Metal Ion-Tetracycline Interactions in Biological Fluids. 10. Structural Investigations on Copper(II) Complexes of Tetracycline, Oxytetracycline, Chlortetracycline, 4-(Dedimethylamino)tetracycline, and 6-Desoxy-6-demethyltetracycline and Discussion of Their Binding Modes

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The present work examines the different binding modes of the Cu^{2+} ion in its complexes with three bioactive tetracycline antibiotics: tetracycline, oxytetracycline, and chlortetracycline (similar studies with doxycline were prevented by precipitation problems). Spectroscopic investigations involving UV-visible absorption and circular dichroism techniques have been used under predetermined conditions of concentrations and pH so that species successively formed in each system could be selectively investigated. Copper interactions with structurally simpler analogs (4-(dedimethylamino)tetracycline and 6-desoxy-6-demethyltetracycline) were also studied as references, which required the determination of corresponding complex formation constants. For all bioactive analogs including 6-desoxy-6-demethyltetracycline, it is shown that the first donor group to bind copper is the O3 atom, which starts deprotonating at relatively low pH, with MLH_2 as the resulting species. When the pH is raised, MLH progressively substitutes for MLH_2, chelation then taking place at the O10–O12 system as the OH12 group dissociates. The ultimate ligand deprotonation at the C4 dimethylammonium group leads to the formation of ML, with a new change in the binding mode in favor of the N4–$OH12_a$ donor set. Concerning the 1:2 metal-to-ligand complexes which coexist with the above species, ML_2H_4 is logically bound in the MLH_2 manner, whereas ML_2H_2, ML_2H, and ML_2 adopt an identical coordination scheme in which alternate bonds are presumed to be formed at both the O10–O12 juncture and the N4–$O12_a$ pinch. The present work further substantiates Albert's former view of a possible influence of metal ions on the pharmacological action of tetracyclines. Following the recent evidence of the important roles of calcium and magnesium in the transport of these drugs in blood plasma, it is now suggested here that copper can act as a cofactor of their antibiotic activity: first, the structural flexibility of copper binary complexes within the three distinct donor sites of bioactive tetracyclines is expected to favor mixed-ligand coordination with bacterial nucleic acids; then, through the formation of such ternary complexes, copper may induce the attack of free radicals known to damage these nucleic acids.

Introduction

Most pharmaceuticals contain electron-donor groups likely to bind metal ions occurring naturally in vivo.[2] Among these, tetracycline antibiotics have long been known to behave as relatively efficient chelating agents.[3] However, it was not until the advent of modern calculation techniques that (i) the identities and stability constants of tetracycline complexes with essential metal ions could be properly assessed[4-11] and (ii) the use of these constants in appropriate simulation models led to reliable evaluations of the biological significance of corresponding species.[4-10,12]

It was shown in these studies that free forms of tetracyclines always occur at insignificant levels in blood plasma during treatment, calcium and magnesium complexes being largely predominant in the fraction of drug not bound to proteins.[4-6] Although giving rise to more stable complexes than calcium and magnesium with tetracyclines, essential trace metal ions like zinc and copper are present in too low concentrations in blood plasma to significantly influence the bioavailability of these drugs.[8,9] Neither can any reciprocal effect be expected from tetracyclines on the fate of these metals, even for drug concentrations well above usual therapeutic levels.[8,9] In contrast, such interactions may be important in the gastrointestinal tract where ligand-to-metal concentration ratios are a priori more favorable. Unlike zinc,[8] however, copper at usual dietary doses should not influence the gastrointestinal absorption of tetracyclines, even though all the derivatives so far investigated are expected to improve its own absorption.[9]

Thus, copper tetracycline complexes seem to be of little relevance to the metabolism of either parent reactants in extracellular biofluids. In comparison, intracellular roles for these species are much more likely. For instance, copper tetracycline interactions have been recently implicated in the degradation of DNA through free radical production.[13] Substantial damage to the DNA molecule was observed following its incubation with a cupric salt and tetracycline (TC) or any of its oxytetracycline (OTC), doxycycline (DOX), and chlortetracycline (CTC) analogs, whereas antibiotics alone had no effect.[13] This is consistent with the earlier observation that copper tetracycline complexes can bind DNA whereas tetracycline itself cannot.[14] Apart from the possibility of being formed de novo from the redistribution of species entering bacteria, copper tetracycline complexes may

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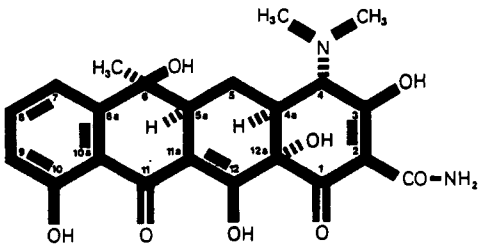
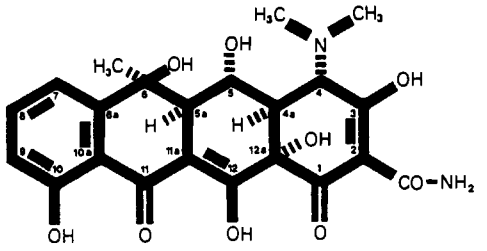
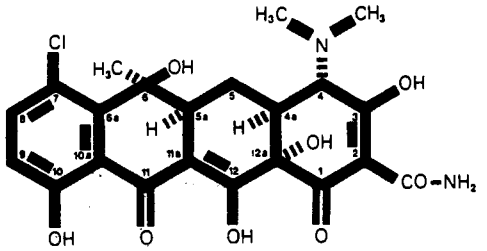
Chart I



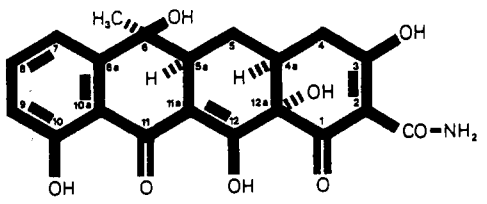
I: tetracycline (TC)



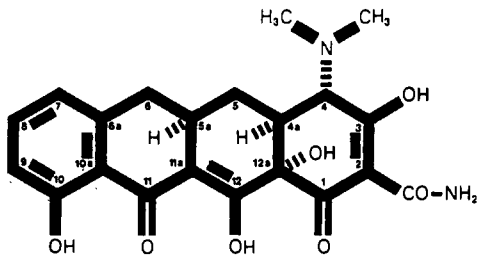
II: oxytetracycline (OTC)



III: chlortetracycline (CTC)



IV: 4-(dedimethylamino)tetracycline (DTC)



V: 6-desoxy-6-demethyltetracycline (DSC)

also penetrate as such from external fluids in which their electrically neutral forms predominate.[9]

Clearly, structural information on the tetracycline copper complexes involved in such processes may help elucidate the factors governing their availability to bacteria as well as their reactivity. With this in view, the present work deals with UV–visible and circular dichroism spectroscopic studies on the formation of copper complexes with TC (see I in Chart I), OTC (see II), and CTC (see III). By analogy with the above-mentioned work,[13] investigations into copper–DOX interactions were also planned, but they were prevented by solubility problems already confronted in an earlier work on copper tetracycline complexes.[9]

In such systems with very intricate complex equilibria, it is desirable that spectroscopic studies be conducted under predetermined conditions of reactant concentrations and pH so that the formation of particular species can be favored in turn. This preselection requires, however, that applicable stability constants have been determined beforehand. Concerning this, studies on copper complex formation with tetracyclines are relatively scarce, and their conclusions have often been controversial.[15-19] This was especially the case of the first works, which, in addition to being restricted to LH forms of the ligands,[15-18] referred to an erroneous tetracycline protonation scheme[20] corrected later.[21] It is only recently that the formation of copper complexes with the dianionic L forms of tetracyclines was reported, with formation constants limited to MLH and ML species.[22] The more recent work by Brion et al. on copper condition with five tetracycline derivatives[9] was the first to simultaneously consider the three forms of the ligand, namely L, LH, and LH_2, as likely to interact with the metal ion over the whole accessible pH range. The constants used to simulate the distribution of TC, OTC, and CTC copper complexes in the conditions of the present studies have thus been taken from that work. Also, as was the case for previous calcium and magnesium studies,[11,23] 4-(dedimethylamino)tetracycline (DTC; see IV in Chart I) and 6-deoxy-6-demethyltetracycline (DSC; see V) simpler inactive analogs have been used as references for structural comparisons. Required formation constants for their own copper complexes have thus been determined.

A second point of importance regarding investigations into the metal bonding modes of tetracyclines is the capacity of these substances to assume different conformations depending on their own chemical structures as well as on diverse external factors. In this respect, conformation equilibria of several tetracycline derivatives as a function of pH have recently been reviewed in the absence and presence of calcium or magnesium at various concentrations.[11,23] Discussions on the present relationships between tetracycline conformational tendencies and nature of the respective copper binding sites will thus refer to information previously collected on the corresponding ligands.[11,23]

Experimental Section

Materials. TC, OTC, and DTC as free bases and DSC as the hydrochloride were kindly supplied by Pfizer Laboratories. CTC hydrochloride was purchased from Sigma Chemical Co. After their analysis for both antibiotic and mineral acid contents, these products were shown to be sufficiently reliable to be used without further purification. On account of the well-documented instability of tetracyclines in aqueous media,[24-26] fresh solutions were systematically prepared every day and kept in the dark under an atmosphere of nitrogen.

Except for DTC, all ligand solutions incorporated hydrochloric acid in sufficient excess (i) to minimize analytical errors on both reactant concentrations and (ii) to have all potential donor groups significantly protonated at the outset of each experiment. For DTC, whose lack of C4 dimethylamino group makes the free molecule exclusively acid—hence poorly soluble in aqueous media at acidic and neutral pH—two complementary strategies were applied. First, very diluted acidic solutions

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of DTC (about 5 mmol·dm^{-3}) were prepared to be titrated in the conventional way. Then, more usual concentrations (near 25 mmol·dm^{-3}) were obtained by dissolving DTC crystals into aqueous sodium hydroxide so that the protons of the two acidic groups were totally dissociated.

Sodium hydroxide stock solutions of specific concentrations were used for potentiometric titrations and spectroscopic investigations. All of these were prepared by diluting BDH concentrates in freshly boiled deionized water under a nitrogen blanket and proved to be carbonate-free by Gran titrations with Prolabo R.P. p.a. potassium phthalate. They were then frequently checked by this technique. Stock solutions of hydrochloric acid, also prepared from BDH or Prolabo R.P. concentrates, were regularly assessed using standardized sodium hydroxide solutions.

Sodium chloride employed as a background electrolyte was a Merck "pro analysi" reagent. So was copper chloride whose stock solutions were obtained by dissolving crystals in diluted hydrochloric acid. Metal and proton concentrations of these solutions were deduced from complexometric titrations involving EDTA[27] and from direct potentiometric readings, respectively.

Potentiometric Investigations. Potentiometric measurements relative to complex formation constant determinations were performed with a Beckman Model 4500 digital mV-meter (precision 0.1 mV). The electrochemical cells consisted of a Beckman glass electrode opposed to a saturated NaCl Ingold Calomel electrode, fitted in an Ingold cell system. The glass electrode was calibrated in the concentration scale following the most recent recommendations.[28] Accordingly, the symbol pH stands for –log [H] throughout this paper.

The temperature of the titration vessel was regulated at 37 ± 0.02 °C, and a stream of purified nitrogen was bubbled through the test solutions. Sodium chloride (0.15 mol·dm^{-3}) was chosen in an ionic strength relevant to that in the physiological medium. In these conditions, the logarithmic value of the ionic product of water was calculated to be –13.31,[29] the electrode slope being found equivalent to its theoretical value.

As is common with tetracycline metal complexes, a slight opacity and foaming were observed during the titrations of copper-containing solutions. In accordance with our usual protocol, experiments were nonetheless pursued until a steady drift became noticeable in the mV readings, this being taken as a criterion for the occurrence of precipitation.

On account of the poor solubility of DTC in acidic and neutral aqueous media (see above), alkaline solutions of this ligand in the absence or presence of copper were back-titrated against hydrochloric acid using a Metrohm 645 Multidosimat buret. More diluted solutions of DTC and all solutions of DSC in hydrochloric acid with or without copper were titrated with sodium hydroxide, successive aliquots being delivered by means of a Radiometer ABU12 Autoburette.

Copper-to-ligand concentration ratios were significantly varied over the sets of experiments performed to help characterize all the complexes in each system. Table I summarizes the experimental conditions of these titrations.

Potentiometric titrations were also performed in parallel to spectroscopic measurements, so as to monitor the conditions under which the formation of individual species could be successively favored. In those cases, concentrations of alkali or acid stock solutions used to adjust preselected pH values were chosen sufficiently high for the added volumes to be negligible with respect to (large) total ones. In this way, required conditions could be met without needing iterative calculations.

For these measurements, all experimental protocols were identical to those described above, except that room temperature was used to match the conditions of spectral determinations. This implies that pH values selected as corresponding to complex maximum percentages calculated from formation constants determined at 37 °C are only approximate.

Spectroscopic Measurements. UV–visible absorption spectra were determined in the 200–850-nm range by means of a Beckman UV5240 or of a Perkin-Elmer 554 double-beam spectrophotometer. Circular dichroism (CD) spectra were recorded over the 210–750-nm region with the help of a Jasco-J20 spectropolarimeter or a Jobin-Yvon Dichrographe III spectrometer, using fused quartz 0.1–10-mm cylindrical cuvettes. CD results are expressed in terms of $\Delta\epsilon = \epsilon_L - \epsilon_R$.

As outlined above, all samples analyzed by these techniques were taken from solutions of predetermined composition and pH so that formation of individual complexes could as much as possible be favored in turn.

Table I. Summary of Titration Data for the Cu–DTC and Cu–DSC Systems[a]

system	C_M	C_L	C_H	C_{OH}	pH range	n
Cu–DTC	0.050	0.501	4.561	5.03	2.61–6.17	46
	0.126	0.501	4.568	5.03	2.71–6.86	73
	0.189	0.501	4.558	5.03	2.70–7.51	61
	0.252	0.501	4.581	5.03	2.68–7.79	60
	0.503	0.501	4.589	5.03	2.54–7.34	66
	1.006	0.501	4.639	5.03	2.60–6.27	63
	0.251	2.535	–4.915	–20.12	8.62–2.71	42
	0.402	2.535	–4.898	–20.12	8.58–2.90	75
	0.653	2.535	–4.871	–20.12	8.51–2.50	72
	1.258	2.535	–4.805	–20.12	8.26–5.03	26
	1.258	1.268	–2.334	–20.12	4.87–3.85	6
Cu–DSC	0.101	0.963	1.963	19.26	2.86–9.66	54
	0.151	0.963	1.968	19.26	2.84–9.65	59
	0.252	0.963	1.979	19.26	2.81–9.59	55
	0.503	0.963	2.007	19.26	2.75–9.84	70
	1.006	0.963	2.061	19.26	2.70–6.12	47
	2.012	0.963	2.171	19.26	2.62–5.09	33

[a] Initial concentrations of copper (C_M), ligand (C_L), and strong acid (C_H) in the titrate as well as hydroxide concentrations (C_{OH}) in the titrant are expressed in mmol·dm^{-3} (negative C_H given for hydroxide concentrations and vice versa indicate that back-titrations with acid were employed); the pH notation stands for –log [H] (see text) and n represents the number of experimental observations in each titration.

Appropriate conditions were selected from simulated distributions of both metal and ligand into their different complexes.

Large ranges of reactant concentrations and concentration ratios were initially scanned within the limits inherent to the above techniques. However, on account of the species distributions obtained for all systems investigated, only the 1:2 metal-to-ligand ratio was finally used, reactant concentrations being adapted to match the sensitivity of each apparatus (as a function of the wavelength range when necessary). Thus, 1 and 2 mmol·dm^{-3} ligand total concentrations were used to record UV absorption and CD spectra relative to tetracycline structures in the absence or presence of copper, whereas copper coordination features were investigated through visible absorption and CD spectroscopies with 0.25 and 3 and 1 mmol·dm^{-3} metal total concentrations, respectively.

As for the pH values selected, they were as far as possible chosen at the peak percentages of the involved species. Regarding this, it is worth noting that the use of reactant distribution profiles simulated outside pH intervals experimentally investigated can be misleading and should thus be avoided. Accordingly, results of simulations above pH 10 in the presence of copper have not been reported.

Calculation Techniques. Our usual approach[4-12] consisting of the two successive optimization and simulation steps was developed to calculate the formation constants of DTC and DSC copper complexes. First, initial estimates deduced from protonation as well as formation curves were refined with the ESTA optimizer[29] and the MINIQUAD program,[30] a large number of species combinations being considered in turn. Then, after a rough selection of the most representative of these combinations on purely numerical grounds, the discrimination of the "best" set of constants among numerically equivalent possibilities was based on graphical comparisons between experimental formation curves and their ESTA-simulated[31] counterparts materializing the hypothesis being tested. For the sake of possible comparisons with previous determinations,[4-12] the best set of constants for each system was ultimately refined with MINIQUAD.

The pH-dependent speciation profiles on which the selection of experimental conditions for the spectroscopic measurements was based were obtained from our plotting with an updated version of the COMICS program.[32] The stability constants used for these calculations are shown in Table II, this including hydrolysis constants of the Cu^{2+} ion selected from the literature.[33]

Results and Discussion

Copper Complex Formation with DTC and DSC. As suggested above, calculations of the formation constants of copper DTC

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Table II. Formation Constants Used in the Calculations

system	complex	log β	R factor	ref
H–DTC	LH	7.715		11
	LH_2	13.216		
Cu–DTC	ML	8.8 ± 0.6	0.01–0.02	this work (n = 221; n = 369)
	MLH	13.5 ± 0.5		
	ML_2H	23 ± 2		
	ML_2H_2	26 ● 2		
H–DSC	LH	9.118		11
	LH_2	16.588		
	LH_3	19.744		
Cu–DSC	ML	11.596 ± 0.019	0.0055	this work (n = 318)
	MLH	17.768 ± 0.013		
	MLH_2	20.494 ± 0.055		
	ML_2	14.788 ± 0.124		
	ML_2H	23.932 ● 0.046		
	ML_2H_2	31.804 ● 0.045		
	M_2LH	20.299 ± 0.084		
H–TC	LH	9.052		4
	LH_2	16.323		
	LH_3	19.485		
Cu–TC	MLH	17.978		9
	MLH_2	20.972		
	ML_2H_2	31.278		
	ML_2H_4	40.673		
H–OTC	LH	8.665		4
	LH_2	15.775		
	LH_3	18.996		
Cu–OTC	ML	12.439		9
	MLH	18.114		
	MLH_2	20.797		
	ML_2H_4	39.102		
	ML_2	15.343		
H–CTC	LH	8.698		7
	LH_2	15.714		
	LH_3	18.794		
Cu–CTC	ML	10.686		9
	MLH	16.672		
	MLH_2	19.957		
	ML_2H_2	29.516		
Cu–hydroxide	MH_{-1}	−8.3		33
	MH_{-2}	−17.6		
	MH_{-3}	−27.8		
	MH_{-4}	−39.0		
	M_2H_{-2}	−10.7		

complexes was made extremely difficult by solubility problems. Determinations in the normal ligand concentration range were subject to errors specifically due to the back-titration procedure. Also, precipitation occurred at neutral pH for metal-to-ligand ratios exceeding 1:2. For direct titrations on the other hand, overall concentrations were so low that experimental errors were magnified. In spite of this, the main species present in this system could be identified and average values of their constants roughly evaluated (Table II). The unusually high MINIQUAD R factors relative to these determinations account for the special conditions encountered.

For the Cu(II)–DSC system, nearly all possible complexes of the mono- and dianionic forms of the ligand have been characterized, MLH and ML species being predominant under the conditions investigated. Corresponding constants along with standard deviations and MINIQUAD goodness-of-fit parameters are reported in Table II. Experimental and simulated formation curves of this system can be compared in Figure 1.

Tetracycline Conformations. Tetracyclines are capable of assuming several conformations in solution[25,26,34–36] as well as in the solid state.[37–39] The relative flexibility of these molecules largely conditions their capacity to accommodate diverse metal bonding modes. Accordingly, a quick recall of their main structural features is necessary before examining the present results.

Conformational variations of tetracyclines in aqueous solution have recently been reviewed by some of the present authors.[11,23] The most important point to be noticed is the key role played by the C4 dimethylamino group of the bioactive analogs in their alternate adopting conformations *A* ("extended"[11]) and *B* ("twisted"[11]) (Chart II) under basic and acidic conditions, respectively.

In basic aqueous solutions, all common tetracyclines (TC, OTC, DOX, CTC) as well as DSC display the all-chair "extended" conformation *A*.[11,23,25] In this form, the C1, C2, C3, and amide carbons lie above the roughly accommodated BCD ring plan, hydrogen bonding being expected to take place between N4 and $OH12_a$ atoms. In acidic and neutral solutions, conformation *A* presumably remains the most stable energetically, but the strong repulsion experienced between solvated C4 dimethylammonium and $OH12_a$ groups is relieved by a slight twisting of the A ring[25] into what was designated from this effect the "twisted" conformation *B*.[11] With the usual reservations in order when transposing X-ray crystal data to solution considerations, the $N4H^+$–O3 hydrogen bond characterized on solid zwitterionic OTC[38] may be considered to still hold in water.

In addition to the two above conformations, tetracycline can assume other structures depending on the nature and position of substituents, nature of the solvent, and metal coordination. On

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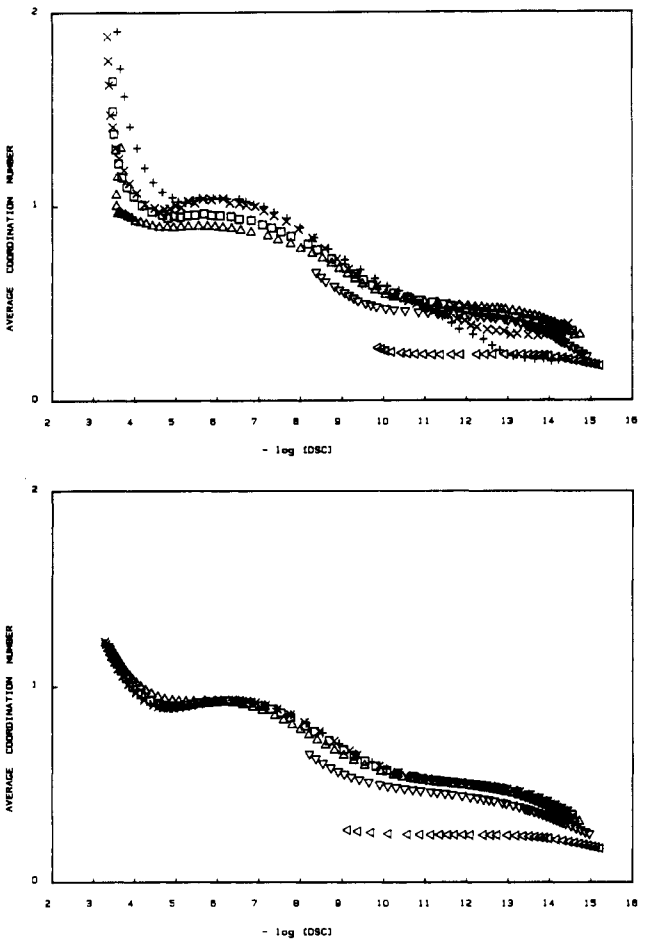


Figure 1. Formation cuves of the copper–DSC system: (a, top) experimental; (b, bottom), simulated as they would result from analytical concentrations in Table I in the hypothesis of formation constants in Table II. Data are represented by the following symbols in the order of the experiments shown in Table I: plus, times, box, up triangle, down triangle, sideways triangle.

binding certain metal ions for example, OH5-substituted derivatives are found to adopt intermediate conformation *C*. In this structure which results from twisting the A ring of conformer *B* until the C4 dimethylamino group becomes equatorial, the A and B rings are forced into higher energy boat forms.[25,26] Likewise, conformation *D*,[25,26] the other all-chair extreme as opposed to *A*, is adopted when inversion of the B ring allows the relief of interactions among perisubstituents in the C4–C7 region, as is the case with the bulky 7-chloro and 6-α-methyl groups of the instable CTC.[23] A slight distortion of *D* leads to conformation *E*[26] seen in X-ray studies,[37-39] referred to as "folded" by Martin,[40] and displayed by the nonionized tautomer of zwitterionic OTC in apolar media.[34-36,41]

(40) Martin, R. B. *Met. Ions Biol. Syst.* **1985**, *19*, 19.
(41) It is worth noting in this respect that conclusions of refs 34–36 may not be so clear-cut as contended. In our opinion, confusion may arise between conformers *A* and *E* in organic solvents of still relatively high dielectric constant. The progressive decrease of the dielectric constant of mixed solvents as their organic fraction grows may not be sufficient to induce the adoption by OTC of the conformation *E* assumed by its unionized form crystallized from benzene or toluene[37,38] but can be important enough to reverse the order of the N4 and O12 protonation steps as has recently been demonstrated.[40] Accordingly, conformation *E* claimed by Hughes et al.[34] may well have been mistaken for *A*. This confusion is all the more possible as the positive Cotton effect at 262 nm attributed to conformation *E* in CD spectra may correspond to conformation *A* as well. The putative role of magnesium in favor of conformation *B* suggested by Everett and Gulbis[35] may also be exerted at the expense of *A* instead of *E*, like calcium complexations through N4–O12a and magnesium through N4–O5 lead to reverse 4-epiTC to its original stereochemistry.[25]

Chart II

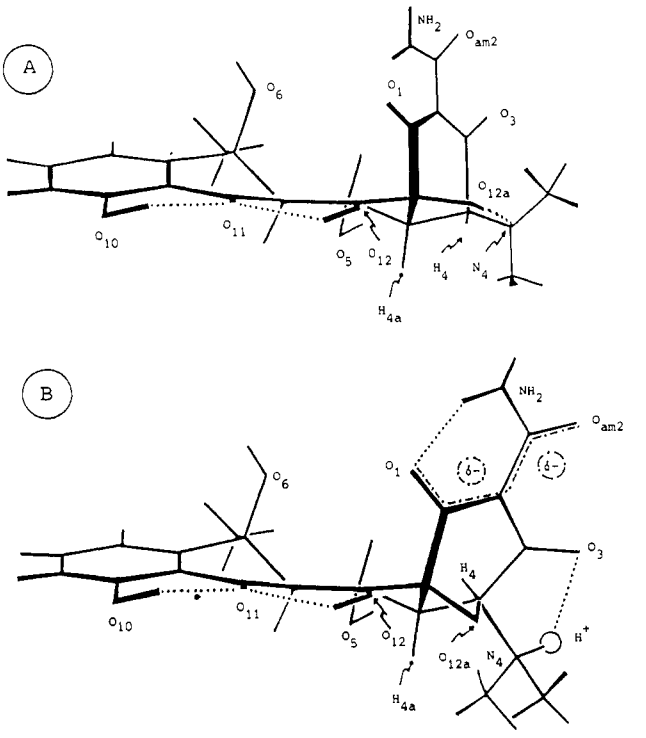
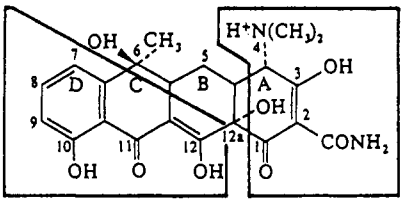


Chart III



Tetracycline Chromophores. In addition to the multiple conformational equilibria of tetracyclines, the noncoincidence between the electron-donor sites and chromophores of these ligands is also a factor of difficulty for interpreting their metal coordination modes. While all common tetracycline analogs possess three proton dissociation centers, namely the C4 dimethylamino, the O10–O12 diketo phenolic group, and the C1–C3 tricarbonyl methane moiety in the increasing acidity order,[21,41] they contain two chromophoric regions only, insulated from each other by the $OH12_a$ group (Chart III).

According to previous spectroscopic data,[25] the highly enolic A-ring chromophore would contribute a π-to-π^* transition in the 260-nm region, the remainder of the peaks being attributable to the BCD-ring one. The UV peak observed at 275 nm is confirmed in CD by two opposite Cotton effects usually observed at 262 nm (negative) and 295 nm (positive) in acidic to neutral aqueous media.[11,23,25,26,34] The most interesting feature of the CD spectra is that the high-intensity negative Cotton effect at 262 nm associated with conformation *B* reverses on increasing the pH: the molecule progressively turns to conformation *A* as the C4 dimethylammonium group deprotonates.[11,23] Also, the appearance of double Cotton effects centered near 385 nm is indicative of joint interactions of the A ring and the BCD system in metal coordination: 370-nm negative and 400-nm positive bands are characteristic of O12–O1 chelation,[23,42] while the same bands with opposite signs correspond to N4–O5 binding for 5-OH derivatives.[23]

Copper Electron Transitions. Among copper electron transitions observable in absorption spectra relative to tetracycline

(42) Newman, E. C.; Franck, C. W. *J. Pharm. Sci.* **1976**, *65*, 1728.

coordination, weak nitrogen-to-copper charge-transfer transitions below 400 nm will not be taken into account since they are likely to be overlapped by more intense intraligand transitions. Likewise, the very intense ligand band which spans up to 460 nm precludes any analysis of phenolate oxygen-to-copper transitions in the absorption. The latter transitions, however, can be clearly assessed in CD spectra near 430–450 nm as the corresponding ligand chromophore is not optically active.

In contrast, ligand field (d–d) transitions relative to copper coordination which appear at longer wavelength (above 500 nm) have been recorded in both absorption and CD spectra.

Copper–DTC System. Because of the solubility problems encountered in related equilibrium investigations, accurate stability constants are not available in this system. Nevertheless, the average orders of magnitude obtained from the direct and back-titrations performed (Table II) are reliable enough to allow useful comparisons.

First of all, it is worth recalling[11] that protonation constants of DTC are significantly higher than those of corresponding donor sites in bioactive analogs (Table II). This effect, which is due to the electron-withdrawing effect of the C4 dimethylamino group in the latter compounds, particularly affects the binding capacity of the O3 atom.[11] Possible copper coordination to O3 and O12 sites will thus be expected to be more stable with DTC than with the other derivatives investigated, more especially if the O3 atom is involved.

If we consider ligand proton interactions to be unchanged in the presence of copper, stabilities of the metal–ligand bonds in protonated species can be estimated from overall formation constants by subtracting appropriate protonation increments. In the MLH complex for example, in which the deprotonated O3 atom is the only possible coordination center, the logarithmic constant relative to the copper–O3 bond can be calculated to be around 6 (Table II). Formation constants for the same bond in complexes of bioactive analogs are thus expected to be significantly inferior to this value. The second binding site of DTC with the deprotonated O12 atom is the O10–O12 system. This site should logically be involved in the ML complex with a logarithmic constant of about 9 (Table II). Formation constants for such bonds with bioactive analogs should thus be found of this order of magnitude or slightly lower. Similar considerations can be applied to the two copper–O3 bonds involved in ML_2H_2, as well as to the copper–O3 and copper–(O10–O12) ones in ML_2H (Table II).

Like the above potentiometric determinations, spectroscopic studies relative to this system were largely thwarted by solubility problems. Precipitates were indeed observed over most of the useful pH range at the copper concentrations required to detect metal ion transitions (see above). As a consequence, data could be obtained in highly alkaline solutions only. Moreover, indications of the CD spectra concerning ligand conformation changes due to complexation are not so clear as with other tetracycline derivatives.[11] Without the C4 dimethylamino group, the DTC molecule cannot follow the above described conformational scheme. Instead, the partial *A* character it assumes in the acidic pH range is superseded by a *B*-like form as the OH12 group dissociates (Figure 2a).[11] No striking change is thus observed in the 260-nm region in the presence of copper. As was the case with magnesium,[11] a reinforcement of the *B* character of the free ligand structure is only noted in alkaline medium whereas the positive band near 240 nm relative to BCD ring interactions[23,25] is attenuated (Figure 2a). This modification of the ligand spectrum is, however, more important than it may seem at first sight since it reflects the influence of the complexed fraction of DTC only (estimated near 7%). This suggests that copper coordination entails conformational changes on the BCD ring system, which confirms the above expectation of a copper bond to the O10–O12 juncture.

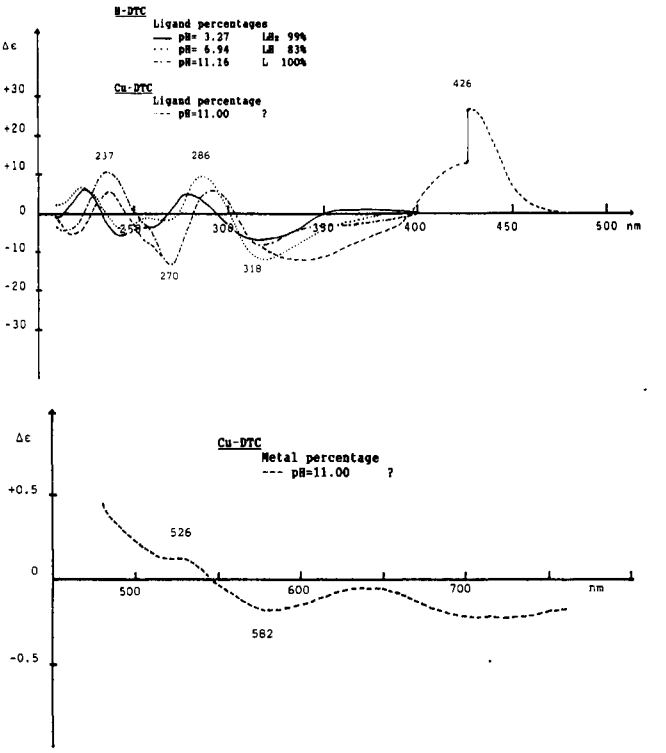


Figure 2. (a) Top: Circular dichroism spectra of DTC (2 mmol·dm^{-3}) in the absence and in the presence of copper (1 mmol·dm^{-3}) as indicated in the table inset. Sensitivities of differential dichroic absorptions are doubled beyond the vertical line. (b) Bottom: Circular dichroism spectrum relative to Cu^{2+} ions (1 mmol·dm^{-3}) in the presence of DTC (2 mmol·dm^{-3}).

Regarding copper electron transitions, the Cotton effects observed in the d–d transition range (Figure 2b), which are characteristic of metal ion interactions with an optically active ligand,[43] confirm that a fraction of copper is bound to DTC at pH 11, presumably in a bidentate manner.[44] In the charge-transfer transition range at the same pH, the CD band at 426 nm (Figure 2a) may correspond to an (O10–O12)-to-metal transition.[43,45,46] A priori, it might also result from the shift of an intraligand transition, but no comparable effect was detected for free DTC over the whole pH range investigated (Figure 2a and ref 11). In addition, both energy and intensity of the d–d transition observed in the absorption spectrum at higher pH (Table III) are reminiscent of those relative to copper tetrahydroxide seen in previous studies.[46]

To conclude with this system, let us note that absorption spectra recorded with a metal concentration of 0.5 mmol·dm^{-3} only (not shown here) confirm that copper complexation is already significant at pH as low as 2 in the form of MLH (22% of DTC). Important variations in the intensities of strong intraligand transitions are indeed observed in the presence of the metal ion.

Copper–DSC System. Among the seven complexes characterized in this system (see above), ML and ML_2 coexist with their mono- and diprotonated forms. An examination of the corresponding stability constants in Table II may thus provide preliminary information on the bonding modes of copper in these two series of species.

Supposing like in the above system that ligand–proton interactions remain unaffected in the presence of copper, stabilities of the metal–ligand bonds formed in the various protonated species can be estimated by subtracting step-protonation increments from

(43) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385.
(44) Larsen, E.; Olsen, I. *Acta Chem. Scand.* **1964**, *18*, 1025.
(45) Pettit, L. D.; Steel, I.; Kowalik, T.; Kozlowski, H. *J. Chem. Soc., Dalton. Trans.* **1985**, 1201.
(46) Kiss, T.; Kozlowski, H.; Micera, G.; Strinna Erre, L. *Polyhedron* **1989**, *8*, 647.

Table III. Visible Absorption Data[a]

system	pH	complex percentages	wavelength (nm)	ϵ
Cu–DTC	11.87	?	574	160.7
Cu–DSC	2.04	MLH_2 56%, M^{2+} 25%	662	44.7
	2.66*	MLH_2 29%, M^{2+} 43%	660	14.0
	3.16*	MLH_2 22%, MLH 57%	658	18.0
	4.95*	MLH 88%	605	85.0
	5.70*	MLH 60%, ML 21%	600	102.0
	11.02	?	613	129.3
	12.03	?	593	155.0
Cu–TC	1.02	MLH_2 45%, M^{2+} 41%	750	16.0
	2.25	MLH_2 54%, ML_2H_4 32%	645	36.7
	3.69	MLH 53%, ML_2H_4 36%	625	53.4
	4.18	MLH 77%, ML_2H_4 20%	625	61.3
	12.17	?	580	166.7
Cu–OTC	1.54	MLH_2 80%	733	34.0
	2.45	MLH_2 57%, MLH 37%	685	35.8
	3.59	MLH 81%	670	72.5
	4.59	MLH 90%	663	78.0
	5.46	MLH 61%, ML 39%	630	108.7
	10.49	?	603	150.0
	11.49	?	605	166.7
Cu–CTC	1.45	MLH_2 61%, M^{2+} 38%	765	22.8
	2.29	MLH 81%	705	36.3
	3.10	MLH_2 59%, MLH 39%	665	65.8
	4.30	MLH 84%	615	118.3
	10.33	?	600	185.0

[a] C_M = 0.00025 (asterisk) and 0.003 mol·dm^{-3}; C_L = 0.0005 (asterisk) and 0.006 mol·cm^{-3}. Species whose percentage is less than 10% are not mentioned.

parent overall formation constants. The respective values obtained in this way for the copper DSC bonds in MLH and MLH_2 (8.65 and 3.91) are very different from each other and much smaller than the ML constant (11.60). This reveals that important changes take place in the copper coordination mode along the pH range investigated. The first two constants approximately correspond to the values expected from the preceding system for copper–O10–O12) and copper–O3 coordinations (see above section); the higher value of the third, relative to the dianionic form of the ligand, suggests a possible participation of the N4 atom. In contrast, estimates of stability constants for the metal–ligand bonds in ML_2H and ML_2H_2, respectively, found to be 14.81 and 13.57 (with $\log \beta_{M(LH)_2} = \log \beta_{ML_2H_2} - 2 \log \beta_{LH}$) or 15.22 (with $\log \beta_{M(LH)_2} = \log \beta_{ML_2H_2} - \log \beta_{LH_2}$), are close to the formation constant of ML_2 (14.79). Unlike 1:1 species, all 1:2 metal-to-ligand ones thus probably involve identical donor groups, especially if alternate sites prevail in the two ligands.

Comparisons of related protonation steps can help solve the latter point, provided corresponding binding sites do not interact with one another. In this respect, stability increments for the successive protonations of ML_2 first into ML_2H (9.14) and then into ML_2H_2 (7.87) compare well with the protonation constants of the dimethylamino (9.12) and diketo phenolic (7.47) groups, respectively. It can thus be presumed that, in the ML_2, ML_2H, ML_2H_2 series, one of the two ligands chelates copper via its O10–O12 system while the other has its N4 atom involved in the copper binding.

Let us now examine the spectroscopic data in the light of the above hypotheses, starting with the CD spectra relative to DSC conformations. As recalled by Figure 3a, the free DSC molecule follows the classical scheme specific to all bioactive tetracyclines: [11] assuming conformation *B* up to pH 8, it then turns to conformation *A* above pH 9, this change inducing the sign inversion of the CD band observed near 260 nm. In the presence of copper at pH 2.2, the 262-nm band is still negative although the intensity of the double Cotton effect centered near 280 nm is lessened. The 22% of DSC involved in MLH_2 are thus presumed to remain in conformation *B*, but this form seems to be somewhat destabilized as it would be the case if the $N4H^+$–O3 hydrogen bond was ruptured following the coordination of copper to O3 as

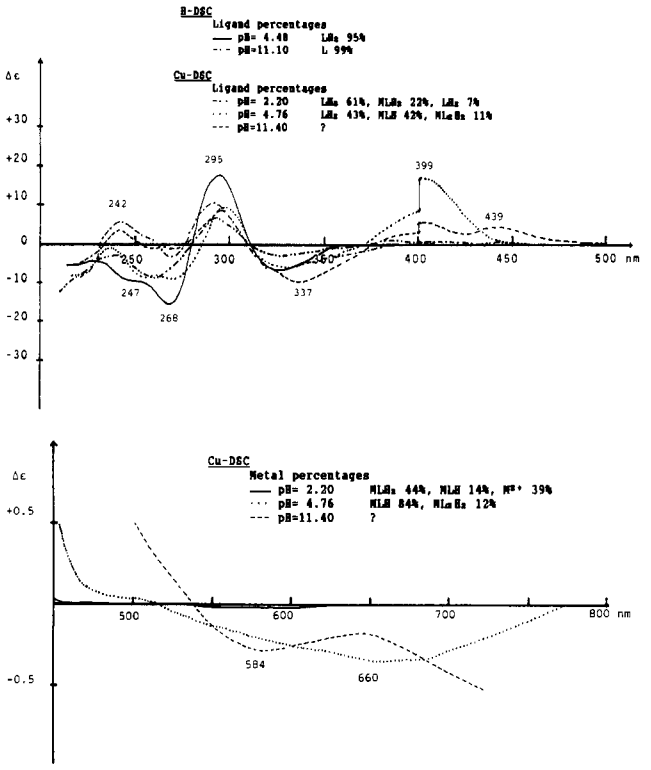


Figure 3. (a) Top: Circular dichroism spectra of DSC (2 mmol·dm^{-3}) in the absence and in the presence of copper (1 mmol·dm^{-3}) as indicated in the table inset. Sensitivities of differential dichroic absorptions are doubled beyond the vertical line. (b) Bottom: Circular dichroism spectrum relative to Cu^{2+} ions (1 mmol·dm^{-3}) in the presence of DSC (2 mmol·dm^{-3}).

this atom deprotonates. At pH 4.76, the 262-nm band is not significantly modified whereas the 255- and 270-nm negative troughs on each side, which are indicative of BCD ring interactions,[25] tend to deepen on the contrary. It thus appears likely that DSC is still in conformation *B* in MLH at least (42% at this pH), with copper probably binding at the O10–O12 site. In basic solutions, the intensities of the bands in the 250–280-nm region become negligible. It is the case in particular at pH 11.40 where the CD spectrum is largely representative of conformation *A* but slightly less so than with free DSC (Figure 3a). This is consistent with most of DSC adopting conformation *A*, which implies that some of the ligand anions involved in ML_2 and ML complexes chelate copper through the N4 and $O12_a$ atoms.[11,23,25]

The examination of the data for copper electron transitions provides complementary information for this discussion. In the absorption spectrum recorded at pH 2.04 for example (Table III), the energy of the d–d transition observed with the metal mainly present as MLH_2 is logically different from that of the cupric aqua ion (800 nm). Moreover, the absence of a Cotton effect in the CD spectrum obtained under similar conditions (Figure 3b) is consistent with copper being coordinated through the O3 atom in a monodentate manner.[44] At pH 4.76, MLH becomes largely predominant in the metal distribution, and the appearance of a CD negative band at 650 nm (Figure 3b) suggests that the Cu^{2+} ion is now chelated,[44] presumably through the O10–O12 site. In addition, the strong CD positive band at 399 nm relative to BCD ring interactions (Figure 3a),[25] which was already observed in calcium coordination,[11,23] probably reflects conformational changes of the ligand due to the binding of copper at its O11–O12 atoms. Correlatively, both energy and intensity of the d–d absorption band (measured at lower reactant concentrations to avoid precipitation) are increased (see Table III at pH 4.95). All this is in line with the above stability constant analysis.

The spectra recorded in very basic solutions also reveal interesting features in line with hypotheses derived from equi-

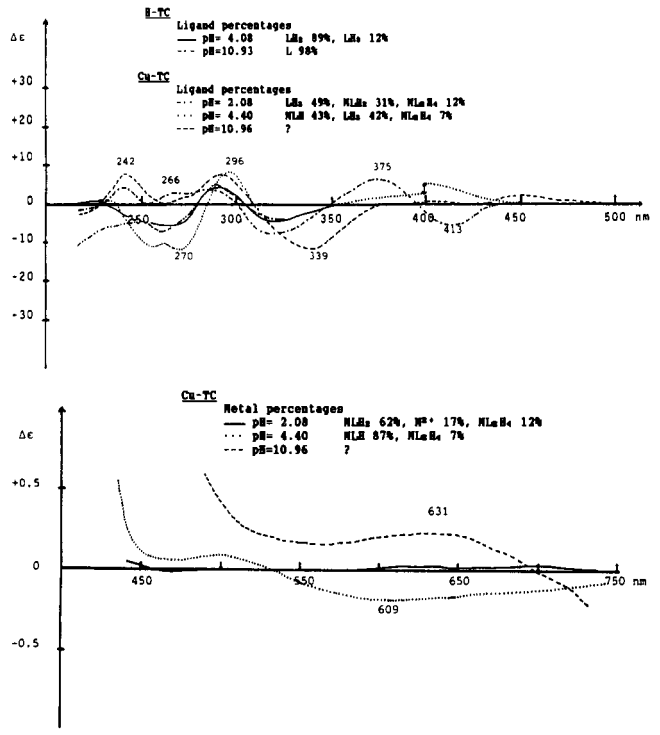


Figure 4. (a) Top: Circular dichroism spectra of TC (2 mmol·dm^{-3}) in the absence and in the presence of copper (1 mmol.dm^{-3}) as indicated in the table inset. Sensitivities of differential dichroic absorptions are doubled beyond the vertical line. (b) Bottom: Circular dichroism spectrum relative to Cu^{2+} ions (1 mmol·dm^{-3}) in the presence of TC (2 mmol·dm^{-3}).

librium constant and ligand conformation considerations. For example, the negative Cotton effect recorded at pH 11.40 in the d–d transition range (Figure 3b) indicates that the copper presumably involved in ML and/or ML_2 is bound differently than in MLH. Chelation through the N4–OH12$_a$ group is likely to take place in ML given the high stability of this species, as is also the case for one of the two ligands in ML_2, whereas the other probably binds through the O10–O12 system. The charge-transfer CD band at 439 nm, which corresponds to that observed at 426 nm in the copper–DTC system, supports this interpretation. Finally, the presence of copper tetrahydroxide at high pH is at the origin of both the hypsochromic shift and increased intensity of the d–d transition seen in the absorption data (Table III).

Copper–TC System. Only protonated species were characterized in our previous studies on this system because of solubility problems,[9] but stabilities of corresponding metal–ligand bonds can be compared to copper–DSC constants used as references. For example, the 8.93 and 4.65 values respectively obtained for the copper–TC bonds in MLH and MLH_2 after deduction of the appropriate protonation constants are of the same order of magnitude as those found with DSC by the same procedure. This implies that TC presumably binds copper through the same donor groups as DSC. Similarly, subtracting $\log \beta_{LH_2}$ from $\log \beta_{ML_2H_2}$ yields 14.96, which is close to the $\log \beta_{ML_2}$ value of the copper–DSC system (14.79). Alternate bonds involving N4 and O10–O12 sites are thus expected in ML_2H_2. The situation is logically different in ML_2H_4, where the constant for the two copper–TC bonds (8.03) suggests that both ligands coordinate copper via their O3 atom as in MLH_2.

As recalled by Figure 4a, TC behaves like DSC from the structural point of view, adopting conformation *B* up to pH 8 and then turning to conformation *A* as the C4 dimethylammonium group deprotonates.[23] At pH 2.08, the CD spectrum obtained with 44% of TC present as MLH_2 and ML_2H_4 is very similar to what it is without metal at pH 4.08. Clearly, TC is in conformation *B*, but the band near 260 nm is less negative than it should be in the absence of copper at pH 2.08.[23] As with DSC, conformation *B* thus appears to be somewhat destabilized; hence, it can be proposed that monodentate bonds are formed at the O3 atom for the two species present. At pH 4.40 with MLH being predominant, the band at 260 nm becomes more negative and two troughs appear on each side at 255 nm and 270 nm, indicative of BCD ring interactions.[25] This situation is also reminiscent of the copper–DSC system: Copper coordination is expected to take place at the O10–O12 site; the N4H$^+$–O3 hydrogen bond is restored, which tends to restabilize conformation *B*.[47] In highly basic solutions, the nonsuperimposition of the CD spectra in the presence or absence of metal shows that copper coordination influences TC conformation (Figure 4a), presumably in ML and/or ML_2 species not characterized by potentiometry for solubility reasons.

Concerning metal ion spectroscopic characteristics, the absorption spectra summarized in Table III show that the d–d transition observed with 45% of copper present as MLH_2 is centered around 750 nm, while with 54% of copper as MLH_2 and 32% as ML_2H_4 this band appears at 645 nm: the more copper that is complexed, the more distant the resulting transition energy is from that of the cupric aqua ion (800 nm). Moreover, as long as MLH_2 and ML_2H_4 are the only complexes in solution, the CD spectra do not display any Cotton effect in the d–d transition range (Figure 4b). All these observations confirm the above hypothesis of copper being monodentately bound through the O3 atom in both MLH_2 and ML_2H_4.

The progressive increase of pH makes MLH the predominant copper species in solution (77% at pH 4.18). The d–d absorption band (Table III) correlatively shifts to 625 nm and becomes more intense, which is consistent with the O10–O12 copper binding. The appearance of a negative d–d band in the CD spectrum obtained at pH 4.40 (Figure 4b) confirms that chelation takes place, the increased energy observed (609 nm) corresponding to the greater percentage of MLH (87%). Like DSC, TC thus probably binds copper via the O10–O12 system in the MLH complex.

At high pH values, complementary pieces of information are obtained from CD and absorption data. At pH 10.96, a positive CD band appears near 630 nm which is by definition indicative of copper binding with an optically active ligand[44] (Figure 4b) whereas the absorption band observed at pH 12.17 is close to that seen for copper tetrahydroxide in previous studies[46] as well as in the copper–DSC system (Table III). In the absence of reliable speciation results in this pH range, one is thus tempted to conclude that the $Cu(OH)_4$ species coexists with ML and/or ML_2 copper–TC complexes as the phenolate oxygen-to-copper charge-transfer band at 452 nm ($\Delta\epsilon = +1.5$) suggests (Figure 4a).

Copper–OTC System. The examination of the complex formation constants in this system leads to hypotheses already proposed for TC and DSC. The values calculated for the copper–OTC bonds in MLH_2 and MLH (respectively 5.02 and 9.45) are higher than those found with TC and DSC, but the ML constant is also higher than that determined in the copper–DSC system by about one logarithmic unit. OTC is thus expected to bind copper through the same donor groups as TC and DSC in the 1:1 complex series: acting as a monodentate ligand through O3 in MLH_2, OTC becomes bidentate via O10–O12 in MLH and then through N4–O12a in ML. Concerning 1:2 metal-to-ligand species, a formation constant of 7.55 can be calculated for the two copper–OTC bonds in ML_2H_4, which clearly corresponds to O3 coordination as in MLH_2. In contrast, nothing clear-cut can a priori be said about the donors involved in ML_2 (15.34) since neither ML_2H nor ML_2H_2 species have been characterized in

(47) Copper chelation through the O3–N4 atoms would also stabilize conformation *B*, but all other parameters (formation and protonation constant comparisons, intraligand transition CD bands near 255 and 270 nm) are in favor of O10–12 coordination.

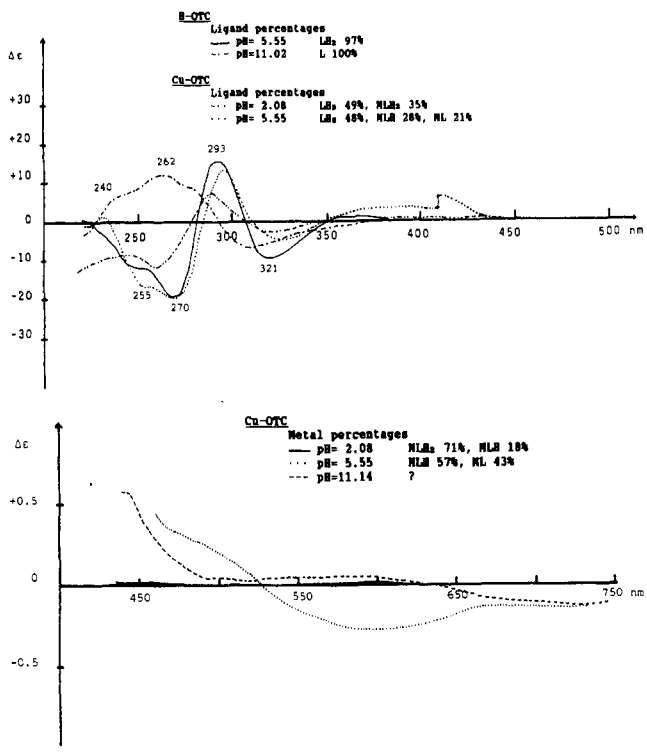


Figure 5. (a) Top: Circular dichroism spectra of OTC (2 mmol·dm^{-3}) in the absence and in the presence of copper (1 mmol·dm^{-3}) as indicated in the table inset. Sensitivities of differential dichroic absorptions are doubled beyond the vertical line. (b) Bottom: Circular dichroism spectrum relative to Cu^{2+} ions (1 mmol·dm^{-3}) in the presence of OTC (2 mmol·dm^{-3}).

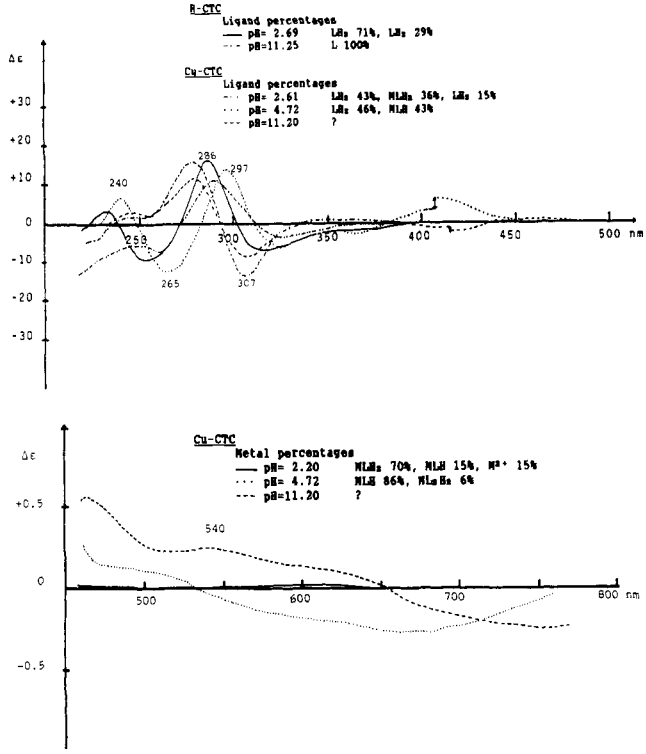


Figure 6. (a) Top: Circular dichroism spectra of CTC (2 mmol·dm^{-3}) in the absence and in the presence of copper (1 mmol·dm^{-3}) as indicated in the table inset. Sensitivities of differential dichroic absorptions are doubled beyond the vertical line. (b) Bottom: Circular dichroism spectrum relative to Cu^{2+} ions (1 mmol·dm^{-3}) in the presence of CTC (2 mmol·dm^{-3}).

this system, but the comparison with DSC (14.79) suggests the participation of alternate binding sites.

From the ligand conformation point of view, Figure 5a confirms that free OTC behaves in the classical DSC and TC manner: conformation *B* is adopted in acidic and neutral medium, but conformation *A* prevails above pH 9 when the C4 dimethylammonium group is deprotonated as the inversion of the Cotton effect near 260–270 nm reveals. Like with DSC and TC near pH 2, a clear destabilization of conformation *B* is observed when 35% of OTC is present as MLH_2 (Figure 5a), indicative of copper coordination to the O3 atom. Then, following a now usual scheme, conformation *B* is restabilized at pH 5.55 as MLH progressively substitutes for MLH_2 with O10–O12 chelation being expected to take place.[47]

Regarding metal ion spectroscopic data, the d–d transition at 733 nm (Table III) as well as the absence of a Cotton effect in this range in very acidic medium (Figure 5b) confirm the O3 unidentate bond of copper to OTC in MLH_2. As the pH is raised, MLH becomes progressively predominant in the metal distribution. The hypsochromic shift of the d–d transition toward 660 nm and the correlative appearance to a negative Cotton effect near 600 nm are consistent with O10–O12 copper chelation in this species. The present negligibility of ML_2H_4 with respect to the copper–TC system may explain the different d–d transition energies observed in absorption (660 nm with OTC; 630 with TC). At pH 5.46, the growing influence of ML in the copper distribution is paralleled by the increase in both energy and intensity of the d–d transition seen in absorption (Table III). A comparison of these parameters with the results obtained with DSC and CTC (Table III) tends to confirm that N4–O12$_a$ coordination takes place in ML. As with MLH above, the results relative to OTC and TC in highly alkaline solutions are also distinct: the energies of the d–d transition bands detected in absorption are lower with OTC (605 nm) than with TC (580 nm), while the two CD positive bands are observed at 570 nm with OTC instead of 631 nm with TC (Figures 4b and 5b). Once again, ML, ML_2, and $Cu(OH)_4$ species probably coexist in solution.

Copper–CTC System. The hypotheses drawn from stability constant comparisons about copper–CTC complexes are in line with those previously proposed for the other bioactive tetracycline analogs. In the 1:1 complex series, copper is presumably bound to O3 in MLH_2 (4.24), via O10–O12 in MLH (7.97), and through N4–O12$_a$ in ML (10.69). The alternate participation of N4 and O10–O12 sites in ML_2H_2 is also likely from a comparison of log $\beta_{ML_2H_2} - \log \beta_{LH_2}$ (13.80) with $\log \beta_{ML_2}$ of the copper–DSC system (14.79), account being taken of the general loss of stability due to the electron-withdrawing effect of the chloro substituent (about one logarithmic unit).

The situation concerning the structural conformations of CTC is quite different. Like other bioactive analogs, CTC does assume conformation *B* in acidic medium.[23] However, when the diketo phenolic group deprotonates, the severe steric hindrance resulting from the interaction of the bulky chlorine at C7 with the 6-α-methyl substituent is presumed to be released by the twisting of the BCN ring system along the C6–C11 axis, so that the pseudoboat form of the C ring is almost totally reversed.[25,26] Thus, the BDc ring system is forced to adopt a structure close to the one it assumes in conformation *D*[23,25,26] (Figure 6a). In the presence of copper at pH 2.61, the 36% of CTC in the form of MLH_2 classically display a somewhat destabilized conformation *B*, the metal ion being presumably bound at the O3 atom. Then, while MLH progressively replaces MLH_2 as the pH is raised, the Cotton effect at 265 nm becomes more negative, indicating a restabilization of conformation *B* due to the chelation of copper through the O10–O12 atoms. Finally, in very basic solutions, CTC turns to the *D*-like conformation in ML as well as in its dianionic free form.

From the metal ion point of view, the d–d transition observed above 750 nm at very acidic pH progressively shifts toward 700

nm as the proportion of copper in the MLH_2 form becomes maximum (Table III). The absence of a Cotton effect in the CD spectrum recorded at pH 2.20 (Figure 6b) confirms the monodentate binding of copper to the O3 atom. Both energy and intensity of the d–d transition band increase as MLH substitutes for MLH_2 in the metal distribution (Table III), presumably reflecting the joint implication of the two oxygen atoms in the copper coordination at the O10–O12 site. That chelation takes place in MLH is ascertained by the correlative appearance of the negative Cotton effect in the 670–680-nm region on the CD spectrum obtained at pH 4.72 (Figure 6b). At pH 10.33 with ML as presumably predominating in the solution, the d–d transition band in absorption shifts to 600 nm and its intensity still grows, confirming the role of the nitrogen atom in the putative N4–$O12_a$ bond.

Conclusion

On the whole, all the conclusions drawn from the present spectroscopic data are consistent with the previsions made from stability constant comparisons. Necessary reservation being made of the limited application range of speciation calculations, determinations of complex formation constants should thus always be considered a prerequisite to structural investigations in solution.

In the present case, the first donor group to bind copper as bioactive tetracyclines start deprotonating is the O3 atom in the MLH_2 species. When the pH is raised, MLH substitutes for MLH_2 with chelation taking place at the O10–O12 system as the OH12 group dissociates. The ultimate deprotonation of the ligand at its C4 dimethylammonium group gives rise to the formation of ML, with a correlative change of the binding mode in favor of N4–$OH12_a$ centers. Concerning the 1:2 metal-to-ligand complexes which coexist with the above species, ML_2H_4 is logically bound in the MLH_2 manner, i.e. to the O3 atom, but ML_2H_2, ML_2H, and ML_2 adopt an identical coordination mode in which alternate bonds are presumed to be formed at both the N4–$O12_a$ pinch and the O10–O12 juncture, because of steric reasons.[11,23]

The present results may have important biological implications. The structural flexibility of bioactive tetracyclines as well as their capacity to coordinate copper via three different donor sites may favor the formation of ternary complexes of this metal with various ligands occurring in vivo. In particular, such mixed-ligand coordination is likely to occur with bacterial nucleic acids. The earlier observation that copper–TC complexes can bind DNA whereas TC itself cannot[14] is in line with this expectation.

As an application of the notion of site specificity of the Fenton reaction,[48] it may be hypothesized that the central Cu^{2+} ions in these ternary complexes can catalyze the formation of free radicals whereby nucleic acids are damaged.[49-51] The capacity of all tetracycline antibiotics to substantially degrade DNA[13] through free radical generation confirms this possibility.

Thus, Albert's former anticipation of a possible role for metal ions in the pharmacological action of tetracyclines now seems fully substantiated. After the important roles predicted for calcium and magnesium in respect of the bioavailability of these drugs,[4-10] it is now suggested that copper may act as a necessary cofactor of their antibiotic activity.

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