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Metal Ion–Tetracycline Interactions in Biological Fluids. 9. Circular Dichroism Spectra of Calcium and Magnesium Complexes with Tetracycline, Oxytetracycline, Doxycycline, and Chlortetracycline and Discussion of Their Binding Modes

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Recent studies relative to coordination equilibria of several tetracycline derivatives in blood plasma have shown that the fraction of these antibiotics not bound to proteins is almost exclusively present as calcium and, to a lesser extent, magnesium complexes. Given the metal-to-ligand ratios involved, electrically charged binuclear complexes are predominantly formed at the expense of neutral diffusible mononuclear species, which tends to reduce the bioavailability of the drug. It has subsequently been postulated that structural variations imposed on the parent tetracycline molecule should diversely influence the bioavailability of resulting derivatives through the preferential formation of either type of complex at will. This hypothesis was tested and effectively proved valid for some particular derivatives. However, before future developments can be envisaged to improve tetracycline bioavailability, it appears necessary that the coordination centers of the biologically relevant complexes of these antibiotics with calcium and magnesium be definitely identified. With the use of newly determined stability constants to select conditions likely to favor the formation of specific species in turn, UV–visible absorption and CD spectroscopic studies have first been developed to characterize the various conformations that tetracycline (TC), oxytetracycline (OTC), doxycycline (DOX), and chlortetracycline (CTC) may adopt in aqueous solutions as a function of pH. Similar investigations have then been undertaken concerning calcium and magnesium binding modes, more specifically aiming at the ML and M_2L plasma predominating species. These biologically relevant complexes have been assigned the following chelation sites: Ca_2TC, N4–O12$_a$ and O12–O1; Mg_2TC, N4–O3 and O10–O12; CaOTC, O12–O1; Ca_2OTC, O12–O1 and N4–O12$_a$; CaDOX, N4–O5; Ca_2DOX, N4–O5 and O10–O12; Mg_2DOX, O12–O1 and N4–O3. The effect by which the chlorine substituent at C7 prevents any coordination of the dianionic form of CTC with the two above metals has been shown to be of essentially steric origin. It should finally be noted that the bonds of the OTC complexes with magnesium could not be assigned, this metal catalyzing the degradation of the antibiotic.

Introduction

Like a great number of organic pharmaceuticals, tetracyclines contain electron-donor groups likely to generate stable complexes with metal ions.[2] It is thus not surprising that resulting interactions can induce significant effects in vivo. For example, the impaired gastrointestinal absorption of these antibiotics in the presence of essential metal ions is well documented.[3-5] More specifically, the capacity of a tetracycline to reach its tissue therapeutic target largely depends on the chemical forms into which it is distributed in blood plasma. Recent simulation studies using newly determined complex stability constants have shown that calcium and to a lesser extent magnesium complexes are expected to play a determining part in this respect, free molecules previously considered to be active[6] being in fact restricted to exceedingly low levels.[7-9] Moreover, given the metal-to-ligand ratios that occur in blood plasma, electrically charged binuclear complexes tend to take up a predominant position over neutral diffusible mononuclear species.[9] It was therefore postulated[9] that the partial blocking of one of the potential coordination sites of the tetracycline (TC) molecule, which would reduce the formation of such binuclear species, should result in a better tissue penetration of the drug. This hypothesis was tested and proved valid on the basis of comparisons between simulated and observed distributions of specific TC derivatives.[10,11]

Clearly, a possible approach for optimizing tetracycline bioavailability would consist of introducing in the TC molecule specific modifications likely to favor the formation of predominant calcium or magnesium plasma complexes having predetermined suitable properties. This requires that the coordination sites as well as the structural features of all tetracycline complexes with these two metals be definitely assigned beforehand. In the above-mentioned studies for instance, the substituents involved in 7-chlorotetracycline (CTC)[10] and 6-methylenetetracycline (MTC)[11] were expected to lower the electron density of the C10–C12 diketophenolic moiety. Although doubt has recently been cast[11,12] on the ubiquity of this mode of bonding, the oxygen atoms of this fraction of the TC molecule are indeed commonly considered as the major coordination site for calcium[13-19] as well as magnesium[13,16,17,19] in neutral aqueous solutions. However, apart from this former general agreement, no clear-cut conclusion has yet been put forward as to the binding mode of these metals in their different tetracycline complexes.[3,17,18] Concerning this, it should be pointed out that structural determinations are made uneasy by the coexistence of many species in solution within a large range of concentrations as well as concentration ratios. Due to their particularly complex structures, tetracycline ligands can form complexes of 2:1, 1:1, and 1:2 stoichiometries, some of them being also protonated.[7-12] It is thus difficult to select experimental conditions likely to favor the formation of a single predominant species so as to analyze its particular structure. At least, this would require that all the possible complexes formed between a given metal ion and the tetracycline derivative under study be quantitatively identified in advance, which was far from being the case at the time of the above-mentioned studies.[13-18]

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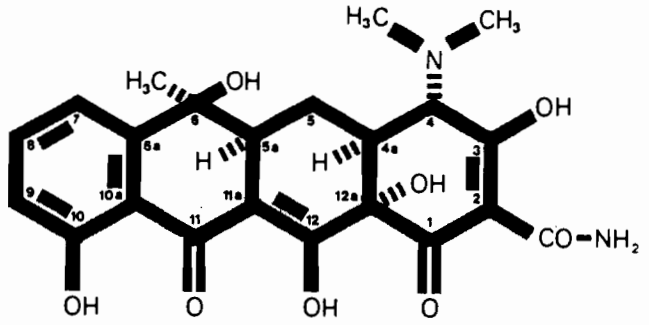
Useful pieces of information were obtained by Stezowski et al.[20] on the conformations displayed by oxytetracycline (OTC) in water, ethanol, and mixed water–ethanol solvents. In their circular dichroism (CD) studies, these authors suggest that the aqueous zwitterionic form of OTC inducing the negative Cotton effect observed near 260 nm should adopt the same conformation as the crystalline free form deduced from an earlier X-ray analysis,[21] which would basically confirm former results obtained in solution by Mitscher et al.[16,17] Nevertheless, no systematic investigation of this kind has been devoted to metal–tetracycline interactions so far. Moreover, it is always hazardous to infer structural features relative to solute species from corresponding X-ray analyses. In the solid state, crystal lattice energies and the necessity of assuming net packing arrangements can indeed render the shape of a molecule substantially different from that it might adopt in solution.

The two main points outlined above have been examined in the preceding paper of this series,[12] where coordinations of calcium and magnesium with two simple TC derivatives, namely 4-(dedimethylamino)tetracycline (DTC) and 6-desoxy-6-demethyltetracycline (DSC), were studied. The specific effects due to the absence of corresponding substituents with respect to TC were rationalized in quantitative (complex stoichiometries, stability constants) as well as qualitative (CD spectra) terms. Also, the available data concerning the main conformations of the various forms of tetracyclines[16,17,21] were critically reviewed.

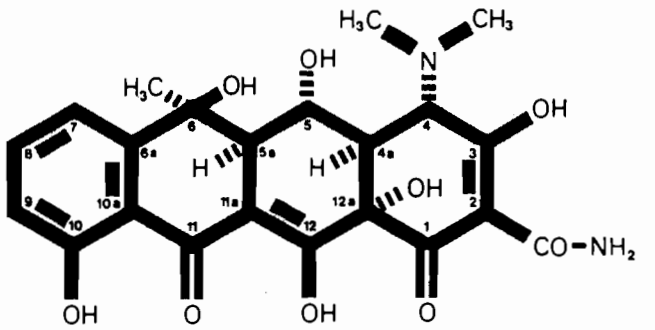
In light of the above results,[12] the present paper deals with the structural analysis of the complexes formed by Ca^{2+} and Mg^{2+} ions with tetracycline (TC; see I), oxytetracycline (OTC; see II), doxycycline (DOX; see III), and chlortetracycline (CTC; see IV) in aqueous solution, using UV–visible absorption and CD spectroscopic techniques.

As outlined above, distinct sets of species may predominate in solution depending on pH as well as on reactant concentrations and concentration ratios. The latter parameters have thus been made to vary so that the largest possible number of biologically relevant complexes were successively examined.

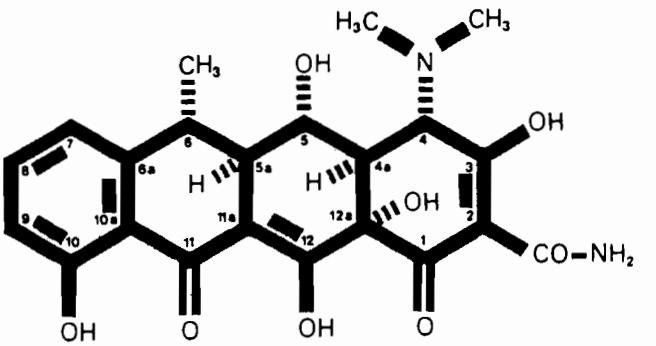
Corresponding conformations and metal binding modes are finally discussed with respect to the present spectroscopic data as well as considerations based on our earlier potentiometric results.[7-10]



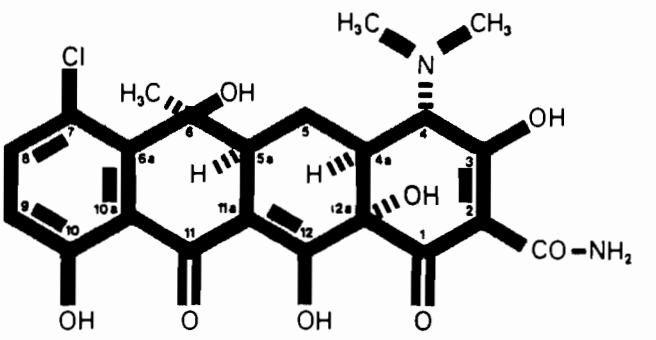
I: tetracycline (TC)



II: oxytetracycline (OTC)



III: doxycycline (DOX)



IV: chlortetracycline (CTC)

Experimental Section

Materials. TC and OTC as free bases and DOX as the hydrochloride were kindly supplied by Pfizer Laboratories, whereas CTC hydrochloride was purchased from Sigma Chemical Co. After their analysis for both antibiotic and mineral acid contents, these products were proved to be sufficiently reliable to be used without further purification. Due to the well-documented instability of tetracyclines in aqueous media,[16,22] especially CTC,[17] fresh solutions were systematically prepared every day and maintained in the dark under an atmosphere of nitrogen.

Sodium hydroxide and hydrochloric acid stock solutions used to impose specific pH values on experimental samples were prepared and standardized as previously described.[12] Sodium chloride employed as a background electrolyte was a Merck pro analysis reagent.

Calcium and magnesium chloride stock solutions were prepared and analyzed as reported in preceding parts of this series.[7-9]

Spectroscopic Measurements. Absorption spectra were determined in the 200–450-nm range by means of a double-beam Perkin-Elmer 554 spectrophotometer. Circular dichroism spectra were recorded with the help of a Jobin-Yvon Dichrographe III spectrometer controlled by an Apple IIe microcomputer, using fused quartz 0.5-mm cylindrical cuvettes. Results are expressed in molar ellipticity $[\theta] = 3300\ \Delta\epsilon$ deg·cm^2·dmol^{-1}.

As specified in the Introduction, reactant concentrations and concentration ratios relative to each metal–ligand system were made to vary within the limits imposed by the sensitivity of the apparatus, so as to define specific conditions suitable for the formation of complexes of predetermined stoichiometries. Practically, pH values and reactant concentrations expected to correspond to the predominance of any of these particular species were selected from simulated distributions of each antibiotic in the absence or presence of metal as a function of pH, using our plotting updated version (COMPLOT) of the COMICS program.[23] The formation constants on which these calculations were based are shown in Table I.

From the experimental point of view, potentiometric titrations had to be carried out simultaneously to spectral measurements for each metal-to-ligand concentration ratio investigated. Successive samples of solution were taken at pH values characteristic of the maximum percentage of each species to be studied. The volumes of these samples were chosen sufficiently small with respect to the titrate initial one for them to be neglected in the COMPLOT calculations.

Potentiometric Measurements. Potentiometric titrations parallel to spectroscopic determinations were carried out by using a Metrohm E 605 pH meter equipped with a Metrohm combined microelectrode. In accordance with previous specifications,[9-12] this electrode was calibrated on the concentration scale in the presence of NaCl, 0.15 mol·dm^{-3}, at room

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Table I. Formation Constants Used in the Calculations

system	complex stoichiometry	log β	ref
proton–TC	LH	9.052	7
	LH_2	16.323	
	LH_3	19.485	
calcium–TC	M_2L	8.671	7
	ML_2	8.731	
	ML_2H	17.618	
	ML_2H_2	25.540	
magnesium–TC	M_2L	7.740	8
	MLH	12.657	
	ML_2	8.698	
	ML_2H	17.597	
	ML_2H_2	25.275	
proton–OTC	LH	8.665	7
	LH_2	15.775	
	LH_3	18.996	
calcium–OTC	ML	4.462	7
	M_2L	7.884	
	ML_2	8.385	
	ML_2H	16.654	
	ML_2H_2	24.625	
magnesium–OTC	ML	4.874	8
	M_2L	8.346	
	ML_2	9.560	
	ML_2H	17.423	
	ML_2H_2	24.095	
	M_2LH	14.970	
proton–DOX	LH	8.676	7
	LH_2	16.090	
	LH_3	19.186	
calcium–DOX	ML	5.600	7
	M_2L	8.885	
	MLH	13.058	
	ML_2H_2	25.263	
magnesium–DOX	M_2L	8.546	8
	MLH	12.988	
	ML_2H	17.420	
	ML_2H_2	25.559	
proton–CTC	LH	8.698	10
	LH_2	15.714	
	LH_3	18.794	
calcium–CTC	MLH_2	17.984	10
	ML_2H_2	25.230	
magnesium–CTC	MLH	11.515	10
	ML_2H_2	23.995	

temperature to match the conditions of the spectral measurements. It is to be noted that the use of these experimental conditions implies that pH values taken as corresponding to the maximum percentages deduced from constants calculated at 37 °C are only approximate.

Results and Discussion

As a foreward to this chapter, it is worth recalling the conclusions reached in the preceding part of this series[12] about the conformations that tetracyclines are likely to assume in aqueous solutions.

If a plane is arranged to approximately accommodate the B, C, and D rings of the TC molecule, its C1, C2, and C3, and amide carbons will lie above this plane in what we have termed the "extended" conformation.[12] This conformation, designated A by Mitscher et al.,[16,17] is adopted by tetracyclines in basic aqueous solutions, with the notable exception of DTC[12] and to a lesser extent OTC, for which the situation is not clear.[17] A distinct conformation is found in acidic to neutral aqueous media, referred to as conformation B by Mitscher et al.[16,17] It consists of a structure similar to conformation A, in which the A ring is somewhat twisted so as to relieve the steric crowding between the bulky solvated C4 dimethylammonium and $C12_a$ hydroxyl groups. This conformation, which we have accordingly called the "twisted" conformation[12] (in contrast with its previous "extended" designation by Martin[19]), is associated with a high-intensity negative Cotton effect at 262 nm.[16]

While there is no need to develop further arguments outlined in the Introduction, it may be worth pointing out that the conformations of Mitscher et al. are pertinent to aqueous solutions and that they may a priori differ[20,24,25] from conformations determined in the solid state.[21,26-29] Nevertheless, it is most likely that the tendency to form the N4H$^+$–O3 hydrogen bond characterized in the crystalline structure attributed to conformation B[27] also holds in water.

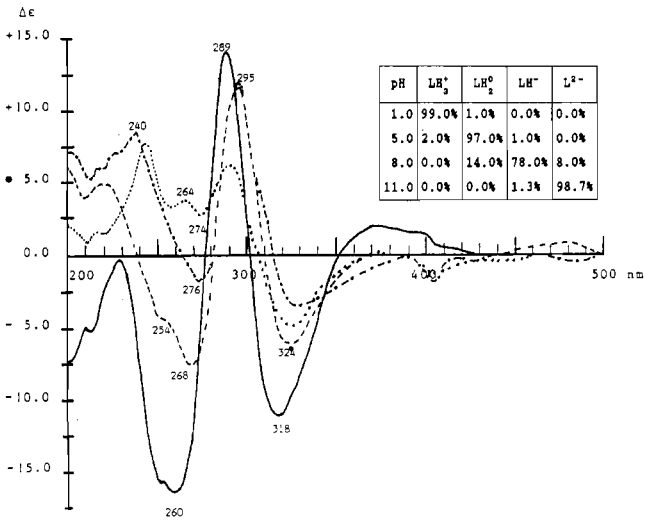

Figure 1. Circular dichroism spectra of TC (C_{TC} = 0.0416 mmol·dm^{-3}) at various pH values: 1.0 (—); 5.0 (- - -); 8.0 (—·—·—); 11.0 (···).

Two important points should also be borne in mind: (i) as pointed out in our recent study,[12] the C10–C12 donor group is to be considered as an important metal coordination center for the tetracycline series, but the N4 atom can easily compete with it for the binding of the first metal ion when the pH is raised, especially in complexes of equimolar stoichiometry; (ii) the chelation of metal ions between the O10 and O12 oxygens has no significant effect on the overall structure of tetracyclines, whereas metal coordination to the A ring donors may stabilize or destabilize conformation A.[12,16,17]

It may finally be worth recalling that the first proton to dissociate from a tetracycline in aqueous solution refers to the OH3 dicarbonylmethane system and that the second is lost by the OH12 of the phenolic diketone moiety, whereas the third one corresponds to the deprotonation of the C4 dimethylammonium group.[19,30]

Let us now examine our own results in light of the above reference data.

Tetracycline. Changes in the CD spectra relative to metal-free TC solutions as a function of pH (Figure 1) are quite characteristic of the conformations involved. Indeed, LH_3^+ as well as LH_2^0 species exhibit a strong negative band at 260 nm, which is indicative of conformation B (see above), while the fully deprotonated L^{2-} form has this band reversed. Like DSC,[12] TC thus occurs in conformation B for pH values less than 8 and then turns to conformation A in more basic solutions where the latter structure is thought to be stabilized by the putative N4–HO12_a hydrogen bond, which forms as the C4 dimethylammonium group dissociates.[16]

As can be seen in Table I, TC gives rise to metal complexes of various stoichiometries. In the first approach, the increments in stability involved in the successive protonation steps of the 1:2 metal-to-ligand ratio species can provide useful information on the possible metal binding modes in this series. For example, the addition of a proton to ML_2 corresponds to an increase in stability of 8.89 for calcium and 8.90 for magnesium, whereas the further protonation of ML_2H into ML_2H_2 implies respective increments

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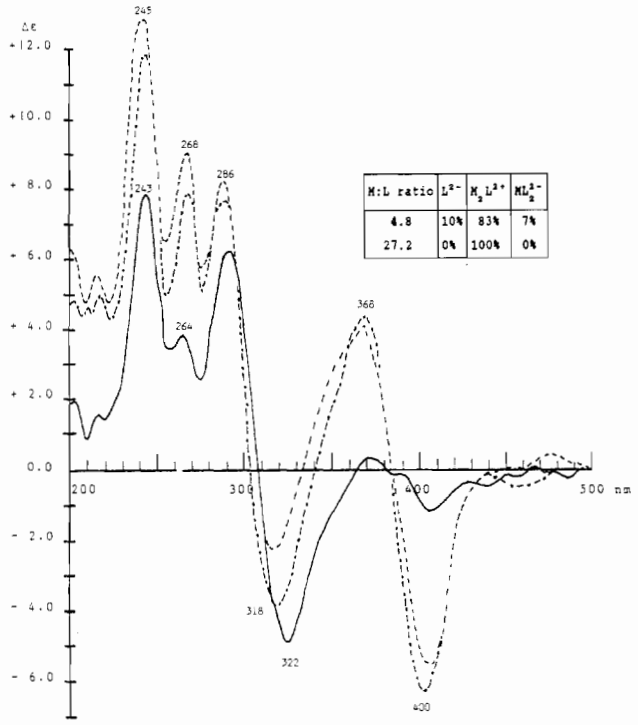

Figure 2. Circular dichroism spectra of TC (C_{TC} = 0.0416 mmol·dm^{-3}) at pH 11.0 in the absence of metal (—) and in the presence of calcium, 0.20 mmol·dm^{-3} (---) and 1.13 mmol·dm^{-3} (-·-·-). Species whose percentage is less than 1% are not mentioned in the table inset.

of 7.92 and 7.68 only. The former values compare well with the protonation constant relative to the C4 dimethylamino group (9.05); in contrast, the latter would rather correspond to that of the O12 site (7.27). If we assume that these two coordination sites are sufficiently distant from each other so that the dissociation process remains unaffected, the above observation suggests that the two ligands involved in the ML_2 species are bound via different modes. In other words, as was previously postulated for DSC,[12] one ligand would coordinate the metal ion via its N4 atom while the other would bind it through its BCD ring system. It would have been of interest to test this hypothesis on spectroscopic grounds, but due to technical difficulties outlined in the Introduction, ML_2H and ML_2H_2 percentages remained too low within concentration limits compatible with the sensitivity of the spectrometer for definite conclusions to be attainable from CD considerations.

Of comparatively much greater biological significance are the binuclear species that take up leading positions in TC plasma distribution,[7,8] and suitable conditions were found to render them predominant in solution. Moreover, their formation results in distinct conformational changes for the TC molecule.

With calcium for example, the shape of the CD spectra obtained at pH 9 and above remains basically the same as that relative to metal-free TC solutions in the wavelength region below 300 nm, but the formation of the M_2L species does entail a very drastic increase of intensity of the positive Cotton effect observed at 268 nm (Figure 2). This result clearly shows that one of the two metal ions bound to TC "pins" its molecule in conformation A, presumably by chelating its N4 and $O12_a$ atoms; it is indeed the most effective way for a metal ion to stabilize this TC conformation. Incidentally, a correlative increase of intensity may be noted for the positive bands near 240 and 285 nm, indicative of BCD ring interactions.[16] In addition, two new Cotton effects, a positive absorption at 368 nm and a negative one near 405 nm, develop from the shoulder around 360 nm and become very intense above pH 9 (Figure 2). These two bands, which are also assigned to the BCD ring chromophore,[14-16] were previously observed by Newman and Franck[15] at pH 7.4 in a 50:50 methanol–water mixture, where they remained at their maximum of intensity after the calcium-to-TC ratio reached 2:1. In contrast, they were only slightly developed at the same pH in water, even with calcium-to-TC ratios as high as 4:1.[15] These bands were also observed by Day et al.[14] for aqueous TC in the presence of a large excess of calcium at pH 9, but not at pH 7.5.[31] Interestingly, the present CD spectra obtained at pH 8 (not shown here) do not significantly differ from those relative to metal-free TC solutions at this pH (Figure 1). At this stage, two pieces of additional information are still necessary to reach clear-cut conclusions: (i) From a comparison made between TC and 2-cyanotetracycline (2-NC-TC) calcium complexes, Newman and Franck[15] logically deduced that the two new Cotton effects above are indicative of an A-ring binding that affects the BCD ring system simultaneously; this led these authors to propose the O12–O1 atoms as the only possible coordination site for the second calcium ion to bind TC.[32] (ii) Why these bands are observed from pH 7.4 in the 50:50 methanol–water mixture while they only appear above pH 9 in aqueous solution also remains to be elucidated: clearly, this must stem from the fact that the order of the O12 and N4 protonation steps (see above) is reversed from one solvent to the other.[19] Indeed, on account of the different dielectric constants of the two media, the water-specific 8:1 ratio in favor of the dissociation of the OH12 site with respect to that of the dimethylammonium group becomes 1:3 in 50% methanol.[19]

The interpretation of our present results (Table I, Figure 2) is now straightforward. The fact that the CD spectra relative to TC are not affected by the presence of calcium up to pH 9 indicates that no coordination occurs as long as the dimethylammonium group remains protonated. Then, as it dissociates, a Ca^{2+} ion binds TC through its N4 and $O12_a$ atoms, which "locks" the molecule into conformation A. As shown from a Dreiding model examination, the O12 and O1 atoms, which pointed in different directions in conformation B, thereby become accessible to the chelation of a second Ca^{2+} ion. This rationale explains why no ML was found in our previous potentiometric investigations;[7] *both Ca^{2+} ions involved in the M_2L species actually bind TC simultaneously as the dimethylammonium group dissociates: one through N4 and $O12_a$; the other via the O12 and O1 atoms.* In addition to the disagreements commented on in ref 32, this interpretation also contradicts other authors' conclusions taking the BCD ring system as the exclusive site for calcium chelation.[13,33] These conclusions were based on the fact that the protonation curve of isochlorotetracycline (ITC), a derivative in which the C11–C12 system is destroyed, was almost not affected by the presence of this metal in solution.[34,35] Regarding this, the following should be noted: (i) At the time of the above studies,[34,35] the TC protonation steps were not properly assigned: namely, the most basic proton dissociation, which was slightly influenced by the presence of calcium,[35] was wrongly attributed to the diketophenolic moiety. (ii) Due to its particular structure, ITC may well be unable to adopt conformation A, which has now been shown to be a prerequisite for calcium coordination.

As far as magnesium is concerned, Martin[13] ruled out the possibility for the N4 atom to bind this metal, but his demonstration was based on former authors' data referring to cobalt and

(31) As outlined in our earlier work,[7] the quantitative approach used by these authors is more than questionable, since they contend that a single ML_2 species is formed under conditions now proved to be specific to M_2L. Nevertheless, their CD spectra do remain a valuable source of information.

(32) From another comparison between TC and DTC, the same authors[15] contend that the first calcium ion to bind TC cannot do it through the N4 atom, since they observe similar CD spectra for both systems. They infer from this observation that the first calcium–TC bond involves the BCD ring system. This conclusion is contradicted by our present interpretation, the more so as (i) our own CD spectra (see Figures 1 and 2 and corresponding ones in ref 12) do not confirm this similarity at any pH in water and (ii) this discrepancy cannot be interpreted in terms of conformational changes due to different solvent compositions since tetracycline conformations are expected to remain identical up to 60% methanol at least.[20] These observations lead us to question the composition of the DTC prepared by the above authors.

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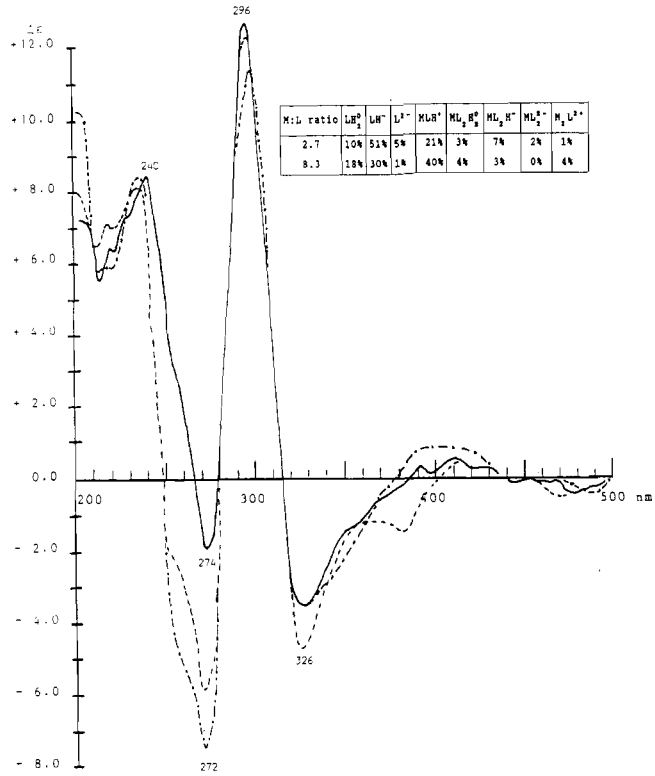


Figure 3. Circular dichroism spectra of TC (C_{TC} = 0.0416 mmol·dm^{-3}) at pH 8.0 in the absence of metal (—) and in the presence of magnesium, 0.113 mmol·dm^{-3} at pH 8.0 (- - -) and 0.347 mmol·dm^{-3} at pH 7.5 (-·-·-).

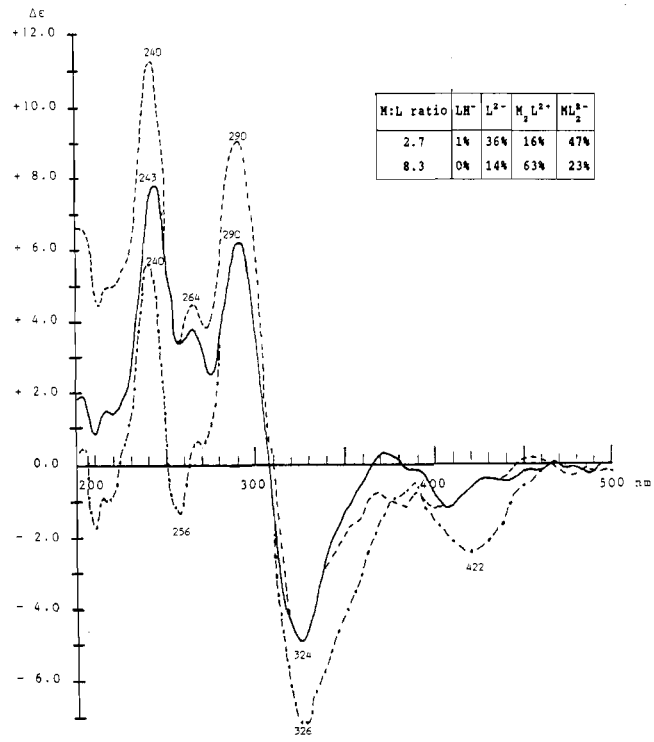


Figure 4. Circular dichroism spectra of TC (C_{TC} = 0.0416 mmol·dm^{-3}) at pH 11.0 in the absence of metal (—) and in the presence of magnesium, 0.113 mmol·dm^{-3} (- - -) and 0.347 mmol·dm^{-3} (-·-·-). Species whose percentage is less than 1% are not mentioned in the table inset.

nickel.[36] He also stated that it was difficult to distinguish between "chelation to the BCD chromophore or to the C1–C2–C3 system of oxygen ligands".[13]

From CD spectra obtained at pH 7.4 in a mixed methanol–water solvent, Newman and Franck[15] concluded that a 1:1 species was formed in which magnesium would coordinate the O11–O12 atoms. Comparing these spectra to those relative to DTC in the presence of magnesium, they excluded the fact that the N4 atom could bind this metal, but as was already noted about calcium,[32] the terms of this comparison seem questionable. From another comparison with 2-NC-TC, the same authors inferred that neither of the A-ring sites involving C1, C2, and C3 could bind magnesium.[15] All of these data were obtained in 90% methanol, but the noticeable features of the magnesium–TC spectrum remained identical at the same pH in water, which led Newman and Franck to contend that *the* Mg^{2+} complex of TC was the same in both aqueous and methanolic solutions. We know now (see Table I) that the actual situation is more complicated as far as the number of species present in solution is concerned. Nevertheless, the fact that no conformational change occurs for TC in the presence of magnesium from 90% methanol to water (as indicated by the observation of the same high-intensity negative Cotton effect at 270 nm in both solvents[15]) is of great interest. It has indeed been clearly established since then[20] that the OTC free molecule displays different conformations in such distinct solvents (water and 90% ethanol), which presumably holds for TC too. This implies that in 90% methanol, magnesium prevents TC from changing conformations from B to A, which is in line with the effect observed by Everett et al.[24] in DMSO. Clearly, an important piece of information can be deduced from these observations: if ever magnesium binds TC via its N4 atom, the $O12_a$ site cannot participate in the involved chelate ring since this would impose conformation A on the TC molecule while it is actually maintained in conformation B. As for the above conclusion about the O1–O3 oxygens,[15] it only concerns 1:1 magnesium species with TC and 2-NC-TC in 90% methanol. It cannot apply to aqueous solutions since the conformation of 2-NC-TC in water (not determined by Newman and Franck) may well be different from that which it assumes in 90% methanol.

In light of the above, the examination of our own CD spectra leads to the following remarks: (i) At pH 8, the presence of 40% of TC in the form of its MLH magnesium complex does not induce any conformational change with respect to the free molecule (Figure 3). Instead, the Cotton effect at 272 nm becomes more negative, which means that conformation B is stabilized. This confirms our above conclusion derived from literature data: *the possibility for magnesium to be involved in a chelate ring combining the N4 and* $O12_a$ *atoms should definitely be ruled out.* (ii) At pH 11 (Figure 4) with ML_2 being predominant (47%), TC displays conformation A, but the positive Cotton effect at 262 nm may reflect the still important percentage of the dianionic form (36%). When M_2L becomes predominant, a considerable decrease of the 262-nm band is observed, indicating that the proportion of TC in conformation A is correlatively reduced. This clearly shows that magnesium and calcium bind TC via different sites in their respective M_2L species. If we consider the above exclusion of the $O12_a$ oxygen as a potential donor site, the only possibility for magnesium to be bound to the dimethylamino group is to coordinate TC through its N4 and O3 atoms, as was previously suggested for the Mg–DSC species.[12] The formation of such a chelate would indeed induce conformation B. The much greater stability constant of the M_2L calcium complex with respect to the magnesium one is also in line with this interpretation. The different binding modes of these two metals to the N4 atom may stem from their distinct ionic radii: indeed, the use of N4 and O3 implies the formation of a five-membered chelate ring for magnesium while N4 and $O12_a$ correspond to a six-membered one for calcium. Logically, the second Mg^{2+} ion should bind TC through its BCD ring system. Since, by comparison with calcium (Figure 2), the participation of the O1 oxygen in the corresponding chelation is precluded, the O10–O12 atoms represent the only possible coordination sites. *Thus, in the magnesium* M_2L *species, one* Mg^{2+} *ion would bind TC via its N4 and O3 donors, while the other would coordinate the O10–O12 system.*

Oxytetracycline. OTC differs from TC by the presence of an OH substituent at C5 (see II). According to Mitscher et al.,[17]

(36) Baker, W. A.; Brown, P. M. *J. Am. Chem. Soc.* **1966**, *88*, 1314.

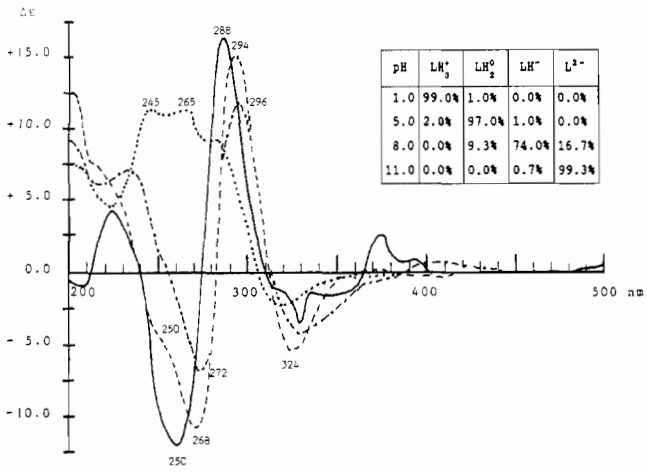


Figure 5. Circular dichroism spectra of OTC (C_{OTC} = 0.0418 mmol·dm^{-3}) at various pH values: 1.0 (—); 5.0 (- - -); 8.0 (-·-·-); 11.0 (···).

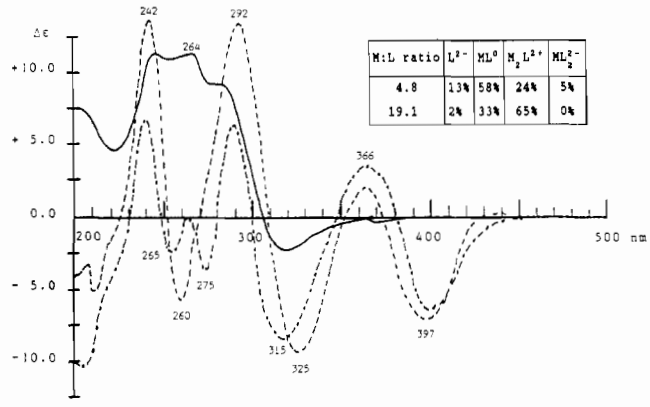


Figure 6. Circular dichroism spectra of OTC (C_{OTC} = 0.0418 mmol·dm^{-3}) at pH 11.0 in the absence of metal (—) and in the presence of calcium, 0.20 mmol·dm^{-3} (- - -) and 0.80 mmol·dm^{-3} (-·-·-). Species whose percentage is less than 1% are not mentioned in the table inset.

this substituent would play a critical role in the conformation as well as in the metal coordination of OTC with respect to the nonhydroxylated subclass of TC derivatives. Regarding this, the above authors noted that, from acidic to neutral pH, there was no evidence in their CD spectra to "support the occurrence of a conformation different from that possessed by DSC"[17] but that, above pH 9, the spectrum of OTC was clearly distinct from those of TC and DSC. The alkaline pH conformation of OTC would thus be either C, D, or E in the classification used by Mitscher et al.,[37] the E one being the most likely. This interpretation was later questioned by Jogun and Stezowski,[18] who stated that the conformation they observed under alkaline conditions displayed "some of the attributes that Mitscher et al. assigned to their proposed conformation for the non-5-hydroxylated tetracycline derivatives", i.e. conformation A. (See Chart I for OTC conformations.)

Our own results show (Figure 5) that the main variations in the CD spectra of metal-free OTC solutions as a function of pH closely follow the formation of the dianionic form of the antibiotic. In particular, the three positive bands at 245, 265, and 286 nm seen at pH 11 strictly correspond to those relative to metal-free TC solutions (Figure 1) as well as to the M_2L calcium species being predominant (Figure 2). These observations support the hypothesis that, like DSC[12] and TC, OTC adopts conformation B from acidic to neutral pH and then turns to conformation A as the C4 dimethylammonium group dissociates. This is in accord with Jogun and Stezowski's above-mentioned conclusions referring to basic aqueous medium[18] and definitely casts doubt on the previous results of Mitscher et al.[17] On account of potential interactions between bulky peri substituents, the presence of the OH group at C5 may affect to some extent the conformation of OTC,[17] but resulting structural modifications cannot be distinguished from the CD spectra shown in Figure 5. This lack of agreement with Mitscher et al.[17] will be elucidated in a next paragraph.

With calcium and magnesium, OTC forms still a larger number of complexes than TC, since ML as well as M_2L have been characterized in both cases (Table I). Besides, M_2LH has also been shown to exist with magnesium only, which seems to indicate that these two metals bind OTC via different sites in their respective binuclear species. Moreover, the increase in stability corresponding to the addition of a proton to the magnesium M_2L complex (6.62) compares well with the step-protonation constant specific to the O10–O12 site (7.11), which suggests that, in this species, one Mg^{2+} ion would chelate OTC through the N4 atom and a second adjacent donor site, while the other would presumably coordinate the O12 and O1 atoms. This is indeed the only possibility for the diketophenolic moiety to dissociate or bind a proton once the M_2L magnesium complex has been formed. The question as to which oxygen among O5, O3, and $O12_a$ will participate in the formation of the N4-based chelate ring remains to be discussed on conformational grounds.

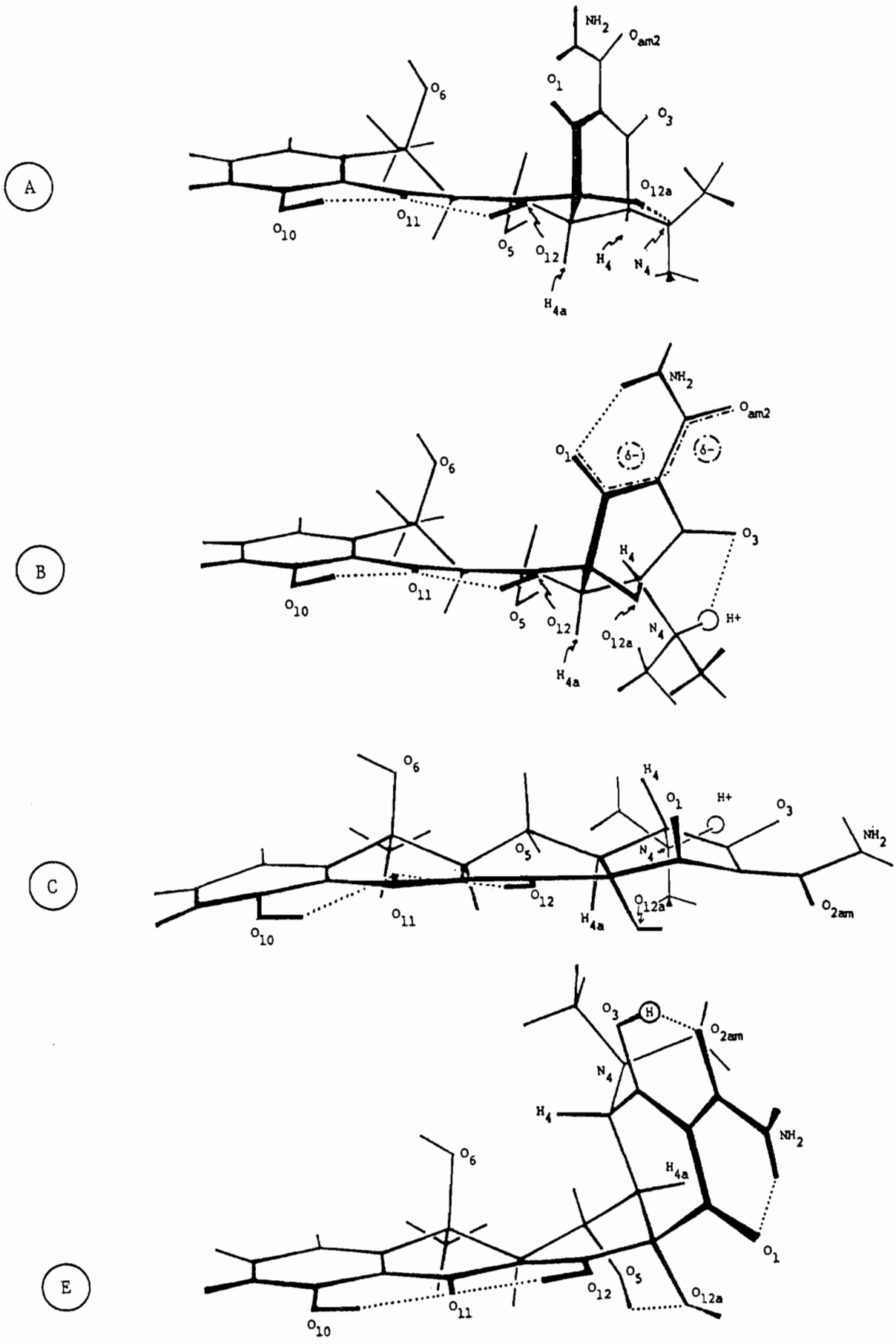
In the series of 1:2 metal-to-ligand ratio species, stability constants corresponding to the successive protonation steps from ML_2 to ML_2H_2 may also bring out some interesting pieces of information. For calcium, the increments relative to the protonation of ML_2 into ML_2H (8.27) and of ML_2H into ML_2H_2 (7.97) are similar and are of the same order of magnitude as the protonation constant of the C4 dimethylamino group (8.66). With magnesium, on the contrary, these two increments are very different from each other (7.87 and 6.67, respectively). This suggests that, unlike what was observed with TC, calcium binds the two ligands of its ML_2 species via the same coordination site (i.e. somewhere at their BCD ring system), whereas magnesium does not. This may explain the greater stability of the ML_2 complex of magnesium with respect to calcium, as opposed to the equivalent stabilities of these species with TC. These variations presumably stem from interactions due to the OH5 substituent. Unfortunately, they could not be analyzed on CD grounds since, for aforementioned reasons, 1:2 metal-to-ligand ratio complexes remained insignificant within the concentration ranges compatible with the spectrometer sensitivity.

In contrast, suitable conditions were found to render calcium ML and M_2L complexes predominant in turn, which is of much greater biological interest, account being taken of the leading part that these species play in OTC plasma distribution.[7,8] In the presence of calcium at pH 8, OTC displays the same conformation (B) as in its metal-free solutions at this pH (Figure 5) since the respective CD spectra (not shown here) are similar in shape and general intensity. For example, with 35% of ML and 15% of M_2L, the OTC spectrum is exactly identical with its homologue in the absence of metal. The still significant percentage of the LH form (32%) may help to maintain the situation unchanged in this case. Nevertheless, it appears that the already significant fraction of OTC bound to calcium does not interact with this ion through the N4 and $O12_a$ atoms; such a chelation would indeed imply conformation A for OTC. From the above discussion on the stability constants in the series of 1:2 metal-to-ligand ratio species and from conclusions of our preceding work on DTC,[12] it may be inferred that calcium coordinates some particular site of the BCD ring system.

At pH 11 with ML predominating (Figure 6) OTC still displays conformation B. In comparison with TC (Figure 2), the two bands at 243 and 294 nm attributed to the BCD chromophore[16] become similarly more positive, which confirms the interaction of this site with calcium, but the band at 262 nm remains negative. Moreover,

(37) Conformation C is the intermediate form that results from a continuous twisting of the A ring from the "extended" all-chair extreme conformation B, until the C4 dimethylamino group becomes equatorial.[17] In this conformation, rings A and B are forced into boat forms. Conformation D represents the second all-chair extreme of the available conformations, as opposed to A. A slight distortion of D leads to conformation E, which is the one seen in X-ray studies[29] and which Martin has named the "folded" conformation.[19]

Chart I. OTC Conformations



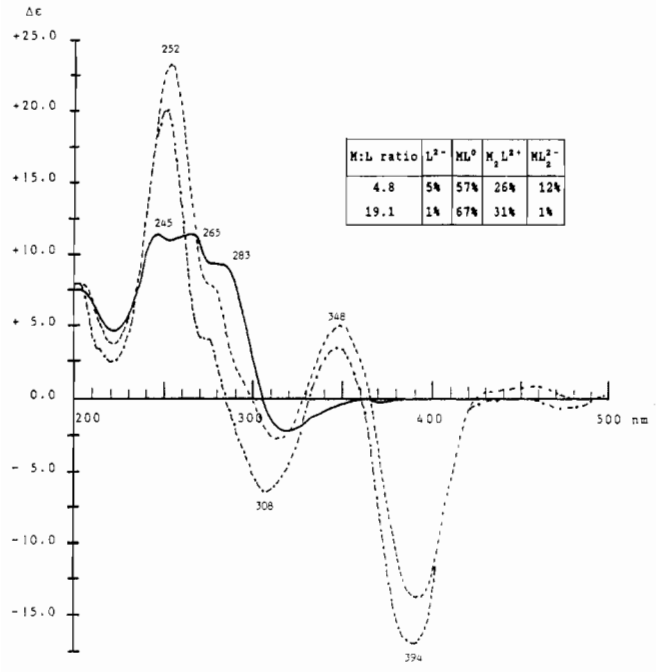


Figure 7. Circular dichroism spectra of OTC (C_{OTC} = 0.0418 mmol·dm^{-3}) at pH 11.0 in the absence of metal (—) and in the presence of magnesium, 0.20 mmol·dm^{-3} (- - -) and 0.80 mmol·dm^{-3} (-·-·-). Species whose percentage is less than 1% are not mentioned in the table inset.

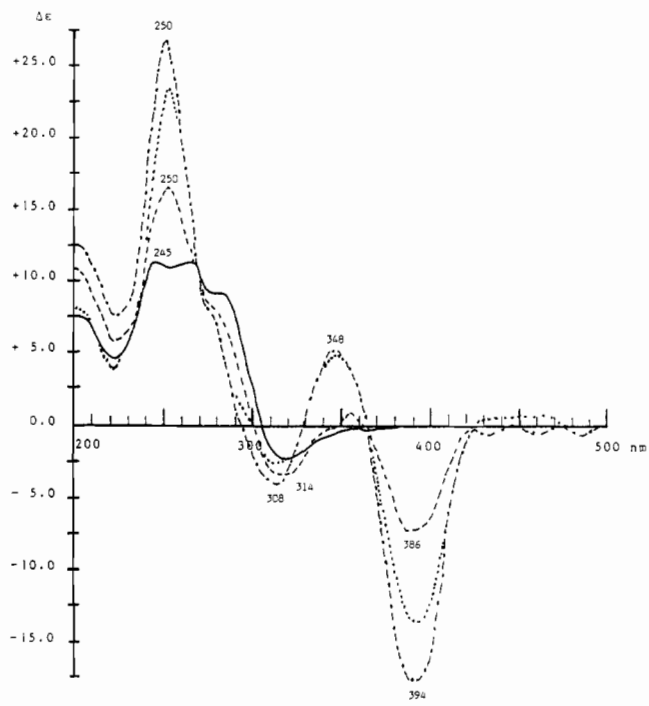


Figure 8. Circular dichroism spectra of OTC (C_{OTC} = 0.0418 mmol·dm^{-3}) at pH 11.0 in the absence of metal for a fresh solution (—), after 5.5 h at pH 5 (- - -), after 7 h at pH 5 (-·-·-), and for a fresh solution in the presence of magnesium, 0.20 mmol·dm^{-3} (···).

the two new Cotton effects observed at 368 and 400 nm for TC also develop, and at the same wavelength. Clearly, calcium interacts with both the diketophenolic moiety and the A-ring chromophore:[15] *calcium thus binds OTC via its O12 and O1 donor sites.*

As M_2L becomes predominant (Figure 6), the negative band at 262 nm is reversed, which indicates that a significant fraction of OTC turns to conformation A. By comparison with our previous study on DSC[12] and with the above discussion on calcium–TC interactions, we can thus conclude that *in M_2L the first Ca^{2+} ion binds OTC between the O12 and O1 oxygens whereas the second coordinates the N4 and $O12_a$ atoms.* It should be noted that this conclusion basically concurs with that of Mitscher et al. on the same system,[17] although these authors failed to characterize the involvement of the O1 site in the coordination.

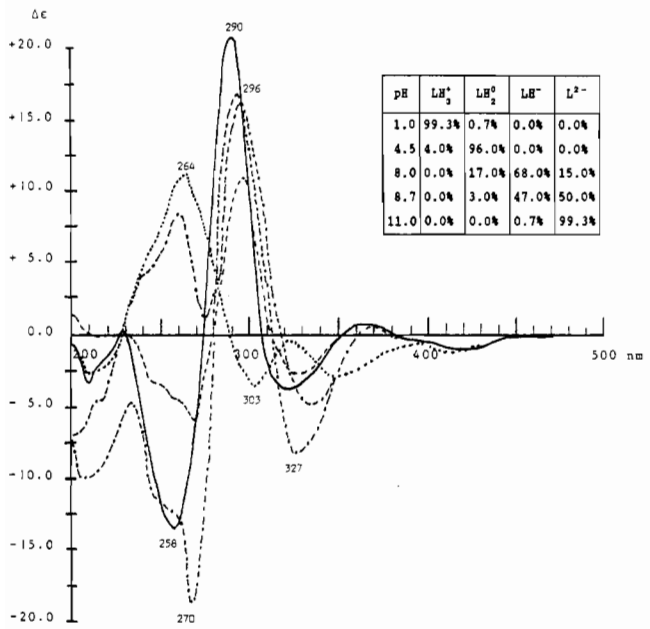
With respect to TC, the influence of the OH group at C5 would thus only consist of splitting the formation of M_2L into two steps, ML being formed separately. This is in line with M_2L being much less stable with OTC than with TC. Incidentally, it is the opposite for magnesium.

Magnesium interactions with OTC are difficult to interpret, due to the specific effect of this metal on corresponding CD spectra. It is worth noting from now on that this effect is at the origin of the misapprehension by Mitscher et al.[17] of the OTC conformation in basic aqueous medium (see above). At pH 8 with 35% of OTC in the form of ML, the CD spectrum (not shown here) remains identical with its homologue without metal in the wavelength region below 300 nm, which means that OTC displays conformation B. Nevertheless, two new Cotton effects, a positive absorption at 350 nm and a negative one at about 390 nm, appear on the shoulder near 360 nm. Although observed at shorter wavelengths, these effects are similar to those noted for calcium with TC and OTC at pH 11, which a priori suggests that Mg^{2+} ions might coordinate OTC through the O12 and O1 atoms. Still at pH 8 with ML reaching 62%, the above effects develop to a greater extent. In addition, the band near 240 nm becomes more positive and the band at 272 nm less negative. These observations concur with those reported by Mitscher et al. under comparable conditions, from which these authors expected magnesium to coordinate to the BCD juncture.[17]

At pH 11, it was impossible to obtain any CD spectrum specific to M_2L separately since, due to its high stability constant (Table I), ML remained predominant for all reactant concentrations likely to favor M_2L. Under these conditions, the peak at 240 nm became still more positive and was shifted to 254 nm (Figure 7). Concurrently, the intensity of the two new opposite Cotton effects near 350 and 390 nm was increased. This also confirmed previous experimental observations of Mitscher et al.[17] From the structural point of view, these authors contended that OTC displayed conformation D, with one Mg^{2+} ion binding between O5 and $O12_a$ and the other coordinating at the BCD juncture.[17]

The latter interpretation is very different from the above suggestion that magnesium could bind to the O12–O1 oxygens. As deduced from Dreiding model considerations, the O12–O1 chelation hypothesis would be consistent with either conformation A or conformation C for the OTC molecule. Moreover, according to our above stability constant analysis, the second Mg^{2+} ion would be bound to the N4 and O5 atoms, which is compatible with conformation C only. Now, this conformation would induce a large negative signal in the 260–280-nm region,[17] whereas a large positive one is actually observed in Figure 7. No definite conclusion could thus be drawn as to the conformations adopted by OTC as well as about the coordination sites involved in its ML and M_2L magnesium complexes.

This lack of consistency between our potentiometric and spectroscopic data, on the one hand, and the striking similarity between the CD spectra of Mitscher et al. on metal-free and magnesium-containing OTC basic solutions,[17] on the other hand, led us to consider the possibility that OTC might undergo significant chemical variations at high pH. An OTC solution was thus prepared at pH 5 and stored in the dark under a nitrogen atmosphere, from which several samples were taken after different periods of time, their pH being then adjusted to various values: 1, 5, 8, and 11. Figure 8 shows the changes with time observed in the CD spectra relative to solutions set at pH 11. None were seen at any other pH value. For the sake of comparison, Figure 8 also shows the CD spectrum obtained in the presence of magnesium at pH 11, already shown in Figure 7. These results call for two important remarks: (i) clearly, OTC undergoes at pH 5 a considerable chemical variation within a few hours that affects its CD spectrum only when the pH is raised up to pH 11; (ii) magnesium favors this degradation at pH 11 since CD spectra in the presence of this metal refer to fresh solutions. Moreover,



pH	LH_2^+	LH_2^0	LH^-	L^{2-}
1.0	99.3%	0.7%	0.0%	0.0%
4.5	4.0%	96.0%	0.0%	0.0%
8.0	0.0%	17.0%	68.0%	15.0%
8.7	0.0%	3.0%	47.0%	50.0%
11.0	0.0%	0.0%	0.7%	99.3%

Figure 9. Circular dichroism spectra of DOX (C_{DOX} = 0.048 mmol·dm^{-3}) at various pH values: 1.0 (—); 4.5 (- - -); 8.0 (–·–·–); 8.7 (-·-·-); 11.0 (···).

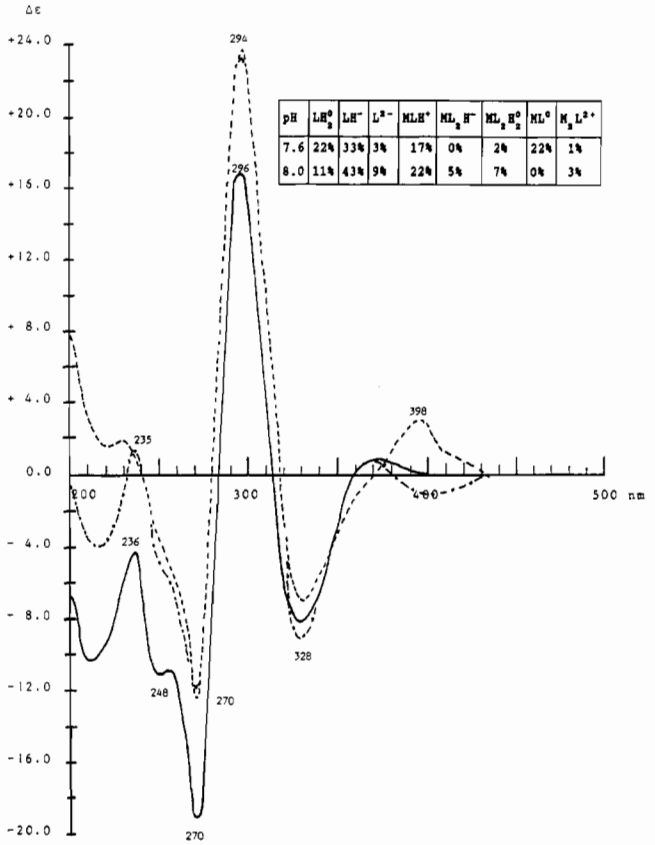
the presence of magnesium makes correlative conformational changes visible from a pH as low as 8 (see above discussions). CD characteristics of TC degradation products have been previously reported,[38,39] but OTC undergoes a supplementary degradation step into α- or β-apo-OTC, for which no CD spectrum could be found in the literature. The degradation product was thus not identified.

On the basis of these observations, it is now easy to explain the discrepancy observed between the CD spectra of Mitscher et al. and our own for metal-free OTC basic solutions: the delay between preparation and CD investigation of the OTC solutions used by these authors was probably too long for OTC to remain chemically unchanged.

Concerning the formation of the M_2L complex, *one would be tempted to suggest from stability constant considerations that one Mg^{2+} ion binds OTC through the O12–O1 oxygens whereas the other is coordinated to the N5 and O5 atoms, but no definite conclusion can finally be drawn since magnesium acts as as a catalyst of the degradation of this antibiotic.*

Doxycycline. DOX is the second 5-hydroxylated derivative of the TC class of antibiotics (see III). As it lacks the OH group at C6, its empirical formula coincides with that of TC itself. In fact, the similarity of the two compounds is only apparent since they exhibit very distinct properties, especially as far as metal coordination is concerned. With respect to OTC, the lack of the OH6 substituent makes DOX much more stable, even in the presence of magnesium, as will be seen later. This may indicate that the OH6 group is necessary to the degradation process undergone by OTC.[40]

The pH dependence of the CD spectra relative to metal-free DOX solutions (Figure 9) is basically the same as that observed with TC and OTC. Once again, the antibiotic molecule displays conformation B form acidic to neutral media and then turns to conformation A as the N4 atom loses its proton. Let us recall that this conformational change is favored by the formation of the putative N4–HO12$_a$ hydrogen bond. With DOX however, an additional effect is to be noted, which progressively develops from pH 8 to 11 along with the dissociation of the C4 dimethylammonium group (spectra not shown here): the positive band at about 300 nm and the negative one near 325 nm tend to reverse. It can be hypothesized that, due to the absence of the OH6 substituent, the interaction between the 6-α-methyl and the 5-α-OH groups may be released by a slight twisting of the C ring along the C6–C11 axis.



pH	LH_2^0	LH^-	L^{2-}	MLH^+	ML_2H^-	$ML_2H_2^0$	ML^0	M_2L^{2+}
7.6	22%	33%	3%	17%	0%	2%	22%	1%
8.0	11%	43%	9%	22%	5%	7%	0%	3%

Figure 10. Circular dichroism spectra of DOX (C_{DOX} = 0.048 mmol·dm^{-3}) at pH 8.0 in the absence of metal (—) and in the presence of calcium, 0.040 mmol·dm^{-3} (- - -), and magnesium, 0.040 mmol·dm^{-3} (–·–·–). Species whose percentage is less than 1% are not mentioned in the table inset.

Before discussion of the structural aspects of DOX–metal coordination on spectroscopic grounds, preliminary information can be drawn from the examination of stability constants in Table I. With calcium for example, the addition of a proton to the ML species involves an increase in stability of 7.46, which is very close to the step-stability constant (7.42) relative to the protonation of O12.[19] This suggests that, in ML, calcium binds DOX to its N4 site, as was previously proposed for DSC.[12] About magnesium the protonation of ML_2H into ML_2H_2 implies an increment in stability of 8.18, which roughly corresponds to the protonation constant assigned to the C4 dimethylamino group. One of the two ligands of ML_2H would thus bind the metal via its O10–O12 system. It is finally worth noting that the M_2L species of calcium is more stable than its magnesium homologue and that its stability constant is 1 order of magnitude above that of the same complex with OTC.

At first sight, DOX probably still displays conformation B in the presence of calcium at pH 8 (Figure 10), as the intense negative Cotton effect at 270 nm already observed with metal-free solutions (Figure 9) tends to indicate. Given (i) the above suggestion that the dimethylamino group is involved in the formation of ML as well as MLH and (ii) the relatively high percentage of DOX present in the form of these two species, it appears logical that O3 or O5 be associated with N4 to chelate calcium. Indeed, the common participation of O12$_a$ and N4 in the coordination is precluded since it would impose conformation A on the DOX molecule. Concerning the discrimination of the oxygen binding site, the positive band of moderate intensity near 400 nm shows that calcium interferes to some extent with the BCD ring system, which lends support to the N4–O5 hypothesis.

(38) Miller, R. F.; Sokoloski, T. D.; Mitscher, L. A.; Bonacci, A. C.; Hoener, B. A. *J. Pharm. Sci.* **1973**, *62*, 1143.
(39) Sokoloski, T. D.; Mitscher, L. A.; Yuen, P. H.; Juvarkar, J. V.; Hoener, B. A. *J. Pharm. Sci.* **1977**, *66*, 1159.
(40) Strittmater, T.; Siewert, M. *Pharm. Ztg.* **1982**, *127*, 1300.

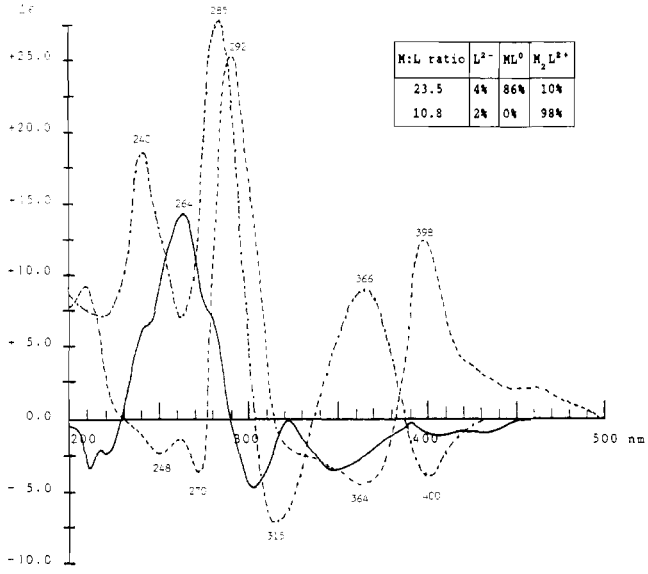


Figure 11. Circular dichroism spectra of DOX (C_{DOX} = 0.048 mmol·dm^{-3}) at pH 11.0 in the absence of metal (—) and in the presence of calcium, 0.113 mmol·dm^{-3} (- - -), and magnesium, 0.520 mmol·dm^{-3} (– ·–·–). Species whose percentage is less than 1% are not mentioned in the table inset.

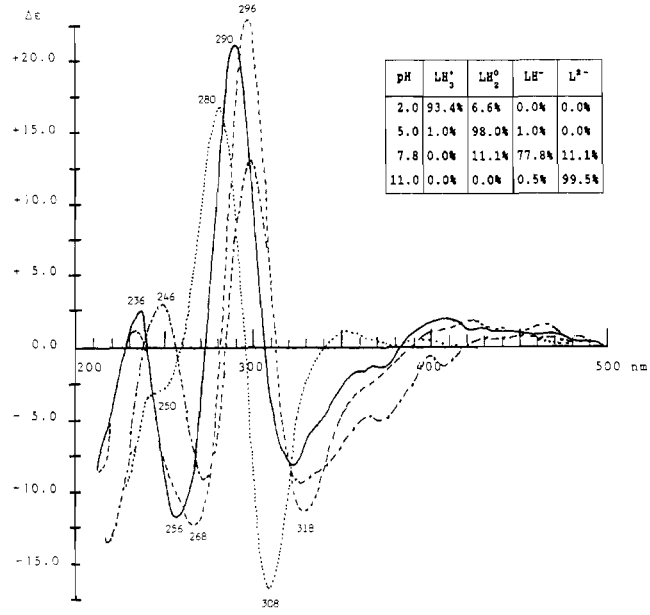


Figure 12. Circular dichroism spectra of CTC (C_{CTC} = 0.042 mmol·dm^{-3}) at various pH values: 2.0 (—); 5.0 (- - -); 7.8 (–·–·–); 11.0 (···).

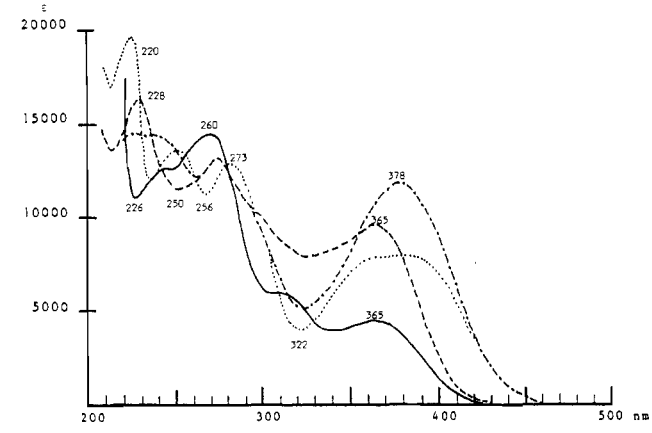


Figure 13. UV–visible absorption spectra of CTC (C_{CTC} = 0.0625 mmol·dm^{-3}) at various pH values: 2.0 (—); 5.0 (- - -); 7.8 (–·–·–); 11.0 (···).

At pH 11, the high stability of the ML complex keeps it predominant regardless of the metal-to-ligand concentration ratios investigated (Figure 11). Under these conditions, DOX still seems to display conformation B, but the increase of the ML percentage in comparison with pH 8 now results in the appearance of a double Cotton effect centered around 385 nm (370 nm negative band; 400 nm positive band), which confirms the interference of the calcium coordination with the BCD ring system. The opposite sign of both the 370- and 400-nm bands with respect to those observed for the M_2L complex of calcium with TC and OTC suggests structures of opposite chiral asymmetry, hence a different mode of bonding. *This definitely supports the hypothesis that calcium coordinates DOX via its N4 and O5 atoms. In such a case, the only possible conformation for DOX is conformation C (see above discussion on Mg^{2+}–OTC interactions). The second Ca^{2+} ion of the M_2L complex should then bind DOX through its O10–O12 system.* With respect to OTC, the specificity of the calcium bonding mode with DOX may derive from the lack of the OH6 group, which would facilitate the planar arrangement of the N4 and O5 coordination sites. Once DOX is in conformation C with one Ca^{2+} ion bound to its N4 and O5 atoms, the projection of the 6-α-methyl group behind the C ring (see above) makes this ring coplanar with the O10, O11, and O12 atoms while the O1 oxygen points in a different direction, which prevents calcium from coordinating to the O12–O1 site.

With magnesium at pH 8 (Figure 10), DOX also seems to display conformation B, as indicated by the trough at 270 nm. However, although of low intensity, a double Cotton effect centered near 385 nm (370 nm positive; 400 nm negative) is observed, which has already been assigned to metal chelation via the O12 and O1 atoms (see above conclusions on calcium with TC and OTC). As this binding mode can only suit conformation A or conformation C, DOX should actually adopt conformation C, as with calcium.

At pH 11 (Figure 11), as M_2L becomes predominant, the intensity of the double Cotton effect is increased, which tends to confirm the O12–O1 hypothesis. The only vacant site compatible with conformation C is then N4–O3. *The above stability constant analysis (M_2L is less stable for magnesium than for calcium), and the present spectroscopic observations are thus consistent with one Mg^{2+} ion binding to the O12 and O1 atoms whereas the other coordinates through the N4 and O3 sites.*

Chlortetracycline. CTC differs from TC by the presence of the bulky chlorine substituent at C7 (see IV). As with TC, OTC, and DOX, the CD spectra recorded for metal-free CTC solutions (Figure 12) are characteristic of conformation B from acidic to slightly basic pH values. Then the CTC molecule seems to turn to conformation A. At pH 8.7 for example, the intensity of the 247-nm positive band observed at pH 7.8 is increased, and the virtual absence of peak at 270 nm suggests that the dianionic form of CTC will adopt conformation A. The similarity of CTC with the other tetracyclines stops there, since above pH 9 its CD spectra become considerably different from those shown in Figures 1, 5, and 9. At pH 11, a very intense double Cotton effect centered around 295 nm (280 nm positive; 307 nm negative) develops, which may be attributed to conformational changes in the BCD ring system.[16] The involvement of the BCD chromophore in this phenomenon is confirmed by the striking difference observed in the CTC UV–visible absorption spectra with respect to those relative to the other tetracyclines: for TC, OTC, and DOX, the large band in the 360-nm region becomes progressively more intense and undergoes a slight bathochromic shift as the pH is raised from pH 2 to 11. This effect is also observed for CTC up to pH 9, but at pH 11, the intensity of the absorption band at 378 nm is drastically decreased (Figure 13). Clearly, the severe steric hindrance that should logically result from the interaction of the chlorine at C7 with the 6-α-methyl substituent is presumably released by the twisting of the BCD ring system along the C6–C11 axis, so that the pseudoboat form of the C ring is almost totally reversed. This effect, which becomes visible only when the diketophenolic group dissociates because of the subsequent electron

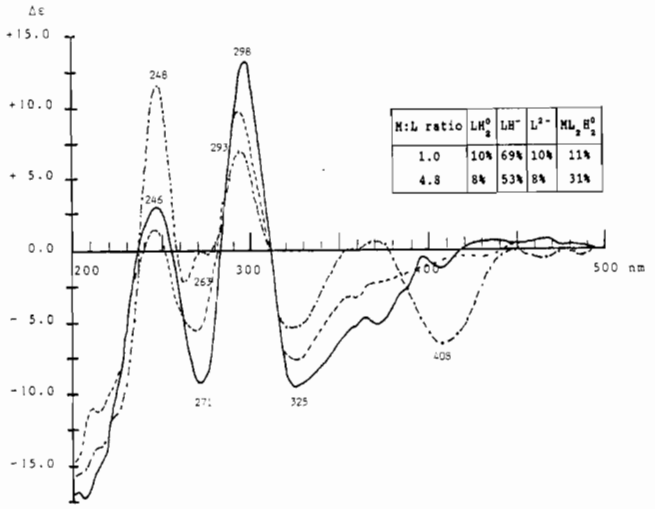


Figure 14. Circular dichroism spectra of CTC (C_{CTC} = 0.042 mmol·dm^{-3}) at pH 7.8 in the absence of metal (—) and in the presence of calcium, 0.042 mmol·dm^{-3} (- - -) and 0.20 mmol·dm^{-3} (-·-·-). Species whose percentage is less than 1% are not mentioned in the table inset.

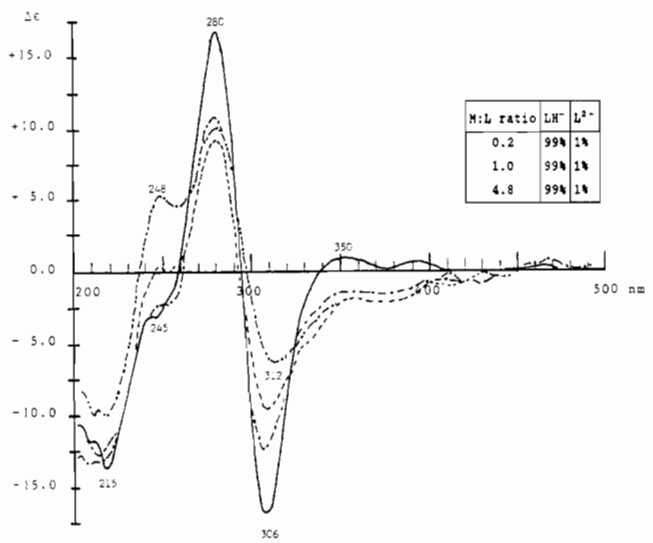


Figure 15. Circular dichroism spectra of CTC (C_{CTC} = 0.042 mmol·dm^{-3}) at pH 11.0 in the absence of metal (—) and in the presence of magnesium, 0.008 mmol·dm^{-3} (-··-··), 0.042 mmol·dm^{-3} (- - -), and 0.200 mmol·dm^{-3} (-·-·-). Species whose percentage is less than 1% are not mentioned in the table inset.

delocalization within the O10–O12 junctures, should result for the BCD ring system in a conformation close to the one it assumes in conformation D.[16,17,37]

As far as the A ring is concerned, given the broadness of the 280-nm band, it is a priori difficult to know whether it simultaneously turns to the structure it adopts in conformation A or remains in a B-like position. The virtual absence of a 270-nm Cotton effect at pH 8.7 would rather be a point in favor of the former alternative.

The most noticeable fact relative to CTC–metal interactions is the failure of its dianionic ligand to form any complex with calcium or magnesium (Table I). It may be worth recalling that the capacity of the Cl7 substituent to lessen the electron density of the O10–O12 system was originally expected to prevent the formation of binuclear complexes of CTC with these two metals.[10] Concerning the Cl7 electron-withdrawing properties, it was previously found that, in addition to the protonation step relative to O12, the one assigned to the C4 dimethylamino group was equally influenced.[10] This unexpected result was tentatively interpreted in terms of solvent effects.[11] The above spectroscopic data would rather point to its possible origination in steric interactions, which may be of some importance to metal coordination as well.

As far as calcium chelation is concerned, the CD spectrum obtained at pH 7.8 for equal concentrations of both reactants (Figure 14) does not significantly differ from its homologue relative to metal-free solutions (Figure 12). For higher metal-to-ligand ratios however, the proportion of complexed CTC is increased, and a double Cotton effect centered near 385 nm (370 nm positive; 408 nm negative) develops, which has previously been recognized to be characteristic of the O12–O1 chelating site.[15] The simultaneous tendency of the 268-nm negative band to reverse suggests that a significant fraction of CTC turns to conformation A as the C4 dimethylammonium group dissociates. This may indicate that *in ML_2H_2, one ligand chelates calcium via the O12–O1 site whereas the other binds it between its N4 and O12$_a$ atoms.* This mode of bonding appears all the more realistic, as it has already been postulated for the ML_2H_2 complex of TC, whose formation constant is approximately equivalent to that of the CTC species. This interpretation is also in accord with the fact that in 80% methanol, where the order of the two most basic protonation steps is presumably reversed,[19] the two signals represented by the positive peak near 270 nm and the double Cotton effect centered around 385 nm have been observed to be considerably more intense than in water.[41] In this lower dielectric constant medium, it is indeed likely that a greater proportion of both ligands involved in the ML_2H_2 species have their C4 dimethylammonium groups deprotonated, which tends to favor conformation A. Nevertheless, the increased intensity of the above double Cotton effect also implies that the O12 and O1 atoms chelate calcium to a very significant extent.

At pH 11, all recorded CD spectra remain basically identical with the preceding one at pH 7.8, regardless of the metal-to-ligand ratio investigated. This result is all the more difficult to interpret, as (i) according to our previous potentiometric investigation,[10] no metal complex is any longer present in the solution at this high pH and (ii) in addition to a slight bathochromic shift of the double Cotton effect centered at 295 nm, these spectra display two major differences from that of free CTC: the highly positive band at 248 nm and the double Cotton effect centered at 390 nm. These two signals indicate that, in the presence of calcium, the conformation of the BCD ring system is different from that of metal-free solutions. This observation still holds with a 5-fold excess of ligand with respect to calcium. The latter point is of special interest, since it means that even though a ML or a M_2L species were to exist with stabilities similar to those of corresponding species with TC or OTC, they could not reach significant percentages under such conditions. This leads us to question the chemical stability of CTC in the presence of calcium above pH 9. Incidentally, striking similarities may be noted with the spectrum of degraded OTC (Figure 8). Such observations suggest that the transformation of CTC into ITC, which is frequent in basic aqueous solutions,[17,42] could be catalyzed by the presence of calcium, presumably through its binding to the O12–O1 site. This rationale would account for the absence of calcium complexes of the dianionic form of CTC.

The presence of magnesium at pH 7.8 induces a significant decrease of the intensity of all the peaks observed in the absence of metal. Nevertheless, except for a slight hypsochromic shift of the positive peak from 296 to 286 nm, the shape of the CD spectrum remains basically the same. The peak at 286 nm is accompanied by a trough at 318 nm. The characteristics of these two signals prefigure those of the double Cotton effect centered at 295 nm, which is observed at pH 11 (Figure 15). Furthermore, the CD spectra obtained at this pH in the presence of magnesium do not fundamentally differ from those of metal-free solutions. Actually, the higher the proportion of metal is, the less distinct the spectra are from those of free CTC. All these observations confirm the very poor capacity of CTC to complex magnesium.[10] It may be hypothesized that the structural change that leads to the specific conformation of the BCD ring system at high pH is

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such that the O10, O11, and O12 atoms are no longer coplanar, which prevents magnesium from binding to this site as it does with TC. *It is thus likely that in both MLH and ML_2H_2, magnesium binds CTC via its N4-O3 site.* This mode of bonding, which is consistent with CTC adopting conformation B, would also explain the following: (i) The ML_2H_2 complex is much less stable than its TC homologue. Let us indeed recall that, with TC, one ligand is thought to bind magnesium through its N4-O3 atoms whereas the other would coordinate it via its O10-O12 system, which presumably confers a higher stability on the resulting complex. (ii) The formation constant of MLH is about 1 unit lower than that of the same species with TC. It seems effectively logical to assume that, in the MLH complex of TC, the proton lies on the N4 atom, while it is associated with the O12 oxygen in the CTC corresponding species.

Conclusion

The present study confirms the considerable complexity of the coordination chemistry of tetracyclines with calcium and magnesium, with which they predominantly interfere in vivo. In particular, given that the free concentrations of Ca^{2+} and Mg^{2+} ions are several orders of magnitude higher than the therapeutic levels of tetracyclines in blood plasma, their complexes represent almost the totality of the fraction of drug not bound to proteins.[7-9] Thus, the ability of tetracyclines to diffuse into tissues first depends on the physicochemical properties of the main complexes of the above metals.

To impose structural variations on the parent TC molecule so that the resulting derivative could form predominant calcium and magnesium complexes having predetermined specific properties therefore appears a logical way of improving the bioavailability of these antibiotics. Since the minimal equipment represented by DSC is necessary to the antibacterial activity of tetracyclines in vivo,[43] the positions remaining available for such variations are confined to the C5-C9 carbons, which a priori seems to open a limited field of possibilities. On the contrary, the results obtained in the present work definitely show that apparently subtle modifications in the nature and positions of the substituent groups at these positions can entail considerable changes in the chelating properties of these substances. The specific use of substituent effects to improve tetracycline bioavailability through metal chelation thus seems to be a promising field of research.

In a recent article, Martin[19] stated about previous quantitative results of ours on calcium and magnesium complex equilibria with TC, OTC, and DOX[7-9] that "there (did) not seem to be any pattern for the variations among the three closely related antibiotics". Great efforts are probably needed before all subtleties of the metal coordination chemistry of tetracyclines under physiological conditions can be brought to light, but it does seem that part of the hidden profile of the above-mentioned pattern is now being delineated.

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Isolation and X-ray Crystal Structure of the Lithiated Phosphoranide $[Li(THF)cyclenP]_x$ and the X-ray Crystal and Electron Diffraction Structures of cyclenPH

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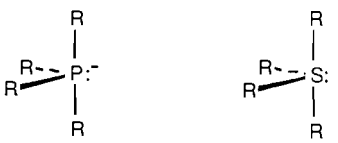
The X-ray crystal structure of the lithiated phosphoranide $[Li(THF)cyclenP]_x$ (**7**) and the X-ray crystal and electron diffraction structures of its precursor, cyclenphosphorane (cyclenPH, **1**), have been determined. Both the gas-phase and solid-state structures of **1** show the geometry to be a distorted trigonal bipyramid (tbp) with the P-N axial bonds longer than the equatorial bonds (the usual case in tbp geometries). However, there is a large difference in the equatorial N-P-N angle: 155° by electron diffraction versus about 138° by X-ray. This difference may reflect the small energy barrier between the tbp and square-pyramidal geometries for pentacoordinate species with crystal packing forces leading to the smaller angle. The structure of **7** is polymeric with each Li(THF) unit bridging two cyclenP units via coordination of the axial nitrogens. The geometry about each lithium is approximately trigonal planar while the phosphorus is a distorted pseudo trigonal bipyramid (ψ-tbp) with a lone pair occupying an equatorial position. The P-N_{ax}-Li angle is only 94°, which may be due to a weak bonding interaction between the phosphorus lone pairs and lithiums throughout the polymeric chain. X-ray data: $C_8H_{17}N_4P$, tetragonal, space group $P4_2/mnm$, a = 6.030 (1) Å, c = 13.740 (3) Å, Z = 2, R = 0.046, R_w = 0.054; $C_{12}H_{24}PON_4Li$, monoclinic, space group $C2$, a = 47.025 (10) Å, b = 10.312 (3) Å, c = 13.802 (3) Å, β = 97.92 (2)°, Z = 18, R = 0.070, R_w = 0.070.

Phosphoranide ions are 10-P-4 phosphorus anions,[1] isoelectronic with the neutral sulfuranes. Structurally, phosphoranides and sulfuranes have similar ψ-trigonal-bipyramidal (tbp) geometries with a lone pair of electrons in the equatorial plane.



However, the presence of a negative charge on the phosphorus

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