Potentiometric and Spectroscopic Study of the Equilibria in the Aqueous Copper(II)–3,6-Diazaoctane-1,8-diamine System and an Equilibrium-dialysis Examination of the Ternary System of Human Serum Albumin–Copper(II)–3,6-Diazaoctane-1,8-diamine [1]

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3,6-Diazaoctane-1,8-diamine (L, trien) has been obtained by a new purification method, and used in a combined potentiometric and spectroscopic study of the total species formed with Cu^{II} (M) in 0.15 mol dm^{-3} NaCl solution at 25 °C over the range pH 3.0—10.0. The species found are MHL, $MH_{-1}L$, ML, MH_4L_2, MH_3L_2, and ML_2, with log stability constants 23.76, 9.39, 20.01, 52.78, 48.52, and 22.87 respectively. The concentrations of the species, over the range pH 3.0—10.0, and their spectroscopic characteristics have been determined. Equilibrium-dialysis studies of the ternary systems: albumin–copper(II)–ligand (ligand = 3,6-diazaoctane-1,8-diamine or D-penicillamine) have been carried out in 0.15 mol dm^{-3} NaCl solution, pH 7.47, at 6 °C, to measure the amounts of low-molecular-weight membrane-diffusable copper(II) species formed. There is a substantial difference between the two ligands in this respect which correlates with their different clinical behaviour when used in the treatment of patients having Wilson's disease.

WILSON'S disease can be successfully treated by the administration of D-penicillamine,[2] which acts by mobilising the excess of body stores of copper through some, as yet, unknown mechanism.[3] In *ca.* 10% of the patients, toxic reactions to penicillamine develop and some alternative drug has to be used. To date the only successful alternative is the well known chelating agent 3,6-diazaoctane-1,8-diamine (L, trien), first used by Walshe [4] in 1968.

The ligand L when bound to copper(II) ions is generally regarded as being quadridentate. However, some doubt has been expressed [5] as to whether, in aqueous solution, it is really quadridentate towards Cu^{II} because of the steric strain imposed when the ligand is constrained to a square-planar geometry. This doubt is partially based on the relatively low energy of the *d–d* absorption maximum (575 nm) reported for neutral 1 : 1 Cu^{II} : L aqueous solutions when compared to other complexes with CuN_4 chromophores, *e.g.* bis(ethylenediamine)copper(II) (550 nm) and (3,7-diazanonane-1,9-diamine)copper(II) (527 nm). It is also partially based on reports [6,7] of pH-dependent species such as $[Cu(HL)(OH_2)]^{3+}$ and $[CuL(OH)]^{+}$, in which L is presumably tridentate. It is also likely, from our experience, that the ligand used in many of these studies was contaminated with other polyamines, *e.g.* the isomeric tris(2-aminoethyl)amine.

In view of the importance of compound L in the treatment of Wilson's disease and the uncertainty of the species formed with Cu^{II}, we have re-examined the aqueous Cu^{II}–L system. The distribution and stability of the species formed was determined by the analytical potentiometric method developed in these laboratories,[8] combined with a detailed spectroscopic examination as a test of the proposed species. This combined potentiometric–spectroscopic approach has been successfully used to delineate the species formed in other copper(II) systems.[9a] As an adjunct to this work, a new method of obtaining the pure ligand, as its tetrahydrochloride salt, was developed and is reported here in detail. The ternary system albumin–copper(II)–L was also examined as a first step in understanding the mode of action of L in mobilising copper from patients having Wilson's disease and is compared to similar studies involving D-penicillamine.

EXPERIMENTAL

Purification of 3,6-*Diazaoctane*-1,8-*diamine* (L, trien).—The criteria used for purity were elemental analysis, pH

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[1] Presented in part at the meeting 'Penicillamine at 21: Its Place in Therapeutics now,' in honour of Dr. J. M. Walshe, Cambridge, September 1976.

[2] J. M. Walshe, *Amer. J. Med.*, 1956, **21**, 487.

[3] S. H. Laurie, T. Lund, and J. B. Raynor, *J.C.S. Dalton*, 1975, 1389.

[4] J. M. Walshe, *Lancet*, 1969, **2**, 1401.

[5] B. Bosnich, R. D. Gillard, E. D. McKenzie, and G. A. Webb, *J. Chem. Soc.* (*A*), 1966, 1331.

[6] H. B. Jonassen, J. A. Bertrand, F. R. Groves, and R. I. Steams, *J. Amer. Chem. Soc.*, 1957, **79**, 4279.

[7] L. Sacconi, P. Paoletti, and H. Ciampolini, *J. Chem. Soc.*, 1961, 5115.

[8] B. Sarkar and T. P. A. Kruck, *Canad. J. Chem.*, 1973, **51**, 3541.

[9] (*a*) T. P. A. Kruck and B. Sarkar, *Canad. J. Chem.*, 1973, **51**, 3549, 3555, 3563; (*b*) Beilstein, Handbuch der Organische Chemie, 4th edn., Springer, Berlin, vol. IV, p. 255.

titration, and electrophoresis. Thin-layer chromatography was found to be unsatisfactory. By these criteria the reported methods of purification *via* the dihydrochloride salt gave impure products. This can be attributed to the extremely high water solubility of L·2HCl rendering separation by recrystallisation an unsuitable procedure. The following method, giving L·4HCl as the final product, gave a purity of $\geqslant 99.8\%$ as judged by the above criteria.

Commercial trien was fractionally distilled *in vacuo* and the middle fraction was collected [*ca.* 100 cm^3, b.p. 105—107 °C (1 mmHg)].* This fraction was diluted with water (150 cm^3), cooled in ice, and H_2SO_4 (230 cm^3, 9 mol dm^{-3}) was added dropwise with continual rapid stirring, maintaining the temperature below 25 °C. As the addition proceeded, L·$2H_2SO_4$ separated as a white solid. The solid was filtered off, washed extensively with ice-cold water, and then recrystallised twice from hot water yielding L·$2H_2SO_4$ (220 g). The white crystalline solid was then slowly added to an ice-cold solution of K[OH] (220 g, slight excess) in water (300 cm^3), keeping the temperature below 40 °C. Potassium sulphate separated out towards the final addition. The mixture was filtered and the precipitate was washed with small volumes of water, alcoholic K[OH], and then ethanol, until the washings gave no blue-violet colour with an aqueous copper(II) solution. The filtrate plus washings were then concentrated on a rotary evaporator leaving a two-phase residue. The top layer contained the base L and was removed by the addition of acetone. The acetone solvent was then removed immediately with a rotary evaporator (prolonged standing of the acetone extract results in the formation of Schiff bases and should be avoided) leaving the pale yellow viscous L liquor. This was transferred to a large beaker, absolute ethanol (200 cm^3) was added, the solution was cooled in ice, and then concentrated HCl (200 cm^3) was added dropwise with continual stirring. Towards the end of the addition, L·4HCl started to separate as a gummy white solid. This was filtered off, washed with ethanol, and then recrystallised twice by suspending the solid in ethanol (200 cm^3) at 65—70 °C, adding the minimum quantity of water to complete solution and then leaving to cool. Finally, the L·4HCl was washed with aqueous ethanol, ethanol, then diethyl ether, and dried in a vacuum desiccator (yield 70—85 g), m.p. 266—269 °C (uncorr.) (lit.,[9b] 266—270 °C) (Found: C, 24.85; H, 7.15; Cl, 48.8; N, 19.25. $C_6H_{22}Cl_4N_4$ requires C, 24.65; H, 7.55; Cl, 48.6; N, 19.15%). High-voltage electrophoresis using phosphate buffer (pH 6.9) showed only one component: 9.58 mg of L·4HCl required 1.30 cm^3 of 0.10 mol dm^{-3} K[OH] (calc. for the neutralisation of 4 H^+: 1.31 cm^3).

Other materials used were of AnalaR or equivalent grade. Copper(II) chloride solutions were standardised against ethylenediaminetetra-acetic acid. Sodium hydroxide solutions were prepared carbonate-free, stored under an argon atmosphere, and standardised against potassium hydrogenphthalate (N.B.S. grade). Human serum albumin was Miles' recrystallised grade and was used without further purification; because of a small amount of haem contamination, the reference solutions for the spectroscopic measurements contained the same concentration of the protein as in the sample cells. The dialysis membrane was obtained from the Visking Co. and was prewashed with 5% aqueous acetic acid and deionised water. Copper-67 was obtained zinc-free as outlined earlier.[10]

Potentiometric Titrations.—All the solutions were prepared from carbonate-free doubly deionised water and kept under an argon atmosphere. Titrations were performed on a Radiometer automatic-titration assembly thermostatted at 25 ± 0.05 °C. The electrodes were calibrated against standard N.B.S. buffers. For the determination of the ligand proton-dissociation constants, L·4HCl solutions (50 cm^3) of concentrations 2.611×10^{-4}, 5.222×10^{-4}, 8.033×10^{-4}, and 10.444×10^{-4} mol dm^{-3} were used. For the metal-variation measurements the L·4HCl was maintained at 5.22×10^{-4} mol dm^{-3} and copper(II) concentrations of 0.432×10^{-4}, 0.618×10^{-4}, 1.080×10^{-4}, 1.944×10^{-4}, and 2.808×10^{-4} mol dm^{-3} were used. For the ligand-variation measurements the copper(II) concentration was maintained at 1.080×10^{-4} mol dm^{-3} and L·4HCl concentrations of 3.133×10^{-4}, 4.178×10^{-4}, 5.222×10^{-4}, 8.355×10^{-4}, and 10.444×10^{-4} mol dm^{-3} were used. All the solutions contained 10^{-4} mol of standardised HCl and 0.15 mol dm^{-3} NaCl. Subsequently it was found that at the starting pH (*ca.* 2.6) of the above solutions there was some complex formation between L and Cu^{II} and so further sets of titrations, as above, were performed in which 5×10^{-4} mol HCl was present and 0.303 mol dm^{-3} Na[OH] was used as titrant. These latter titrations, carried out over the range pH 2.0—6.0, were in excellent agreement with the earlier sets using 0.1090 mol dm^{-3} Na[OH]. All the calculations were made on a series GE400 computer.

Spectroscopic measurements were made with a Cary model 15 spectrophotometer thermostatted at 25 ± 0.1 °C.

Equilibrium dialysis was carried out as described earlier [11] using solutions buffered at pH 7.47 with 0.10 mol dm^{-3} *N*-ethylmorpholine–hydrochloric acid buffer and containing 0.15 mol dm^{-3} NaCl. The solutions were allowed to equilibrate for 6 d at 6 °C. For the one set of experiments, one half-cell contained a known amount of copper(II)–albumin, with a 10% molar excess of albumin to ensure that all the Cu^{II} was complexed, and was labelled with ^{67}Cu. The other half-cell contained known amounts of L·4HCl. In the second set, one half-cell contained physiological concentrations of albumin and Cu^{II}, labelled with ^{67}Cu, and known amounts of either L·4HCl or D-penicillamine. The other half-cell contained 0.10 mol dm^{-3} of the buffer (pH 7.47) and 0.15 mol dm^{-3} NaCl. Copper-67 activity was assayed before and after dialysis with a Picker Nuclear gamma counter, allowance being made for the isotope's 61.9-h half-life.

CONDITIONS AND RESULTS

Stability Constants and Species Distribution.—The general equilibrium involving a metal ion M, a ligand L, and proton H, can be written in the form (1). The stabilities of the

$$pM + qH + rL \rightleftharpoons M_pH_qL_r \qquad (1)$$

species formed may be expressed as their stoicheiometric equilibrium constants β_{pqr} in terms of concentrations at constant ionic strength, temperature, and pressure as in (2)

$$\beta_{pqr} \rightleftharpoons \frac{[M_pH_qL_r]}{m^p h^q l^r} \qquad (2)$$

where m, h, and l are the concentrations of free metal ion, hydrogen ion, and completely deprotonated ligand respectively. Equations (3)—(5) define the total state of the

* Throughout this paper: 1 mmHg ≈ 13.6 × 9.8 Pa.

[10] N. Marceau, T. P. A. Kruck, D. B. McConnel, and N. Aspin, *Internat. J. Appl. Radiation Isotopes*, 1970, **21**, 667.

[11] S. Lau and B. Sarkar, *J. Biol. Chem.*, 1971, **246**, 5938.

system as regards the species and their variation in concentration, where c_M, c_H, and c_L represent the total metal-ion,

$$c_M = m + \sum_p p\beta_{pqr}\, m^p h^q l^r \quad (3)$$

$$c_H = h - o + \sum_q q\beta_{pqr}\, m^p h^q l^r \quad (4)$$

$$c_L = l + \sum_r r\beta_{pqr}\, m^p h^q l^r \quad (5)$$

total hydrogen-ion, and total ligand concentrations, respectively, participating in a complex-formation reaction, and o represents the amount of free hydroxyl ion. Relations (6)

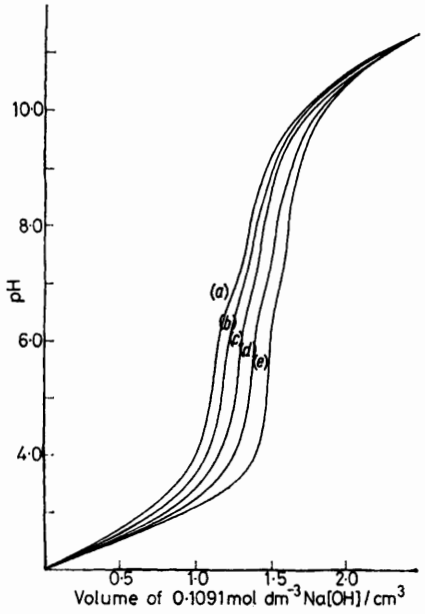

FIGURE 1 Titration curves for the copper(II)–3,6-diazaoctane-1,8-diamine (L) system (metal variation). [L] = 5.222×10^{-4} mol dm^{-3}. [Cu^{II}] = 0 (*a*), 0.432×10^{-4} (*b*), 1.080×10^{-4} (*c*), 1.944×10^{-4} (*d*), and 2.808×10^{-4} mol dm^{-3} (*e*)

and (7) were used to obtain the values of the unbound portions of metal ion and ligand in solutions at any specified pH value, where pM = −log (free metal M), pL = −log (free

$$pM = pM_i + \int_{pH_i}^{pH_f} (\delta H_l^+/\delta c_M)\mathrm{d}pH \quad (6)$$

$$pL = pL_i + \int_{pH_i}^{pH_f} (\delta H_l^+/\delta c_L)\mathrm{d}pH \quad (7)$$

ligand L), and H_l^+ = moles of $[OH]^-$ consumed to titrate hydrogen ions liberated in the complex-formation reaction. The subscripts i and f refer to the initial and final states of the system throughout the titration.

The proton-liberation data and the amounts of unchanged reagents were determined by processing the original titration data with the computer program PLOT 2. The data so obtained were then processed by programs GUESS 2 and LEASK 2 * to obtain the distribution of the species and their stability constants. A detailed account of the data processing has been previously reported.[8]

Proton–3,6-Diazaoctane-1,8-diamine System.—A typical titration curve is shown in Figure 1(*a*). The two protonation equilibria at *ca.* pH 3—4 and 6—7 were sufficiently well separated to be treated by the Henderson–Hasselbach equation. The two equilibria in the pH 9—10 region were solved by treating the original data with PLOT 2 to obtain $\delta H_l^+/\delta c_L$. From these results the dissociation constants were determined by using $\bar{n}_H$ data [equation (8)], where Q =

$$\bar{n}_H = Q - (\delta H_l^+/\delta c_L) \quad (8)$$

maximum number of protons bound to the ligand throughout the titration range. In this case Q = 2 for the higher pH equilibria, and hence we obtain (9). Using increments

$$\bar{n}_H = \beta_{011}(1 - \bar{n}_H)h + \beta_{021}(2 - \bar{n}_H)h^2 \quad (9)$$

of 0.20 pH units, simultaneous sets of equations (9) were set up and processed by program LEASK 2 to obtain the constants β_{011} and β_{021} of the species HL and H_2L respectively. These values, given as pK values for ease of comparison with literature values, are shown in Table 1. The values are in good agreement with other literature data.

Proton–3,6-Diazaoctane-1,8-diamine–Copper(II) System.—The metal-variation and ligand-variation titrations are shown in Figures 1 and 2. From these the unbound metal, pM, and unbound ligand, pL, as a function of pH and at concentrations $c_M = 1.080 \times 10^{-4}$ and $c_L = 5.222 \times 10^{-4}$ mol dm^{-3}, were obtained by digitising the data and processing with program PLOT 2. Values of $\delta H_l^+/\delta c_M$ and $\delta H_l^+/\delta c_L$ as functions of pH were also obtained from PLOT 2 and

TABLE 1

pK_a and log stability constants (β_{pqr}) of complex species $M_pH_qL_r$ (M = Cu^{II}, L = 3,6-diazaoctane-1,8-diamine) in 0.15 mol dm^{-3} NaCl at 25 °C; standard deviations are shown in parentheses

			This work		Other
p	q	r	pK_a	log β_{pqr}	references
0	4	1	3.59 (0.015)		3.25,[a] 3.32 [b]
0	3	1	6.77 (0.015)		6.55,[a] 6.67 [b]
0	2	1	9.22 (0.020)		9.08,[a] 9.20 [b]
0	1	1	9.81 (0.020)		9.74,[a] 9.92 [b]
1	0	1		20.01 (0.02)	20.1,[c] 20.08 [d]
1	1	1		23.76 (0.02)	23.6,[d] 23.8 [a,e]
1	−1	1		9.39 (0.02)	9.93 [e,f]
1	0	2		22.87 (0.025)	21.09 [e,g]
1	3	2		48.52 (0.025)	
1	4	2		52.78 (0.025)	

[a] 25 °C, I = 0.1 mol dm^{-3}; D. W. Margerum, D. B. Rorabacher, and J. F. G. Clarke, *Inorg. Chem.*, 1963, **2**, 667. [b] 20 °C, I = 0.1 mol dm^{-3}; G. Schwarzenbach, *Helv. Chim. Acta*, 1950, **33**, 974. [c] 25 °C, I = 0.1 mol dm^{-3}; C. N. Reilly and R. W. Schmid, quoted in ref. 12. [d] 25 °C, I = 0.1 mol dm^{-3}; ref. 7. [e] Recalculated using the pK_a values from this work. [f] 25 °C, I = 0.1 mol dm^{-3}; ref. 14. [g] Ref. 16.

are shown in Figure 3. The presence of likely metal–proton–ligand species was tested for by using the following values: p = 1 or 2; q = 4, 3, 2, 1, 0, −1, or −2; r = 1 or 2. Input of these values together with the pM and pL values into program GUESS 2 reduced the possible species, in the range pH 3—10, to MH_3L, MH_2L, MHL, $MH_{-1}L$, ML, MH_5L_2, MH_4L_2, MH_3L_2, MH_2L_2, MHL_2, and ML_2. Initial processing with LEASK 2 to fit β_{pqr} values eliminated the species MH_3L, MH_2L, MH_2L_2, and MHL_2. The species MH_5L_2 was later eliminated by its incompatability in the visible-absorption spectral fit. So the final species which gave a minimum-error solution with program LEASK 2 and which satisfied the spectral fit were MHL, $MH_{-1}L$, ML, MH_4L_2, MH_3L_2,

* These three programs are available in both FORTRAN-IV and APL languages. All enquiries regarding these programs should be directed to Dr. B. Sarkar.

and ML_2. Their β values are given in Table 1 and their percentage distribution, as a function of pH, in Figure 4.

Visible-absorption Spectroscopy.—Solutions of copper(II)

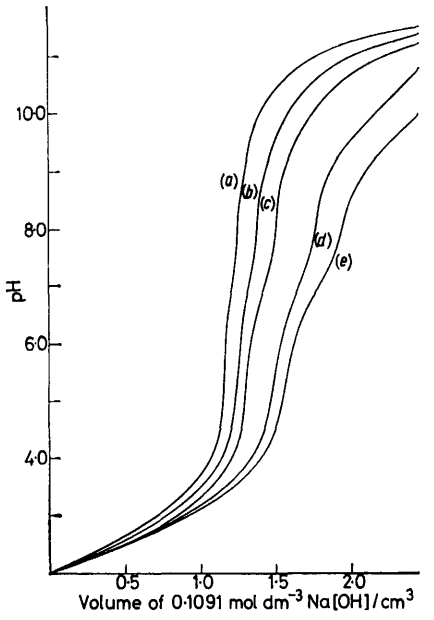

FIGURE 2 Titration curves for the copper(II)-3,6-diazaoctane-1,8-diamine (L) system (ligand variation). $[Cu^{II}] = 1.080 \times 10^{-4}$ mol dm^{-3}. $[L] = 3.133 \times 10^{-4}$ (*a*), 4.178×10^{-4} (*b*), 5.222×10^{-4} (*c*), 8.355×10^{-4} (*d*), and 10.444×10^{-4} mol dm^{-3} (*e*)

and 3,6-diazaoctane-1,8-diamine (L) obeyed the Beer–Lambert law over the tested range $[Cu^{II}] = 2.5 \times 10^{-4}$—$5.0 \times 10^{-3}$ mol dm^{-3}; so it may be safely assumed that the same species distribution pertains to the solutions used for the spectroscopic study as to the more dilute solutions used

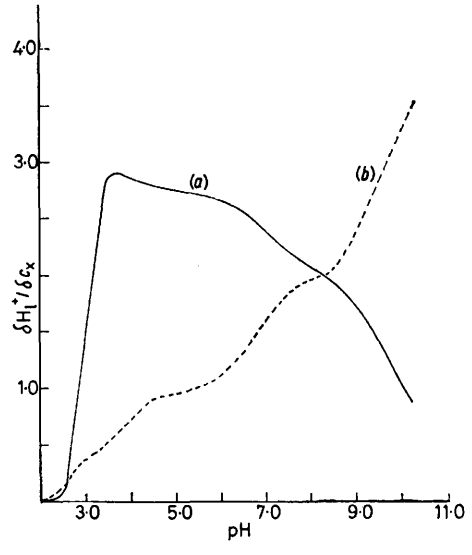

FIGURE 3 Proton displacement $\delta H_l^+/\delta c_X$ as a function of pH for copper(II)–3,6-diazaoctane-1,8-diamine (L): X = M (*a*) and L (*b*). $c_M = 1.080 \times 10^{-4}$ mol dm^{-3}, $c_L = 5.222 \times 10^{-4}$ mol dm^{-3}

in the potentiometric study. The spectrum of a solution containing a Cu^{II} : L ratio of 1 : 4.83, as used in the species determination from the pH data, and 0.15 mol dm^{-3} NaCl was recorded as a function of pH. The total copper(II) concentration was 5.00×10^{-3} mol dm^{-3}. Representative spectra are shown in Figure 5. Over the range pH 6.5—9.0

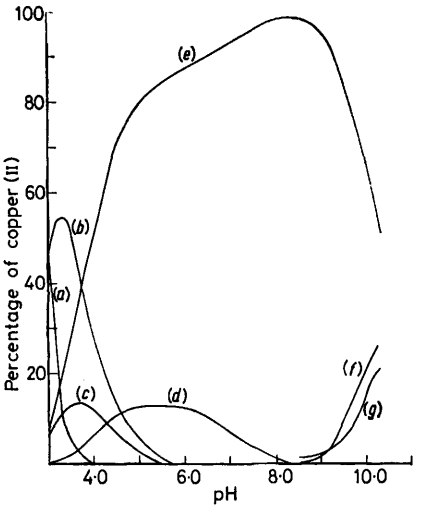

FIGURE 4 Species distribution as a function of pH for copper(II)–3,6-diazaoctane-1,8-diamine (L) in 0.15 mol dm^{-3} NaCl at 25 °C. $c_M = 1.080 \times 10^{-4}$ mol dm^{-3}, $c_A = 5.222 \times 10^{-4}$ mol dm^{-3}. Curve: (*a*) unbound Cu^{II}; (*b*) MHL; (*c*) MH_4L_2; (*d*) MH_3L_2; (*e*) ML; (*f*) ML_2; (*g*) $MH_{-1}L$

the spectrum was invariant corresponding to $\lambda_{max.}$ 580 nm and ε 160 dm^3 mol^{-1} cm^{-1} for the main species ML. Changing the metal-to-ligand ratio had little effect on the absorption maxima but caused significant changes in the absorbance values, particularly in the ranges pH 3—6 and >9, the more marked changes being produced on changing from a 1 : 1 to a 1 : 2 ratio. Fitting of the species from the pH study

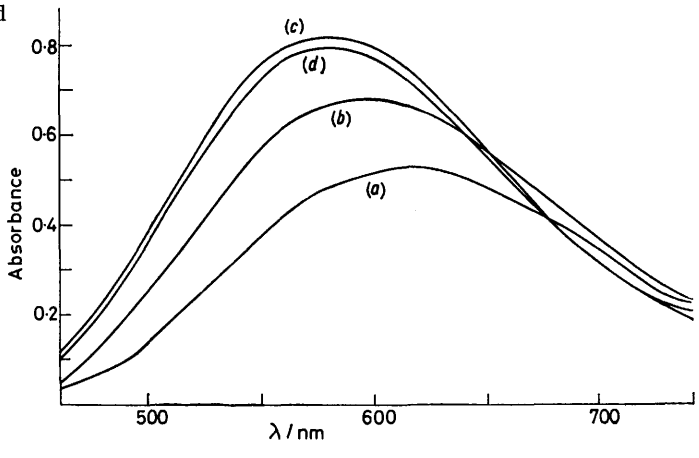

FIGURE 5 Visible-absorption spectra of the copper(II)–3,6-diazaoctane-1,8-diamine (L) system in 0.15 mol dm^{-3} NaCl at 25 °C. $[Cu^{II}] = 5.00 \times 10^{-3}$ mol dm^{-3}, L = 2.50×10^{-2} mol dm^{-3}, l = 1 cm. pH 2.83 (*a*), 3.35 (*b*), 5.98 (*c*), and 7.68 (*d*)

to the measured spectra was accomplished by using the program LEASK 2 to solve the Beer–Lambert expression (10),

$$A_l^{\lambda} = \Sigma \varepsilon_{\lambda,l,pqr}[M_pH_qL_r] \qquad (10)$$

where A_l^{λ} is the measured absorbance for a 1.0-cm pathlength at some specified wavelength and pH, and ε_{pqr} and $M_pH_qL_r$ are the molar absorption coefficient and concentration of each species present in solution at the specified pH.

A matrix was set up at each pH value for a set of A values (20-nm intervals), and $M_pH_qL_r$ values were determined from the species distribution obtained from the potentiometric study. The procedure was simplified by recording the spectrum at pH 8.4 where ML is the sole species (see Figure 3) and hence obtaining the ε values for ML, and then measuring the spectrum at pH 5.70 where ML and MH_3L_2 are the only significant species and hence obtaining the ε values for the latter. The spectroscopic characteristics of the various species are given in Table 2.

TABLE 2

Spectroscopic characteristics of the MHL species (M = Cu^{II}, L = 3,6-diazaoctane-1,8-diamine) in 0.15 mol dm^{-3} NaCl at 25 °C

Species	$\lambda_{max.}$/nm	ε ±5 dm^3 mol^{-1} cm^{-1}
ML	580	159
MHL	620	180
$MH_{-1}L$	560	123
ML_2	580	202
MH_3L_2	580	200
MH_4L_2	540	132

Equilibrium-dialysis Studies.—The distribution of ^{67}Cu activity at equilibrium showed the ligand L to be particularly effective in competing with albumin for Cu^{II} (Figure 6). Thus, at equimolar concentrations, 86% of the Cu^{II} was bound to L; this is in marked contrast to D-penicillamine which was ineffective in forming low-molecular-weight copper(II) species at the same concentration. Using more physiologically relevant concentrations of albumin and copper(II) (Table 3) as found in human-blood serum,* D-penicillamine was again ineffective in forming low-molecular-weight copper(II) species. Very similar results were obtained using non-dialysed human serum in place of the buffered albumin solutions.

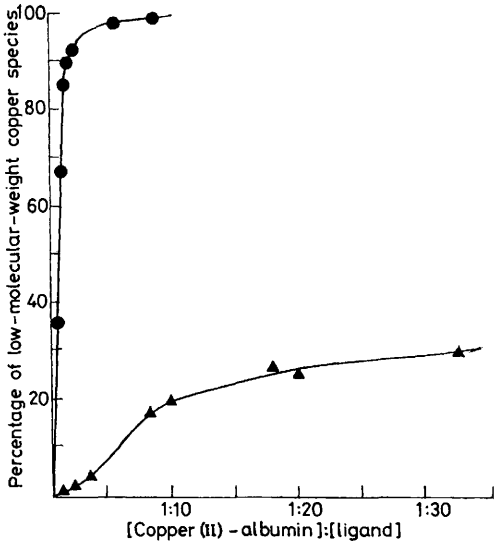

FIGURE 6 Formation of low-molecular-weight species in the systems albumin–copper(II)–3,6-diazaoctane-1,8-diamine (●) and albumin–copper(II)–D-penicillamine (▲). Data from equilibrium-dialysis studies at 6 °C, 0.15 mol dm^{-3} NaCl, and pH 7.47

* The concentration ranges of ligand used in these studies are those found in serum of patients 1—3 h after an oral dose of D-penicillamine (0.5—1.0 g) (Dr. A. Sass-Kortsak, unpublished work).

DISCUSSION

By comparison with the pK_a values of ethylenediamine (en) and 3-azapentane-1,5-diamine (L') [12] we conclude that the first two deprotonations of $[H_4L]^{4+}$ (pK_a 3.59 and 6.77) correspond to deprotonation of the secondary amine groups and hence the species $[H_2L]^{2+}$ and $[HL]^+$ are those protonated at the terminal amino-groups. The species ML, MHL, and $MH_{-1}L$ [ML(OH)] (M = Cu^{II}, L = 3,6-diazaoctane-1,8-diamine) have been recorded previously and the stability constants for these species reported here are in good agreement with the literature values (Table 1).

The proton-liberation data (Figure 3) show the rapid onset of complex formation as the pH increases from 2.6 to 3.6; this reveals the extremely high thermodynamic stability of the species formed since in this region the ligand L is still highly positively charged, *e.g.* at pH 3.0 the amounts of L and $[HL]^+$ present are *ca.* 10^{-22} and 10^{-12} mol dm^{-3} respectively. The enhanced stability of the major ML species (log β_{101} 20.01) relative to that of the tridentate 3-azapentane-1,5-diamine complex (log β_{101} *ca.* 16) [12] is consistent with L being tetradentate in nature. This is also supported by the observation of Sacconi *et al.*[7] that the same species has an unusually large entropy of formation. Furthermore, if L was only tridentate in nature then ML would correspond to the aqua-ion $[CuL(OH_2)]^{2+}$ and in this case we would expect to see the formation of the hydroxo-species $MH_{-1}L$ at a much lower pH (1.5—2 units lower) than is actually observed. The relatively low-energy $\lambda_{max.}$ of 580 nm of the quadridentate $[CuL]^{2+}$ species, commented on earlier, can be attributed to two possible causes: (1) replacement of primary by secondary amino-nitrogens resulting in a lowering of the the ligand-field strength around the copper(II) ion as compared to that in, say, $[Cu(en)_2]^{2+}$; (2) due to steric

TABLE 3

Formation of low-molecular-weight (l.m.w.) species from equilibrium-dialysis experiments in 0.15 mol dm^{-3} NaCl, pH 7.47 buffer, at 6 °C. All concentrations are in 10^{-5} mol dm^{-3}

Serum			% L.m.w. species	
Albumin protein	Cu^{II}	Ligand	D-Penicillamine	3,6-Diazaoctane-1,8-diamine
58.04 [a]	0.12 [a]	0	0	0
58.04 [a]	0.12 [a]	2.12	0	71.1 ±0.2
58.04 [a]	0.12 [a]	5.31	0.5	92.1
58.04 [a]	0.12 [a]	8.49	0.5	95.5
58.04 [a]	0.12 [a]	12.73	4.8	95.3
29.6 [b]	0.60 [b]	2.2	1.4 ±0.5	
29.6 [b]	0.60 [b]	5.5	3.1	
29.6 [b]	0.60 [b]	10.9	0.4	
29.6 [b]	0.60 [b]	12.1	0	

[a] Typical concentrations in normal human blood serum. [b] Typical concentrations in the blood serum of patients having Wilson's disease (before treatment).

[12] 'Stability Constants of Metal–Ion Complexes,' eds. A. E. Martell and L. G. Sillén, *Special Publ.*, The Chemical Society, London, 1964, No. 17.

strain, L forms a distorted square-planar geometry around the copper(II) ion which results in a lowering of the ligand-field strength. This latter effect is consistent with the observation that the insertion of an extra carbon atom into the ligand backbone, which gives greater flexibility and hence a square-planar geometry, results [5] in the lowering of $\lambda_{max.}$ from 580 to 527 nm.* Furthermore, in the crystal structure [13] of [CuL(SCN)][NCS] the CuN_4 chromophore is distorted from a square plane with the copper(II) ion 37 pm above the approximate plane defined by the 4N atoms.

The species MHL corresponds to $[Cu(HL)(OH_2)]^{3+}$ in which HL is tridentate. This is consistent with the observed $\lambda_{max.}$ of 620 nm, a value close to that obtained by Sacconi *et al.*[7] by an extrapolation procedure for the same species, and also close to that (610 nm) of the $[CuL'(OH_2)]^{2+}$ ion. There has been some speculation in the earlier literature [14] as to whether, in the species ML(OH) or $[CuL(OH)]^+$, the $[OH]^-$ ion replaces one of the co-ordinated primary amino-groups; such a conclusion is feasible if the substitution results in a decrease in the ligand strain. Alternatively, the $[OH]^-$ ion may be axially co-ordinated to $[CuL]^{2+}$; either form is consistent with the observed $\lambda_{max.}$ 560 nm.

The most surprising feature of this work is the establishment of bis complexes, although such species have been well established [12] with the tridentate 3-azapentane-1,5-diamine (L') ligand and therefore should not be unexpected with the protonated forms of 3,6-diazaoctane-1,8-diamine. Earlier workers, however, had concluded [15] on the basis of spectroscopic measurements (Job's plot and absorption maxima) that only 1 : 1 species formed. On the other hand, Youness,[16] from similar observations, deduced that the species $[CuL_2]^{2+}$ existed and had a log K_2 value of 1.08. Using our pK_a values this corresponds to a log β_{102} 21.09, in reasonable agreement with our value of 22.87. On purely statistical grounds the formation of the species ML_2 should not be unexpected when relatively large concentrations of completely deprotonated ligand are present, such is the case in our work at pH > 10. It is apparent from the close similarity of the absorption spectra of the various species found in this work, particularly in their $\lambda_{max.}$ values, that a Job's plot would not necessarily reveal any bis species.

The most unexpected species, perhaps, is MH_3L_2 which accounts for 13% of the total Cu^{II} at pH 4.7—5.5. Its presence is evident by the shoulder in the plot of $\delta H_1^+/\delta c_M$ against pH in the pH 5 region (Figure 3). Further deprotonation of this species does not give MH_2L_2 but leads to ML.†

Equilibrium dialysis shows 3,6-diazaoctane-1,8-diamine (L) to be very effective in removing Cu^{II} from the copper(II)–albumin complex at neutral pH. The pattern of the copper(II) mobilisation is similar to that of the peptide derivative glycylglycyl-L-histidine-N-methyl amide which was designed as a synthetic molecule to mimic the copper(II) specific transport site of human albumin.[17a,b] In fact L is more effective than the peptide. Interestingly, the array of nitrogen ligands around Cu^{II} in the L complex at physiological pH is rather similar to that found in the peptide complex.[17c] The efficiency of L reflects the high thermodynamic stability of the $[CuL]^{2+}$ species although the kinetics of the copper(II) exchange from the protein–copper(II) complex to the L complex is relatively slow (*ca.* 15—20 min to establish equilibrium at 25 °C). The reason for this slow exchange is not known at this time.

In contrast to 3,6-diazaoctane-1,8-diamine, D-penicillamine is unable to form low-molecular-weight species in blood serum, except at high ligand : albumin ratios.[1] The formation of low-molecular-weight species is essential for the removal of copper from the blood stream as such species are membrane diffusable.[18] These findings correlate with the clinical findings of Walshe [19] that L and D-penicillamine, which are both effective in 'decoppering' patients with Wilson's disease, act on different pools of copper in the body. From the results presented here and elsewhere [1] we conclude that 3,6-diazaoctane-1,8-diamine is effective in removing excess of copper from the blood stream whilst D-penicillamine is more effective in removing copper from tissues, *e.g.* the liver. These conclusions are also in agreement with those of Williams [20] obtained from computer-based predictions. Interestingly, both these compounds have recently been found [21] to be very effective in removing nickel from nickel-loaded rats.

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* Decreases in ligand strain on going from five- to six-membered chelate rings have been noted in similar polydentate systems: R. Barbucci, L. Fabbrizzi, and P. Paoletti, *J.C.S. Dalton*, 1972, 1529; *Inorg. Chim. Acta*, 1973, **7**, 157.

† In the absence of any evidence to the contrary we have assumed in all cases that the N donor atoms are confined to a near square plane around the copper(II) ion and are not involved in axial co-ordination.

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