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# STABLE SUPEROXIDE DISMUTASE (SOD)-MIMETIC TERNARY HUMAN SERUM ALBUMIN–Cu(II)(3,5-DIISOPROPYLSALICYLATE)$_2$/Cu(II)$_2$(3,5-DIISOPROPYLSALICYLATE)$_4$ COMPLEXES IN TISSUE DISTRIBUTION OF THE BINARY COMPLEX

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**Abstract**—Copper(II)$_2$(3,5-diisopropylsalicylate)$_4$ [Cu(II)$_2$(3,5-DIPS)$_4$] has been found to have anti-inflammatory, antiulcer, anticancer, anticonvulsant, antimutagenic, antidiabetic, analgesic, and radiation protection and recovery activities. It has also been found to reduce ischemia-reperfusion injury. Because of these activities it was of interest to understand how this compound is transported in the body to affected tissues. Evidence supporting the suggested formation of ternary human serum albumin (HSA)–Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$ complexes was obtained using ultraviolet spectrophotometry, dialysis, and atomic absorption spectrophotometry or atomic emission spectroscopy. Superoxide dismutase (SOD)-mimetic activity was also determined using the xanthine/xanthine oxidase/cytochrome *c* system. Ultraviolet spectra of aqueous solution mixtures of Cu(II)$_2$(3,5-DIPS)$_4$ ⇄ 2Cu(II)(3,5-DIPS)$_2$ and HSA as well as aqueous solutions of solid Cu(II)$_2$(3,5-DIPS)$_4$ obtained by stirring the solid with an aqueous solution of HSA showed no obvious change in absorbance to indicate ternary complex formation. However, comparison of ultraviolet spectra taken before and after dialysis supports the suggested bonding of Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$ to HSA. Comparison of copper concentrations before and after dialysis also supports the suggested bonding of Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$ to HSA. Based upon these data it is plausible that Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$ form stable ternary complexes with HSA. These stable ternary complexes were also found to have SOD-mimetic activity.

Human serum albumin (HSA¶) is an important plasma protein that functions in the transport of drugs to all tissues [1]. The concentration of HSA in plasma ranges between 3.5 and 5.0 g/100 mL (0.5 to 0.8 mM) [2–4]. Aqueous solubility of HSA is attributed to the large number of titratable acidic and basic amino acids, 117 and 99, respectively, which impart a large net negative charge of −18 at pH 7.5 [3]. In addition to these functional groups albumin contains 1 thiol group and 17 disulfide (cystine) groups. Adsorption of lipophilic substances to hydrophobic sites on albumin and the water solubility of albumin at physiologic pH account for the fact that many water-insoluble substances will "dissolve" in plasma. A large portion of drug transport in plasma can be accounted for by the fact that most drugs actually bond to HSA [1, 4] and pharmacological efficacy of some drugs has been found to be dependent on drug–albumin bonding interactions [4–6]. The interaction of HSA and drugs in drug transport and tissue distribution has, therefore, been a field of intensive study [6, 7].

To date copper(II)$_2$(3,5-diisopropylsalicylate)$_4$ [Cu(II)$_2$(3,5-DIPS)$_4$] (Registry No. 72841-56-6) has been found to have antiinflammatory [8], antiulcer [8], anticonvulsant [9, 10], anticancer [11, 12], anticarcinogenic [13], antimutagenic [14], antidiabetic [15], analgesic [16], and radiation protection and recovery activities [17, 18]. This binary complex is also as effective as the copper-dependent superoxide dismutase ($Cu_2Zn_2SOD$) in preventing ischemia-reperfusion injury [19]. All of these activities have been attributed to the marked lipid

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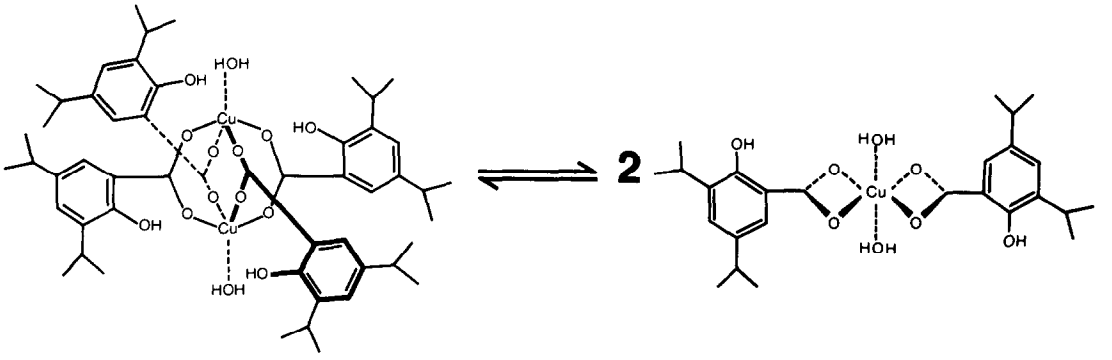
¶ Abbreviations: CL, chemiluminescence; HSA, human serum albumin; BSA, bovine serum albumin; SOD, superoxide dismutase; Cu(II)(salicylate)$_2$, bis-salicylatocopper(II); 3-5-DIPS, 3,5-diisopropylsalicylic acid; Cu(II)$_2$(3,5-DIPS)$_4$, copper(II)$_2$(3,5-diisopropylsalicylate)$_4$; Cu(II)(3,5-DIPS)$_2$, bis-3,5-diisopropylsalicylatocopper(II); Cu(II)(L-histidinate)$_2$, bis-histidinatocopper(II); Cu(II)$_2$(indomethacinate)$_4$, tetrakis-$\mu$-1-(5-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetodicopper(II); Cu(II)$_2$(aspirinate)$_4$, tetrakis-$\mu$-acetylsalicylatodicopper(II); Cu(II)(salicylate)$_2$, bis-salicylatocopper(II); Cu(II)(PuPy$_2$), bis-1,4-di(2-pyridyl)-2,7-diazooctadiene-1,7-copper(II); and Cu(II)(PuIm$_2$), bis-1,4-di(2-imidazolyl)-2,7-diazooctadiene-1,7-copper(II).

solubility of this SOD-mimetic complex. While SOD-mimetic activity may have some role in accounting for the above pharmacological effects, it is more likely that these effects are due to facilitation of *de novo* syntheses of copper-dependent enzymes and proteins.

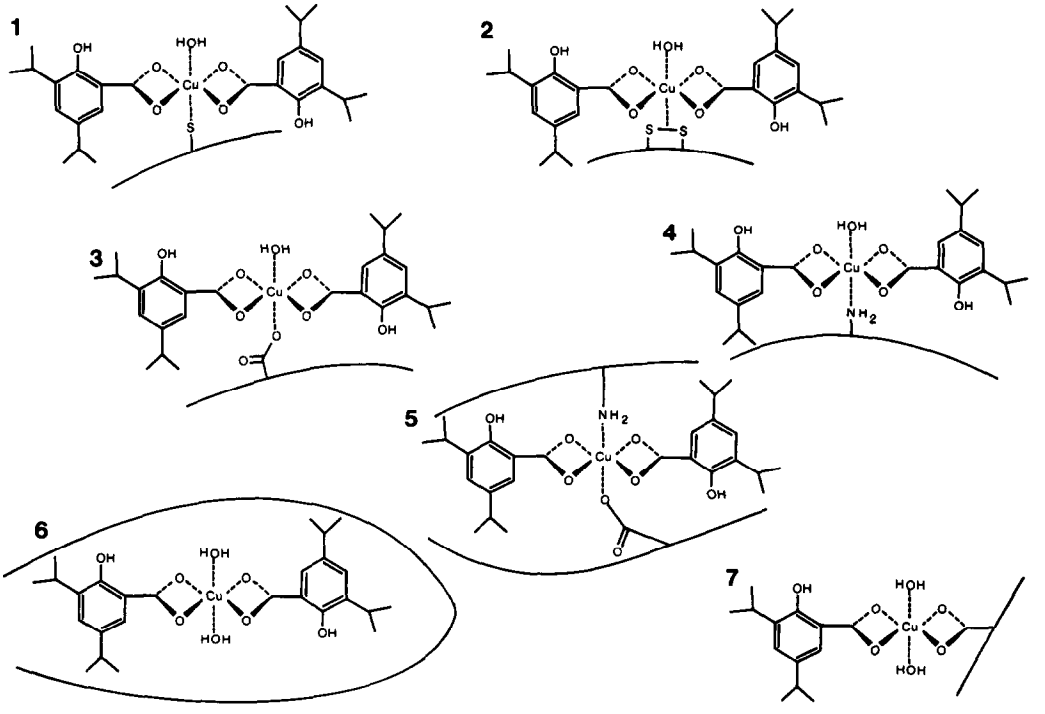
The solid state binuclear form of $Cu(II)_2(3,5\text{-}DIPS)_4$, which exists in nonpolar media, gives the mononuclear $Cu(II)(3,5\text{-}DIPS)_2$ form in polar media [20] as shown in the following equilibrium.



These lipid and aqueous solubility properties are likely to enable distribution of this complex to nonpolar and polar compartments, translocation across cell membranes, and facilitation of *de novo* synthesis of copper-dependent enzymes.

Due to the varied pharmacological activities of $Cu(II)_2(3,5\text{-}DIPS)_4$, it is of interest to examine its physical and chemical properties in accounting for these activities. It has long been known that a small portion of plasma copper is bonded to HSA [21] as a physiologically important form of copper which is in equilibrium with copper-containing components of tissues [22]. The occurrence of ternary amino acid–HSA coordination complexes has been suggested to be important in biological chemistry [23–26], offering a mechanism for the transfer of copper to cellular macromolecules as binary amino acid copper complexes.

Copper(II) $(salicylate)_2$ is known to have the same stability constant, $K = 10^{19}$, as $Cu(II)(histidinate)_2$ [27] which is the most abundant binary low molecular mass chelate in plasma [28]. The ternary HSA–Cu(II) (histidinate) complex has a stability constant which is five orders of magnitude greater than the binary complex [23]. The question as to whether or not binary $Cu(II)(3,5\text{-}DIPS)_2$ or $Cu(II)_2(3,5\text{-}DIPS)_4$ complexes might bond to HSA with the formation of ternary complexes led to these studies of the interaction between $Cu(II)(3,5\text{-}DIPS)_2$ and $Cu(II)_2(3,5\text{-}DIPS)_4$ with HSA. It is suggested that these interactions yield stable ternary complexes, illustrated below for the mononuclear complex, that can disproportionate superoxide.



## MATERIALS AND METHODS

3,5-Diisopropylsalicylic acid (3,5-DIPS) (Aldrich Chemical Co.), gelatin (W. H. Curtain Co.), Cation-Cal (Baxter Healthcare Corp.), sodium phosphate (Fisher), catalase (Boehringer Mannheim GmbH), sodium xanthanate, grade IV xanthine oxidase (0.52 U/10 g of protein), and type IV horse heart cytochrome *c* (all from the Sigma Chemical Co.) were used as purchased without further purification. Copper(II)$_2$(3,5-DIPS)$_4$ was prepared with 3,5-DIPS and $CuCl_2$ according to published methods [8]. HSA, 25% (American Red Cross), was purified by dialysis before use in studies of ternary complex formation. Ultraviolet spectra with absorbance ranging from 0 to 2 were obtained with a Shimadzu Bauch and Lomb Spectronic 200 ultraviolet spectrophotometer and a Fisher recordal series 5000 recorder. Deionized water (pH 7.5) was used to adjust zero absorbance prior to obtaining all ultraviolet spectra. SOD-mimetic activity was determined with an Aminco 2W-2UV/VIS spectrophotometer. Copper was determined with a Thermo Jarrel Ash ICAP 61E atomic emission spectrometer or an IL 157 atomic absorption spectrophotometer. A diluted solution of Cation-Cal, for which analytical results are routinely ± 5% of the expected copper content, was used to ensure accuracy in these determinations.

Dialysis tubing was boiled in a 1% solution of sodium bicarbonate in deionized water for 1 hr. The tubing was flushed with deionized water and then boiled with deionized water (pH 7.5) for 1 hr. After cooling, the pH of the water was adjusted to 7.5 and left to stand overnight prior to use.

One hundred milliliters of a 25% HSA solution was dialyzed against 100 mM EDTA at 3.5° in a 1-L graduated cylinder with stirring over a period of 7 days to remove preservatives, sodium *n*-acetyl tryptophanate and sodium caprylate, and bonded metalloelements including zinc and copper usually present in commercial preparations. The initial 100 mM $Na_2EDTA$ solution was changed on three successive days followed by daily changes of deionized water (pH 7.5) for three successive days in order to remove the remaining EDTA. Dialyzed HSA was then filtered through a 0.22 millimicron Corning filter system (model 25944) to sterilize it and then it was pipetted using a sterile technique into sterile 5-mL polypropylene tubes which were then capped and stored in a refrigerator. The concentration of the dialyzed HSA was then determined by ultraviolet spectrophotometry to be 1.36 mM using a molecular mass of 66,000 Da and an adsorptivity of 35,000 $M^{-1}cm^{-1}$ at 280 nm [29].

Three milliliters of a 2.25 mM ethanol solution of Cu(II)$_2$(3,5-DIPS)$_4$($H_2O$)$_2$ (molecular mass 1048 Da), 6.7 μmol, was dropped slowly into 40 mL of stirred deionized water (pH 7.5) contained in a 50-mL volumetric flask and brought to volume with this water after removing the stirring bar. The final concentration of this solution was 135 μM. All concentrations of Cu(II)$_2$(3,5-DIPS)$_4$ are based upon the binuclear form of this complex since this is the form of the complex used to prepare these solutions.

The first series of spectra were obtained with solutions containing 27, 54, 81 or 108 μM Cu(II)$_2$(3,5-DIPS)$_4$ and 27 μM HSA. These solutions were prepared by mixing 1, 2, 3, or 4 mL of the aqueous 135 μM HSA solution of Cu(II)$_2$(3,5-DIPS)$_4$ with 0.1 mL of 1.36 mM HSA solution in sterile polypropylene culture tubes and diluting to 5 mL with pH 7.5 deionized water before capping and shaking.

Bonding interactions between 3,5-DIPS and HSA were also studied. A 270 μM solution of 3,5-DIPS was prepared by dissolving 3 mg in 40 mL of deionized water with 10% KOH, adjusted to pH 7.5 with 10% HCl, and brought to volume in a 50-mL volumetric flask with deionized water (pH 7.5). Solutions of 54, 108, 162, or 216 μM 3,5-DIPS and 54 μM HSA were prepared by mixing 1, 2, 3, or 4 mL of 270 μM 3,5-DIPS with 0.2 mL of 1.36 μM HSA and diluting to 5 mL with deionized water (pH 7.5) before capping and shaking.

Spectra were also obtained for solutions in which the concentration of Cu(II)$_2$(3,5-DIPS)$_4$ was held constant and the concentration of HSA increased. Solutions of 27, 54, 108, 162, 216, or 270 μM HSA and 54 μm Cu(II)$_2$(3,5-DIPS)$_4$ were prepared by mixing 2 mL of 135 μM Cu(II)$_2$(3,5-DIPS)$_4$ with 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 mL of 1.36 mM HSA and diluting to 5 mL with deionized water (pH 7.5) before capping and shaking.

Solutions of 29, 58, 87, or 116 μM Cu(II)$_2$(3,5-DIPS)$_4$ in 29 μM HSA were prepared by stirring 4.2, 8.4, 12.6, or 16.8 mg of solid (Cu(II)$_2$(3,5-DIPS)$_4$($H_2O$)$_2$ in 4 mL of dialyzed 1 mM HSA, diluted 36-fold with pH 7.5 deionized water, and stored at refrigerator temperature in capped sterile polypropylene culture tubes. A glass capillary tube was used to break up particules of Cu(II)$_2$(3,5-DIPS)$_4$ to affect solution. Increasing the concentration of Cu(II)$_2$(3,5-DIPS)$_4$ increased the duration of vortex stirring required to obtain solutions. The two highest concentrations may have been solution/suspensions. These solution/suspensions actually represent the upper limits of Cu(II)$_2$(3,5-DIPS)$_4$ "solution" in 1 mM HSA.

Five milliliters of a solution to be dialyzed was placed in prepared dialysis tubing and the tied tubing placed in a 250-mL Erlenmeyer flask containing 250 mL of deionized (pH 7.5) water. This flask was closed with parafilm and capped with thick aluminum foil. The capped flasks were then agitated with a Thomas Shaking Apparatus for 25 hr at 3.5°. These Erlenmeyer flasks had been scrupulously cleaned by scrubbing with Citronox (Alconox, Inc.) and thoroughly rinsed with deionized water before use.

The rate of cytochrome *c* reduction by superoxide produced with the xanthine/xanthine oxidase system was determined by measuring the initial time-dependent increase in absorbance of reduced cytochrome *c* at 550 nm minus background absorbance at 540 nm.

The cuvette reaction mixture was composed of 3 mL of 1% gelatin in 0.05 M phosphate buffer saturated with sodium xanthanate (pH 7.8), 20 μL of catalase solution, 150 μL of 1 mM cytochrome *c*, and 100 μL of test solution, which gave final concentrations of 0.8, 1.6, 2.4, or 3.2 μM Cu(II)$_2$(3,5-DIPS)$_4$ in 0.8 μM HSA, and 100 μL of xanthine oxidase which was used to initiate the reaction. Test

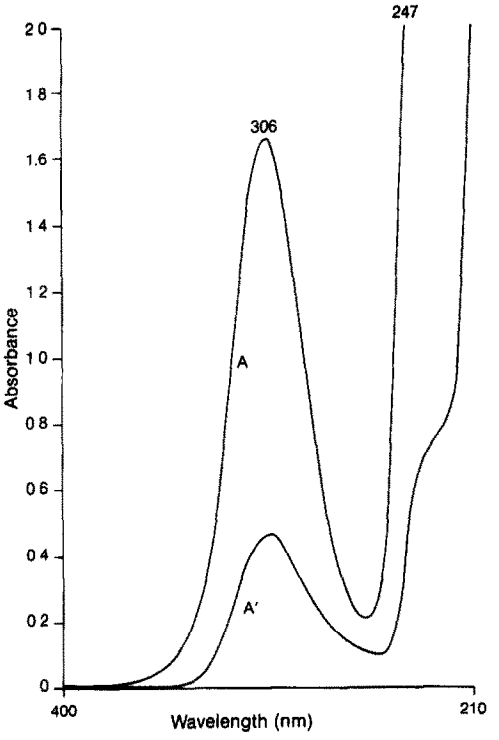



Fig. 1. Spectra showing absorbances at 306 and 247 nm for non-dialyzed (A) and dialyzed (A') solutions of 108 $\mu$M $Cu(II)_2(3,5\text{-}DIPS)_4$ in $H_2O$ (pH 7.5).

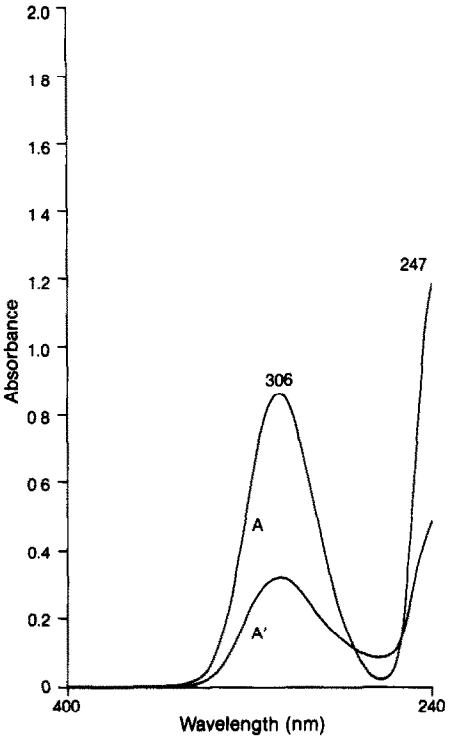



Fig. 2. Spectra showing absorbances at 306 and 247 nm for non-dialyzed (A) and dialyzed (A') solutions of 216 $\mu$M 3,5-DIPS in $H_2O$ (pH 7.5).

solutions prepared with solid $Cu(II)_2(3,5\text{-}DIPS)_4$ contained 1.2, 2.1, 2.7, or 3.3 $\mu$M $Cu(II)_2(3,5\text{-}DIPS)_4$ in 0.89 $\mu$M HSA.

Percent inhibition of cytochrome *c* reduction was calculated based upon the initial rate of reduction observed for 1 min following reaction initiation with the addition of 100 $\mu$L of phosphate buffer (0% inhibition-control) or test solution to the reaction mixture using the formula: initial control solution (without SOD-mimetic) rate minus the initial test solution (with SOD-mimetic) rate divided by initial control rate multiplied by 100 = % inhibition of the initial rate of reduction. Increases in measured absorbance were linear over the 5–10 min period of measurement. Percent inhibition versus concentration of $Cu(II)_2(3,5\text{-}DIPS)_4$ in the test solution was plotted to obtain the concentration producing 50% inhibition of cytochrome *c* reduction ($IC_{50}$).

## RESULTS

Spectra obtained for $Cu(II)(3,5\text{-}DIPS)_2/Cu(II)_2(3,5\text{-}DIPS)_4$ (Fig. 1) and 3,5-DIPS (Fig. 2) contained a maximum at 306 nm for aromatic $\pi$ to $\pi^*$ transitions and a shoulder at 242 nm and maximum at 210 nm for the respective carbonyl *n* to $\pi^*$ and $\pi$ to $\pi^*$ transitions. Ligand to copper charge transfer transitions and *d* to *d* transitions for $Cu(II)(3,5\text{-}DIPS)_2/Cu(II)_2(3,5\text{-}DIPS)_4$ are weak and were not observed for concentrations of this complex used in these studies. The spectrum of 54 $\mu$M HSA (Fig. 3)

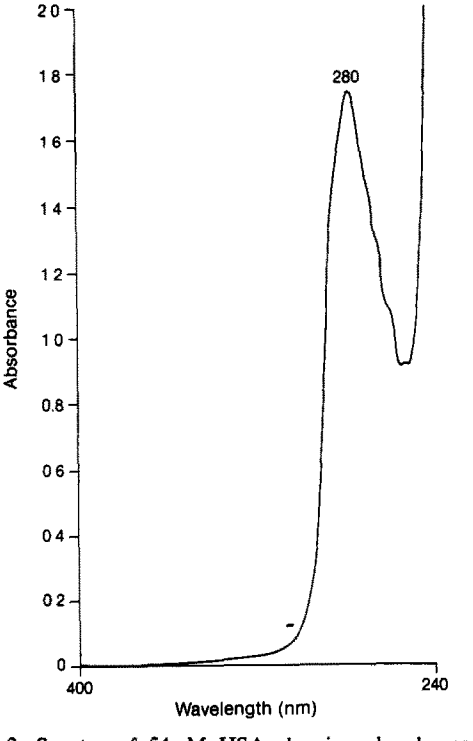



Fig. 3. Spectra of 54 $\mu$M HSA showing absorbance at 280 nm.

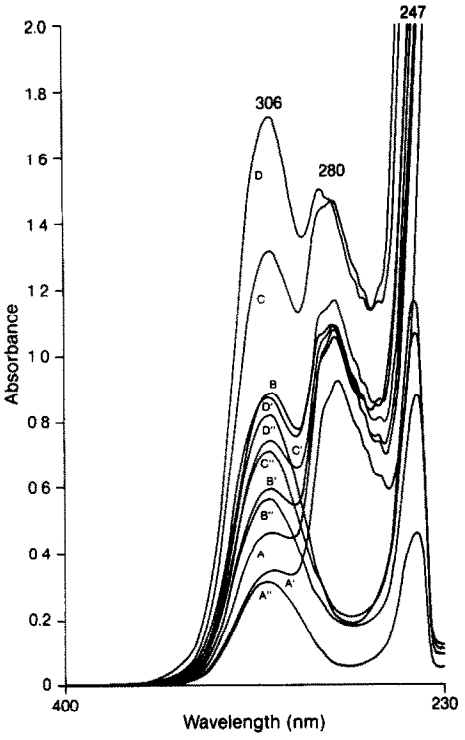



Fig. 4. Spectra of non-dialyzed solutions of 27 $\mu$M (A), 54 $\mu$M (B), 81 $\mu$M (C), and 108 $\mu$M (D) Cu(II)$_2$(3,5-DIPS)$_4$ in 27 $\mu$M HSA. Spectra of dialyzed solutions of 27 $\mu$M (A'), 54 $\mu$M (B'), 81 $\mu$M (C') and 108 $\mu$M (D') Cu(II)$_2$(3,5-DIPS)$_4$ in 27 $\mu$M HSA. Difference spectra of dialyzed solutions of 27 $\mu$M (A''), 54 $\mu$M (B''), 81 $\mu$M (C''), and 108 $\mu$M (D'') Cu(II)$_2$(3,5-DIPS)$_4$ in 27 $\mu$M HSA, obtained with 27 $\mu$M HSA in the reference cuvette.

had a maximum at 280 nm assigned to peptidyl aromatic amino acid, principally tyrosine, $\pi$ to $\pi^*$ transitions, and a second maximum between 250 nm and 200 nm assigned to carbonyl transitions.

Absorbances of aqueous solutions of Cu(II)(3,5-DIPS)$_2$/Cu(II)$_2$(3,5-DIPS)$_4$, 3,5-DIPS, and HSA were linear throughout the concentration ranges used in these studies. Spectral data obtained for solution mixtures of Cu(II)(3,5-DIPS)$_2$/Cu(II)$_2$(3,5-DIPS)$_4$ and HSA (Fig. 4 and Table 1) or 3,5-DIPS and HSA (Table 2) revealed no new unique absorbance attributable to a salicylate aromatic ring pertubation and were essentially composite spectra.

Difference spectra obtained with a solution of Cu(II)(3,5-DIPS)$_2$/Cu(II)$_2$(3,5-DIPS)$_4$ or 3,5-DIPS in the reference cell contained the 280 nm maximum for HSA with a cut-off of the carbonyl transition region. Difference spectra obtained with a solution of HSA in the reference cell revealed maxima at 247 nm (Fig. 4 and Tables 1 and 2); however, this is a salicylate carbonyl absorbance and the sharpness of this maximum and the complete elimination of this maximum with increasing concentration of HSA are consistent with a partial to complete cut-off of the carbonyl transition region by increasing concentrations of HSA. Consequently, this "new maximum" is an artifact and cannot be attributed to a new absorbance assignable to ternary complex formation.

Spectrophotometric evidence consistent with the presence of ternary complexes was obtained following dialysis. Comparison of absorbances in pre-dialysis and post-dialysis spectra obtained for 27, 54, 81, and 108 $\mu$M solutions of Cu(II)$_2$(3,5-DIPS)$_4$ in 27 $\mu$M HSA (Fig. 4 and Table 3) support the possibility that

Table 1. Absorbances for aqueous solutions of Cu(II)$_2$(3,5-DIPS)$_4$ and difference spectra obtained with HSA in the reference cuvette

| Concentration of Cu(II)$_2$(3,5-DIPS)$_4$ ($\mu$M) | Concentration of HSA ($\mu$M) | Absorbances | | Absorbances for difference spectra | |
|---|---|---|---|---|---|
| | | 306 nm | 280 nm | 306 nm | 247 nm |
| 27 | 27 | 0.46 | 1.07 | 0.43 | 0.63 |
| 54 | 27 | 0.89 | 1.17 | 0.87 | 1.11 |
| 81 | 27 | 1.35 | 1.47 | 1.26 | 1.40 |
| 108 | 27 | 1.73 | 1.48 | 1.91 | 1.56 |
| 54 | 54 | 0.95 | ⩾2.00 | 0.86 | 0.00 |
| 54 | 108 | 0.96 | ⩾2.00 | 0.90 | 0.00 |
| 54 | 162 | 1.16 | >2.00 | 0.92 | 0.00 |
| 54 | 216 | 1.26 | >2.00 | 0.93 | 0.00 |
| 54 | 270 | 1.36 | >2.00 | 0.93 | 0.00 |

Table 2. Absorbances for aqueous solutions of 3,5-DIPS and HSA

| Concentration of 3,5-DIPS ($\mu$M) | Concentration of HSA ($\mu$M) | Absorbances | | Absorbances for difference spectra | |
|---|---|---|---|---|---|
| | | 306 nm | 280 nm | 306 nm | 247 nm |
| 54 | 54 | 0.28 | 1.66 | 0.20 | <0.10 |
| 108 | 54 | 0.46 | 1.88 | 0.41 | 0.36 |
| 162 | 54 | 0.53 | >2.00 | 0.52 | 0.39 |
| 216 | 54 | 0.96 | >2.12 | 0.80 | 0.54 |

Table 3. Decrease in absorbance at 306 and 280 nm following dialysis of the HSA, HSA–$Cu(II)_2(3,5\text{-DIPS})_4/Cu(II)(3,5\text{-DIPS})_2$, $Cu(II)_2(3,5\text{-DIPS})_4/Cu(II)(3,5\text{-DIPS})_2$, or HSA-3,5-DIPS and 3,5-DIPS solutions

| Concentration ($\mu$M) | Concentration of HSA ($\mu$M) | % Decrease of 306 nm absorbance | % Decrease of 280 nm absorbance |
|---|---|---|---|
| $Cu(II)_2(3,5\text{-DIPS})_4$ | | | |
| 0 | 54 | 0 | 0 |
| 27 | 27 | 22 | 13 |
| 54 | 27 | 33 | 9 |
| 81 | 27 | 44 | 25 |
| 108 | 27 | 49 | 26 |
| 54 | 27 | 33 | 9 |
| 54 | 54 | 20 | * |
| 54 | 108 | 8 | * |
| 108 | 0 | 72 | 65 |
| 3,5-DIPS | | | |
| 216 | 54 | 23 | 8 |
| 216 | 0 | 62 | 29 |

* Pre- and post-dialysis absorbances were greater than 2 and the percent decrease could not be calculated.

$Cu(II)(3,5\text{-DIPS})_2$ or $Cu(II)_2(3,5\text{-DIPS})_4$ formed stable ternary complexes with HSA. As anticipated, the 280 nm absorbance for a solution containing only HSA did not change on dialysis while solutions containing only $Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ or 3,5-DIPS, without HSA, lost 72 or 62%, respectively, of their absorbance at 306 nm during the period of dialysis. The loss of 22–49% of the 306 nm absorbance with dialysis of HSA–$Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ solutions, wherein the concentration of $Cu(II)_2(3,5\text{-DIPS})_4$ was increased from 27 to 108 $\mu$M and the concentration of HSA was held constant at 27 $\mu$M, was attributed principally to the adsorption of ternary complex to the inside of the dialysis tubing, which is consistent with the loss of copper with dialysis (*vide infra*). There may also have been some undetected precipitation of the ternary complex with the addition of 3 and 4 equivalents of $Cu(II)_2(3,5\text{-DIPS})_2$ to HSA. The possibility that some, although it is likely to be a relatively small amount, of the retained 306 nm absorbance was due to ligand exchange released 3,5-DIPS which subsequently bonded to HSA cannot be discounted. In collateral experiments percent losses of the 306 nm absorbance due to dialysis of 29, 58, or 87 $\mu$M $Cu(II)_2(3,5\text{-DIPS})_4$, prepared with solid $Cu(II)_2(3,5\text{-DIPS})_4$, in 29 $\mu$M HSA were 13, 22, or 41%, respectively. Less than 1% of the expected 306 nm absorbance was found in these dialysis solutions. Adsorption of complex to the inner surface of the dialysis tubing in accounting for the loss of the 306 nm absorbance for these ternary complex solutions was also consistent with the corresponding loss of the 280 nm absorbance (Table 2) of the dialyzed solutions. Losses of the 280 nm absorbance for these dialyzed HSA–$Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ solutions were smaller than losses of the 306 nm absorbance due to smaller losses of the HSA component of these solutions.

Holding the concentration of $Cu(II)_2(3,5\text{-DIPS})_4$ addition constant at 54 $\mu$M and increasing the concentration of HSA from 27 to 108 $\mu$M caused a linear decrease in loss of the 306 nm absorbance with increasing concentration of HSA (Table 3). This is consistent with decreased adsorption to the dialysis tubing or precipitation from solution with increasing concentration of HSA.

Increasing the concentration of $Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ and holding the concentration of HSA constant at 27 $\mu$M increased the amount of absorbed complex even though the loss of absorbance at 306 nm with dialysis increased from 22 to 49% (Table 3). Solutions for which the concentration of $Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ was held constant at 54 $\mu$M and the concentration of HSA increased from 27 to 108 $\mu$M increased in 306 nm absorbance, and the percent decrease in this absorbance with dialysis decreased. The relatively small loss of 3,5-DIPS from the solution of 216 $\mu$M 3,5-DIPS and 54 $\mu$M HSA (Table 3) shows that free ligand also bonds with HSA, which is likely to occur at hydrophobic sites or at protonated amino groups through anion exchange.

It is also interesting to note that the most bonding of $Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ to HSA occurred with the addition of equivalent amounts of $Cu(II)_2(3,5\text{-DIPS})_4$ and HSA with 82% retention of the 306 nm absorbance upon dialysis. This result may be most easily accommodated by the formation of approximately equal amounts of mononuclear ternary complexes wherein one ternary complex forms at one site on HSA and contains both 3,5-DIPS ligands while at another site the ternary complex contains only one 3,5-DIPS ligand. The addition of larger quantities of $Cu(II)(3,5\text{-DIPS})_2/Cu(II)_2(3,5\text{-DIPS})_4$ to HSA with increasing absorbance at 306 nm may be accommodated by various combinations of bonding involving these and other possibilities which conceivably include bonding of Cu(II) and 3,5-DIPS at different sites on HSA. However, the latter possibility is unlikely or accounts for only a small fraction of bonded Cu(II) and 3,5-DIPS to HSA since independent carboxyl or amino coordination of Cu(II) or even large ring carboxyl

Table 4. Decrease in copper content following dialysis of the HSA–Cu(II)$_2$(3,5-DIPS)$_4$/Cu(II)(3,5-DIPS)$_2$ solutions

| Concentration of Cu(II)$_2$(3,5-DIPS)$_4$ ($\mu$M) | Concentration of HSA ($\mu$M) | Copper added ($\mu$g/mL) | Copper found ($\mu$g/mL) | % Decrease |
|---|---|---|---|---|
| 0 | 54 | 0.00 | 0.13 | |
| 27 | 27 | 3.43 | 1.68 | 51 |
| 54 | 27 | 6.86 | 3.03 | 56 |
| 81 | 27 | 10.29 | 2.88 | 72 |
| 108 | 27 | 13.72 | 4.05 | 70 |
| 54 | 27 | 6.86 | 3.03 | 56 |
| 54 | 54 | 6.86 | 4.13 | 40 |
| 54 | 108 | 6.86 | 4.44 | 35 |
| 108 | 0 | 13.72 | 0.74 | 95 |

and amino chelate coordination in competition with 3,5-DIPS is not favored due to the much greater salicylate chelate stability. It is hoped that ongoing ESR and X-ray crystallographic studies will help resolve questions as to how much of each type of Cu(II), Cu(II)(3,5-DIPS)$_2$, and Cu(II)$_2$(3,5-DIPS)$_4$ bonding to HSA there is in these solutions.

Data in Table 4 show the copper concentration found in dialyzed solutions. The percent loss of copper with dialysis increased from 50 to 70% with increasing additions of Cu(II)$_2$(3,5-DIPS)$_4$ while the concentration of HSA was held constant at 27 $\mu$M. Increasing the HSA concentration from 27 to 108 $\mu$M increased the retention of copper when the addition of Cu(II)$_2$(3,5-DIPS)$_4$ was held constant at 54 $\mu$M. It is noteworthy that there was a 95% loss of copper from the 108 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$ solution in the absence of HSA. These losses in copper content were also observed when HSA-Cu(II)(3,5-DIPS)$_2$/Cu(II)$_2$(3,5-DIPS)$_4$ solutions were prepared with solid Cu(II)$_2$(3,5-DIPS)$_4$(H$_2$O)$_2$ and dialyzed (Table 5). These losses paralleled losses of 306 nm absorbance. However, they were always less than the apparent loss of copper. Data presented in Table 5 also show a marked loss of copper with dialysis of a 135 $\mu$M solution of Cu(II)$_2$(3,5-DIPS)$_4$ in the absence of HSA. This loss can only be due to adsorption to the dialysis tubing.

A negative matrix interference for the determination of copper in the presence of HSA and losses of copper and the 306 nm absorbance due to storage of these solutions in polypropylene culture tubes were ruled out as causes for these losses. A plausible rationale consistent with results of both studies is that ternary HSA complexes adsorbed to the inner surface of the dialysis tubing and this adsorption was decreased with increasing concentration of HSA.

Large losses of copper complex to the inner surface of dialysis tubing in the absence of HSA and retention of copper with the presence of HSA suggest that hydrogen bonding of the phenolic hydroxyl groups and/or dative coordinate-covalent bonding to Cu(II) of Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$ by this cellulose membrane is overcome by bonding to HSA. As the stronger bonding sites on HSA become saturated, the weaker bonding to cellulose occurs and losses on dialysis increase with increasing concentration of Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$. Larger losses of copper in comparison to losses of 306 nm absorbances suggest that the loss of copper was due to bonding of Cu(II)(3,5-DIPS)$_2$ or Cu(II)$_2$(3,5-DIPS)$_4$ and ternary HSA complexes to the dialysis membrane since a loss of copper must be interpreted as a loss of some complexed form of copper from these solutions. The dialysis tubing did cause an interference in these studies.

Data in Fig. 5 show that the addition of HSA to an aqueous solution of Cu(II)(3,5-DIPS)$_2$/

Table 5. Mass balance of copper associated with dialysis of 29, 58, 87, or 116 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$ in 29 $\mu$M HSA, 135 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$, and HSA

| Calculated Copper content ($\mu$g/mL) | Dialyzed solution ($\mu$g/mL) | Dialysate ($\mu$g/mL) | Total ($\mu$g/mL) | Loss ($\mu$g/mL) | % Loss |
|---|---|---|---|---|---|
| Cu(II)$_2$(3,5-DIPS)$_4$ in HSA | | | | | |
| 3.8 | 2.0 | 0.3 | 2.3 | 1.5 | 39 |
| 7.5 | 3.6 | 0.6 | 4.2 | 3.3 | 44 |
| 11.2 | 4.7 | 0.7 | 5.4 | 5.8 | 52 |
| 15.0 | 5.6 | 1.4 | 7.0 | 8.0 | 53 |
| 135 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$ | | | | | |
| 17.5 | 1.2 | 5.5 | 6.7 | 10.8 | 62 |
| 29 $\mu$M HSA | | | | | |
| 0.0 | 0.1 | 0.0 | 0.1 | 0.0 | 0 |

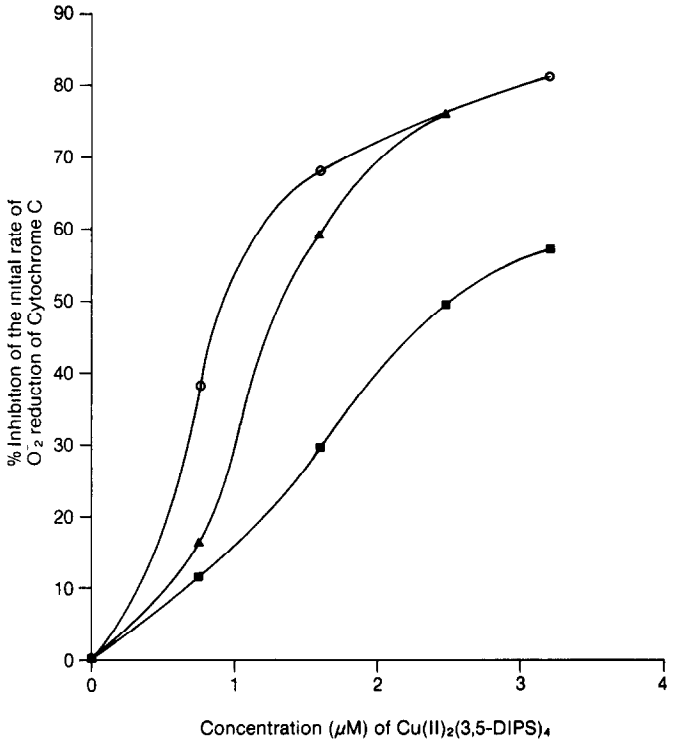



Fig. 5. Plots of SOD-mimetic activity for (○) aqueous solutions of $Cu(II)_2(3,5\text{-DIPS})_4$; (△) non-dialyzed solutions of $Cu(II)_2(3,5\text{-DIPS})_4$ bonded to 0.8 μM HSA; and (■) dialyzed solutions of $Cu(II)_2(3,5\text{-DIPS})_4$ bonded to 0.8 μM HSA.

$Cu(II)_2(3,5\text{-DIPS})_4$ increased the $IC_{50}$ for SOD-mimetic activity from 1 μM (2 μM based on the mononuclear complex) for an aqueous solution of $Cu(II)_2(3,5\text{-DIPS})_4$ to 1.4 μM for the non-dialyzed 0.8 μM HSA solution or to 2.4 μM for the dialyzed 0.8 μM HSA solution. The $IC_{50}$ found for dialyzed HSA solutions prepared with solid $Cu(II)_2(3,5\text{-DIPS})_4$ was essentially the same value, 2.1 μM. Increasing the concentration of HSA in solution decreased superoxide disproportionation, whereas decreasing the concentration of HSA increased superoxide disproportionation. Addition of larger amounts of HSA caused an increase in $IC_{50}$ as a result of the loss of copper-reaction-sites. The 71% increase in $IC_{50}$ for dialyzed solutions versus non-dialyzed solutions was also consistent with the adsorption of ternary complexes to the inner surface of the dialysis tubing and the artifactual loss of copper complexes associated with this procedure.

## DISCUSSION

It is well known that all measurable copper in biological systems exists as complexes. Plasma copper is distributed between ceruloplasmin (9.8 μM), transcuprein (2.1 μM), albumin (2.1 μM), amino acids (1.2 μM) [30], and a calculated ionic fraction ($10^{-12}$ μM) [28]. The amount of ionic copper in tissues is far too small to measure with any existing equipment.

It was not understood why small molecular mass copper complexes with thermodynamic stability constants in a range which would allow ready exchange with ligands in biological systems, with $\beta$ values ranging from $10^{15}$ to $10^{25}$ at pH 7.4, exhibit remarkably potent and varied pharmacological effects. It was plausible that ternary copper–albumin complexes (albumin–$Cu(II)_2(L)_4$, albumin–$Cu(II)L_2$, and/or albumin–Cu(II)L) formed *in vivo* following absorption of the binary complex ($Cu(II)_2(L)_4$ or $Cu(II)L_2$) and that these ternary complexes had a role in accounting for the observed pharmacological effects.

Although it has been long known that HSA contains copper, the suggested speciation of this copper as a ternary complex and its plausible role in copper transport and utilization have been overlooked by some. The formation of a ternary HSA complex was first demonstrated with the addition of HSA to the binary Cu(II)(L-histidinate)$_2$ complex [27]: HSA + Cu(II)(L-histidinate)$_2$ → HSA-Cu(II)L-histidinate + L-histidinate. The HSA–Cu(II)L-histidinate complex was suggested to mediate copper transport. Other ternary HSA–Cu(II) complexes formed with L-threoninate [31], L-cysteinate, and glutathionate [32] have also been suggested to be transport forms of copper and the latter two binary complexes were demonstrated to form ternary complexes with albumin *in vivo* following intravenous injection of $Cu(II)Cl_2$ [33].

Recent studies suggest that the ternary HSA–Cu(II)L-histidinate complex has a role in modulating copper uptake by fibroblasts [32, 34, 35], hepatocytes [36, 37], and neurons [36]. A role for ternary HSA–

Cu(II) amino acid complexes in restricting rapid uptake of copper by the liver and allowing peripheral distribution of copper [35] is consistent with the suggestion that albumin complexes are mobile extracellular storage forms of copper [37]. Translocation of copper may be mediated by other endogenous chelating agents [38] or amino acids [37], L', present in the extracellular matrix: HSA-Cu(II)L + L' → HSA + L'-Cu-L, and the steady-state cellular concentration may depend upon still other intracellular copper bonding ligands which may regulate cellular retention and copper utilization [39].

Receptor activation by medium containing micromolar concentrations of albumin–copper complexes has been suggested to account for cellular uptake of copper by neoplastic cells [40]. Receptor activation by BSA–copper complex and transduction has been used to account for the suppression of murine and human peripheral blood lymphocyte mitogen responsiveness [41]. This mechanistic effect was suggested to be pharmacologically important in accounting for the antiinflammatory activities of copper complexes of non-steroidal antiinflammatory drugs including Cu(II)$_2$(3,5-DIPS)$_4$.

The possibility that ternary HSA complexes of Cu(II)(3,5-DIPS)$_2$ may form *in vivo* and account for an increase in thermodynamic stability as well as its various pharmacological effects led to the present *in vitro* study. Results of this study suggest that thermodynamically stable ternary HSA–Cu(II)$_2$(3,5-DIPS)$_4$, HSA–Cu(II)(3,5-DIPS)$_2$ and/or HSA–Cu(II)(3,5-DIPS) complexes are formed when aqueous solutions of HSA and solid Cu(II)$_2$(3,5-DIPS)$_4$, or an aqueous solution of Cu(II)$_2$(3,5-DIPS)$_4$/Cu(II)(3,5-DIPS)$_2$ are combined at pH 7.5.

Our observation of ternary HSA–Cu(II)(3,5-DIPS)$_2$/HSA–Cu(II)(3,5-DIPS) complex formation *in vitro* is consistent with reports of *in vitro* as well as *in vivo* formation of ternary albumin complexes formed with copper complexes of other non-steroidal antiinflammatory agents: Cu(II)$_2$(indomethacinate)$_4$ [42], Cu(II)$_2$(aspirinate)$_4$ [43], Cu(II)$_2$(lonazolacate)$_4$ [44], and Cu(II)(salicylate)$_2$ [45] as well as other potential antiinflammatory copper complexes, Cu(II)(PuPy$_2$) and Cu(II)(PuIm$_2$) [45]. These ternary complexes formed following interaction with whole blood, serum, or albumin. In retrospect, the copper complex of indomethacin isolated following treatment of an animal model of inflammation with indomethacin [42] was a ternary albumin or other whole blood protein complex [45].

It is well known that small molecular mass copper complexes including Cu(II)$_2$(3,5-DIPS)$_4$ have SOD-mimetic activity (see citations in Ref. 46). The $IC_{50}$ for Cu(II)$_2$(3,5-DIPS)$_4$/Cu(II)(3,5-DIPS)$_2$ has been reported to be of the order of 1–3 $\mu$M and the rate of superoxide disproportionation is the same diffusion-limited rate found for $Cu_2Zn_2SOD$, $10^9\ M^{-1}\ sec^{-1}$ [17]. Disproportionation of superoxide by small molecular mass copper complexes depends upon the interaction of superoxide with an available copper-reaction-site. Assertions that Cu(II) bonds avidly to many proteins and SOD-mimetic Cu(II) complexes such as Cu(II)$_2$(3,5-DIPS)$_4$ are unlikely to retain SOD-mimetic activity *in vivo* in the presence of the mixture of proteins in blood plasma or within cells or *in vitro* [47–50], which may have been misleading [49, 51, 52], have also been examined in the present studies.

Our data showing that ternary HSA complexes of Cu(II)$_2$(3,,5-DIPS)$_4$/Cu(II)(3,5-DIPS)$_2$, HSA–Cu(II)$_2$(3,5-DIPS)$_4$/HSA–Cu(II)(3,5-DIPS)$_2$/HSA–Cu(II)(3,5-DIPS), have SOD-mimetic activity are consistent with reports that the SOD-mimetic $IC_{50}$ for Cu(II)$_2$(3,5-DIPS)$_4$ was not altered by the presence of 15 $\mu$M BSA and less than 15% inhibition of mimetic activity was observed for a mixture containing 50 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$ and 150 $\mu$M BSA [53]. Our data are also consistent with the report that a 150 $\mu$M concentration of calf serum was necessary to cause a 95% loss of the SOD-mimetic activity of a 10 $\mu$M solution of Cu(II)$_2$(3,5-DIPS)$_4$ [54]. Further support comes from the observation that concentrations of 50–400 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$ in calf serum, which contains 500–600 $\mu$M albumin, were more effective in disproportionating superoxide than the maximally effective concentration of $Cu_2Zn_2SOD$ [55]. Effective SOD-mimetic activity has also been reported for Cu(II)(salicylate)$_2$ and Cu(II)$_2$(3,5-DIPS)$_4$ in BSA [31, 48], Cu(II)(salicylate)$_2$, Cu(II)$_2$(indomethacin)$_4$, and Cu(II)$_2$(lonazolacate)$_4$ in BSA or human serum [44], and Cu(II)(serinate)$_2$, Cu(II)(salicylate)$_2$, Cu(II)(PuPy$_2$), and Cu(II)(PuIm$_2$) in BSA or whole human blood [45]. In all of these reports it was found that increasing the amount of serum protein decreased SOD-mimetic activity. It is most likely that this decrease in SOD-mimetic activity is due to a decrease in the number of collisions at copper-reaction-sites, equatorial bonding positions, in these ternary copper complexes. These bonding interactions between albumin or other blood proteins and copper no doubt account for total blockade in the event that bonding by albumin or other blood proteins occupies all of the remaining reaction sites on copper of Cu(II)$_2$(L)$_4$ or Cu(II)(L)$_2$. This situation, however, would markedly increase the apparent *in vivo* stability of the binary complex, which would allow tissue distribution and dissociation of the binary complex, i.e. HSA–Cu(II)(3,5-DIPS)$_2$ → HSA + Cu(II)(3,5-DIPS)$_2$, in the extracellular matrix of affected tissues and subsequent cellular translocation of Cu(II)(3,5-DIPS)$_2$ in accounting for the remarkable pharmacological effects of these complexes.

Our studies emloyed copper-free HSA which had no SOD-mimetic activity. This copper-free form of albumin would not serve as an effective extracellular antioxidant. However, it is likely that ternary HSA–Cu(II) complexes would be excellent extracellular antioxidants [56]. In support of this suggestion, it has been found that a whole feline plasma solution of Cu(II)$_2$(3,5-DIPS)$_4$ is as effective as $Cu_2Zn_2SOD$ in preventing feline mesenteric capillary ischemia-reperfusion injury at a dose of 25 $\mu$mol Cu(II)$_2$(3,5-DIPS)$_4$/kg [19].

In addition to disproportionating superoxide, copper complexes also disproportionate hydroperoxyl radical and hydrogen peroxide [57, 58]. Both catalase-mimetic and peroxidase-mimetic activities are known for copper complexes [58]. In addition

to disproportionating superoxide in the presence of albumin, ternary BSA–Cu(II)$_2$(3,5-DIPS)$_4$/BSA–Cu(II)(3,5-DIPS)$_2$/BSA–Cu(II)(3,5-DIPS) complexes produced in 15 $\mu$M BSA actually cause a significant increase in the rate of hydrogen peroxide disproportionation in comparison to the rate of disproportionation with Cu(II)$_2$(3,5-DIPS)$_4$/Cu(II)(3,5-DIPS)$_2$ alone [53], which can be interpreted as activation by bonding at only one equatorial position. In 150 $\mu$M albumin this reaction was inhibited by 50% [53] consistent with the reduction in reactive-copper-sites by the additional BSA bonding. In addition to this catalase-mimetic activity, Cu(II)$_2$(3,5-DIPS)$_4$ also has peroxidase-mimetic activity. The $V_i$ for a 25 $\mu$M Cu(II)$_2$(3,5-DIPS)$_4$ solution was 85 $\mu$M $H_2O_2$ min$^{-1}$. The addition of 15 $\mu$M BSA slightly reduced this peroxidase-mimetic activity to 74 $\mu$M $H_2O_2$ min$^{-1}$ and addition of 150 $\mu$M BSA markedly reduced this activity to 3 $\mu$M $H_2O_2$ min$^{-1}$, again consistent with a reduction in reactive sites by protein bonding.

Finally, copper complexes also reduce hydroxyl radical to hydroxide [57], and they may convert singlet state oxygen to triplet state oxygen [59].

Concomitant superoxide and hydrogen peroxide disproportionation, SOD-mimetic, and catalase-mimetic activities may account for the reduction in chemiluminescence (CL) found for a xanthine/xanthine oxidase/luminol system used to study the interaction of BSA with a complex mixture of Cu(II)$SO_4$, histidine, and salicyclic acid [60]. These chemical reactivities of copper complexes may also account for the reduction of CL in phorbol ester activated porcine and human polymorphonuclear leukocytes (PMNLs)/lucigenin system with ternary BSA complexes of Cu(II)(serinate)$_2$, Cu(II)(salicylate)$_2$, Cu(II)(thiocin), Cu(II)(PuIm$_2$), and Cu(II)(PuPy$_2$) [45]. A 0.1 $\mu$M solution of the complex mixture caused a 45% reduction in CL whereas a 1.0 $\mu$M solution of this mixture containing 20 $\mu$M bovine albumin produced 50% inhibition of CL. The $IC_{50}$ values for Cu(II)$SO_4$ and the above complexes in the PMNL/lucigenin system were essentially unaffected by the addition of 3 $\mu$M BSA. However, their $IC_{50}$ values increased up to 110 $\mu$M with the addition of up to 600 $\mu$M BSA and the order of efficient inhibition of CL was Cu(II)(PuPy$_2$) > Cu(II)(PuIm$_2$) > Cu(II)(thiocin) > Cu(II)(salicylate)$_2$ > Cu(II)(serinate)$_2$ > Cu(II)$SO_4$. It was also pointed out that the activity of Cu(II)$SO_4$ was not due to aquated ionic copper since its concentration would be less than $10^{-15}$ M in the presence of BSA and $10^{-8}$ M aquated Cu(II), which actually formed a complex with the buffer, caused only 5% inhibition of CL.

It is plausible that ternary HSA–Cu(II)$_2$(L)$_4$, HSA–Cu(II)(L)$_2$, or HSA–Cu(II)L complexes have roles in tissue distribution to all extracellular fluids. In these fluids these ternary complexes may be in equilibrium with small molecular mass complexes produced by endogenous ligands (L'): i.e. HSA–Cu(II)L + L' $\rightarrow$ HSA + Cu(II)LL', and these small molecular mass complexes either activate receptors on the extracellular surfaces of cells or undergo translocation and cellular utilization. Cellular utilization may involve either facilitation of *de novo* synthesis of copper-dependent enzymes or activation of inactive apoenzyme, e.g. *de novo* synthesis of $Cu_2Zn_2SOD$ or activation of the second subunit of $CuZn_2SOD$ which may not be copper-activated under normal cellular metabolic circumstances. Conversion of $CuZn_2SOD$ to $Cu_2Zn_2SOD$ as a result of ligand exchange may only be required under conditions of cellular stress associated with particular cells in particular disease states. This suggestion is consistent with observations that the copper-dependent SOD concentration and/or activity are known to be less than normal in chronic arthritic diseases, cancers, diabetes, and seizures which may also pertain to other acute and chronic diseases including gastrointestinal ulcers, stroke, myocardial infarction, cardiovascular disease, kidney diesease, other ischemia-reperfusion injuries, and infections (see specific disease states in Ref. 46). The above suggestion is also consistent with a marked elevation in plasma copper-containing components that occurs as a physiological response to these disease states [46] which may facilitate remission when this response and all others are sufficient. All of this supports the notion that pharmacological uses of small molecular mass copper complexes offer a physiological approach to preventing and/or treating acute and chronic diseases. It is also concluded that ternary albumin–copper complexes are useful formulations for intraarterial or intravenous administration in preventing or treating ischemia-reperfusion injury and organ preservation.

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