the Mg^{+2} complex with tetracycline. The positive peaks at 246 and 264 nm were enhanced and shifted to 252 and 265 nm, respectively, while the positive Cotton effect at 283 nm was greatly increased in intensity and shifted to 289 nm. The negative peak at 318 nm was slightly increased in intensity and shifted to 316 nm. All these changes were complete at a metal-ion to ligand ratio of 1:1.

A comparison of the circular dichroism spectra of the magnesium chelates of tetracycline and dedimethylaminotetracycline indicates that they are identical; thus, the same complexation site is used in both cases. This precludes binding at any site utilizing the dimethylamino group of C-4.

The changes in the circular dichroism spectrum of 2-cyanotetracycline (Fig. 11) with addition of Mg^{+2} were similar to those of the Mg^{+2} complexes with tetracycline and dedimethylaminotetracycline. The positive Cotton effect at 225 nm was increased in size and shifted to 235 nm. The 260-nm region gave rise to the two negative peaks at 251 and 271 nm, which were increased in intensity, masking the positive peak at 259 nm. The positive peak at 286 nm was shifted to 284 nm and slightly decreased in intensity along with the negative Cotton effect at 320 nm.

Most of these changes took place when the metal-ion to ligand ratio reached 1:1, but minor changes occurred as more Mg^{+2} was added. These data indicate that the 1:1 complex between Mg^{+2} and 2-cyanotetracycline is not as favorably formed as 1:1 complexes of Mg^{+2} with tetracycline and dedimethylaminotetracycline. A comparison of the circular dichroism spectrum of the Mg^{+2} complex with 2-cyanotetracycline to that of tetracycline shows that they are essentially the same and thus indicates the same complexation site. The fact that 2-cyanotetracycline forms this complex indicates that neither of the A-ring sites (C-1, C-2 or C-2, C-3) is used.

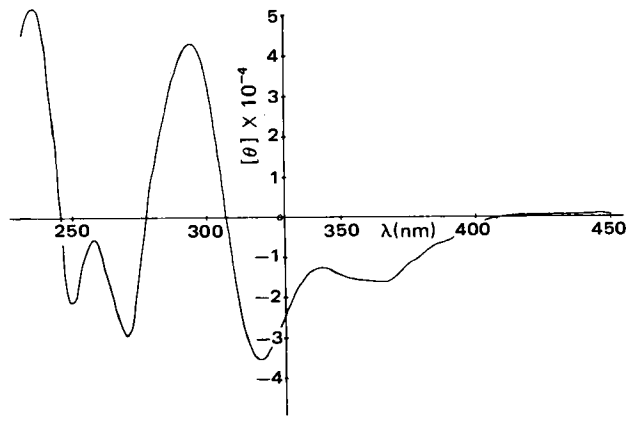

Figure 11—*Circular dichroism spectrum of the magnesium complex of 2-cyanotetracycline in methanol–water (90:10).*

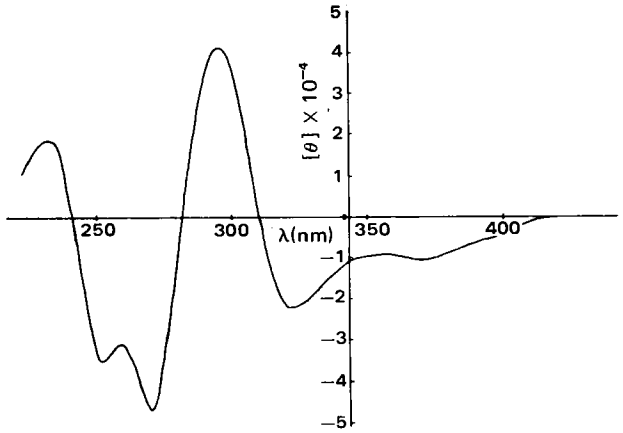

Figure 12—*Circular dichroism spectrum of the magnesium complex of tetracycline in aqueous solution.*

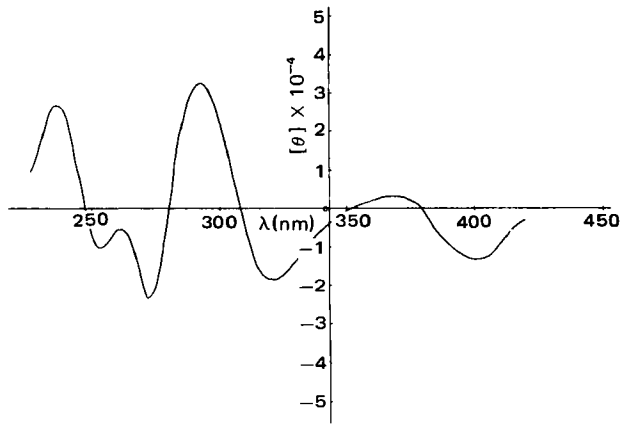

Figure 13—*Circular dichroism spectrum of the calcium complex of tetracycline in aqueous solution.*

Since tetracycline, dedimethylaminotetracycline, and 2-cyanotetracycline all form the same 1:1 complex with Mg^{+2}, there are only three possible sites of complexation: C-10, C-11; C-11, C-12; and C-12, C-1. As stated previously, the calcium ions bind at the C-10, C-11 and C-12, C-1 sites and the Mg^{+2} complex binds at a different position, as indicated by the circular dichroism spectra. Thus, the Mg^{+2} complex of tetracycline binds at the C-11, C-12 β-diketone site.

Comparison of Solvent Systems—The circular dichroism spectrum of the Mg^{+2} complex of tetracycline in aqueous solution (Fig. 12) buffered to pH 7.4 was similar to the corresponding spectrum in 90% methanol. However, the formation of the complex was not favorable in the aqueous solution, since a fourfold excess of Mg^{+2} was necessary to force the complexation. The 225-nm positive Cotton effect was shifted to 232 nm and increased in intensity, the negative peaks at 251 and 272 nm were significantly increased in intensity, and the negative peaks at 293 and 323 nm were slightly affected by complexation. It is apparent from these data that the Mg^{+2} complex of tetracycline is the same in both aqueous and methanolic solutions.

In pH 7.4 buffered solution, the circular dichroism spectrum of the Ca^{+2} complex of tetracycline (Fig. 13) was similar to that of tetracycline in 90% methanol in the wavelength region below 340 nm. But the two Cotton effects at 370 and 403 nm were only slightly developed with a metal-ion to ligand ratio of 4:1. Thus, it appears that the second Ca^{+2} is not complexed significantly in aqueous solution. The positive peak at 225 nm was shifted to 234 nm and increased in intensity, and there was an increase in intensity of the positive 260-nm peak, as attested by the change of the 251- and 270-nm peaks. The positive peak at 292 nm was slightly decreased, while the 322-nm peak was unchanged. As with the Mg^{+2} complex in aqueous solution, the complexation of even the first Ca^{+2} was not as favorable a process in aqueous solution as in methanolic solution.

In pH 7.4 methanolic buffers, tetracycline and several related compounds formed different complexes with Mg^{+2} and Ca^{+2}. Magnesium complexed in a 1:1 ratio at the C-11, C-12 β-diketone site. The complex in 90% methanol was much stronger than the same complex in water, requiring only stoichiometric quantities of metal ion and ligand to achieve essentially 100% complexation. In aqueous solution at pH 7.4, higher concentrations of Mg^{+2} were necessary to complete the complexation.

The calcium chelate of tetracycline in 90% methanol was a 2:1 metal-ion to ligand complex. The two metal ions added stepwise, the first at the C-10, C-11 site and the second at the C-12, C-1 site. Formation of this 2:1 complex was very favorable, requiring only stoichiometric quantities of metal ion and tetracycline to achieve essentially 100% complexation. However, formation of the calcium chelate in pH 7.4 aqueous solution was not as favorable as in 90% methanol. An excess of Ca^{+2} was necessary to force the addition of the first cation onto the C-10, C-11 site, and the second cation did not add to any significant extent. Apparently, in aqueous solution, the removal of water molecules from the hydration sphere of the metal ion is much more difficult; therefore, both steps in the formation of the complex are more difficult. Since the first metal ion must add to the C-10, C-11 site before the C-12, C-1 site is capable of accommodating a cation, incomplete addition of this first cation inhibits addition of the second cation.

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