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Copper(II) Interaction with 3,5-Diisopropylsalicylic acid (Dips): New insights on its role as a potential ·OH Inactivating Ligand (OIL)

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Abstract

The purpose of this study was to identify the low-molar-mass complexes formed between copper(II) and 3,5- diisopropylsalicylic acid (Dips) in physiological conditions. Copper(II)-Dips complex equilibria were determined using glass electrode potentiometry and their solution structures checked by UV-visible spectrophotometry. Because of the low solubility of Dips in water, the equilibria were investigated in different water/ethanol mixtures. Formation constants were extrapolated to 100% water and then compared with the values obtained for the other anti-inflammatory drugs previously studied. Given the prime role of histidine as the copper(II) ligand in blood plasma, copper(II)-histidine-Dips ternary equilibria were studied under similar experimental conditions. Computer

simulations of copper(II) distribution relative to different biofluids, gastrointestinal (g.i.) fluid and blood plasma, show that like salicylic and anthranilic acids, Dips favors g.i. copper absorption, but cannot exert any significant influence on plasma copper distribution. Moreover, Dips can mobilize increasing fractions of copper(II) as the pH decreases. In conclusion, Dips seems to correspond to the notion of $\cdot\text{OH}$ -inactivating ligand (OIL) as determined for anthranilic acid.

Keywords: Copper complexation, Inflammation, Salicylic acid derivatives, Copper-mediated oxidation, Anti-inflammatory agents.

Abbreviation used: Ant, 2-aminobenzoic acid; Dips, 3,5-diisopropylsalicylic acid; l.m.m., low molecular mass; OIL, $\cdot\text{OH}$ -inactivating ligand; NSAIDs, nonsteroidal anti-inflammatory drugs; NDP, number of dissociable protons, Sla, salicylic acid.

1. Introduction

Like iron, copper can trigger Fenton-type reactions which produce *in vivo* $\cdot\text{OH}$ radicals responsible for inflammation. Yet, copper is known to have anti-inflammatory properties [1]. Indeed, the paradox that it can play both anti-inflammatory and pro-oxidant roles is related to the nature of the biological molecules bound to the metal *in vivo*. In fact, its pro-oxidant action in Fenton chemistry may result in anti-inflammatory effect if the copper(II) complex is involved in the Haber-Weiss reaction and the ligand acts as “lure” for hydroxyl radicals, which corresponds to the definition of an $\cdot\text{OH}$ inactivating ligand (OIL) [2]. To be active in this kind of action, a potential OIL must firstly mobilize copper at the inflamed site.

Presently, several hundred copper complexes have been proposed as potential anti-inflammatory agents. The results of these studies have been reviewed [1, 3] and confirm that many complexing agents, which are pharmacologically inactive by themselves, display anti-inflammatory properties in association with copper(II) [1, 4], such as anthranilic acid [5] and its methoxy derivatives [6]. Among inactive substances activated by copper(II), 3,5-diisopropylsalicylic acid (Dips) has attracted great interest because of its radioprotectant, anti-inflammatory, anticancer, antimutagenic, antidiabetic, analgesic, antineoplastic and anticonvulsant activities [7-10].

Among the species formed between copper(II) and Dips, the complex $[\text{Cu}(\text{Dips})_2]_n$ ($n=1,2$) have shown potent anti-inflammatory activity [11]. Superoxide dismutase (SOD) mimetic activity was originally suggested as a possible underlying process, because these complexes catalyze the dismutation of superoxide with a Cu,Zn-SOD-like activity [9]. However, superoxide disproportionation is not sufficient to insure complete protection against $\cdot\text{OH}$ radicals [12-15]. Other biological mechanisms have been proposed also, such as induction of lysyl oxidase, modulation of prostaglandin synthesis, stabilization of lysosomal membrane, modulation of histaminic activity, of T-lymphocyte responses and of enzymatic activities [1]. In particular, down regulation of nitric oxide synthase has been proposed [7], and catalase- and peroxidase-mimetic activities have been characterized *in vitro* for $[\text{Cu}_2(\text{Dips})_4]$ [16]. Finally, the Copper(II)-Dips system possesses anti-tumor activity by inhibiting the promotion stage of carcinogenesis [4]

More recent studies showed that Dips could exhibit antioxydant activity as a scavenger for alkylperoxyl radicals in association with other metal ions, such as Fe(III) and Mn(II) [17], and its complexes with Zn(II) show anticonvulsant activity [18].

In spite of the wide pharmacological data, the interpretation of the role of copper(II)-Dips species in the anti-inflammatory activity is still a matter of conjecture. Apart from structural studies relative to the $[\text{Cu}(\text{Dips})_2]_n$ ($n = 1, 2$) species, performed with different spectroscopic methods [19], copper(II)-Dips complex equilibria have never been investigated, probably hampered by the insolubility of the ligand in water. The speciation of copper(II)-Dips system undergoing tissue distribution *in vivo* remains unknown and the important question of stability and fate of administered complexes *in vivo* remains unanswered.

In order to understand how Dips exerts its pharmacological activity in association with copper(II), the complexes formed under physiological conditions, that can be active *in vivo*, must be identified. This step requires preliminary *in vitro* speciation studies under experimental conditions closed to the physiological medium. In this paper, we report the formation constants of the metal complexes, which could pass through the lipidic membranes and then be active in the main biofluids (g.i. fluid and blood plasma). Because L-histidine is the main l.m.m. (low molecular mass) ligand of copper(II) in plasma, ternary equilibria of Cu(II)-His-Dips have also been investigated. Finally, simulations of the distribution of copper(II)-Dips species under different *in vivo* conditions were simulated, based on these constants, and the corresponding results were interpreted for their biological implications.

2. Experimental

2.1 Reagents

3,5-Diisopropylsalicylic acid (98%, Aldrich Chemie) was dissolved in water and ethanol in varying proportion from 40 to 70 % , w/w. L-histidine (>99% pure) was a Merck reagent. Absolute ethyl

alcohol (99.98%, Prolabo) was used. Commercial products were used without further purification, the exact content of each ligand solution was systematically checked by potentiometry.

The stock solution of copper(II) chloride ($\sim 0.1 \text{ mol}\cdot\text{L}^{-1}$) (Merck p.a.) was prepared in diluted aqueous hydrochloric acid ($\sim 0.01 \text{ M}$). Its metal content was determined by complexometric titrations against EDTA and the acid concentration was deduced from direct potentiometric measurements.

The sodium chloride, used as a background electrolyte ($0.15 \text{ mol}\cdot\text{L}^{-1}$), was a SDS analytical grade reagent. Titrant stock solutions of 0.1 M sodium hydroxide were prepared by diluting the contents of Prolabo R.P. Normapur grade ampoules with freshly boiled deionized water under a nitrogen atmosphere. The alkali titre and the absence of carbonate were periodically checked through Gran titrations [20] against potassium hydrogenophthalate (Prolabo). Acid solution, prepared from Merck Titrisol hydrochloric acid, were titrated against standardized sodium hydroxide.

All stock solutions were kept in the dark under a nitrogen atmosphere.

2.2. Potentiometric measurements

The electrochemical cells used for potentiometric titrations opposed a Corning glass electrode to an Ingold calomel electrode. The pair of electrodes was fitted to a Methrom cell system and calibrated in terms of hydrogen-ion concentrations in each mixture [21]. The corresponding pK_w values were determined in each water/ethanol mixture and reported in Table 1. Electromotive force (emf) variations were monitored by means of a Consort pH-meter. The temperature inside reaction cells was maintained at $37.00 \pm 0.02^\circ\text{C}$ by circulating thermostated water, and bubbling purified nitrogen was supplied in test solutions throughout all titrations.

The solutions used in the potentiometric measurements were prepared in the appropriated water/ethanol mixture to maintain the homogeneity of the experimentation. Initial test solutions to be titrated were made sufficiently acidic for all dissociating groups to be substantially protonated at the outset of the experiments. Accurate volumes of sodium hydroxide were successively added with a Metrohm 645 multi-Dosimat burette. Metal-to-ligand ratios were varied significantly over the set experiments performed, to the extend allowed by solubility, to favor the formation and detection of all

possible complexes. Corresponding values are reported in Tables 2 and 3, along with useful pH² ranges investigated.

2.3. Calculation Procedures

Formation constant calculations were performed with our usual approach involving optimization and simulation steps successively [22-25]. For binary complexes of the M_rL_pH_s and M_rX_qH_s types, experimental formation curves representing the average coordination number

$$\bar{p} = \frac{C_L - [L](1 + \sum \beta_{LH_s} [H]^s)}{C_M} \quad (1)$$

as a function of $-\log [L]$, with

$$[L] = \frac{C_H - [H] + [OH]}{\sum s \beta_{LH_s} [H]^s} \quad (2)$$

were used to estimate possible stoichiometries and approximate constants of the species likely to be present in solution.

Formation constants were calculated by means of ESTA [26] and MINIQUAD [27] programs, the final values for each system being refined with MINIQUAD for the sake of coherence with our data bank.

In titrations relative to the copper(II)-Dips system, precipitation occurred in relatively acidic medium, which limited the useful pH range of investigation. In a first approach, MINIQUAD was run with data including for each experiment a few titration points beyond precipitation so as to identify the solid species.

Footnote: ²The pH notation must be understood in terms of $-\log[H]$ throughout this paper.

Then, MINQUAD calculations were rerun considering as the last point in each experiment the point obtained just before precipitation—as detected from the characteristic drift in millivoltmeter readings. Copper hydrolysis constants used throughout were adapted from Sylva and Davidson [28].

For mixed ligand ternary systems $M_rL_pX_qH_s$, protonation curves were employed

$$\frac{-}{S} = \frac{C_H + NDP_L \cdot C_L + NDP_X \cdot C_X - C_{OH} + [OH] - [H]}{C_L + C_X} \quad (3)$$

Protonation and complex formation constants derived from experimental protonation and formation curves were refined with the optimization module of the ESTA program library and MINQUAD. A first selection between the possible models was made by comparing statistic parameters (sum of squared residuals s and Hamilton R factors).

Binary constants in water for the copper(II)-L-histidine system used in calculations relative to ternary equilibria were taken from a previous study [29].

2.4. Spectroscopic measurements

Solutions to be investigated were freshly prepared under the same conditions as for potentiometric titrations. Experimental conditions of reactant concentrations and pH were chosen from speciation calculations so that all species characterized from potentiometric investigations and being of spectroscopic interest could be made successively predominant in solution. The different pH values investigated were imposed on tests solutions by adding negligible volumes of concentrated NaOH or HCl.

UV-visible absorption spectra were recorded at room temperature in the 200-800 nm range using double beam Lambda 2 Perkin-Elmer (equipped with 1 and 10 mm quartz cells depending on the ligand concentration used). Ethanol/water (50% w/w) blank was used as a reference. Results were expressed as ϵ (molar absorption coefficient) with respect to molar concentrations of salicylic acid derivatives.

2.5 Simulation Studies

The anti-inflammatory activity reported for 3,5-diisopropylsalicylate in combination with copper was obtained following oral or parenteral administration [30]. Potential copper(II)-Dips interactions in the gastrointestinal (g.i.) fluid were investigated using the adapted version of the SPE program [31], which provides percentage distribution profiles of metal or ligand as a function of pH. In this kind of approach, special attention is paid to the formation of neutral complexes, which are usually considered capable of diffusing freely across the mucosal membrane. Therefore, a higher percentage of metal and/or ligand present, especially spanning a large g.i. pH range, is expected to induce a better g.i. absorption of the reactant under consideration.

Blood plasma simulations were performed with the help of the ECCLES program [32] after incorporating the newly determined constants into our current data bank. An interesting application of ECCLES is its possible use to predict the effect of a particular ligand on the distribution of the l.m.m. fraction of one or several metal ions in a given biofluid. In blood plasma, the capacity of a ligand to mobilize the metal ion under study at the expense of its labile protein-bound pool may be quantified in terms of the plasma mobilizing index (PMI) [33], which expresses the ratio by which the metal l.m.m. fraction is increased in the presence of drug compared to normal plasma. Of course, the influence of individual complexes on these global variations may also be analyzed. Applications of all ECCLES facilities are not restricted to blood plasma and can be extended to other biofluids, provided the composition of these is known with enough precision. In the present case, these have been used to gain an insight into how copper(II)-3,5-diisopropylsalicylate interactions may be influenced by inflammation-induced pH changes in the synovial fluid.

3. Results and Discussion

3.1. Determination of formation constants

Tables 4 and 5 report constants determined in these studies. The identification of the active species *in vivo* requires the preliminary identification of the species formed *in vitro* under experimental conditions as closed as possible to the physiological medium. One main problem in the potentiometric measurements is the low solubility of Dips in water [11], because of the presence of two hydrophobic alkyl groups on the molecular skeleton of salicylic acid (Fig. 1), resulting in water solubility lower than 10^{-6} M. To overcome this problem, different percentages of ethanol are added to the titration solutions. The use of the hydrogen ion-responsive glass electrode using water/ethanol mixture has been reported some years ago [34]. It was shown that the water solvation of the chemical species was efficient until 60% ethanol (w/w) [35]. Under these conditions, reproducible results comparable to standard reference state in water and a good response of the glass electrode was observed. Above 60% ethanol, ion pair formation, predominant in organic solvents, did not allow a correct extrapolation to 100% water [35].

No thermodynamic constants of either protonation or copper(II) complexation of Dips have been found in the literature, to compare with our experimental values. Derivative of salicylic acid, Dips contains two potential protonation sites (Fig. 1) whereas only one protonation constant is accessible experimentally. In fact, dissociation of the phenol group takes place, in all the mixture used, at a pH too high (>10) to be measured with accuracy. In analogy with the previous study on salicylic acid [29], we report here the only protonation constant of the carboxylic group in the NDP = 1 hypothesis (Table 4). However, in order to allow a direct comparison with copper(II)-Sla constants determined under similar conditions, we have also considered the NDP = 2 hypothesis, and, by analogy with salicylic acid [11], we have used in the calculations the estimated value of 13.0 for the pKa of the phenol group³.

Footnote: ³Guy Berthon explained “there are thus two possibilities to account the metal-salicylate equilibria: (i) to consider the number of dissociable protons (NDP) of the ligand equal to 2 and calculate the two protonation constants with the risk of a large systematic error on the first and hence on the derived metal-salicylate constants or (ii) to consider NDP=1 and calculate the only protonation constant accessible with accuracy (that of the COO group), with the consequence of a priori mistaking the coordination of a metal ion to the phenolate oxygen for that of a hydroxide ion from dissociated water (both alternatives are mathematically equivalent) (Ref. [29])”.

Comparing the protonation constant of the carboxylic group for Dips ($\log \beta_{\text{LH}} = 3.1$) (Table 6) and for Sla ($\log \beta_{\text{LH}} = 2.790$), the small difference observed is in agreement with the electron-releasing effect of the two aliphatic groups, which reduces the acidity of the carboxylic group in the *meta* position. This value is more basic than that of the carboxylic group for anthranilic acid ($\log \beta_{\text{LH}} = 2.186$) because of the electron-withdrawing effect of the amino function. In the comparative analysis of the molecular structures of Dips and Sla, it is also expected that the aliphatic groups, in *ortho* and *para* positions, reduce the acidity of the phenol group with a $\log(\text{constant})$ that should be probably higher than 13 [36].

Table 4 reports the constants determined for the copper(II)-Dips binary system. The experiments are performed in water/ethanol mixture in varying proportion from 40 to 70% (w/w) then linearly extrapolated to 100% water (Table 6). However, because of the precipitation of some species formed, the potentiometric measurements are limited in the pH ranges. Fig. 2 represents some experimental and simulated formation curves, in the $\text{NDP} = 2$ hypothesis, in 50% water, solvent in which the measurements are possible at highest pH values, and a good agreement between experimental and simulated values is noted (the total set of data is reported in Fig. S1). Similar correspondence is observed for the other titrations in each mixture used. As shown in Table 4, the same chemical species are identified in all the water/ethanol ratios used, confirming the non participation of ethanol in the solvation sphere below 60% ethanol [34]. Concerning the extrapolation of the constants to 100% water, a good linearity of the values with standard deviations smaller than 8% is observed, with the exception of MX_2H_2 complex (Table 6).

The most interesting species for our simulations in biological fluids are MX and MX_2H_2 , which are electrically neutral and hence likely to diffuse into tissues. The latter complex corresponds to $[\text{Cu}(\text{Dips})_2]$ [11]. At slight acidic pH, we note also the presence of MXH. MX and MXH have been identified in the case of salicylic and anthranilic acid [5, 29], however, these complexes are more stable for Sla and Dips than for Ant. MX_2 does not exist for Dips probably because of the steric hindrance of the alkyl groups, which favors binuclear species like $\text{M}_2\text{X}_2\text{H}_2$.

Table 5 reports all the constants determined for ternary system copper(II)-Dips-His. In order to verify the validity of the extrapolated values for the ternary systems, the copper(II)-His binary system is also studied in different water/ethanol mixtures (Table 5) and the results, after extrapolation to 100% water (Table 6), compared to the constants previously found in water [37]. We find a very good correlation between these values, excepted for ML_2H_{-1} . It may be noticed that, as in the case of Dips, the correlation is not so good for the species formed at basic pH values.

$MLXH$ and $MLXH_2$ are the species identified in the ternary system formed by copper(II), Dips (X) and histidine (L). Taking into account the protonation pKa of Dips, these species could be written as $ML(XH)$ and $M(LH)(XH)$ where XH indicates Dips deprotonated only at the carboxylic function. In order to calculate the stabilization of the ternary system compared to the statistical values of the binary systems, we cannot use the well-known Eqs (4) and (5) [38]

$$\log\beta_{MLXH} = \frac{1}{2} (\log\beta_{ML_2} + \log\beta_{MX_2H_2}) + \log 2 \quad (4)$$

$$\log\beta_{MLXH_2} = \frac{1}{2} (\log\beta_{ML_2H_2} + \log\beta_{MX_2H_2}) + \log 2 \quad (5)$$

In fact, using the constant values in Table 6, the difference $\log\beta_{MX_2H_2} - 2 \log\beta_{MXH}$ gives +1 low-estimated, which has no thermodynamic meaning. In the NDP=2 hypothesis, this could be related to the value of 13 for the pKa of the phenolic function. However, the mixed-ligand complexes in this system is confirmed by the corresponding $\Delta\log K$ stability increments calculated as +0.72 and +0.60, respectively

$$\Delta\log K = \log\beta_{MLXH} - \log\beta_{MXH} - \log\beta_{ML} \quad (6)$$

$$\Delta\log K = \log\beta_{MLXH_2} - \log\beta_{MXH} - \log\beta_{MLH} \quad (7)$$

These values are in agreement with the results obtained for acetylsalicylic acid [29]. This, however, does not infer any definite conclusion as to the relative importance of the corresponding mixed-ligand complexes in biological media where reactant concentration and pH are largely determining factors.

3.2 UV-visible spectra

Table 7 reports the spectroscopic features of the copper(II)-Sla and -Dips systems at different pH values, in the absence and presence of copper(II). Reactant concentrations are different than those used in potentiometric titrations to ensure that all complexes under study are significantly formed in solution.

In the 190-600 nm region, at acidic pH, salicylic acid displays three absorption bands centered at around 205, 237 and 304 nm (Table 7). There are all $\pi \rightarrow \pi^*$ transitions corresponding to the $A_{1g} \rightarrow E_{1u}$, $A_{1g} \rightarrow B_{1u}$ and $A_{1g} \rightarrow B_{1g}$ transitions in the benzene ring, corrected by the bathochromic shift due to the auxochromic 1,2-substituted groups. With reference to the UV-vis spectra of anthranilic acid [6], the OH auxochromic group, compared to the NH_2 group, has a higher bathochromic shift on the E bands than on the B band. The presence of isopropyl groups on the aromatic skeleton of salicylic acid, as expected, increases the bathochromic shifts of all the $\pi \rightarrow \pi^*$ transitions (Table 7) [11]. Compared to anthranilic acid, the $n \rightarrow \pi^*$ transitions of the carboxyl function are present as a shoulder at 226 and 238 nm for Sla and Dips, respectively. As the pH is increased, the deprotonation of the carboxyl function, which destroys the hydrogen bond between the phenol group and the adjacent carboxylate, induces a small hypsochromic shift in the $A_{1g} \rightarrow B_{1g}$ transition. Above pH 13, the deprotonation of the phenol function in Sla results in a bathochromic shift of the E_2 transition at around 250 nm. Moreover, a shoulder at around 238 nm appears, the B band splits and the phenolate function induces a small bathochromic shift at around 335 nm. However, the lower acidity of the phenol function in Dips does not allow observation of such as a similar deprotonation under our experimental conditions.

When copper(II) ions are added to a Sla solution at pH below 4.00, the ligand is mostly in the XH form. The addition of the metal ion results in a small hyperchromic effect and in a hypsochromic shift of the B and E_2 bands at around 296 and 257 nm respectively, indicating the formation of MXH species. Above pH 3.29 isosbestic points are present at 210, 245, 300 and 325 nm. However, above pH 4.93, precipitation occurs with the concomitant formation of the MX complex and the precipitation of metal hydroxides, resulting in a decrease in the intensity of the spectrum and a general

diffusion of light, prevents spectral resolution. If the experiments are performed with a lower metal-to-ligand molar ratio (0.55 vs 5.5) to minimize the precipitation of the metal hydroxides, the formation of the MX species is less favored, however, at pH 6.37, the presence of a shoulder at around 350 nm indicates the deprotonation of the phenol.

The addition of Cu^{2+} to a Dips solution does not result in strong modifications in the UV-vis spectra. However, small modifications in the intensity and in the wavelength occurs as a function of the pH. The hypsochromic shift at around pH 4 for the E_2 band indicates the presence of MXH whereas above pH 7.5, the B band at 308 nm indicates the formation of $\text{M}_2\text{X}_2\text{H}_2$. In order to observe the MX species, a lower concentration of Dips is used. In these experimental conditions the precipitation of neutral species deforms the spectra, however, the main spectral features at pH 5.0 are bands at 250 nm and shoulders at 208, 300 and 425 nm; this last band corresponds to the LMCT band of the phenolate function, which reveals the formation of MX species. It is to be noticed that this band is not related to the carboxylic function [11], because it does not appear in the spectra at pH 4, where MXH is predominant. Furthermore, MX_2H_2 is present in a very low amount in these experimental conditions and it cannot be differentiated from other species [11].

For a better understanding, in MXH and MX_2H_2 complexes, salicylic acid derivative are bound through the carboxylic function, whether in MX and MX_2 , the phenolate function is also involved in the metal coordination sphere. Moreover, in the binuclear complex $\text{M}_2\text{X}_2\text{H}_2$, Dips is twice deprotonated (X) -in agreement with our spectroscopic results- and the metal ions are probably bridged by two deprotonated water molecules (H_2 or bis- μ -OH).

3.3. Simulation Studies and biological implications

Two biofluids mainly influence the copper(II)-Dips interactions on the metabolism: the gastrointestinal (g.i.) fluid and blood plasma. The potential influence of the simultaneous oral administration of Dips and copper(II) on the bioavailability of each of these two reagents was investigated. Calculations were run for a concentration of copper(II) equal to 1.6×10^{-4} mol.L⁻¹, which corresponds to a dose of 2 mg of metal dissolved into 200 mL of water, the Estimate Safe and

Adequate Daily Dietary Intake of copper being 1.5 to 3.0 mg [40]. The concentration of Dips is equal to $4.8 \cdot 10^{-4}$ M which corresponds to 11.7 mg of the drug in 200 mL of water by analogy with anthranilic acid [5].

Fig. 3 reports the simulated the species distribution. It has been noticed that only electrical neutral species can favor the g.i. absorption of copper(II), i.e., MX and MX_2H_2 . In the pH range from 5 to 8 the main complex present is MX (16%) whereas MX_2H_2 is present in 0.2% only at pH 4.36. For instance, MX_2H_2 could be present in an appreciable amount (43%) only at a concentration of Dips equal 1.13×10^{-2} M (equivalent to 500 mg, corresponding to the pharmaceutical dose of acetylsalicylic acid), not allowed by the low solubility of Dips in water ($<10^{-6}$ M).

In order to evaluate the impact of Dips on the distribution of copper(II) in the extracellular fluid under physiological and inflammatory conditions, a blood plasma model was used [29]. The lower limit, from which the concentration of Dips was scanned, was taken as 1×10^{-6} M, which corresponds to 0.12 mg/dm³. The upper limit has been arbitrarily fixed at 0.1 M, which largely exceeds any potential therapeutic dose by reference to the parent anthranilate [5]. The simulation was performed taking into account the MLXH and MLXH_2 ternary species. Fig. 4 shows the P.M.I. results of the ECCLES scan at pH 7.4 and 5.5, compared with those obtained for anthranilic and salicylic acids. Speciation calculations suggest that, as in the case with anthranilate, Dips copper(II) binding is negligible in plasma but tends to increase with decreasing pH. Dips is less efficient than Ant [5] in mobilizing copper(II) at pH 5.5, i.e., in the intercellular space at inflammatory sites, however it is better than Sla [29], and it does not virtually affect its original distribution under physiological conditions. Unfortunately, reactivity studies in order to better understand the anti-inflammatory properties of Dips, such as the determination of the redox potential and the detection of the hydroxyl radical production [6, 41], are precluded by complex precipitation at anti-inflammatory pH (5,5) and poorly representative at pH 7,4.

The attempts made in this respect in SDS aqueous solutions have had limited success. However, the results obtained by potentiometry and the simulations clearly indicate that Dips can act as a potential OIL.

4. Conclusion

The simulations show that Dips cannot mobilize a significant fraction of copper(II) in blood plasma. Unlike salicylic acid, which is totally inefficient to mobilize copper(II) regardless of the pH *in vivo*, Dips binds increasing fractions of copper(II) as the pH decreases -i.e., as inflammation grows. This behavior, which is comparable to that of anthranilic acid, makes Dips a potential OIL. Although it displays a lesser capacity than anthranilic acid to bind copper(II) in extracellular fluid under inflammatory conditions. Dips benefits in compensation from a much higher lipophilicity, hence a higher solubility in membranes where oxygen concentrates, which may explain its high pharmacological activity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jinorgbio.2006.11.017

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TABLES AND FIGURES

Table 1: . Values of pK_w as a fonction of water percentage.

System	70%	60%	55%	50%	40%
Proton-Dips	13.37	13.88	n. d.	14.00	14.32
Copper-Dips	13.37	13.88	n. d.	14.00	14.32
Proton-His	13.76	13.90	13.97	14.02	14.16
Copper-His	13.76	13.90	13.97	14.02	14.16
Copper-His-Dips	13.37	13.88	n. d.	14.00	14.16

n.d. : not determined

Table 2. Titration Data used to calculate stability constants for the copper(II)-Dips system in different aqueous ethanol mixtures^a

System	C _M	C _L	C _X	C _H	pH range ^b	n	%water
Proton-Dips			1.546	0.000	3.32-9.16	26	70%
			3.094	0.000	3.14-9.46	46	
			3.365	4.991	2.99-9.59	42	
			4.129	0.000	3.09-9.70	92	
Copper-Dips	1.010		3.758	0.100	3.04-5.54	52	
	2.012		4.551	0.195	2.95-5.18	72	
	1.010		1.945	0.100	3.19-5.00	26	
	2.012		3.453	0.195	3.02-5.14	63	
	3.363		4.695	0.326	2.89-4.45	56	
	2.012		1.756	0.195	3.16-5.02	32	
	5.024		3.576	0.487	2.90-3.72	22	
Proton-Dips			2.254	0.000	3.29-9.85	46	60%
			4.510	0.000	3.13-9.87	76	
			4.510	4.945	3.00-9.94	55	
			6.018	0.000	3.08-10.01	103	
Copper-Dips	1.341		5.946	0.130	3.02-5.56	91	
	1.006		4.446	0.097	3.10-5.63	65	
	1.006		2.226	0.097	3.25-5.31	38	
	2.012		4.482	0.197	3.06-5.32	55	
	2.012		2.254	0.195	3.19-4.77	34	
	4.024		4.510	0.390	2.98-4.85	62	
	2.012		1.128	0.295	3.32-4.46	20	
Proton-Dips			6.236	0.000	3.13-10.01	62	50%
			6.236	6.211	2.76-10.10	68	
			12.476	0.000	2.97-10.01	112	
			12.476	12.408	2.75-9.88	102	
Copper-Dips	1.251		24.600	12.733	2.95-6.15	114	
	1.251		12.405	12.554	3.02-5.64	72	
	1.251		6.236	6.312	2.79-5.32	50	
	2.503		12.476	12.651	2.76-5.30	65	
	2.503		6.236	6.454	2.77-4.94	55	
	3.128		6.236	5.273	2.74-4.86	51	
	6.249		12.476	11.787	2.79-4.48	53	
	2.503		2.497	2.727	3.23-4.42	17	
	6.249		6.236	6.818	2.77-4.23	44	
	6.249		3.122	3.091	2.88-3.92	29	
Proton-Dips			5.003	0.000	3.26-10.00	47	40%
			5.003	4.929	2.64-10.03	52	
			12.493	4.929	2.61-9.94	61	
Copper-Dips	1.255		12.314	5.181	2.61-5.47	57	
	2.510		12.493	5.160	2.59-5.10	53	
	1.255		2.502	4.995	2.60-4.76	28	
	2.008		2.502	5.073	2.58-4.37	15	
	2.510		2.494	2.630	2.66-4.26	23	
	5.019		2.502	5.406	2.61-3.93	22	

^a The initial total concentrations of the ligands L (C_L) or X (C_X), copper(II) (C_M), mineral acid (C_H) in the titrate as well as the hydroxide concentrations (C_{OH}) in the titrant are expressed in mM ; n represents the number of experimental observations in each titration.

Abbreviations are as follows: Dips = 3,5-Diisopropylsalicylic acid.

^b To be understood as $-\log[H]$ (see text).

Table 3. Titration data used to calculate stability constants for copper(II)-His and copper(II)-His-Dips systems in different aqueous ethanol mixtures^a

System	C _M	C _L	C _X	C _H	pH range ^b	n	%water
Proton-His		4.976		24.784	1.77-9.86	135	70%
		5.022		9.937	2.44-9.80	67	
		10.040		9.938	4.03-9.78	109	
		10.060		19.880	2.21-9.89	128	
Copper-His	2.001	10.000		10.125	2.28-9.75	90	
	2.001	7.009		12.609	2.33-9.93	126	
	2.000	5.000		10.242	2.33-9.13	85	
	4.977	9.985		12.871	2.40-8.60	135	
	4.987	7.010		12.895	2.24-6.48	101	
	4.999	5.140		12.808	2.13-5.87	112	
	10.000	5.000		5.966	2.46-5.15	72	
Copper-His-Dips	2.020	2.497	1.508	5.174	3.13-7.07	63	
	5.044	4.995	3.244	5.482	2.86-6.07	65	
	5.044	4.995	3.309	5.482	2.95-5.98	57	
	2.020	4.995	1.621	5.189	3.10-9.97	82	
	1.683	1.665	4.371	3.482	3.04-6.38	54	
	2.020	4.995	3.415	5.189	3.09-10.00	71	
	4.040	2.497	1.778	5.370	3.02-5.66	42	
Proton-His		5.038		9.975	2.44-10.48	80	60%
		5.027		24.842	1.88-10.92	84	
		10.050		9.973	3.90-10.75	106	
		10.050		19.962	2.22-8.86	105	
Copper-His	2.000	10.050		10.130	2.78-10.09	99	
	2.000	7.044		12.600	2.30-9.01	88	
	2.025	5.025		10.130	2.31-9.13	81	
	4.990	10.030		12.701	2.36-6.90	87	
	5.000	7.121		12.867	2.20-6.21	93	
	5.000	5.025		12.731	2.10-5.63	100	
	10.000	5.080		5.991	2.40-4.90	76	
Copper-His-Dips	1.006	1.012	0.883	4.887	3.07-6.47	51	
	2.012	2.525	2.443	4.790	2.81-6.99	103	
	1.006	2.022	0.913	4.972	3.06-8.86	66	
	1.006	1.012	2.051	4.895	2.73-6.66	63	
	1.006	2.525	2.282	4.945	2.99-8.78	76	
	2.012	1.011	1.025	4.930	3.04-5.52	47	
Proton-His		2.030		4.970	2.62-9.93	110	55%
		5.080		4.970	4.16-9.95	117	
		5.080		9.940	2.48-10.06	169	
		10.160		24.810	2.04-9.92	239	
Copper-His	1.250	10.160		24.960	2.00-10.0	111	
	1.250	5.080		10.060	2.39-10.00	95	
	2.500	5.080		10.180	2.32-9.00	86	
	1.250	2.030		5.090	2.51-6.78	33	
	5.000	5.080		10.430	2.24-5.48	55	
	2.500	2.030		5.200	2.46-5.32	46	
	5.000	2.030		5.460	2.39-4.43	39	

Proton-His		2.6115		24.895	2.72-9.39	65	50%
		5.0785		14.937	2.12-9.70	84	
		5.1223		24.895	1.781-9.389	108	
Copper-His	1.000	10.000		24.905	2.40-9.40	75	
	1.000	5.000		24.905	1.80-9.00	120	
	2.000	5.000		25.092	2.40-8.79	58	
	5.000	10.000		24.944	1.93-5.83	52	
	5.000	5.000		24.488	1.75-5.35	58	
	10.000	5.000		24.934	179-4.92	75	
Cu-His-Dips	2.503	2.508	2.498	5.212	2.48-5.81	73	
	5.005	5.019	4.994	5.455	2.51-5.71	77	
	2.503	2.508	6.173	2.727	2.73-6.24	79	
	5.005	5.020	1.248	5.455	2.48-5.88	101	
	1.251	2.508	1.249	2.606	2.93-9.58	70	
	2.503	5.019	2.498	5.212	2.70-9.645	81	
	1.251	2.508	2.498	2.598	2.91-9.47	83	
	2.503	5.019	6.235	5.212	2.67-9.26	91	
	2.503	1.005	2.498	2.727	2.63-5.18	42	
	2.503	1.005	1.249	2.720	2.64-5.28	50	
Proton-His		4.990		0.000	6.90-9.87	35	40%
		2.000		4.930	2.640-9.74	70	
		9.990		9.860	3.96-10.03	89	
Copper-His	1.250	10.130		24.740	2.00-9.94	113	
	2.510	10.130		10.100	2.71-9.86	80	
	2.010	2.030		5.130	2.43-5.58	35	
	5.020	5.060		12.810	2.09-5.32	57	
	5.020	2.030		5.420	2.37-3.10	17	
Cu-His-Dips	2.008	2.028	2.502	5.111	2.43-6.13	55	
	5.019	5.064	5.003	5.421	2.48-5.57	58	
	2.133	5.064	2.502	5.123	2.70-9.88	64	
	2.510	2.028	5.003	5.160	2.37-5.65	45	
	5.019	2.028	2.502	5.406	2.34-5.02	40	

^a The initial total concentrations of the ligands L (C_L) or X (C_X), copper(II) (C_M), mineral acid (C_H) in the titrate as well as the hydroxide concentrations (C_{OH}) in the titrant are expressed in mmol dm^{-3} ; n represents the number of experimental observations in each titration. Abbreviations are as follows: Dips = 3,5-Diisopropylsalicylic acid.

^b To be understood as $-\log[H]$ (see text).

Table 4. Stability Constants $\beta_{pqrs} = [M_r L_p X_q H_s] / [M]^r [L]^p [X]^q [H]^s$ of the complexes characterized in these studies for the copper(II)-Dips system (37°C and I = 0.15 M NaCl) in different aqueous ethanol mixtures ^a

System	p	q	r	s	log β	S	R	n	%Water.	Ref
Copper-hydroxide	0	0	1	-1	-7.60					[28]
	0	0	2	-2	-10.00					
	0	0	3	-4	-20.00					
Proton-Dips (NDP = 1)	0	1	0	1	3.625±0.002	0.571E-7	0.00294	206	70	This work
Copper-Dips (NDP = 2)	0	1	1	0	9.494±0.007	0.160E-7	0.00100	323		This work
	0	1	1	1	14.992±0.008					
	0	2	1	2	29.828±0.034					
	0	2	2	-2	10.655±0.055					
Proton-Dips (NDP = 1)	0	1	0	1	3.833±0.001	0.593E-7	0.00188	280	60	This work
Copper-Dips (NDP = 2)	0	1	1	0	9.392±0.003	0.555E-8	0.00051	365		This work
	0	1	1	1	14.682±0.008					
	0	2	1	2	28.806±0.137					
	0	2	2	-2	9.777±0.038					
Proton-Dips (NDP = 1)	0	1	0	1	4.031±0.001	0.411E-6	0.00205	344	50	This work
Copper-Dips	0	1	1	0	9.113±0.012	0.237E-6	0.00100	550		This work
	0	1	1	1	14.834±0.008					
	0	2	1	2	29.298±0.030					
	0	2	2	-2	8.876±0.042					
Proton-Dips (NDP = 1)	0	1	0	1	4.204±0.001	0.105E-6	0.00188	160	40	This work
Copper-Dips (NDP = 2)	0	1	1	0	9.390±0.029	0.367E-7	0.00089	198		This work
	0	1	1	1	15.238±0.009					
	0	2	1	2	29.348±0.099					
	0	2	2	-2	10.372±0.260					

^a L = L-Histidine ; X=Dips

S = MINQUAD sum of squared residuals; R = Hamilton R-factor; n = number of experimental observations.

Table 5. Stability Constants $\beta_{pqrs} = [M_r L_p X_q H_s] / [M]^r [L]^p [X]^q [H]^s$ of the Complexes Characterized in these Studies (37°C and I = 0.15 mol dm⁻³ NaCl).

System	p	q	r	s	log β	S	R	n	Ref.	%water
Proton-His	1	0	0	1	8.602±0.001	0.272E-6	0.0018	439	This work	70%
	1	0	0	2	14.189±0.001					
	1	0	0	3	16.146±0.003					
Copper-His	1	0	1	0	10.283±0.003	0.702E-6	0.00281	721	This work	70%
	2	0	1	0	18.206±0.006					
	1	0	1	1	14.052±0.004					
	2	0	1	1	23.579±0.005					
	2	0	1	2	26.897±0.014					
	2	0	1	-1	7.557±0.038					
	1	0	1	-1	3.086±0.031					
Copper-His-Dips	1	1	1	1	25.144±0.027	0.408E-6	0.00376	434	This work	70%
	1	1	1	2	29.149±0.037					
Proton-His	1	0	0	1	8.554±0.001	0.184E-6	0.001537	375	This work	60%
	1	0	0	2	14.032±0.001					
	1	0	0	3	16.026±0.003					
Copper-His	1	0	1	0	10.561±0.004	0.124E-5	0.003660	624	This work	60%
	2	0	1	0	18.613±0.010					
	1	0	1	1	14.206±0.005					
	2	0	1	1	23.842±0.007					
	2	0	1	2	27.138±0.015					
	2	0	1	-1	7.499±0.133					
	1	0	1	-1	3.680±0.031					
Copper-His-Dips	1	1	1	1	25.684±0.036	0.161E-6	0.00437	406	This work	60%
	1	1	1	2	29.128±0.187					
Proton-His	1	0	0	1	8.557±0.001	0.374E-6	0.00195	635	This work	55%
	1	0	0	2	14.041±0.001					
	1	0	0	3	16.147±0.002					
Copper-His	1	0	1	0	10.486±0.004	0.292E-6	0.00264	465	This work	55%
	2	0	1	0	18.360±0.012					
	1	0	1	1	14.217±0.005					
	2	0	1	1	23.818±0.009					
	2	0	1	2	27.184±0.021					
	2	0	1	-1	6.894±0.265					
	1	0	1	-1	3.608±0.058					
Proton-His	1	0	0	1	8.497±0.004	0.365E-6	0.003921	257	This work	50%
	1	0	0	2	13.941±0.005					
	1	0	0	3	15.987±0.008					
Copper-His	1	0	1	0	10.475±0.005	0.844E-6	0.003102	438	This work	50%
	2	0	1	0	18.275±0.017					
	1	0	1	1	14.152±0.004					
	2	0	1	1	23.667±0.011					
	2	0	1	2	26.753±0.042					
	2	0	1	-1	9.058±0.042					
	1	0	1	-1	4.075±0.058					

Copper-His-Dips	1	1	1	1	25.259±0.024	0.355E-5	0.00514	753	This work	50%
	1	1	1	2	29.175±0.051					
Proton-His	1	0	0	1	8.492±0.001	0.629E-7	0.00176	194	This work	40%
	1	0	0	2	13.854±0.002					
	1	0	0	3	16.127±0.015					
Copper-His	1	0	1	0	10.809±0.011	0.919E-6	0.00413	302	This work	40%
	2	0	1	0	19.404±0.040					
	1	0	1	1	14.469±0.013					
	2	0	1	1	24.446±0.025					
	2	0	1	2	27.949±0.026					
	2	0	1	-1	8.205±0.243					
	1	0	1	-1	4.075±0.136					
Copper-His-Dips	1	1	1	1	25.264±0.073	0.305E-6	0.00339	262	This work	40%
	1	1	1	2	29.504±0.107					

S = MINIQAD sum of squared residuals; R = Hamilton R-factor; n = number of experimental observations.

Table 6. Stability Constants $\beta_{pqrs} = [M_r L_p X_q H_s] / [M]^r [L]^p [X]^q [H]^s$ of the complexes characterised in these studies in water (37°C and I = 0.15 M NaCl)^a

System	p	q	r	s	log β	S	R	n	Ref.
Proton-Dips ^b (NDP = 1)	0	1	0	1	3.1±0.3				This work
Proton-Dips ^b (NDP = 2)	0	1	0	1	13.0				This work
	0	1	0	2	16.1				
Proton-His	1	0	0	1	8.759±0.002	0.196E-6	0.00186	349	[37]
	1	0	0	2	14.602±0.002				
	1	0	0	3	16.283±0.004				
Proton-His ^b	1	0	0	1	8.72±0.04				This work
	1	0	0	2	14.51±0.02				
	1	0	0	3	16.13±0.07				
Proton-Sla (NDP = 2)	0	1	0	1	13.000				[29]
	0	1	0	2	15.790				
Proton-Ant	0	1	0	1	4.679				[5]
	0	1	0	2	6.865				
Copper-Dips ^b (NDP = 2)	0	1	1	0	9.6±0.8				This work
	0	1	1	1	14.5±0.3				
	0	2	1	2	30.0±1.3				
	0	2	2	-2	11.0±2.4				
Copper-His	1	0	1	0	9.748±0.014	0.291E-5	0.00395	420	[37]
	2	0	1	0	17.494±0.015				
	1	0	1	1	13.788±0.010				
	2	0	1	1	23.056±0.011				
	2	0	1	2	26.286±0.063				
	2	0	1	-1	6.324±0.109				
	1	0	1	-1	2.264±0.210				
Copper-His ^b	1	0	1	0	9.8±0.2				This work
	2	0	1	0	17.1±0.5				
	1	0	1	1	13.7±0.2				
	2	0	1	1	22.8±0.4				
	2	0	1	2	25.9±0.5				
	2	0	1	-1	6.3±0.8				
	1	0	1	-1	2.2±0.4				
Copper-Sla	0	1	1	0	10.198±0.004	0.185E-06	0.00302	646	[29]
	0	2	1	0	18.032±0.015				
	0	1	1	1	14.739±0.018				
	0	2	1	1	24.613±0.077				
Copper-Sla (NDP = 1)	0	1	1	-1	-2.820				[29]
	0	2	1	-2	-7.968				
	0	1	1	0	1.739				
	0	2	1	-1	-1.387				
Copper-Ant	0	1	1	0	3.696±0.006	0.393E-08	0.00167	204	[5]
	0	1	1	1	6.364±0.071				
	0	2	1	0	6.455±0.059				
	0	1	1	-1	-2.867±0.071				
	0	2	1	0	-11.28 (solid)				

Copper-His-Dips ^b	1	1	1	1	25.02±0.07					This work
	1	1	1	2	28.80±0.02					
Copper-His-Sla	1	1	1	0	18.690±0.017	0.459E-06	0.00302	646		[29]
	1	1	1	1	23.911±0.119					
	1	1	2	-2	8.506±0.082					
Copper-His-Ant	1	1	1	0	12.831±0.024	0.956E-08	0.00171	239		[5]
	1	1	1	1	16.673±0.111					

^a L = L-Histidine ; X = Dips ; S = MINQUAD sum of squared residuals ; R = Hamilton R-factor ; n = number of experimental observations

^b values extrapolated to 100% water

Table 7. Spectroscopic features of the copper(II)-Sla and copper(II)-DIPS systems in aqueous ethanol solution (5.795×10^{-3} ; $C_{\text{NaCl}} = 0.15 \text{ M}$).

Ligand	pH	Complex	$\pi \rightarrow \pi^*$ E_1^a	$n \rightarrow \pi^*$ (COOH)	$\pi \rightarrow \pi^*$ E_2^a	$\pi \rightarrow \pi^*$ B^a	$\pi \rightarrow \pi^*$ B'^a
Sla	1.42	XH ₂	205 (11730)	226 sh (1500)	237 (8560)	304 (3670)	-
	3.48	XH	203 (16050)	228 sh (6250)	235 (6940)	300 (3470)	-
	>13 ^b	X	n.i. ^c	238 sh (6700)	250 sh (2800)	298 (3670)	335 sh (5000)
+ Cu ²⁺	3.56	XH	204 (22570)	Submerged	237 (10417)	301 (3470)	-
	4.42	MXH/XH	209 (24300)	231 sh (10590)	257 sh (3472)	296 (3820)	-
	6.37 ^d	MX/MX ₂	n.i. ^c	Submerged	230 (10400)	296 (7640)	350 sh (4000)
Dips	1.40	XH ₂	210 (27430)	238 sh (5900)	246 (7750)	317 (4230)	-
	4.55	XH	208 (22600)	Submerged	240 sh (5700)	309 (3819)	-
+ Cu ²⁺	3.08	XH ₂ /XH	208 sh (13750)	Submerged	245 (10560)	315 (4050)	-
	4.06	MXH/XH	208 (10760)	Submerged	250 (6380)	313 (1175)	-
	5.88 ^e	MX ^f	208 sh (7080)	Submerged	250 (4375)	300 sh (3700)	-
	7.84 ^e	M ₂ X ₂ H ₂	208 (10170)	Submerged	240 sh (10070)	308 (8000)	-

^a For the designation of the bands employed see ref. [39].

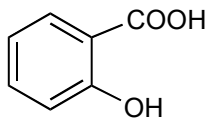
^b Spectra measured in 1 M aqueous NaOH.

^c n.i. = not identified

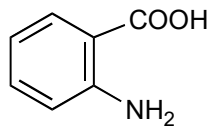
^d Similar spectral features were observed in the 6.00-9.50 pH range.

^e Measured with the following experimental conditions: $C_X = 1.20 \times 10^{-4} \text{ M}$; $C_M = 7.95 \times 10^{-3}$.

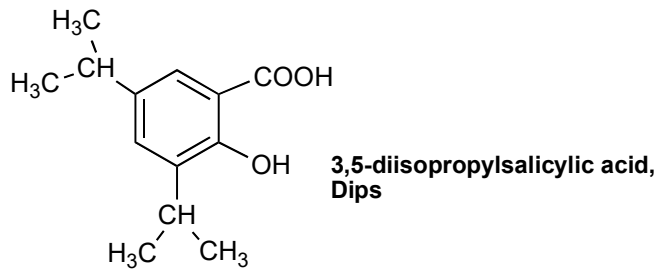
^f Precipitated species



salicylic acid, Sla



anthranilic acid, Ant



**3,5-diisopropylsalicylic acid,
Dips**

Fig. 1: Structures of the ligands.

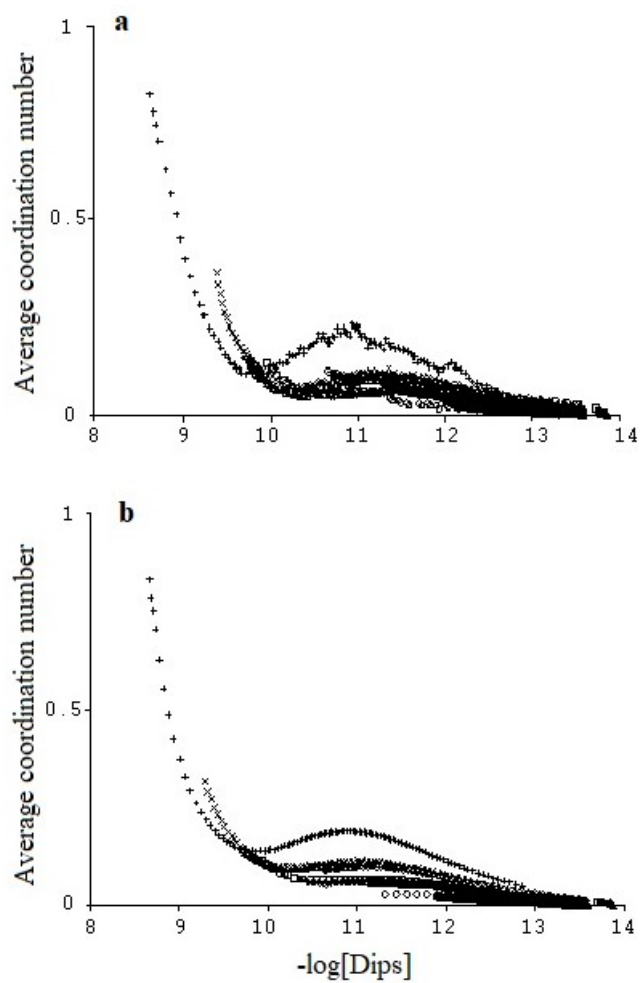


Fig. 2: Experimental (a) and simulated (b) –though ESTA calculations using constants in Table 4– formation curves of the copper(II)-Dips system for NDP = 2. The following data symbols +, x, □, Δ, ◇, ○, ■, ▲ refers to experiments in the order shown in Table 2 for 50 % of water.

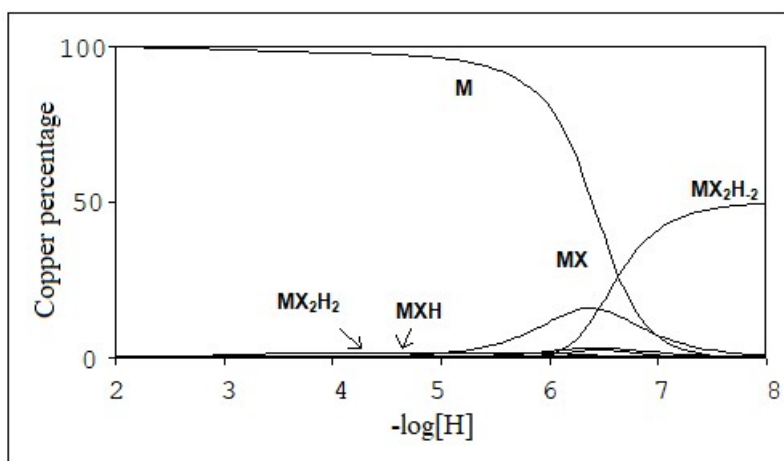


Fig. 3: Distribution of copper(II) into its free and complexed forms ($C_{\text{Cu(II)}} = 1.60 \times 10^{-4} \text{ M}$,

$C_{\text{Dips}} = 4.80 \times 10^{-4} \text{ M}$; $C_{\text{NaCl}} = 0.15 \text{ M}$).

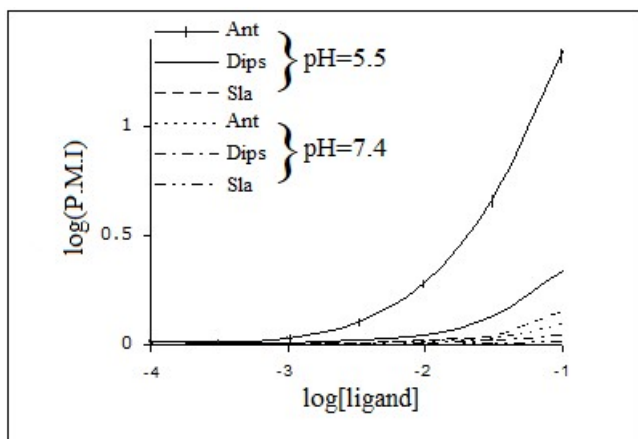


Fig. 4: Mobilization of copper(II) in simulated extracellular fluid in the presence of Dips, Ant and Sla at physiological (7.4) and inflammatory (5.5) pH values.