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^{1}H NMR STUDIES OF REACTIONS OF COPPER COMPLEXES WITH HUMAN BLOOD PLASMA AND URINE

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Abstract—Reactions of the copper complexes $Cu(II)Cl_2$, $[Cu(II)(EDTA)]^{2-}$, $[Cu(II)_2(DIPS)_4]$ and $[Cu(I)(DMP)_2]^+$ (where DIPS is 3,5-diisopropylsalicylate and DMP is 2,9-dimethylphenanthroline) with human blood plasma and urine have been studied by 500 MHz ^{1}H NMR spectroscopy, and CD spectroscopy has been used to monitor the transfer of Cu(II) onto albumin in plasma. The rate of transfer of Cu(II) from $[Cu(II)(EDTA)]^{2-}$ onto albumin as measured by CD ($T_{1/2}$ 26 min, 0.5 mM Cu, 21°), was similar to the rate of Cu(II) binding to amino acids and citrate, and to the rate of formation of $[Ca(II)(EDTA)]^{2-}$ in plasma. Reactions of $Cu(II)Cl_2$ and $[Cu(II)_2(DIPS)_4]$ in plasma followed a similar course, but were more rapid. The latter complex also appeared to give rise to the displacement of lactate from protein binding. Reactions of copper complexes in plasma therefore involve a range of low M_r ligands as well as albumin, and the ligands play a major role in determining the kinetics of the reactions. These factors, as well as the partitioning of both complexes and displaced ligands into lipoproteins, are likely to play important roles in the molecular pharmacology of copper-containing drugs. In urine, His and formate were involved in EDTA and DIPS displacement from their respective copper complexes, and peaks for free DIPS and $[Ca(II)(EDTA)]^{2-}$ were observed. The complex $(Cu(I)(DMP)_2]^+$ appeared to be relatively stable in both plasma and urine.

Despite the known importance of the essential element copper in biochemical processes, only a few copper compounds are currently used as drugs: copper salts such as $Cu(II)CO_3$ are constituents of mineral supplements, and Cu(II)–amino acid complexes and Cu(II)EDTA have been used to treat Menke's kinky hair syndrome [1–3], a disease in which the serum copper levels are low.

Although ligand dissociation and exchange processes can occur readily, it is clear that the biological activity of copper complexes depends strongly on the nature of the ligands and on the oxidation state of copper, Cu(I) or Cu(II). For example, only certain copper complexes exhibit significant anticancer activity. These include [Cu(II)(KTS)],† which is thought to undergo intracellular reduction with release of the KTS ligand [4], and $[Cu_2(DIPS)_4]$, a lipophilic complex which also has radioprotectant, anticonvulsant and antiarthritic properties [5]. The stable lipophilic Cu(I) complex $[Cu(DMP)_2]NO_3$ has antimycoplasmal activity [6] and is also cytotoxic, but incorporation of sulphonyl groups on the periphery of the DMP rings, with consequent increase in hydrophilicity, reduces the activity [7, 8]. In addition, the cytotoxicities of several organic agents such as bis(diphenylphosphino)ethane and 4′-(9-acridinylamino)methanesulphon-*m*-anisidide are considerably enhanced by the presence of copper [9, 10], which may also play a direct role in the activity of many other organic drugs such as antibiotics [11].

In blood plasma, most of the endogenous copper is strongly bound to caeruloplasmin, probably as a trinuclear Cu(II) cluster, together with three intensely blue type-I copper centres [12]. These bound copper ions appear to be kinetically inert under normal conditions. Another strong protein binding site for Cu(II) is thought to be at the N-terminus of albumin: Asp-Ala-His-, which provides four N ligands: the terminal amino group, two deprotonated peptide nitrogens and a His imidazole N [13]. Copper(II) in this site may also form ternary complexes with small ligands such as histidine, and endogenous ligands in plasma may play a role in controlling the kinetics of copper uptake and release by albumin [14, 15]. However, the significance of this site in copper metabolism is not understood, and is the subject of debate [16].

Calculations have suggested that about 5–10% of total serum copper (*ca.* 17 μM) is present in a "non-caeruloplasmin pool" which is likely to be more reactive [17, 18]. However, recent assays based on the degradation of DNA by phenanthroline-chelatable copper suggest that this pool is much smaller (<0.6% of the total copper), except in Wilson's disease [19]. The control of serum copper levels is important; copper(II)-induced oxidation of low density lipoproteins (LDL) *in vitro* can modify their recognition by receptors [20]. Calculations of the distribution of copper in biofluids are often complicated by the lack of knowledge of all the appropriate stability constants, and, in addition, predictions of the kinetics of reactions may not be possible, especially since many fluids are inhomogeneous. Therefore there is

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† Abbreviations: KTS, 3-ethoxyl-2-oxobutyraldehyde-bisthiosemicarbazone; DMP, 2,9-dimethyl-1,10-phenanthroline; DIPS, 3,5-diisopropylsalicylate; HSA, human serum albumin; LDL, low density lipoproteins; pH*, pH meter reading in D_2O; T_2, spin–spin relaxation time; TSP; sodium trimethylsilyl-d_4-propionate; DMSO, dimethyl sulphoxide.

a need for experimental investigations of the interaction of copper complexes with blood plasma and other biological fluids.

In this work we explore the use of proton NMR spectroscopy to study the interactions of lipophilic and hydrophilic complexes of Cu(II) and Cu(I): $Cu(II)Cl_2$, $[Cu(II)(EDTA)]^{2-}$, $[Cu(II)_2(DIPS)_4]$ and $[Cu(I)(DMP)_2]^+$ with blood plasma and urine. Cu(II) complexes are usually paramagnetic and the copper-bound ligands give rise to broadened ^1H NMR resonances, whereas free ligands are expected to give sharp peaks since they are diamagnetic. Ligand binding to Cu(I) usually induces small shifts of peaks but little broadening. In this way NMR can be used to follow ligand displacement reactions. We have used CD spectroscopy to complement the NMR data and monitor the transfer of copper onto albumin in plasma [21]. In some cases we have been able to follow both the extent and kinetics of the reactions.

MATERIALS AND METHODS

Materials. $[Cu(H_2EDTA)]\cdot 0.5H_2O$, $[Cu_2(DIPS)_4]$ and $[Cu(DMP)_2]NO_3$ were prepared by standard methods [22–24] and had satisfactory elemental analyses (within ±0.4% of calculated values). $CuCl_2\cdot 2H_2O$ was purchased from BDH (Poole, U.K.), amino acids and human serum albumin (essentially fatty acid free, A3782) were obtained from the Sigma Chemical Co. (Poole, U.K.).

Venous blood from healthy volunteers was collected in vials containing lithium heparin. The cells were removed by centrifiugation and the plasma was always used immediately for spectroscopic experiments without further storage. Low M_r ultrafiltrates (<10 kDa) were prepared using Amicon Centrifree devices. Urine was obtained after overnight fasts.

NMR measurements. NMR Spectra were recorded at 25° in 5 mm tubes on JEOL GX500 and GX270 spectrometers at 500 and 270 MHz, respectively, and some spectra were also recorded on Bruker WM400 and AM500 instruments at 400 and 500 MHz, respectively. Either single pulse or Hahn spin-echo sequences (90°-τ-180°-τ-collect fid, $\tau = 60$ msec) were used with gated irradiation to suppress the water signal. Typical pulsing conditions for single-pulse spectra were: 40° (3 μsec) pulses, 2.7 sec acquisition time, 2.3 sec pulse delay, 32 k computer points, and 64 transients. Exponential functions equivalent to a line-broadening of 0.5–1 Hz were used for processing. For quantitative work, a concentric capillary containing an external standard of TSP (1.9 mM in D_2O) was employed as a concentration standard, chemical shift reference and lock signal.

Peak assignments are based on standard additions and previous reports of shifts and couplings [25, 26]. Additions of copper complexes were made as microlitre aliquots of fresh stock solutions: $CuCl_2\cdot 2H_2O$ 30 mM in D_2O, $[Cu(H_2EDTA)]\cdot 0.5H_2O$ 35 mM in D_2O adjusted to pH* 7 with NaOD (referred to as $[Cu(EDTA)]^{2-}$), $[Cu_2(DIPS)_4]$ 50 mM in d_6-DMSO, and $[Cu(DMP)_2]NO_3$ 97 mM in d_6-DMSO.

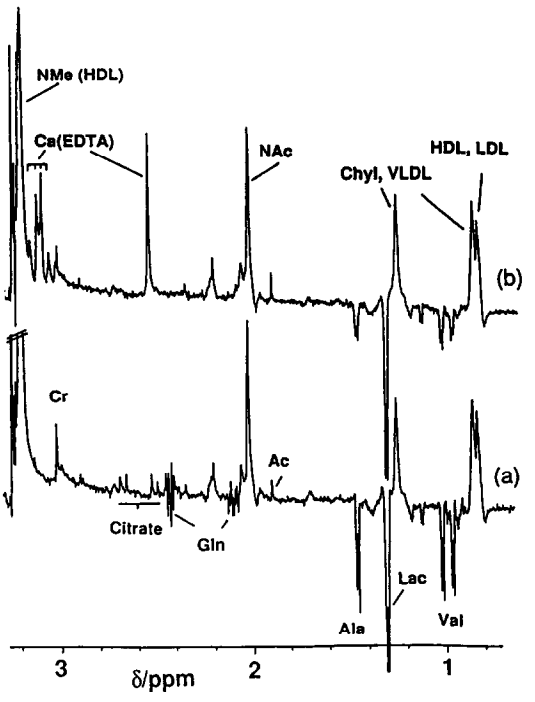

Fig. 1. 500 MHz Hahn spin-echo ($\tau = 60$ msec) ^1H NMR spectra of the aliphatic region of heparinized human blood plasma: (a) control; (b) 28 min after the addition of 1 mM Cu(II)EDTA. Assignments: lipoproteins—Chyl, chylomicrons; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. Val, valine; Lac, lactate; Ala, alanine; Ac, acetate; NAc, *N*-acetyls of glycoproteins; Gln, glutamine; Cr, creatine; NMe, methyls of choline headgroups of HDL phospholipids; Ca(EDTA), $[CaEDTA]^{2-}$.

Measurements of pH were made with a Corning 145 meter equipped with a combination electrode.

RESULTS

Reactions of three Cu(II) complexes $Cu(II)Cl_2$, $[Cu(II)(EDTA)]^{2-}$ and $[Cu(II)_2(DIPS)_4]$ and one Cu(I) complex $[Cu(I)(DMP)_2]^+$ with human blood plasma and urine *in vitro* were studied by ^1H NMR spectroscopy. The transfer of copper onto albumin was also monitored by CD spectroscopy, and for $[Cu(EDTA)]^{2-}$ the time course of the reaction in plasma was followed by NMR and CD spectroscopies.

Plasma

A 500 MHz Hahn spin-echo ^1H NMR spectrum of heparinized human blood plasma is shown in Fig. 1. The spin-echo method filters out broad peaks with associated short T_2 values from high M_r proteins such as albumin, leaving only sharp peaks from mobile small molecules and mobile regions of macromolecules (such as the glycan chains of glycoproteins). No signals from the anticoagulant heparin appear in the spectrum, and, under the conditions used ($\tau = 60$ msec), only weak peaks remain in the aromatic region.

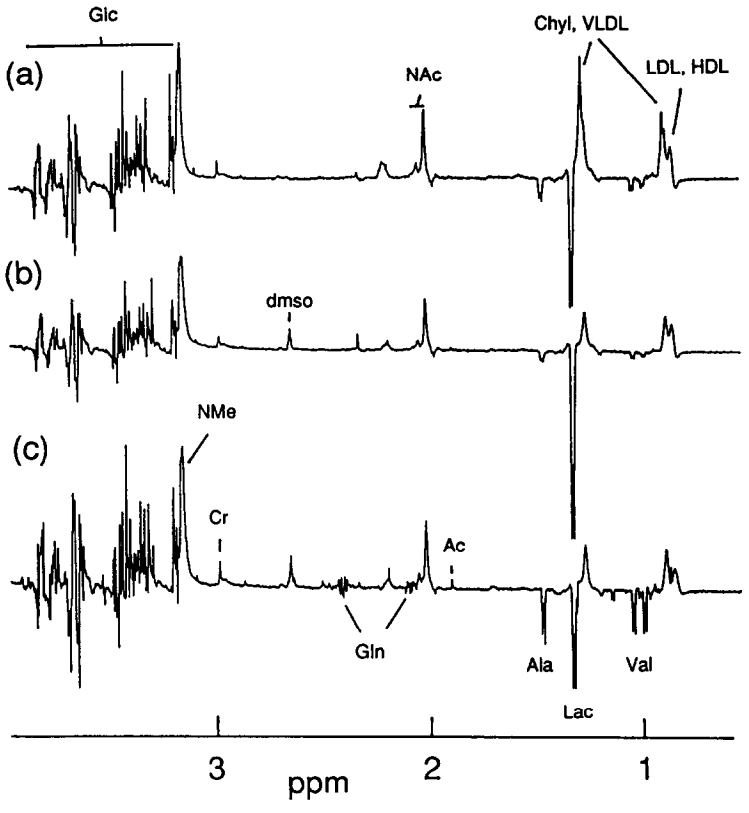

Fig. 2. 500 MHz Hahn spin-echo ($\tau = 60$ msec) ^{1}H NMR spectra of the aliphatic region of heparinized human blood plasma in the presence of (a) 0.75 mM $Cu(II)Cl_2$; (b) 0.5 mM $Cu(II)_2(DIPS)_4$; and (c) 1 mM $[Cu(I)(DMP)_2]NO_3$. Assignments: as Fig. 1, Glc, glucose. The diamagnetic Cu(I) complex has little effect on the spectrum, whereas the paramagnetic Cu(II) complexes decrease the intensities of amino acid peaks (shortening of T_2 values). Note the high intensity of the lactate peak in (b). The control spectra for each sample were similar although not exactly the same (same donor, different days).

Addition of $[Cu(II)(EDTA)]^{2-}$ (0.5–2 mM) leads to a decrease in the intensity of specific resonances (Fig. 1b), notably those for the amino acids Val, Ala and Gln, and for citrate. The other notable change in the spectrum is the appearance of a singlet at 2.56 ppm and an AB quartet at 3.05 ppm assignable to the $—NCH_2—$ and $—CH_2CO_2—$ protons, respectively, of $[Ca(EDTA)]^{2-}$ [27]. The changes in peak intensities were time-dependent and were monitored in detail with 0.5 mM added $[Cu(II)(EDTA)]^{2-}$ (*vide infra*).

Similar decreases in peak intensities were observed after the addition of $Cu(II)Cl_2$ (0.25–1 mM) to blood plasma (Fig. 2), but the effects were rapid and no time dependence was seen.

Similarly, $[Cu(II)_2(DIPS)_4]$ (0.25–1 mM) broadened peaks for Val, Ala, Gln and citrate, but in this case the peaks for lactate in the spin-echo spectrum significantly increased in intensity (Fig. 2b), a feature seen when lactate is released from binding to proteins, for example on addition of ammonium sulphate [28]. With 0.25 mM of added $[Cu(II)_2(DIPS)_4]$ present, the intensity of the Lac methyl peak increased by a factor of *ca.* 2.5 with little change in the Gln, Val and Ala peaks. With 0.5 mM added complex [i.e. 1 mM Cu(II)] significant decreases in intensities of Ala, Val, Gln and citrate peaks were observed, and with 1 mM complex present these peaks were broadened beyond detection, but the lactate peaks remained sharp. No peaks for the free ligand DIPS were observed.

In contrast, the peaks observed for small molecules all remained sharp when $[Cu(I)(DMP)_2]^+$ was added (0.5–2 mM) to plasma (Fig. 2c). No peaks for the free ligand DMP were observed.

CD spectra of plasma samples similar to those used for NMR measurements were recorded, Fig. 3. The CD spectrum of blood plasma is dominated by bands at 400 and 460 nm for bilirubin bound to albumin [21, 29]. A characteristic bisignate pair of bands associated with the 525 nm absorption band of Cu(II)–albumin [30] appeared rapidly on addition of $CuCl_2$ (Fig. 3b), and similar new bands were seen after addition of $[Cu(II)_2(DIPS)_4]$ (Fig. 3c). The intensity of the CD bands for Cu–albumin in the latter spectrum suggested that after addition of 0.25 mM $[Cu(II)_2(DIPS)_4]$ to plasma most of theCu(II) was transferred to albumin.

The transfer of Cu(II) from $[Cu(II)(EDTA)]^{2-}$ (0.5 mM) to albumin in plasma was slow enough to be followed with time by monitoring the CD band at 565 nm (Fig. 4), from which a half-time of 26.4 min

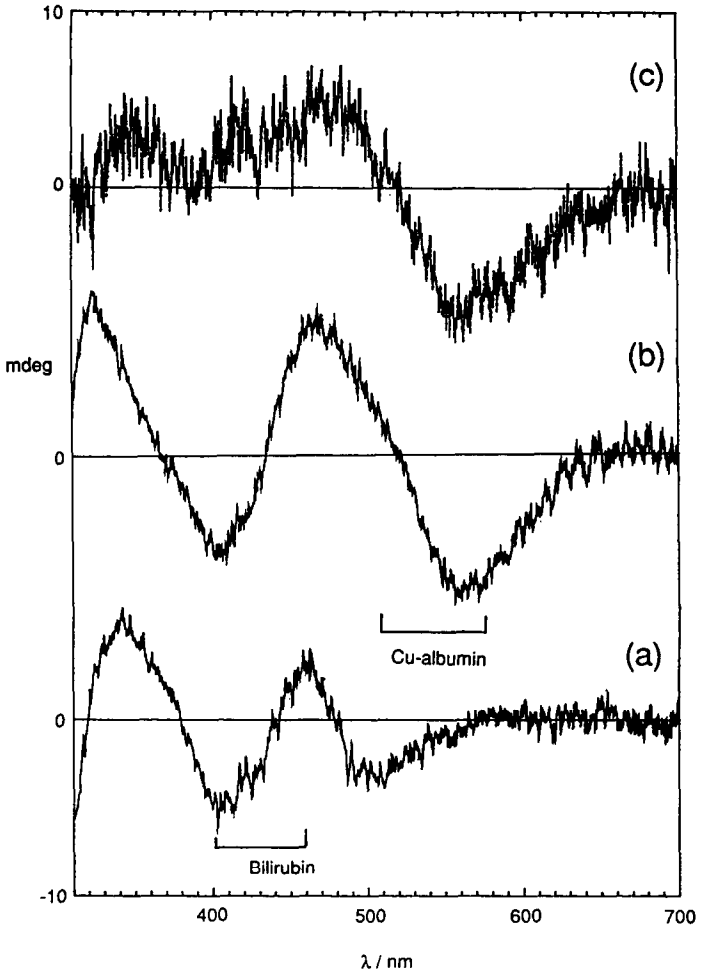

Fig. 3. Circular dichroism spectra of heparinized human blood plasma: (a) control; (b) after addition of $CuCl_2$ (0.5 mM); and (c) after addition of $[Cu(II)_2(DIPS)_4]$ (in DMSO, 0.25 mM) showing the appearance of bands characteristic of Cu(II)–albumin.

was calculated (21°). This can be compared with the half-time for appearance of $[Ca(EDTA)]^{2-}$ in the ^1H NMR spectrum of 20.8 min (25°). At higher concentrations of $[Cu(II)(EDTA)]^{2-}$ the half-time for Cu–albumin formation plateaued: 10.6, 9.8 and 8.7 min at 0.75, 1.0 and 1.25 mM added $[Cu(II)(EDTA)]^{2-}$, respectively. Also shown in Fig. 4 are the time dependencies for the decreases in intensities of the ^1H NMR peaks for Val and Ala; in contrast there is little change in intensity of the Lac peaks. It can be seen that the time courses for the appearance of $[Ca(EDTA)]^{2-}$, formation of Cu–albumin, and broadenings of peaks for Val and Ala are similar.

In order to assess the effect of macromolecules on copper binding, reactions of $[Cu(II)(EDTA)]^{2-}$ with low M_r ultrafiltrates of plasma were studied using single pulse spectra (Fig. 5). In the presence of 0.5 mM $[Cu(II)(EDTA)]^{2-}$ broadenings of peaks for Val, Ala, Gln, citrate, His, Tyr and Phe were observed (Fig. 5b), together with the appearance of peaks for $[Cu(II)(EDTA)]^{2-}$. To this solution, albumin was added (0.5 mM) and peaks for Val, Ala and Gln clearly sharpened again (Fig. 5c). These data were recorded soon (minutes) after the additions were made, and the time-dependence (if any) was not studied.

When $[Cu(II)(EDTA)]^{2-}$ (0.5 mM) was added to a mixture of amino acids, citrate and lactate of similar composition to blood plasma (Ala 0.38 mM, Cys–Cys 0.04 mM, His 0.09 mM, Lac 1.79 mM, citrate 0.11 mM, Val 0.23 mM, pH* 7), peaks for the amino acids broadened but the citrate and lactate peaks remained sharp. No sharp peaks for EDTA were seen suggesting that $[Cu(EDTA)]^{2-}$ was involved in the formation of ternary complexes with amino acids in this solution.

Urine

Addition of low concentrations of $CuCl_2$ caused dramatic effects on certain peaks in ^1H NMR spectra of urine. Single-pulse spectra were recorded since the protein content of urine is low and therefore no spin-echo filtering was necessary. With 0.15 mM

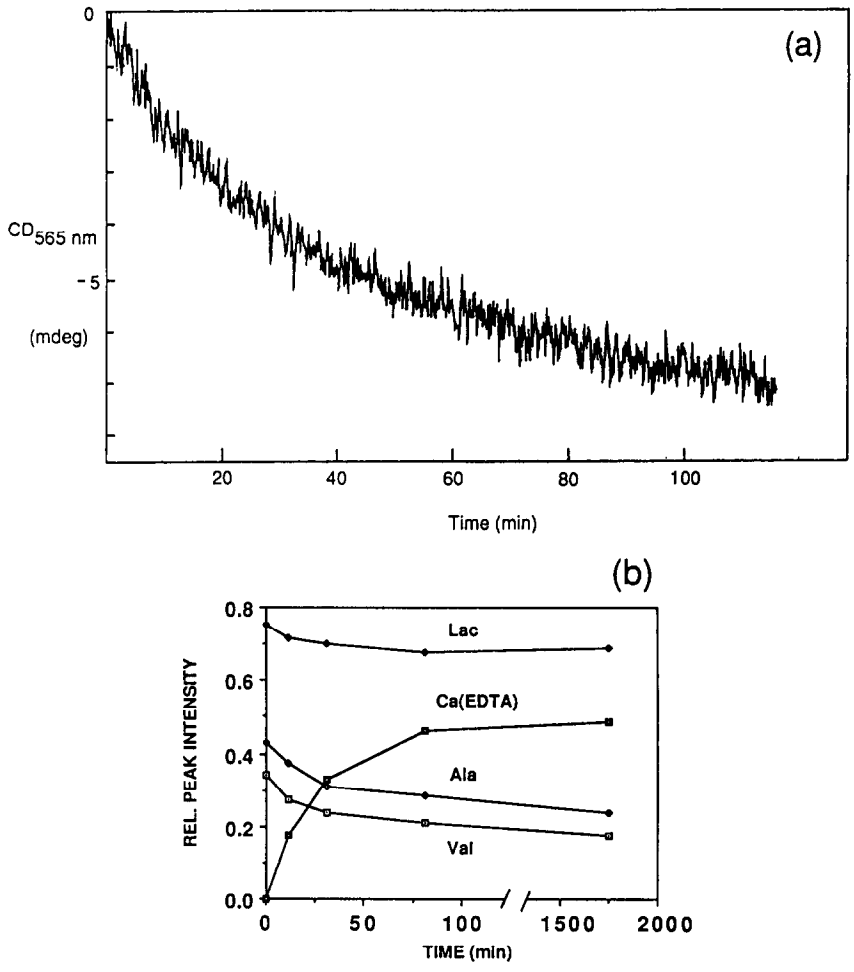

Fig. 4. Plots showing the time-dependence of the reaction of $[Cu(EDTA)]^{2-}$ (0.5 mM) with human blood plasma: (a) the appearance of CD bands for Cu(II)–albumin (monitored at 565 nm, see Fig. 3b); (b) the appearance of ^{1}H NMR peaks for $[Ca(EDTA)]^{2-}$, and decrease in intensity of peaks for Val and Ala (see Fig. 1b).

Cu(II) present, the C2H (8.37 ppm) and C4H (7.24 ppm) peaks of His were severely broadened together with those of formate (8.46 ppm), citrate and an unassigned singlet in the aromatic region at 8.23 ppm. With 0.3 mM of added Cu(II) present, His and citrate peaks were broadened beyond detection. The titration was continued to 0.6 mM Cu(II) and it was notable that peaks for many molecules still remained sharp, e.g. those for hippurate and indoxyl sulphate.

When $[Cu(II)(EDTA)]^{2-}$ was added to urine (0.5, 1.0, 1.5 and 2.0 mM), citrate peaks broadened (as with $CuCl_2$, the low field doublet of the AB quartet broadened more than the high field doublet), and new peaks for $[Ca(II)(EDTA)]^{2-}$ appeared at 2.56 and 3.13 ppm, the former overlapping the high-field doublet of citrate. Again resonances for formate, His and the unassigned peak at 8.23 ppm also broadened. Even at the lowest $[Cu(II)(EDTA)]^{2-}$ concentration studied (0.5 mM), the His peaks were broadened almost beyond detection.

After addition of $[Cu(II)_2(DIPS)_4]$ (0.5, 1.0 and 2.0 mM) to urine, peaks for His, formate, citrate and the unassigned peak at 8.23 ("X" in Fig. 6b) were severely broadened, and new peaks assignable to the ligand DIPS appeared in the aliphatic (1.2 ppm, isopropyl methyls) and aromatic (7.3, 7.6 ppm, 4,6-ring protons) regions (Fig. 6). A plot of the amount of free ligand, measured from the intensity of the isopropyl methyl peak, versus the amount of added Cu(II) suggested that an average of one DIPS ligand per dimer was displaced through reaction with urine under these conditions (Fig. 7).

Detailed studies of the effects of $[Cu(I)(DMP)_2]NO_3$ on spectra of urine were not made since the solubility of the complex was low; no effect was observed with 0.5 mM of the complex (added in DMSO) present.

DISCUSSION

The aim of the present study was to explore the use of ^{1}H NMR spectroscopy for investigation of reactions of Cu(I) and Cu(II) complexes of

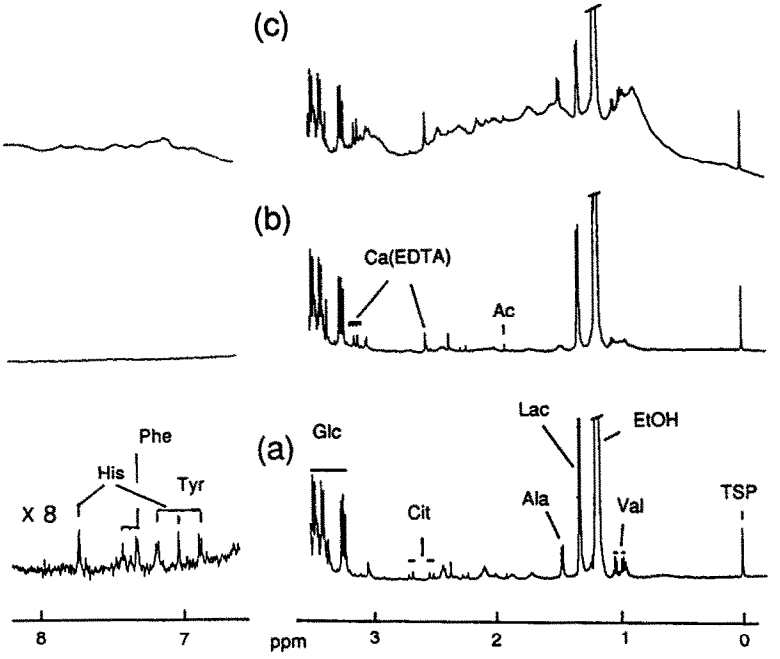

Fig. 5. 500 MHz ^{1}H NMR spectra of the lowM_r fraction (<10 kDa) of human blood plasma: (a) control; (b) in the presence of 0.5 mM Cu(EDTA); and (c) after the further addition of albumin (0.5 mM) to (b). Assignments: as Fig. 1, Cit, citrate EtOH, ethanol impurity from dialysis membranes used for separation; His, histidine (C2H and C4H protons); Tyr, tyrosine; Phe, phenylalanine.

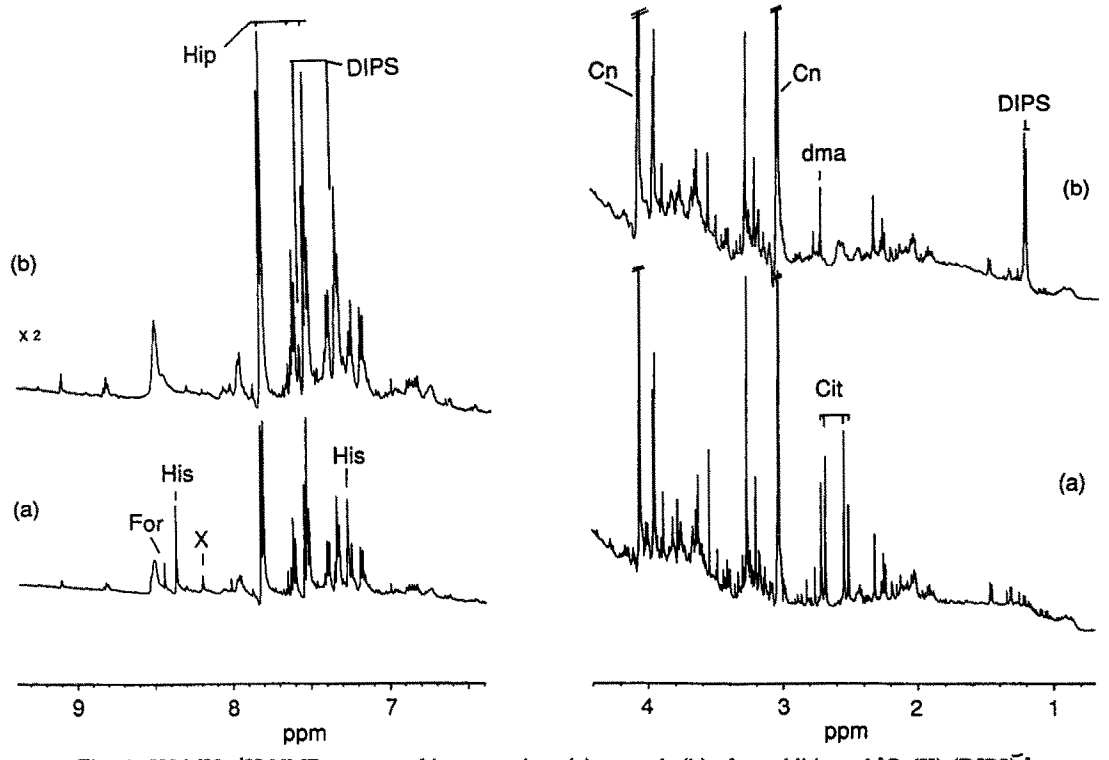

Fig. 6. 500 MHz ^{1}H NMR spectra of human urine: (a) control; (b) after addition of $[Cu(II)_2(DIPS)_4]$ (in DMSO, 0.25 mM). Assignments: as Figs 1–3, and dma, dimethylamine; Hip, hippurate; Cn, creatinine; For, formate; "X", unassigned. Left: aromatic region; right: aliphatic region.

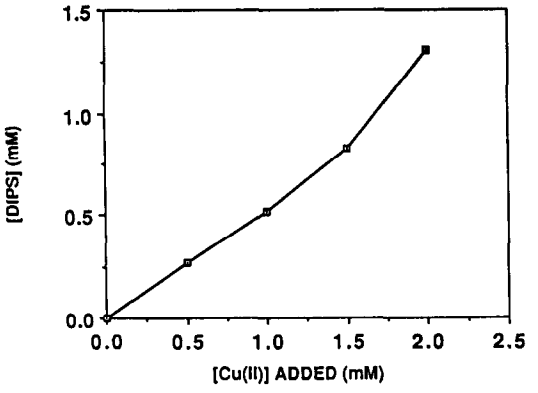

Fig. 7. Plot of the concentration of DIPS in urine (as determined by ^{1}H NMR) versus the amount of Cu(II) added as $[Cu(II)_2(DIPS)_4]$.

pharmacological importance with intact body fluids. Human blood plasma and urine were chosen as representative fluids, the former being a complicated hetereogeneous mexture of particles, proteins and low M_r substances [31], whereas the latter has a low protein content. Since a major acceptor site for Cu(II) in blood plasma is on albumin, which is not readily detectable in plasma by NMR under normal conditions, CD spectroscopy was used as a complementary technique.

The effect of copper on NMR peaks is dependent on its oxidation state. Copper(I) ($3d^{10}$) complexes are diamagnetic and often tetrahedral in geometry. In contrast, Cu(II) ($3d^9$) is paramagnetic with a long electron spin relaxation time. Its normal coordination geometry is square-planar, with two more-weakly bound axial ligands. Ligand exchange reactions, especially from the axial coordination positions, are rapid and Cu(II) is therefore an effective relaxant for nuclear spins, broadening the resonances from protons within a few Ångstroms. Dipolar paramagnetic effects have a $1/r^6$ dependence on distance (r) from Cu(II) to the proton concerned, and there may also be through-bond (contact) effects caused by the presence of unpaired electron spin density on the ligand [32, 33]. In the present study, resonances may also be broadened by chemical exchange processes arising, for example, from binding of a small molecule to a macromolecule or a diamagnetic metal ion (e.g. Ca^{2+}), and also by slow molecular tumbling, especially in blood plasma. Only highly mobile protons with concentrations $>ca.$ 50 μM give rise to observable ^{1}H NMR resonances, and this has to be borne in mind when interpreting the binding data.

When aqua Cu(II) ions were added to plasma in the range 0.25 to 1 mM resonances for several amino acids and for citrate broadened rapidly. These broadenings are likely to arise from paramagnetic effects, due to the formation of Cu(II)–amino acid and Cu(II)–citrate complexes, but secondary effects involving increased binding of the small molecules to proteins in the presence of Cu(II) are difficult to rule out. No information about aromatic amino acids (His, Tyr, Phe, Trp) was obtained from NMR studies of intact plasma since these give rise to very weak peaks, partly because of binding to plasma proteins (*vide infra*). The new CD bands which appear after addition of aqua Cu(II) ions to plasma are characteristic of Cu(II)–albumin, and are associated with the 525 nm absorption band. This arises from Cu(II) bound to the strong N-terminal site (log K 16.2 [34]) Asp-Ala-His- with square-planar coordination geometry provided by the terminal amino group, two deprotonated amide nitrogens, and histidine ring nitrogen [30, 35, 36]. Albumin is the most abundant plasma protein with a concentration of *ca.* 0.63 mM [13]. Amino acids and citrate are known to form strong complexes with Cu(II) and may be involved in the transfer of Cu(II) to the N-terminal site of albumin. Typical log K values (conditional constants, pH 7, in brackets) are 14.9 (12.2), 14.2 (12.2) and 14.9 (12.4) for 1:2 complexes of Cu(II) with Ala, Gln and Val, respectively, and 14.8 for 2:2 complexes with citrate [37]. Interactions between Cu(II) and these small ligands may also occur via the formation of ternary complexes with Cu(II)–albumin. The formation constant for the ternary complex Cu(II)–(L-His)–(HSA) has been reported to be high: log K 21.9 [34]. Copper(II) bound to the strong terminal site of albumin is not removed by treatment with Chelex-100 resin, unlike Cu(II) bound to four additional weaker (K *ca.* 10^7 lower) sites [38].

The observation of saturation kinetics for the transfer of Cu(II) from $[Cu(II)(EDTA)]^{2-}$ to albumin is consistent with the involvement of an intermediate binding step, probably the formation of Cu(EDTA)–albumin. Gao *et al.* [39] have studied the kinetics of the reverse reaction, the mobilization of Cu(II) from Cu(II)HSA with EDTA. They interpreted their kinetic data with a scheme involving the ternary complex Cu(II)(HSA)(EDTA); the on and off rates for EDTA and Cu(II)EDTA, respectively, were similar. Calcium(II) would not be expected to displace Cu(II) from EDTA directly since it binds much more weakly, log K 10.6 compared to 18.7 for Cu(II) (conditional log K values 7.3 and 15.4, respectively, at pH 7) [37, 40], but Ca(II) (along with amino acids) may play a role in the mechanism of displacement of EDTA from the intermediate Cu(EDTA)–albumin complex. No peaks for $[Mg(II)(EDTA)]^{2-}$ were seen in our spectra. However, Ca(II) (plasma concentration *ca.* 2 mM) was always in excess over added $[Cu(II)(EDTA)]^{2-}$ and the stability constant for the $[Cu(II)(EDTA)]^{2-}$ is about two orders of magnitude greater than that for the $[Mg(II)(EDTA)]^{2-}$ (conditional log K values of 7.3 and 5.3, respectively). When excess disodium EDTA is added to blood plasma (e.g. as an anti-coagulant), then peaks for both $[Cu(II)(EDTA)]^{2-}$ and $[Mg(II)(EDTA)]^{2-}$ are seen [27].

Arena *et al.* [41] have determined the formation constants for ternary complexes of Cu(EDTA) with amino acids and have predicted that when $[Cu(EDTA)]^{2-}$ is added to blood plasma, mixed ligand amino acid complexes such as [Cu(H-EDTA)(amino acid)] should account for a significant fraction (up to 10%) of the total copper. They did not include Cu(II) binding to albumin in their

calculations. In our NMR experiments on low M_r (<10 kDa) plasma ultrafiltrates, aromatic and aliphatic amino acids were clearly involved in reactions with $[Cu(II)(EDTA)]^{2-}$ and the formation of $[Ca(EDTA)]^{2-}$. The binding to aliphatic amino acids in ultrafiltrates was reversed on addition of albumin suggesting that amino acids together with citrate and Ca(II) can displace EDTA from Cu(II) and that Cu(II) can be transferred from Cu(II) amino acid and citrate complexes to albumin. Curiously, citrate did not interact with $[Cu(II)(EDTA)]^{2-}$ in a simple mixture of amino acids which suggests that either the broadening of citrate peaks seen in spectra of intact plasma is dependent on mediation by Ca(II) or other components of plasma, or that strong [Cu(EDTA)-(amino acid)] ternary complexes form in the simple mixture. $[Ca(EDTA)]^{2-}$ was also formed from $[Cu(EDTA)]^{2-}$ in urine, and the reaction appeared to involve binding of Cu(II) to histidine, formate, citrate, and some unidentified molecules.

$[Cu(II)_2(DIPS)_4]$ exhibits a wide range of biological effects including radioprotectant, antiinflamatory, antineoplastic and anticonvulsant activities [5]. It is a lipophilic complex, insoluble in water, but soluble in ether as well as polar solvents such as DMSO. The tan-coloured solid is thought to contain carboxylate-bridged dimers, although the X-ray structure has not been determined. In polar solvents, including DMSO, which was used as a solvent for the complex in the present studies, it has been suggested, on the basis of EPR data, that the complex dissociates into monomers, $[Cu(II)(DIPS)_2X_2]$, with two bidentate diisopropylsalicylates, coordinated through carboxylate and protonated phenolate groups and two weakly bound solvent molecules X [42]. The NMR and CD data obtained here suggest that the ligand DIPS is readily displaced from $[Cu(II)_2(DIPS)_4]$ in blood plasma by albumin assisted by amino acids and citrate. This leads to the release of protein-bound lactate, presumably through direct displacement of lactate by the lipophilic carboxylate DIPS. We have previously observed a similar displacement of lactate on addition of ammonium sulphate to plasma [28]. Candidate binding sites include the guanidinium side-chains of arginine residues on proteins such as albumin. No peaks for free DIPS were seen suggesting that it is indeed relatively immobile and bound to proteins or lipoproteins. In urine, on the other hand, where the protein concentration is low, sharp peaks for DIPS were seen, and the distinct broadening of resonances for His and citrate suggest that these ligands are involved in DIPS displacement from Cu(II). The intriguing range of biological activities of $[Cu_2(DIPS)_4]$ may therefore be due not only to the effects of the intact complex but also to the independent effects of Cu(II) and the ligands. The pharmacological properties of the complex may be highly dependent on the relative *rates* of partitioning of both the complex and displaced ligand onto macromolecules and into lipoprotein particles. It would therefore be interesting to study the relationship between biological activity and formulation (e.g. in liposomes).

The tetrahedral Cu(I) complex $[Cu(I)(DMP)_2]^+$ has a high redox potential since the methyl groups destabilize the square-planar geometry favoured by Cu(II). As expected therefore, this cytotoxic complex did not appear to be easily oxidized to Cu(II) in plasma since no broadening of resonances for amino acids nor peaks for free ligand were seen. However, the ligand is lipophilic and, if released, would be expected to bind strongly to macromolecules and give rise to broadened resonances.

CONCLUSION

We have shown that ^1H NMR studies can provide an insight into reactions of copper complexes of pharmacological interest with biofluids. In blood plasma, both the rate and extent of transfer of Cu(II) from $[Cu(II)(EDTA)]^{2-}$ to the N-terminal site of albumin can be monitored, a process which involves both amino acids and citrate, and the formation of $[Ca(EDTA)]^{2-}$. However, ^1H NMR spectroscopy alone cannot provide a complete picture of such reactions. In the present case CD spectroscopy was used as a complementary technique to monitor the formation of Cu(II)–albumin. Reactions of Cu(II) complexes with more weakly bound ligands such as $CuCl_2$ and $[Cu_2(DIPS)_4]$ followed a similar course but were more rapid. No resonances were seen for the ligands of the lipophilic complexes $[Cu_2(DIPS)_4]$ and $[Cu(DMP)_2]^+$ in plasma, probably because they are bound to macromolecules and relatively immobile. In urine, on the other hand, peaks for DIPS are readily observed and the broadening of peaks implicated histidine, formate and citrate in the displacement process. Since the phosphate concentration in urine is high it would be interesting to extend these studies to include ^{31}P NMR spectroscopy. Investigations of the interactions of copper complexes with biofluids have a special significance for the *in vitro* screening of copper complexes, e.g. for anticancer and antiviral activity, much of which is now being carried out with cell cultures. The complexes may be transformed in the culture media before they enter cells.

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