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Copper–Ligand Interactions and Physiological Free Radical Processes. Part 3. Influence of Histidine, Salicylic acid and Anthranilic Acid on Copper-Driven Fenton Chemistry *In Vitro*

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With a view to the possible use of copper(II)–•OH inactivating ligand (OIL) complexes as regulators of inflammation, the reactivity of the copper(II)–ascorbate system with hydrogen peroxide has been investigated in the presence of three key substances: histidine (the main copper(II) low molecular mass ligand in extracellular fluid), salicylic acid (the well-known non-steroidal antiinflammatory drug, previously shown to be *potentiated* by copper(II) in animal models of inflammation), and anthranilic acid (an inactive substance by itself, known to be *activated* by copper(II) in the same models) at physiological pH (7.4) and inflammatory pH (5.5).

Such substances may affect the amount of TBARS detected in solution following copper-mediated Fenton-like reactions through three distinct mechanisms: (i) by decreasing the Cu(II)/Cu(I) redox potential, i.e. at the expense of •OH radical *production*, (ii) by scavenging •OH radicals in the body of the solution, and/or (iii) by acting as a true OIL, i.e. at the expense of •OH *detection*. Redox potential measurements of initial solutions have been performed in parallel to TBARS determinations to help discriminate between different ligand influences. Computer-aided speciation has been used to understand the role of copper(II) distribution on the ligand effects characterised.

Contrary to previous interpretations, histidine has been found to mainly affect •OH production by lowering the redox potential of the Cu(II)/Cu(I) couple. Salicylate, which has no effect on •OH production, has been confirmed to mainly scavenge •OH radicals in the body of the solution. Anthranilate, which both increases •OH production and decreases •OH detection, behaves as a potential OIL.

These results tend to confirm our previous hypothesis that copper *potentiation* of antiinflammatory substances is indirect, i.e. independent of any interaction between metal and drug, whereas copper *activation* of substances that are inactive by themselves results from specific metal–substance interactions taking place at inflammatory sites.

Keywords: Copper-mediated oxidation, hydroxyl radicals, copper complexation, histidine, salicylic acid, anthranilic acid

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INTRODUCTION

Copper plays an ambivalent role with respect to inflammation. On the one hand, the anti-inflammatory properties of the metal and its complexes are well-known.[1,2] Experiments on animal models of inflammation have shown that copper(II) complexes of inactive substances are more antiinflammatory than parent inorganic copper salts and that copper(II) complexes of non-steroidal antiinflammatory drugs (NSAIDs) are more active than these drugs by themselves.[3,4] Based on these observations, Sorenson advanced the hypothesis that the active metabolites of NSAIDs are their copper complexes[3,4] – a hypothesis that still remains to be tested.[5,6] On the other hand, the notion is now generally accepted that copper can, like iron, trigger Fenton-type reactions *in vivo* to produce hydroxyl radicals[7] that are key mediators of the inflammatory process.[8] Attention has even been drawn to the potential risk of using therapeutic copper in this context.[9]

It may seem paradoxical that copper complexes can both display antiinflammatory properties and act as catalysts in Fenton-type reactions. In fact, the paradox is apparent only.[10] While copper uniformly behaves as a catalyst of highly site-specific Fenton-type reactions,[11] this *pro-oxidant* action may either result in a deleterious, i.e. *inflammatory*, effect if the ligand bound to copper(II) ions at the outset of the Haber–Weiss-type reaction *in vivo* gives rise to radical derivatives at least as offending to essential biomolecules as the hydroxyl radical itself, or a protective, i.e. *antiinflammatory*, effect if the Fenton-type reaction yields stable, non-radical ligand metabolites. The notion of •OH inactivating ligand (OIL) has been proposed to designate any substance that can (i) bind metal ions involved in •OH formation, (ii) maintain the redox activity of these ions in the resulting complexes, and (iii) inactivate •OH radicals as they are formed by producing innocuous metabolites.[10]* A possible therapeutic role has been anticipated for Cu(II)–OIL complexes in the control of inflammation, these complexes being expected to act as "lures" for the hydrogen peroxide involved in iron-mediated Fenton chemistry.[10]

Having a knowledge of how copper complexes may influence the course of the inflammatory process is a prerequisite to the design of any new copper-based antiinflammatory strategy. In this respect, testing Sorenson's hypothesis relative to the fate of NSAIDs copper complexes *in vivo* [3,4] was the first objective to address. As metal doses observed to produce antiinflammatory effects in animal experiments were situated far below toxic levels,[3] these were first considered to correct a copper deficiency or inadequacy disease.[12] The protective role characterised for copper against acute inflammation[5] is in line with this physiological interpretation,[13,14] but the fact that rheumatoid arthritis patients do not seem to be copper deficient[15] tends to contradict it. Furthermore, the observation that the antiinflammatory activity depends on the nature of the ligand and not on the total amount of metal[3,4] indicates that part of the therapeutic action is due to the ligand itself.

The ligand in this context may first act as a simple carrier delivering copper to the therapeutic target regardless of its possible interactions with other ligands present at the inflammatory site. It may also, alternately or jointly, endow copper with the capacity to interfere with some step of the inflammatory process. In this respect, much attention was paid to the superoxide dismutase-like activity of copper(II) complexes during the last two decades, but with little success eventually.[6,16,17] The ligand may also exert a direct influence on copper Fenton reactivity and/or its consequences for surrounding biomolecules. First, copper complexation may

*In other words, a M_{ox}–OIL complex would exert a pseudo-catalase-like action, with H_2O_2 producing innocuous oxidised metabolites of the OIL (instead of water with catalase).

entail a decrease in the Cu(II)/Cu(I) redox potential at the expense of the Fenton-type reaction. Second, even though copper is known to trigger highly site-specific Fenton-type reactions,[11] the ligand may also, above a certain concentration at least, act as a bulk •OH scavenger in the body of the solution. Finally, it may behave as a true OIL, i.e. in a pseudo-catalase-like manner.[10]

The evaluation of the carrier properties of a given ligand falls within the field of computer-aided speciation.[18,19] For example, by contrast with the report of a direct relationship between the trapping of •OH radicals produced with copper complexes and the extent of Cu(II)–salicylate chelation[7] (an observation apparently in line with Sorenson's above hypothesis[3,4]), it has been established by this technique that salicylate is unlikely to exert its antiinflammatory action through copper *in vivo*, plasma therapeutic levels of the drug being unable to mobilise more than 0.1% of the ultrafiltrable metal.[20] More recently, anthranilic acid has also been found to be unable to significantly mobilise plasma copper up to a concentration of 0.1 M, i.e. far above realistic drug levels.[21] Thus, anthranilic acid (an inactive substance by itself activated by copper in animal models of inflammation[3,4]) and salicylic acid (the reference NSAID known to be potentiated by copper in the same models[3,4]) display a similar affinity for copper in normal blood plasma. However, the analysis of their capacity to mobilise the metal under different conditions has revealed a fundamental difference: whereas salicylate remains a poor ligand of copper(II) in extracellular fluids *whatever the pH*, anthranilate on the contrary is expected to mobilise increasing copper(II) fractions as the pH decreases from 7.4 to 5.5, i.e. the more inflammation, the more copper bound to it.[21] Based on these results, a beginning of rationale to the pharmacological activity of copper complexes has been proposed in relation to Sorenson's hypothesis:[3,4] copper potentiation of NSAIDs would be independent from any association between metal and drug, whereas substances inactive by themselves would become antiinflammatory through binding copper on inflammatory sites.[21]

Regarding copper(II) reactive properties in the presence of the same ligands, preliminary studies led at room temperature in unbuffered solutions have indicated that the decrease by salicylate of the detected amount of copper-generated •OH radicals would only be due to its bulk •OH scavenging effect[22] but that the effect of anthranilate, like that of histidine,[23] tends to parallel the fraction of complexed copper.[22] Histidine and anthranilate have therefore been suggested to act as potential OILs,[21–23] such a possibility being ruled out for salicylate.[20,22]

In order to improve our current knowledge of the role of complexation in copper-mediated Fenton chemistry, the present work further investigates the influence of histidine, salicylic acid and anthranilic acid on the production/inactivation of copper-produced •OH radicals at 37°C in buffered aqueous solution at physiological pH 7.4 and at pH 5.5 (considered to mimic inflammatory media).[24] Redox potential determinations likely to help discriminate the ligand effects on •OH production and inactivation[10] have also been performed. Finally, the reciprocal influence of histidine and each of the two acids on the detected amount of •OH radicals generated by corresponding mixed-ligand copper complexes has been analysed.

MATERIALS AND METHODS

Materials

L-histidine was a Merck biochemical grade reagent (> 99% pure) and orthophosphoric acid a Merck Suprapur reagent. Salicylic acid and anthranilic acid were Aldrich-Chemie products (99% and 98% pure, respectively). $CuSO_4 \cdot 5H_2O$, sodium hydroxide and trichloroacetic acid were Normapur Prolabo reagents. Ethylenediaminetetraacetic acid (EDTA) was obtained from

Fluka (puriss. p.a., $\geq 99.5\%$). 2-deoxy-D-ribose, L-ascorbic acid and 2-thiobarbituric acid (TBA) were from Sigma. Hydrogen peroxide was from Gifrer.

All solutions were prepared from triply deionised, freshly deaerated water. Fresh 3% (w/v) (10 vol.) H_2O_2 stock solutions were prepared just before use from the 30% (w/v) (110 vol.) commercial product. Carbonate-free sodium hydroxide solutions were prepared by dissolving previously washed pellets under a nitrogen atmosphere.

Spectrophotometric analyses were performed by means of a Perkin-Elmer Spectrometer Lambda 2. pH determinations were carried out with a Consort P602 pH meter equipped with a Metrohm combined glass electrode.

Hydroxyl Radical Production and Detection

As suggested from previous investigations,[25] $^{\bullet}OH$ radicals were generated by adding hydrogen peroxide to a mixture of copper(II) ions and L-ascorbic acid used as a reductant. In accordance with Rowley and Halliwell's reference work,[26] equimolar concentrations were used for these three reactants.

A particular objective of this work was the analysis of $^{\bullet}OH$ production and detection as a function of the complex species present in solution at the outset of the Fenton reaction. As metal and ligand speciation is highly pH-dependent, the pH had to be controlled in all experiments. The choice of a buffer in such a situation is not an easy task, however. Ideally, the buffering substance should not (i) interfere with the reaction process under study, (ii) complex the metal ions involved in free radical production, and (iii) scavenge free radicals. In practice, this amounts to select one among several disadvantages. Phosphate was finally preferred to Tris whose scavenging activity was judged too important, the extent of copper(II) coordination by phosphate being taken into account in the speciation calculations whenever necessary. A serious drawback about phosphate was its poor buffering capacity at pH 5.5. However, this capacity is notably improved at 37°C (due to the decrease in the corresponding pK), and 30 mM phosphate was shown to prevent significant pH variations of the solutions even in the presence of ascorbate and hydrogen peroxide at the concentrations used.

Detection of copper-generated hydroxyl radicals can easily be biased because of the site specificity inherent in copper Fenton chemistry.[23,27] Deoxyribose, which has a low affinity for copper, was chosen as a detector to minimise this effect.

For binary system investigations, each reaction tube contained a 2 ml solution including successively: 30 mM phosphate buffer (previously adjusted to the desired pH), 2.5 mM deoxyribose, 0.125 mM $CuSO_4$, a concentration of the ligand investigated within the 0–0.625 mM range, 0.125 mM ascorbic acid, and finally 0.125 mM hydrogen peroxide to trigger the Fenton reaction. After adding hydrogen peroxide, each tube was securely stopped, vortexed, and incubated at 37°C for 90 min in the dark. Following incubation, each tube was added 1 ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05 N NaOH, 0.1 ml of 6.25 mM EDTA and 1 ml of 2.8% (w/v) trichloroacetic acid (TCA), and heated at 100°C for 15 min to allow colouration to develop. It was then cooled and the pink chromogen corresponding to TBA-reactive substances (TBARS) was assessed against appropriate blanks by measuring related absorbances at 532 nm. (The EDTA added after reaction completion was used in place of cation exchange resins to make the test metal compatible[28] after it was checked that the absorption band due to the Cu(II)–EDTA complex at 532 nm could be neglected.) Mixed-ligand ternary system determinations were conducted in the same way as above, except that a concentration of 0.250 mM of reference ligand coexisted with variable concentrations (0.063–0.625 mM) of the second ligand investigated. All determinations were made in triplicate.

Redox Potential Determinations

Redox potential measurements were carried out with a 713 Metrohm mV-meter equipped with a Metrohm platinum electrode and an Ingold calomel reference electrode fitted to an Ingold cell system. The temperature inside the reaction cell was maintained at $37 \pm 0.02°C$ by circulating thermostated water, and test solutions were kept under a constant bubbling of purified and thermostated nitrogen.

Determinations referred to the zero time of Fenton-type reactions. Accordingly, the test solutions were composed of phosphate, deoxyribose, copper sulfate, ligand and ascorbate at the above concentrations. They also contained 0.15 M sodium chloride to maintain the ionic strength constant near physiological conditions.

Speciation Calculations

Speciation calculations referring to test solutions before the addition of ascorbic acid and hydrogen peroxide were carried out using the SPEC task of the ESTA simulating module.[29] The formation constants on which these calculations were based were taken from recent works of our group for copper(II) complexes with histidine,[20] salicylic acid,[20] and anthranilic acid,[21] and from literature data for copper(II)-phosphate equilibria.[30]

RESULTS AND DISCUSSION

Copper–Ligand Binary Systems

Figures 1 and 2 show the percentage evolution of the extent by which the amount of TBARS detected in the absence of ligand is reduced in the presence of increasing concentrations of histidine, salicylate and anthranilate at pH 7.4 and 5.5, respectively. Tables I and II display the stoichiometries and copper percentages of the copper(II) complexes formed by histidine, salicylate and anthranilate in the initial reaction mixtures under the same respective conditions. Figure 3 shows the variations of the Cu(II)/Cu(I) redox potential due to the presence of increasing concentrations of histidine, salicylate and anthranilate in the test solutions just before triggering Fenton-type reactions at pH 5.5.†

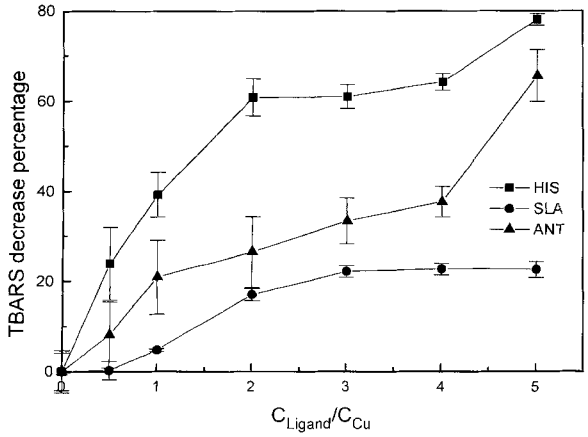

FIGURE 1 Decrease percentages of TBARS observed in the presence of histidine (HIS), salicylate (SLA) and anthranilate (ANT) under physiological conditions (pH 7.4) as a function of the ligand-to-copper concentration ratio. (The concentration of copper(II) was 0.125 mM in all test solutions.)

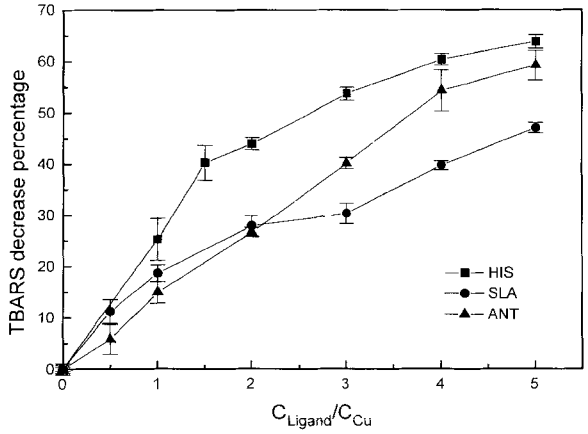

FIGURE 2 Decrease percentages of TBARS observed in the presence of histidine (HIS), salicylate (SLA) and anthranilate (ANT) under inflammatory conditions (pH 5.5) as a function of the ligand-to-copper concentration ratio. (The concentration of copper(II) was 0.125 mM in all test solutions.)

† Steady drifts indicative of a slow precipitation process were observed in redox potential readings at pH 7.4. These were thus discarded.

TABLE I Stoichiometries and copper fractions (in percentages) of the predominant copper(II) complexes with histidine, salicylate and anthranilate in initial reaction mixtures under physiological conditions (pH 7.4) at the different metal-to-ligand ratios (M:L) investigated. Free Cu^{2+} ions, phosphate complexes as well as complexes with other ligands whose percentages are inferior to 0.1% are not mentioned. (Copper(II) concentration as in Figures 1 and 2)

M:L	Histidine	%	Salicylate	%	Anthranilate	%
1:1/2	ML	17.7	ML	3.4	ML	0.3
	MLOH	14.6	MLOH	0.1	MLOH	2.3
	ML_2	3.5				
	ML_2H	0.1				
1:1	ML	25.2	ML	6.5	ML	0.7
	MLOH	20.8	MLOH	0.2	MLOH	4.5
	ML_2	19.1	ML_2	0.1	ML_2	0.2
	ML_2H	0.3				
1:2	ML	3.6	ML	11.8	ML	1.2
	MLOH	2.9	MLOH	0.3	MLOH	8.2
	ML_2	89.9	ML_2	0.4	ML_2	0.2
	ML_2H	1.3				
1:3	ML	0.3	ML	16.1	ML	1.6
	MLOH	0.3	MLOH	0.4	MLOH	11.3
	ML_2	97.7	ML_2	0.8	ML_2	0.3
	ML_2H	1.4				
1:4	ML	0.2	ML	19.7	ML	2.0
	MLOH	0.1	MLOH	0.5	MLOH	13.9
	ML_2	98.1	ML_2	1.3	ML_2	0.5
	ML_2H	1.4				
1:5	ML	0.1	ML	22.6	ML	2.4
	MLOH	0.1	MLOH	0.6	MLOH	16.2
	ML_2	98.3	ML_2	1.9	ML_2	0.8
	ML_2H	0.5				

Histidine

A marked decrease is observed in the detected amount of TBARS as the concentration of histidine is raised. At pH 7.4, the apparent inhibition percentage amounts to as much as 61% for the 1:2 metal-to-ligand ratio, reaching 78% for the 1:5 ratio (Figure 1). The same effect is induced by histidine at pH 5.5 (Figure 2), but to a lesser extent (44% and 64% for 1:2 and 1:5 metal-to-ligand ratios, respectively).

As can be seen from Tables I and II, there appears to be a clear relationship between the histidine inhibitory effect and the fraction of copper(II) bound to histidine. However, this relationship may not be univocal. The histidine apparent inhibitory effect may *a priori* originate in one or all of the three following causes: histidine may (i) lower the Cu(II)/Cu(I) redox potential through complexation and inhibit $^{\bullet}OH$ radical production, (ii) scavenge $^{\bullet}OH$ radicals in the body of the solution (speciation calculations not shown here reveal that free histidine increases as total histidine is raised in excess of copper(II) and the fraction of histidine-complexed copper(II) is increased), or/and (iii) inactivate $^{\bullet}OH$ radicals in a site-specific manner as a true OIL (see Introduction). A comparison between apparent inhibitory effects and redox potential variations may help discriminate among these effects. Clearly, the increase in the histidine inhibitory effect at pH 5.5 (Figure 2) is paralleled by a decrease in the Cu(II)/Cu(I) redox potential in test solutions (Figure 3), inhibition percentages and redox potential variations being found in linear relationship (Figure 4). Although this observation does not negate the possibility for histidine to act as an $^{\bullet}OH$ bulk scavenger or OIL, i.e. to influence $^{\bullet}OH$ *detection*, it definitely establishes that at least part of its inhibitory effect is exerted at the expense of $^{\bullet}OH$ *production*. This tends to question the commonly accepted principle that histidine would prevent copper-generated free $^{\bullet}OH$ radicals from being released into the solution by reacting with these in a site-specific manner as soon as they are formed.[31]

The above observation may be chemically interpreted in the following terms. Imidazole has a well-known tendency to increase the redox potential of the Cu(II)/Cu(I) couple whereas amino-containing ligands tend to decrease it.[32] The complexation of copper(II) by histidine would simply reflect this. Virtually all the bonds formed in the different copper(II)–histidine complexes have been characterised from thermodynamic data:[33] copper(II) is bound to histidine through imidazole and carboxyl groups in MLH and through amino, imidazole and carboxyl groups in ML and MLOH (with a additional dissociated water in MLOH). ML_2H_2 is coordinated in the MLH mode, and ML_2H combines the MLH and

TABLE II Stoichiometries and copper fractions (in percentages) of the predominant copper(II) complexes with histidine, salicylate and anthranilate in initial reaction mixtures under inflammatory conditions (pH 5.5) at the different metal-to-ligand ratios (M:L) investigated. Free Cu^{2+} ions, phosphate complexes as well as complexes with other ligands whose percentages are inferior to 0.1% are not mentioned. (Copper(II) concentration as in Figures 1 and 2)

M:L	Histidine %		Salicylate %		Anthranilate %	
1:1/2	Not determined		ML	0.4	ML	2.9
					MLOH	0.3
					ML_2	0.1
1:1	ML	51.8	ML	0.8	ML	5.6
	MLH	1.8	MLH	0.1	MLOH	0.5
	ML_2	7.3			ML_2	0.3
	ML_2H	8.4				
1:3/2	ML	49.3	Not determined		Not determined	
	MLH	1.7				
	ML_2	15.9				
	ML_2H	18.4				
1:2	ML	39.0	ML	1.6	ML	10.2
	MLH	1.3	MLH	0.2	MLOH	0.9
	ML_2	24.3			ML_2	1.2
	ML_2H	28.1				
1:3	ML_2	22.5	ML	2.4	ML	14.0
	MLH	0.8	MLH	0.2	MLOH	1.5
	ML_2	34.3			ML_2	3.9
	ML_2H	39.6				
1:4	ML	14.8	ML	3.2	ML	17.1
	MLH	0.5	MLH	0.3	MLOH	1.5
	ML_2	38.6			ML_2	3.9
	ML_2H	44.5				
1:5	ML	10.9	ML	3.9	ML	21.6
	MLH	0.4	MLH	0.4	MLOH	1.9
	ML_2	40.7			ML_2	7.4
	ML_2H	46.9				

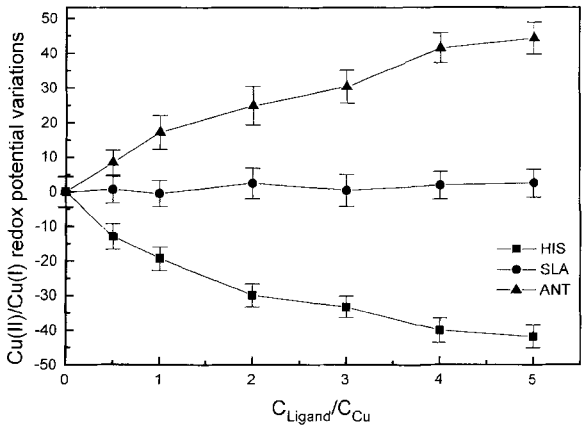

FIGURE 3 Variations of Cu(II)/Cu(I) redox potentials induced by the addition of histidine (HIS), salicylate (SLA) and anthranilate (ANT) at pH 5.5.

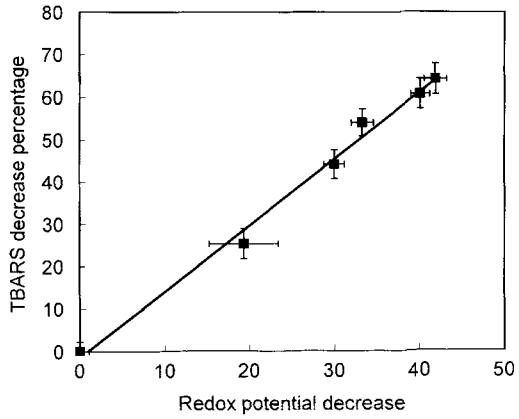

FIGURE 4 Relationship between TBARS decrease percentage and redox potential decrease for various histidine-to-copper concentration ratios at pH 5.5.

ML modes. In ML_2, one of the two available carboxyl groups is considered to coordinate copper (one histidine binding in the ML mode, the other in the histamine-like mode),[33] although a double histamine-like mode has also been suggested.[34] Based on these assignments, a relative decrease in the redox potential of the Cu(II)/Cu(I) couple is expected to be seen in relation to the fraction of copper bound to the amino group, i.e. in relation to the copper percentage present as ML_2. The decrease in the redox potentials (Figure 3) observed at pH 5.5 in parallel to the progressive formation of ML_2 as the histidine concentration is raised (Table II) corroborates this. Likewise, the effective competition of ML_2H at the expense of ML_2 at pH 5.5 (Table II) as compared to pH 7.4 (Table I) should normally entail a lesser decrease of the redox potential of the Cu(II)/Cu(I) couple at pH 5.5 than at pH 7.4 (the latter being unfortunately impossible to determine), hence the lesser inhibitory effect actually observed. The strong decrease in the copper-mediated oxidation of tryptophan recently reported to occur as a function of tryptophan concentration[35] is also in line with this interpretation.

Salicylic Acid

The decrease in TBARS caused by salicylate at pH 7.4 appears to be much less important than that of histidine, being approximately one-third of the latter at the most (Figure 1). At pH 5.5, however, this difference is less significant and the inhibition due to salicylate reaches more than two-thirds of that brought about by histidine at the 1 : 5 metal-to-ligand ratio (Figure 2). Unlike histidine, salicylate seems to exert its apparent inhibitory effect regardless of its capacity to coordinate copper(II) ions since the maximum inhibition is observed at pH 5.5 where the percentage of copper bound to salicylate tends to be insignificant (Table II). There is also no systematic variation of the redox potential of the Cu(II)/Cu(I) couple depending on the concentration of salicylate (Figure 3). Thus, salicylate seems to act as an $^{\bullet}OH$ bulk scavenger. This contradicts a former assertion relating salicylate $^{\bullet}OH$ radical trapping with the degree of Cu(II)–salicylate coordination,[7] but corroborates our own preliminary findings on copper reactivity in Fenton chemistry[22] and is in line with our previous speciation results.[20]

Anthranilic Acid

In the presence of anthranilate, the detected amount of TBARS is significantly reduced at both pH values investigated, the effect being observed about half-way between histidine and salicylate (Figures 1 and 2). At first sight, the mode of action of anthranilate is similar to that of histidine as a clear correlation appears between apparent inhibitory effect and fraction of copper bound to anthranilate (Tables I and II). The similarity stops there, however, as, unlike histidine which significantly decreases the redox potential of the Cu(II)/Cu(I) couple, anthranilate on the contrary tends to increase it (Figure 3). In other words, even though reducing the net amount of $^{\bullet}OH$ radicals finally detected by deoxyribose, anthranilate tends to increase the $^{\bullet}OH$ radical initial production. This behaviour corresponds to that expected from a potential OIL,[10] which concurs with the conclusions of our recent speciation studies.[21]

Copper–Histidine–Second Ligand Ternary Systems

Influence of Salicylate and Anthranilate on the Histidine Effect

The reducing effect of histidine on TBARS is very significant at the 1 : 2 copper-to-histidine ratio and above (Figures 1 and 2). The 1 : 2 ratio has therefore been used as a reference to investigate the influence of salicylate and anthranilate on the histidine individual effect at pH 5.5. As Figure 5 shows, the decrease percentage of the TBARS detected in the presence of each of the two acids in addition to histidine is uniformly greater than with histidine alone and progressively increases as the acid concentration is raised, especially for anthranilate. This effect, however, is relatively less important than that induced by each acid by itself (Figure 2) since histidine remains the main ligand of copper(II) even at high acid-to-metal concentration ratios (speciation results not shown here). As histidine reduces $^{\bullet}OH$ radical *production*, the relative effect of each acid on the *detection*

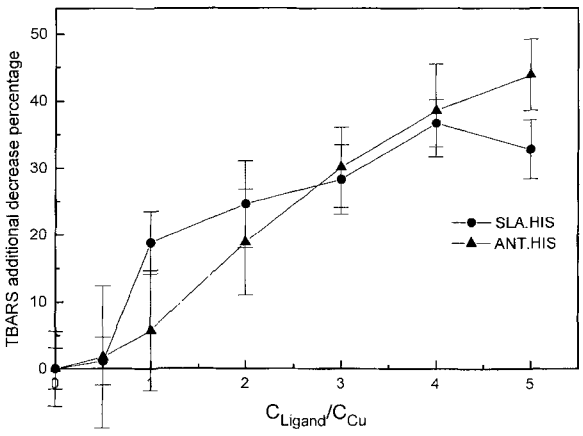

FIGURE 5 TBARS additional decrease percentages brought about at pH 5.5 by increased concentrations of salicylate (SLA) and anthranilate (ANT) in the presence of constant copper and histidine (HIS) concentrations (0.125 mM and 0.250 mM, respectively).

of TBARS operates in virtually equal terms – even though, according to our above analysis, salicylate is thought to compete with deoxyribose for •OH radicals in the body of the solution whereas anthranilate is expected to compete with histidine for copper(II) coordination. (In the latter respect, it is to be noted that while mixed-ligand coordination between histidine and salicylate is negligible over the salicylate concentration range investigated (1.3% max. for the 1 : 5 metal-to-acid ratio), copper(II)–histidine–anthranilate ternary complexes reach 4.6%, 8.7%, 12.4%, 15.5% and 18.5% for 1 : 1, 1 : 2, 1 : 3, 1 : 4 and 1 : 5 ratios, respectively.)

Influence of Histidine on Salicylate and Anthranilate Effects

Even though the effect of the two acids on TBARS detection is more progressive over the whole acid concentration range (Figure 2), the 1 : 2 metal-to-ligand ratio was also taken as a reference to investigate the influence of histidine on that of each acid at pH 5.5 (Figure 6). Again, the effect of the ligand mixture is more important than that induced by each ligand individually. This time, however, the effect added by histidine to that exerted by salicylate is virtually identical to that of histidine alone (see Figures 2 and 6 profiles), which confirms the different modes of action of the two substances. (Speciation calculations show that all the copper is virtually bound to histidine from the lowest histidine-to-copper ratio investigated.) The interpretation of the influence of histidine with respect to that of anthranilate is also relatively straightforward. Based on our hypothesis of a possible OIL behaviour of anthranilate (see above), the fact that the apparent inhibitory effect added by histidine is significantly inferior to that of histidine itself suggests that anthranilate competes with histidine for copper, even at high histidine concentrations. This tends to be confirmed by speciation calculations: copper(II)–histidine–anthranilate ternary complexes account for 8.7% of total copper at the 1 : 2 copper to histidine ratio and still represent 2.8% of copper at the 1 : 5 ratio.

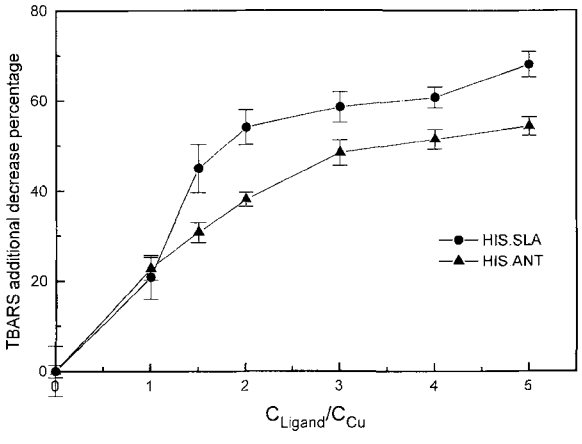

FIGURE 6 TBARS additional decrease percentages brought about at pH 5.5 by increased concentrations of histidine (HIS) in the presence of constant copper and salicylate (SLA) or anthranilate (ANT) concentrations (0.125 mM and 0.250 mM, respectively).

CONCLUSION

A significant step has been taken in this study regarding the understanding of the ligand influence on copper-mediated Fenton chemistry in relation to the antiinflammatory activity of copper complexes. Ligands may *a priori* affect copper reactivity and/or its effects on surrounding biomolecules through three possible mechanisms: (i) by decreasing the Cu(II)/Cu(I) redox potential at the expense of the Fenton-type reaction, (ii) by scavenging •OH radicals in the body of the solution, and (iii) by acting as a true OIL.[10] Three ligands of copper(II) have been investigated in this context: histidine, the main low-molecular-mass ligands of copper(II) in blood plasma,[19] salicylic acid, the reference NSAID whose specific antiinflammatory properties are potentiated by copper,[3,4] and anthranilate, an inactive substance that is activated by copper in animal models of inflammation.[3,4] From previous speciation studies, the hypothesis was put forward that copper potentiation of NSAIDs (e.g. salicylate) would be independent from any association

between metal and drug, whereas substances inactive by themselves would become antiinflammatory through binding copper on inflammatory sites (e.g. anthranilate).[21] Parallel preliminary investigations on copper(II) reactivity in Fenton chemistry led to the conclusion that salicylate reduced the amount of detected TBARS in solution through its bulk •OH scavenging properties only[22] but that both histidine and anthranilate,[22,23] whose effect was a function of the fraction of complexed copper, could behave as potential OILs.[21–23]

The present work, which completes the above investigations, leads to the following conclusions:

In contrast to the former assertion that salicylate •OH radical trapping is related to the degree of Cu(II)-salicylate coordination,[7] salicylate is confirmed to mainly scavenge •OH radicals in the body of the solution, i.e. regardless of copper complexation – which strengthens the significance of related speciation results;[20]

- Contrary to the common thought that histidine prevents copper-generated free •OH radicals from being released into the solution by site-specific scavenging,[31] histidine has been found to mainly affect •OH radical *production* by lowering the redox potential of the Cu(II)/Cu(I) couple, its effect on •OH radical *detection* being apparently of minor importance. As the predominant ligand of copper(II) in extracellular fluids,[19] histidine therefore seems to play a copper-specific antioxidant role *in vivo*;
- Anthranilate has been shown both to enhance •OH radical *production* by increasing the Cu(II)/Cu(I) redox potential and to decrease •OH radical *detection* by surrounding molecules through OIL site-specific activity. As such, anthranilate behaves as a potential OIL.[10]

These results globally confirm our previous hypothesis[21] that copper *potentiation* of substances that are antiinflammatory by themselves (like salicylate) is indirect, i.e. independent of any interaction between metal and drug, whereas copper *activation* of substances that are inactive by themselves (like anthranilate) results from metal–substance interactions that take place at inflammatory sites. From a more general point of view, they bring to light the high complexity of the effects to be expected from small ligands on the reactivity of copper in Fenton chemistry and its consequences for surrounding biomolecules *in vivo*.[36] They finally confirm that, in addition to quantitative investigations into copper(II) mobilising capacities of ligands in biological fluids,[37] detailed reactivity studies will be necessary before the profiles of new OIL-based antiinflammatory copper complexes can be outlined.

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