Metal Ion–Tetracycline Interactions in Biological Fluids. Part 6.* Formation of Copper(II) Complexes with Tetracycline and some of its Derivatives and Appraisal of their Biological Significance

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Abstract

It was previously established by computer simulation that the interactions of tetracyclines with calcium and magnesium play a paramount role in the bioavailability of these antibiotics in blood plasma. More recently, the influence of zinc was found to be insignificant in this biofluid at drug therapeutic levels but clinical data, relative to the gastrointestinal absorption of tetracyclines as well as zinc in the presence of each other, could be interpreted on the basis of the appropriate simulations.

The present paper deals first with the experimental determination of the formation constants for the complexes of copper(II) with tetracycline, oxytetracycline, doxycyline, minocycline, chlortetracycline and demethylchlortetracyclin in aqueous NaCl 0.15 mol dm^{-3} at 37 °C.

The data was then used for simulating the distribution of copper and each of these drugs in blood plasma as well as in gastrointestinal fluid. No influence can be expected from copper on the bioavailability of tetracyclines in blood plasma, the reverse also being true. For the therapeutic doses under consideration, copper cannot interfere with gastrointestinal absorption of these antibiotics but the presence of the latter tends to favour copper absorption to a determining extent.

Introduction

It is now common knowledge that a number of the biological effects displayed by tetracyclines originate in interactions of these substances with endogenous metal ions. In this respect, the involvement of metal ions in the deleterious impact of tetracyclines on mineralizing tissues has drawn much attention [1–4] but their impairment of the gastrointestinal drug absorption [5–15] as well as their implications in various aspects of the antibacterial activity of this class of antibiotics [16–19] are also well-documented.

At the time of these biological studies, physicochemical investigations were carried out which led to the discovery of the high affinity of tetracycline and its derivatives for many metal ions [20–29]. However, in spite of several attempts to correlate the extent of those biological effects with the coordinative properties of these ligands [18, 30–33], it was not until significant progress was made in computing science that: (i) stoichiometries and stabilities of the various complexes giving rise to tetracyclines with metal ions could be reliably assessed, (ii) the quantitative simulation of the distribution of the relevant species in various biofluids allowed an insight into potential roles of metal–drug interactions *in vivo*.

In particular, studies reported in previous parts of this series showed that calcium and magnesium should exert a determining role in the bioavailability of tetracyclines in blood plasma [34–38]. Actually, more than 99% of the fraction of antibiotic not bound to proteins is expected to be complexed with either or both of these metals at usual therapeutic levels of the drug. Lately, earlier observations made by clinicians concerning the antagonistic role of zinc on the absorption of tetracycline [8, 10, 11] and doxycycline [8] was explained, using computer simultations based on the pertinent clinical data [39].

Available reports concerning copper–tetracycline interactions are relatively scarce. Albert's former investigations into the avidity of tetracyclines for metallic cations mention the existence of stable complexes of copper(II) with chlortetracycline [20], oxytetracycline [20] and tetracycline, itself [21]. Since then, other copper complexation studies involved tetracycline [23, 24], oxytetracycline [22, 23], chlortetracycline, and demethylchlortetracycline

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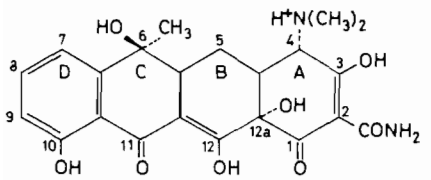
[23, 24], but these often led to controversial results as to the stoichiometries of the characterised species. More recently, it has been demonstrated that complexes formed by cupric ions with tetracycline are effective in binding DNA [40], which suggests that copper might play a part in the mechanism of tetracycline action in biological systems. The fact that copper compounds have now been recognized as potent anti-inflammatory agents [41–43] constitutes additional incentive for the study on the interactions of this metal with tetracyclines.

Complexes formed between copper and tetracyclines may indeed be stable enough to mobilize a significant part of the low-molecular-weight fraction of this metal ion and, hence, facilitate its tissue penetration or its excretion [44, 45]. The possible interference of the antibiotic with copper metabolism appears to be of special interest since plasma concentrations of this metal are known to fluctuate during the evolution of inflammatory diseases [43].

The extent of the dependence of copper bioavailability on plasma tetracycline concentrations can be assessed by computer simulations, provided that stoichiometries and stabilities of the pertinent complexes have been ascertained beforehand under suitable *in vivo* conditions. Thus, this paper reports on the determination of the formation constants for copper(II) complexes of tetracycline (TC = (I)), oxytetracycline* (OTC), doxycycline* (DOX), minocycline* (MIN), chlortetracycline* (CTC), and demethylchlortetracycline* (DMC) under biological conditions.



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These parameters are then used to simulate the distribution of copper and antibiotic in blood plasma at different levels of these drugs.

Finally, the physiological significance of the potential interactions between cupric ions and the above tetracyclines in the gastrointestinal fluid were also assessed from computer simulations.

*Oxytetracycline = 5-hydroxytetracycline, doxycycline = 6-deoxy-5β-hydroxytetracycline, minocycline = 6-demethyl-6-deoxy-7-dimethylaminotetracycline, chlortetracycline = 7-chlorotetracycline, demethylchlortetracycline = 6-demethyl-7-chlorotetracycline.

Formation Constant Determinations

Experimental

Reagents

Tetracycline and oxytetracycline as free bases and doxycycline as the hydrochloride were donated by Pfizer Lab.; minocycline and demethylchlortetracycline hydrochloride were kindly supplied by Lederle Lab., whereas chlortetracycline hydrochloride was purchased from Sigma Chemical Co. Solutions of free bases were obtained by dissolving the reagent in a sufficient amount of aqueous hydrochloric acid.

On account of the well-documented instability of tetracyclines in aqueous medium [16, 33, 46, 47], solutions of all these antibiotics were stored in the dark under a nitrogen atmosphere. In spite of these precautions, fresh solutions had to be frequently prepared, according to the following criterion. For every new solution, the ligand protonation curve is generally determined in order to check the antibiotic and mineral acid contents through GRAN plots. In the present case, this curve was also systematically plotted whenever using a given solution for metal complexation purposes, any noticeable lack of superimposability with the previous plot being regarded as a test for the evolution of the involved tetracycline with time.

The metal stock solution was prepared by dissolving crystals of *'pro analysi'* Merck copper chloride in diluted hydrochloric acid. Its respective metal ion and proton concentrations were deduced from complexometric titrations involving EDTA [48] and from direct potentiometric readings.

The stock solution of sodium hydroxide, obtained by diluting BDH concentrated volumetric solution vials in freshly boiled and degassed double deionized water, was standardized and proved carbonate-free through titrations with Prolabo R.P. p.a. potassium hydrogenphthalate [49].

Constant ionic strength (I = 0.15 mol dm^{-3}) and isotonicity with blood plasma were ensured by Merck *'pro analysi'* sodium chloride used as a background electrolyte.

Apparatus and technique

The titration unit was basically the same as the one described in previous parts of this series [36–39]. It mainly consisted of a digital mV-meter Beckman Model 4500, which was used for monitoring the evolution of the e.m.f.s of an electrochemical cell of the type

G.E./Antibiotic, Cu^{2+}, NaCl (0.15 mol dm^{-3})/
NaCl(saturated)/Hg_2Cl_2 – Hg (1)

TABLE I. Summary of Titration Data Used in Formation Constant Calculations. Initial Total Concentrations of Copper (C_M), Antibiotic (C_L), Mineral Acid (C_H) in the Titrate, Concentrations of Sodium Hydroxide (C_{OH}) in the Titrant, and pH[a] Range Investigated. All Concentrations are Expressed in mmol dm^{-3}

Antibiotic	C_M	C_L	C_H	C_{OH}	pH range
Tetracycline	0.503	5.305	9.960	19.65	2.22–5.44
	1.006	5.305	10.014	19.65	2.16–5.96
	1.509	5.315	9.974	19.65	2.14–6.27
	2.515	5.305	10.178	19.65	2.08–5.71
	5.030	5.245	10.356	19.65	2.02–4.50
	5.030	5.245	10.356	19.65	2.02–4.49
	5.030	2.652	5.499	19.65	2.25–5.10
Oxytetracycline	0.503	5.000	9.865	50.00	2.21–8.92
	1.006	5.000	9.919	50.00	2.17–6.18
	1.509	5.000	9.974	50.00	2.13–5.72
	2.515	5.000	10.083	50.00	2.08–5.54
	5.030	5.000	10.356	50.00	1.96–4.71
	5.030	2.500	5.451	50.00	2.24–4.45
Minocycline	0.503	4.912	9.585	49.90	2.69–9.04
	1.056	4.912	9.645	49.90	2.60–6.20
	1.509	4.912	9.694	49.90	2.51–6.23
	2.515	4.912	9.803	49.90	2.37–5.55
	5.030	4.912	10.076	49.90	2.22–4.90
	5.030	2.456	5.311	49.90	2.40–4.32
Chlortetracycline	0.201	2.029	3.963	20.05	2.58–7.70
	0.402	2.029	3.985	20.05	2.55–7.13
	0.604	2.029	4.006	20.05	2.51–5.92
	1.006	2.029	4.050	20.05	2.48–5.17
	2.012	2.029	4.159	20.05	2.42–4.72
	2.012	1.014	2.189	20.05	2.68–4.75
Demethylchlortetracycline	0.251	2.379	4.969	10.00	2.48–8.57
	0.503	2.379	4.997	10.00	2.45–8.58
	0.805	2.379	5.029	10.00	2.42–6.07
	1.257	2.379	5.079	10.00	2.39–5.74
	2.515	2.379	5.215	10.00	2.34–4.38
	2.515	1.189	2.744	10.00	2.52–4.33

[a]pH stands for $-\log [H]$ (see text).

The Beckman glass electrode and Corning calomel electrode were fitted in an Ingold cell system in which successive aliquots of sodium hydroxide were delivered by means of a Metrohm Multidosimat 645 burette. The temperature was maintained at 37 ± 0.02 °C and experiments were performed under a constant bubbling of purified nitrogen.

Solutions to be titrated contained metal, ligand, and a sufficient amount of hydrochloric acid for all donor groups of the ligand to be protonated at the outset of the experiment. It is worthwhile mentioning that, as noticed earlier for other metals [34–39], these solutions became opaque and foaming occurred as the pH was raised, sometimes from the very beginning of the titration, depending on the copper concentration. In spite of this phenomenom, experiments were pursued as long as stable potentials were observed, the observation of a steady drift in the mV-meter readings being regarded as characteristic of the appearance of a precipitate in the solution. It must, nevertheless, be pointed out that, because of the precipitation problem, the initially planned investigation of the copper–doxycycline system proved impossible. Indeed, precipitation of the initial solution occurred for concentrations as low as 1 mmol dm^{-3}.

Full details on the titration data are available elsewhere [50]. Hence, Table I only reports initial concentrations and pH range for each experiment. The electrode system being calibrated in terms of concentrations, the pH notation will stand for $-\log [H]$ throughout this study.

Calculation procedure

The optimization/simulation two-stage approach developed in earlier studies [51, 52, 39] was used.

TABLE II. Formation Constants $\beta_{pqr} = [M_qL_pH_r]/[M]^q[L]^p[H]^r$ of the Complexes Characterized in These Studies at 37 °C and $I = 0.15$ mol dm^{-3} NaCl.

Antibiotic	p	q	r	log β	S	R	n
Tetracycline	1	1	2	20.972 ± 0.062	5.32E − 07	0.0046	200
	1	1	1	17.978 ± 0.036			
	2	1	2	31.278 ± 0.089			
	2	1	4	40.673 ± 0.059			
Oxytetracycline	1	1	2	20.797 ± 0.089	4.56E − 07	0.0043	180
	1	1	1	18.114 ± 0.066			
	1	1	0	12.439 ± 0.111			
	2	1	0	15.343 ± 0.236			
	2	1	4	39.102 ± 0.170			
Minocycline	1	1	3	25.391 ± 0.023	2.66E − 07	0.0026	261
	1	1	2	23.065 ± 0.016			
	1	1	1	18.316 ± 0.028			
	1	1	0	12.523 ± 0.054			
	2	1	4	41.730 ± 0.174			
Chlortetracycline	1	1	2	19.957 ± 0.026	1.80E − 07	0.0067	264
	1	1	1	16.672 ± 0.038			
	1	1	0	10.686 ± 0.099			
	2	1	2	29.516 ± 0.119			
Demethylchlortetracycline	1	1	2	19.821 ± 0.077	1.41E − 07	0.0054	289
	1	1	1	16.829 ± 0.059			
	1	1	0	9.690 ± 0.080			
	2	1	2	29.169 ± 0.083			
	2	1	4	38.148 ± 0.108			
	1	2	1	19.604 ± 0.102			

S = sum of squared residuals; R = R factor as defined in ref. 53; n = number of experimental observations.

Initial approximations of the complex stability constants to be refined by the MINIQUAD programme [53] were deduced from the relevant formation curves representing the experimental average formation degree

$$\bar{p} = \frac{C_L - [L] - \sum_{1}^{R} [LH_r]}{C_M} \quad (2)$$

as a function of the logarithm of the free ligand concentration [L].

The final choice of the 'best' set of constants, made among species combinations giving rise to similar numerical fits, was based on the comparison of this experimental formation curve with its simulated homologues obtained by means of the PSEUDOPLOT programme [54].

Ligand protonation constants being held constant during the MINIQUAD refinements were taken from previous studies in this series [34, 38], whereas the value of pK_w was considered to be 13.31 [55].

Results

Formation constants determined in these studies are reported in Table II. It may be worth recalling that, due to precipitation problems, the copper–doxycycline system could not be investigated.

In comparison with what was previously obtained for zinc [39], the presence of copper in the test solution induced far more important deviations of all the ligand protonation curves [50]. Moreover, such deviations were also observed above $\bar{r} = 2$ for CTC and DMC, which was not the case for zinc. It was inferred from this observation that a diprotonated complex of copper was presumably formed with TC, OTC, CTC and DMC. Similarly, a triprotonated species was expected for MIN since the additional protonation step displayed by this ligand was also affected, to a large extent, by the presence of copper.

The existence of all these species was clearly confirmed by the MINIQUAD analysis. A close examination of Table II actually shows that, except in the case of ML for tetracycline which was discarded as being a minor complex, all 1:1 species

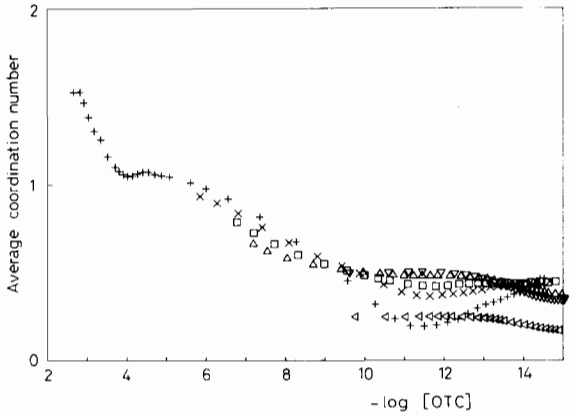

Fig. 1. Experimental formation curve of the copper–oxytetracycline system. The following symbols are in the respective order of the experiments shown in Table I: +, ×, □, △, ▽, ◁.

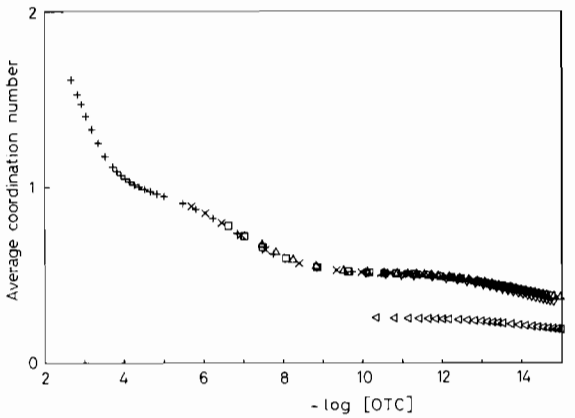

Fig. 2. Simulated formation curve of the copper–oxytetracycline system as based on the results reported in Table II. Symbols are the same as in Fig. 1.

formed by each ligand in all of its possible forms (L^{2-}, LH^{-}, LH_2° and LH_3^{+} for MIN) have been unequivocally characterized. Species corresponding to the 1:2 metal to ligand ratio were also shown to exist: ML_2H_4 was found in all systems except the copper–CTC one; ML_2H_2 was characterized for TC, CTC and DMC, whereas ML_2 was characterized for OTC only.

In the latter case, in spite of its low significance for the whole set of data (see the standard error in Table II), the ML_2 species proved necessary to simulate the upper end of the formation curve (Figs. 1 and 2). It can be seen from these figures that, especially for low free ligand concentrations, curves relative to the 1:10 – and to a lesser extent 1:5 – metal to ligand ratio experiments (see Table I) are subject to the largest error repercussion. This is basically due to the low concentration of the complexed ligand with regard to its protonated fraction. It should also be pointed out that the determination of a significant fraction of these formation curves (Fig. 1) results from pH measurements lower than 3, which explains that the simulation of these curves is not as perfect as it is generally expected [34–39]. In the present case, where such stable acidic species are observed, simulating the whole shape of the formation curves must be considered as satisfactory. Finally, it is noteworthy that OTC is the only ligand to give rise to $\bar{p}$ values over 1 with copper.

As mentioned above, results in Table II involve all possible forms of each ligand and as such, do not confirm conclusions of earlier studies according to which only the LH^{-} form would complex copper [20, 21, 23, 24]. Nevertheless, the fact that isochlorotetracycline (in which the C_{11}–C_{12} system is destroyed) was shown to bind cupric ions [23] is in line with our findings that MLH_2 species do exist. According to the Leeson *et al.* protonation sequence [56], the binding of LH_2 to copper would presumably occur at the C_1–C_2–C_3 system, but the C_{12a} hydroxyl group and the dimethylamino group are more likely to be involved [23], all the more so as copper is particularly prone to bind nitrogen atoms.

It is finally worth mentioning that the existence of the previously characterized Cu_2OTC complex [22] was not confirmed in the present studies, its formation constant being made negative during MINIQUAD refinement.

Computer Simulation Studies

Our prime objective was to assess the extent to which copper and antibiotics can affect the bioavailability of each other in blood plasma at the therapeutic level of the drug. In addition, since copper may also be orally administered to fulfill specific needs [57, 58], the same problem was subsequently examined for gastrointestinal conditions.

Blood Plasma Investigations

Constants in Table II were added to the current blood plasma databank, subsequent simulations thus taking into account the tetracycline interactions with proton, calcium, magnesium, zinc, and copper simultaneously. Ternary constants relative to mixed-ligand complexes, formed by each antibiotic with low-molecular-weight ligands occurring normally in plasma, were used in these simulations. They were estimated from statistical considerations. The ECCLES programme [59] was used, throughout. Free metal ion concentrations were taken as in our latest study in this series [39]. The total concentration of each tetracycline was scanned from 1×10^{-7} to 1×10^{-3} mol dm^{-3} which encompasses the therapeutic concentration previously measured around 1×10^{-5} mol dm^{-3} [34].

TABLE III. Dependence of the Distribution of the Low-molecular Weight Copper Fraction on the Concentration of Various Tetracyclines in Blood Plasma. The Plasma Mobilising Index (P.M.I.) is Defined in the Text

Antibiotic	Plasma concentration of antibiotic in mol dm^{-3} [a]	Copper-mobilised fraction of antibiotic in percentage	log P.M.I.
Tetracycline	1.00×10^{-5}	0.0	0.00
	3.16×10^{-5}	0.2	0.00
	1.00×10^{-4}	0.5	0.00
	3.16×10^{-4}	2.0	0.01
	1.00×10^{-3}	5.9	0.03
Oxytetracycline	3.16×10^{-6}	0.1	0.00
	1.00×10^{-5}	0.4	0.00
	3.16×10^{-5}	1.3	0.01
	1.00×10^{-4}	4.0	0.02
	3.16×10^{-4}	11.4	0.05
	1.00×10^{-3}	27.0	0.14
Minocycline	1.00×10^{-5}	0.0	0.00
	3.16×10^{-5}	0.1	0.00
	1.00×10^{-4}	0.2	0.00
	3.16×10^{-4}	0.6	0.00
	1.00×10^{-3}	1.8	0.01
Chlortetracycline	1.00×10^{-5}	0.1	0.00
	3.16×10^{-5}	0.4	0.00
	1.00×10^{-4}	0.8	0.00
	3.16×10^{-4}	2.1	0.01
	1.00×10^{-3}	4.2	0.01
Demethylchlortetracycline	1.00×10^{-5}	0.0	0.00
	3.16×10^{-5}	0.0	0.00
	1.00×10^{-4}	0.1	0.00
	3.16×10^{-4}	0.4	0.00
	1.00×10^{-3}	1.4	0.01

[a]Concentrations lower than 1.00×10^{-5} mol dm^{-3} are not mentioned whenever the corresponding copper-mobilised fractions are not superior to 0.0%.

For each tetracycline concentration considered in turn, Table III shows the corresponding fraction of antibiotic bound to copper as well as the logarithmic value of the Plasma Mobilising Index (PMI), this parameter being defined as the ratio by which the low-molecular weight fraction of copper in normal plasma is increased in the presence of the drug.

It can be seen from these results that interactions between copper and each of these antibiotics are utterly insignificant at usual drug therapeutic level. No influence can thus be expected from tetracyclines on copper bioavailability in blood plasma, the reverse being also true. It may, nevertheless, be worth mentioning that, for increased drug concentrations, the predominant complexes so formed are electrically neutral, hence diffusible into tissues. Regarding this, further work would be required to determine the stoichiometry of those major complexes prevailing in the intracellular fluid and to assess their significance.

Gastrointestinal Interactions

It has been known, for some time, that the gastrointestinal absorption of tetracyclines may be impaired by simultaneous ingestion of various substances, especially those containing metal ions [7–14, 25].

Among these substances, antiacids exert a specific effect. Tetracyclines have their maximum solubility at low pH values and the raising of gastric pH is logically expected to reduce their dissolution [10, 13, 60]. On the other hand, tetracycline absorption being presumably due to passive diffusion [61], the formation of metal ion complexes of different electrical charges is likely to affect this process to a significant extent. This is substantiated by the fact that the effect of a given metal ion crucially depends on the nature of the predominant anion [7, 8, 11], and is also confirmed by the observation of a correlative impairment of the metal ion absorption [14, 62].

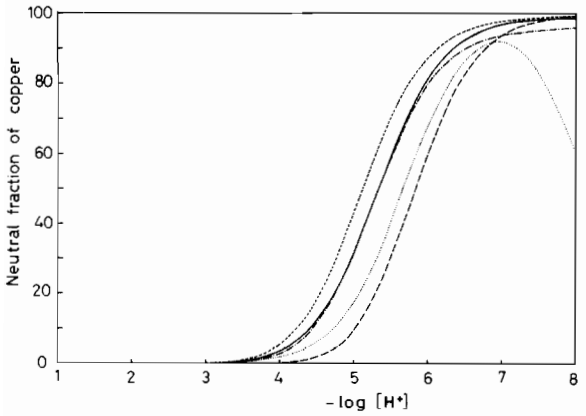

Fig. 3. Antibiotic influence on copper bioavailability in gastrointestinal fluid, expressed as neutral complexed fractions of copper. $C_{Cu} = 1.6 \times 10^{-4}$ mol dm^{-3} and $C_{antibiotic} = 5.2 \times 10^{-3}$ mol dm^{-3} as explained in the text. Symbols are as follows: TC ———, OTC ·····, MIN – – –, CTC ------, DMC –·–.

Contrary to many studies involving zinc, for which clinical data are available in the literature [9–11], *in vivo* interactions of copper with tetracyclines have not been investigated, so far. Yet, indications for oral copper administration can be found [57, 58], a and single doses between 2 and 5 mg have been recommended for long-course treatment [58]. As is also the case for tetracyclines, for reasons given above [8], the metal dose should preferably be taken separately from meals [58]. It may thus be of interest to assess the extent of their mutual influence in an empty stomach.

Potential interactions of copper with each of the above tetracyclines were thus analysed using the NEUPLOT programme [63], which plots the electrically neutral fraction of each reactant as a function of pH. A standard concentration of 5.2×10^{-3} mol dm^{-3} was chosen for all antibiotics, which corresponds to the dose of 500 mg of TC hydrochloride mixed in 200 cm^3 of water, used for *in vivo* absorption studies [9, 11]. Copper concentration was considered to be 1.6×10^{-4} mol dm^{-3}, as would result from the addition of 2 mg of elemental copper to the same volume of water [58].

As could be expected from the relative order of magnitude of the above metal and antibiotic concentrations, the influence of copper on the extent of the neutral fraction of drug proved negligible. However due to the same concentration ratio, copper ions are most significantly complexed into neutral species at pH values as low as 4. Figure 3 shows the neutral fraction of copper complexes formed by each tetracycline under the present conditions. These results may be interpreted in terms of a better absorption of copper, since this neutral fraction is especially favorable at duodenal pH (6.5) in all cases. Nevertheless, it should be borne in mind that the lipophilicity of neutral species may induce their precipitation in aqueous media. Accordingly, the present interpretation clearly needs to be put in perspective with the data collected in Table I.

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