THE ACTION OF CHELATING AGENTS IN THE REMOVAL OF COPPER FROM CERULOPLASMIN

An in vitro study

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1. Introduction

The total copper content of biofluids such as blood plasma is composed of three fractions:

(i) Inert metalloproteins (95% of Cu in blood).
(ii) Exchangeable protein copper complexes (5%).
(iii) Low molecular weight (low mol. wt) copper complexes (< 1%).

It has been clearly established that it is the minority low mol. wt complexes which are responsible for the excretion and for the distribution of the metal ions into body tissue [1,2].

Computer models of the distribution of metal-ions amongst complexes in blood plasma have been published [3–5]. Essentially these are equilibrium calculations based upon stability constants and the respective metal and ligand concentrations. These models have enabled us to explain and to predict the action of different agents used in chelation therapy. Also they have made it clear that chelation from serum albumin is not the 'modus operandi' through which D-penicillamine liberates copper(II) into low mol. wt form [5]. The apparent inability of this agent to procure albumin-bound copper cannot be correlated with the substantial cupruresis induced when this drug is administered to patients [6]. This had led us to investigate the possible mechanisms of activity of chelating agents and in particular the influence of D-penicillamine upon ceruloplasmin in saline solution and in blood plasma. Although ceruloplasmin represents the major portion of copper in plasma the metal is inertly bound and is not normally available to the labile equilibrium system [7]. Hence any small liberation of copper from ceruloplasmin (e.g. 1%) will produce a large increase in the labile copper fraction (in the e.g., up to a 20% increase).

Much research has been reported concerning the amount and availability of copper in ceruloplasmin [8–11]. The review [7] concludes that ceruloplasmin contains 8 copper atoms, 4 of which are exchangeable when reduced. At low pH all the copper atoms can be reversibly removed [12]. More recent work has tended to suggest that there are only 6 tightly held copper atoms per ceruloplasmin molecule and possibly one exchangeable copper atom [13]. The underlying reasons for these dichotomies are the uncertainty in the mol. wt (132 000–150 000) of the protein and the ease with which it is degraded [13,14]. Thus we have chosen experimental methods which are independent of these two factors.

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2. Materials and methods

Three copper-containing protein solutions, human serum, plasma and a pure ceruloplasmin solution, were treated with chelating agents and the resulting low mol. wt species separated by ultrafiltration. Copper concentrations in each fraction were then determined by atomic absorption spectrophotometry and colorimetric analysis.

2.1. *Chemicals*

Ethylenediaminetetraacetic acid (EDTA) triethylenetetramine (Trien) and ascorbic acid (Asc) were obtained from BDH Biochemicals, diethylenetriaminepentaacetic acid (DTPA) from R. N. Emanuel Ltd, sodium diethyldithiocarbamate (DDC) from Fisons Ltd and D-penicillamine (D-Pen) from Koch-Light Labs, human serum and plasma (citrated) from Ninewells Hospital, Dundee, and ceruloplasmin solution from Serva Fein Biochemica.

2.2. *Ultrafiltration and atomic absorption*

To pooled human serum or plasma (10 ml samples) chelating agents were added (to final conc. 25 mmol.dm^{-3}) and the solution left to stand for 1 h, then filtered with stirring (Millipore filtration apparatus type XX4202510, 25 mm) using filters having a nominal mol. wt cut-off of 25 000 (Millipore PTGC 02510) and an overpressure of 25 psi of nitrogen, giving a filtration rate of 2 ml/h. The same volume of filtrate was collected in each investigation (2 ml) and analysed for total copper content (Perkin-Elmer 360 atomic absorption spectrophotometer with digital read-out and deuterium background corrector). Standard copper solutions for these analyses were prepared in 10% glycerol [15]. Ceruloplasmin solutions (5%) were diluted 100-fold with phosphate buffer at pH 7.4. A background concentration of 150 mmol.dm^{-3} NaCl was used in the preparation of the ceruloplasmin and the chelating agent solutions. The results of the copper analyses are listed in table 1. They are accurate to better than 5% with the exception of Trien (25 mmol.dm^{-3}) which is better than 10%.

2.3. *Spectrophotometric determinations*

Exchangeable copper in serum and ceruloplasmin solutions was determined by complexation with DDC and spectrophotometric analysis [16]. A Unicam SP800B instrument was used. In general the results are in agreement with those determined by atomic absorption. However, the concentrations in question are near to the limits of detectability by spectral approaches and so atomic absorption is considered to be the much more reliable method. The commercially supplied ceruloplasmin solutions were found to contain a large amount of exchangeable copper. DDC had previously been used to extract total ceruloplasmin copper at low pH [12] but to remove solely exchangeable copper at higher pH [16]. Since we were working at pH 7.4 it must be concluded that the ceruloplasmin solutions supplied had suffered considerable degradation during or since their manufacture [13].

3. Results and discussion

The ability of the series of agents used in this study to mobilize the copper of blood plasma, serum and ceruloplasmin solutions into a low mol. wt form is shown in table 1. Since the ultrafiltrable component may be raised substantially above the 5% of the total copper corresponding to the albumin-bound fraction in blood plasma and serum, it can be concluded that in these cases the additional copper at least is derived from ceruloplasmin. This is supported by the similarity in percentage copper mobilized in all three fluids investigated.

The effects of the agents roughly parallel their copper binding ability, i.e.:

Trien > DTPA > EDTA > D-Pen, DDC, Asc

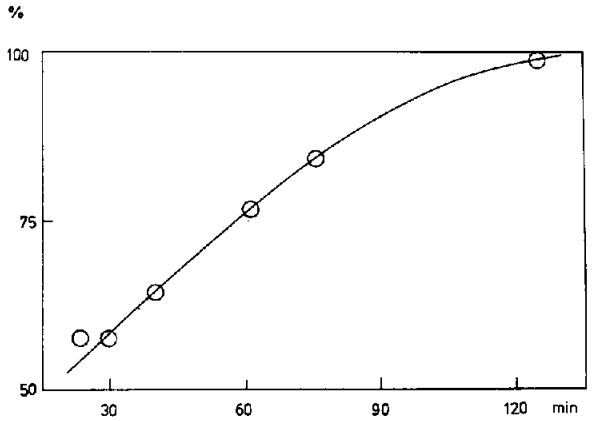
This suggests that chelation is an important step in the irreversible degradation of the metalloprotein. Our results are in agreement with the copper mobilizing order [8]; for ceruloplasmin solutions at lower pH they found EDTA>>D-Pen. We also noted the increased mobilization of copper from plasma under a nitrogen atmosphere which they obtained from ceruloplasmin solution although the possibility that D-Pen liberates ceruloplasmin copper simply by reduction is negated by comparing the D-Pen and Asc results.

Figure 1 indicates that a kinetic factor is involved when Trien removes copper from ceruloplasmin; this

Table 1
Atomic absorption analyses on various ultrafiltration samples of serum, plasma and ceruloplasmin solution

Sample	Copper concentrations						
	Serum		Plasma		Ceruloplasmin		
	ppm	%	ppm	%	ppm	%	Δ%
Blanks							
Total Cu	1.05	100	0.75	100	2.18	100	–
Filtrate	0.03	2.7	0.00	0.0	0.55	25.2	0.0
Experiments							
Additions to protein solutions							
D-Pen	0.07	6.6	0.00	0.0	0.80	36.7	6.4
EDTA	0.18	17.1	0.12	16.7	0.90	41.3	11.0
Trien	0.43	41.0	0.31	41.3	1.55	71.0	40.7
DTPA	0.32	29.1	–	–	–	–	–
DDC	0.00	0.0	–	–	0.66	30.3	0.0
D-Pen + EDTA	0.50	47.6	0.27	36.7	1.00	45.9	15.6
D-Pen + Trien	0.60	57.1	0.44	58.7			
D-Pen + DTPA	0.36	32.7					
D-Pen + DDC	0.16	14.6					
Asc + DDC	0.0	0.0					
Asc + EDTA	0.14	12.7					

I, 150 mmol dm^{-3} NaCl. %, % total copper Δ%, (% – background reading from DDC results)



may arise either from steric hindrance to the agent binding the metalloprotein copper or from a Trien-promoted chemical degradation of the ceruloplasmin. However, the concentration dependence of Trien-liberated copper, shown in fig.2, is difficult to reconcile with a solely kinetic phenomenon. Rather, it appears indicative of some mechanism involving chelation.

Fig.1. The kinetics of copper mobilization from serum using Trien. The percentage of total copper in low mol. wt form is plotted versus the time (in min).

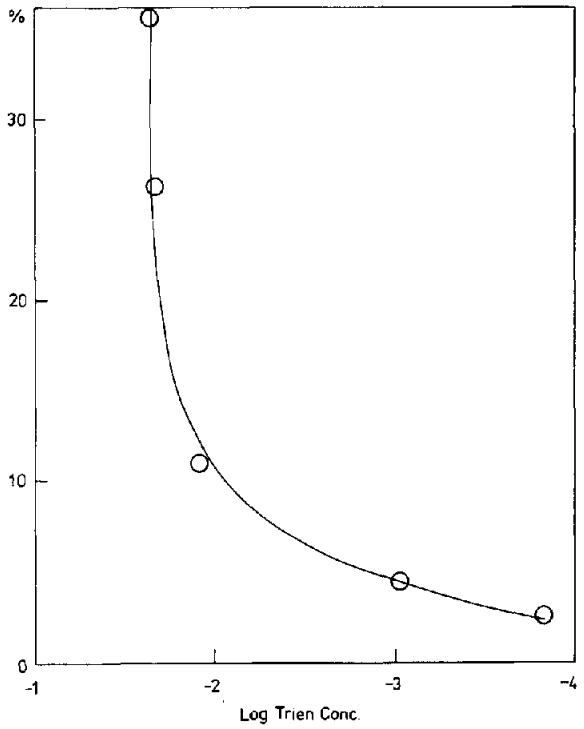


Fig.2. The effect of Trien concentration upon the degree of copper mobilization. The percentage of copper in low mol. wt form is plotted versus log (total Trien conc.).

Our results with respect to D-Pen show that this agent alone causes very little increase in low mol. wt copper. Thus the drug is incapable of removing the metal from ceruloplasmin and/or is not able to maintain any additional exchangeable copper in a low mol. wt form. The synergistic effects shown when two chelating agents are introduced simultaneously suggest that D-Pen does release a small amount of copper from ceruloplasmin, transferring it to serum albumin in the absence of some more powerful chelating agent, with only a small proportional increase of the low mol. wt fraction [17]. These conclusions are at variance with [18] where 32.3% of the total copper in serum was found to be ultrafiltrable after the addition of D-Pen. One explanation for this difference may lie in the type of molecular filters used: the collodion filters may not have been sufficiently discriminating and allowed serum albumin to pass through whereas our filters have been checked for this [19].

These studies still cannot explain the very large cupruresis produced by D-Pen therapy relative to EDTA and Trien [20]. It seems likely that whatever the mode of action of D-Pen, it ultimately increases the low mol. wt copper concentration in plasma. This is in spite of its inability to degrade ceruloplasmin or to compete effectively with labile copper binding proteins in the biofluid. If this proposition is correct, an alternative improved treatment for Wilson's Disease is possible: simultaneous administration of D-Pen and Trien should produce a synergistic cupruresis. The Trien would hold the copper labilized into plasma by D-Pen as a low mol. wt charged complex [5] thereby considerably facilitating its renal excretion. Such a therapeutic strategy is expected to lower the required D-Pen dosage and so minimise the side effects for which this drug is often responsible.

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