A Comparative Study of *in vitro* and *in vivo* Interaction of D-penicillamine and Triethylenetetramine with Copper

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In 1956, Walshe used D-penicillamine (pen) to treat patients with Wilson's disease (Walshe 1956). Because of this discovery, suffering from this disease has been greatly alleviated. However, there are instances where patients do not respond to pen and conditions are not improved by this agent. Again, in 1969, Walshe used triethylenetetramine (trien) for the first time to treat Wilson's disease and met with success in patients who did not respond to penicillamine (Walshe 1969). We have studied the interaction of pen and trien with copper ions, and their equilibrium reaction with copper bound to human albumin. Mobilization, organ distribution and excretion of copper by these two agents were investigated in detail in order to gain an understanding of their mode of action in the removal of excess copper in Wilson's disease.

Interaction of Copper with Pen and Trien

Copper-pen: Two types of complexes are formed when copper and pen are mixed. The complexes formed are dependent on the copper:pen molar ratio and counter ion used. One is a pink coloured complex and the other is a colourless complex. Both complexes are polymeric in nature.

The pink coloured complex with λ_{max} at 525 nm is formed in the presence of halide ion. Chloride ion was shown to be an integral part of the complex by using ^{67}Cu, ^{36}Cl and pen at pH 7.4. The complex was separated by thin-layer chromatography according to the method used in identifying various copper-amino acid complexes (Sarkar & Kruck 1967). The thin-layer plate was first exposed for ^{67}Cu and then for ^{36}Cl after ^{67}Cu had been allowed to decay. Both ^{67}Cu and ^{36}Cl were detected in the same complex. The results are in accord with a similar report about the halide ion dependence of the complex (Wright & Frieden 1975). Recently, X-ray crystallography of a purple complex formed by copper and pen showed a mixed valence cluster with composition $[Cu(I)_8\ Cu(II)_6\ Pen_{12}\ Cl]^{5-}$. The Cl^- ion is located at the centre of the cluster (Birker & Freeman 1976).

Copper-trien: Trien is one of the classical polyamine type of chelating agents known to inorganic chemists for several decades. It is generally regarded as a tetradentate ligand which can adopt one of several conformations when bound to a metal ion; with copper, it is constrained to a near square-planar conformation when all four nitrogen atoms are bound to the central cupric ion.

However, some doubts have been expressed in the literature whether, in aqueous solution, trien is tetradentate or tridentate towards the copper ion (Bosnich *et al.* 1966). The reasons for this conflict arise, we believe, because (a) impure trien has been used in many cases, and (b) there has been no attempt to characterize the species present in aqueous solution.

We finally obtained pure trien in the form of trien 4HCl via fractional distillation of commercial trien and intermediate crystallization of the trien $2H_2SO_4$ salt. Purity was greater than 99.8% as judged by elemental analysis, electrophoresis and potentiometric titration. Attempts to synthesize trien, as a route to obtaining ^{14}C-labelled material, have so far been unsuccessful.

We have also completed a potentiometric-spectroscopic analysis to determine the species present and their stability constants in the aqueous Cu(II)-trien system (25°C, 0.15 M NaCl) by the method outlined by Sarkar and Kruck (1973). Over the pH range 4.0–10.2, the 1:1 $[Cu(trien)]^{2+}$ complex is the major (but not the only) species present and is undoubtedly the tetradentate form of the ligand ($\log \beta = 20.01$). Other species, particularly bischelate forms, are also present; thus at pH 7.40 a 1:5 $CuCl_2$:trien solution comprises 96% $[Cu(trien)]^{2+}$ and 4% $[Cu(trien\ H_2)(trien\ H)]^{5+}$.

Equilibria in Albumin-copper-pen and Albumin-copper-trien Systems

In these competition studies, the ligand was placed on one side of the membrane (Visking type) and copper(II) chloride with a slight molar excess of albumin was placed on the other side. Both solutions contained 0.15 M NaCl and were buffered with 0.1 M N-ethylmorpholine-HCl buffer at pH 7.47. Under these conditions, it can be assumed that all the copper is initially bound to the specific copper-transport site of albumin. The dialysis cells were agitated at 6°C for five to six days to ensure complete equilibration, after which aliquots were removed from both sides of the cell and the ^{67}Cu activity was assayed on a Picker Autowell gamma counter. In calculating the amount of low molecular weight species formed, allowance was made for the diffusion of such species through the membrane and hence

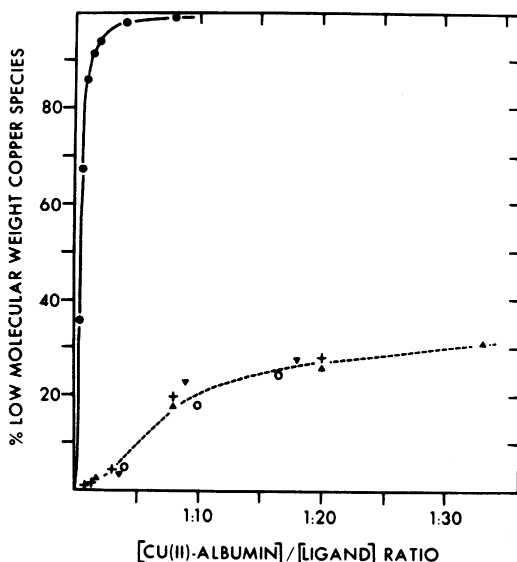

Fig 1 *Formation of low molecular weight copper species in the system: albumin-^{67}Cu-D-penicillamine, L-cysteine or triethylenetetramine. Data from equilibrium dialysis studies at 6°, 0.15 M NaCl and pH 7.47. D-penicillamine: deoxygenated solutions* (+), *oxygen-saturated solutions* (▲), *in presence of nitrate ion* (▼). *L-cysteine in deoxygenated solutions* (○). *Triethylenetetramine* (●)

an equal distribution (^{67}Cu activity) of the low molecular weight species on both sides of the membrane.

The results are shown graphically in Fig 1. Now one could envisage that if any chelating agent is to be therapeutically useful, then one of its functions would be to mobilize copper in the bloodstream as membrane-diffusable low molecular weight species. Pen, as can be seen in Fig 1, is poor in this respect, only 30% of the Cu(II) bound to albumin being released in the form of low molecular weight species in the presence of a large excess of pen. In contrast, trien is very efficient, 86% of the Cu(II) being mobilized by an equimolar amount of the ligand.

Several explanations can be offered for the relatively poor pen performance: (1) there is some binding of the pen to the albumin (either by salt linkage or disulphide bond formation); (2) there is some unfavourable steric interaction which prevents formation of an intermediate ternary complex albumin–copper–pen; (3) the pen forms high molecular weight polymeric complexes with copper ions; and (4) the pen is undergoing prior oxidation to the disulphide form.

Equilibrium dialysis experiments involving no copper showed negligible binding between the protein and pen under the above conditions and hence ruled out (1). Explanation (2) appeared unlikely, since other larger molecules, e.g. tripeptides, have been shown to remove copper quite effectively from albumin (Lau *et al.* 1974). Explanation (4) was eliminated by carrying out the dialysis with solutions which had been thoroughly de-oxygenated and the cells sealed under argon; this had only a negligible effect on the copper removal. This leaves (3) as being the most likely explanation. The formation of the copper polymer has been shown to be dependent upon the presence of chloride ion, and so we also carried out dialyses in which nitrate replaced chloride ion; this produced only a minor change in the results (*see* Fig 1). To check further for polymer formation, the high molecular weight side after dialysis was chromatographed on Sephadex G-75 using 0.1 M N-ethylmorpholine buffer as eluant and assaying for ^{67}Cu in the fractions.

As seen in Fig 2, two peaks are evident, one at the void volume (≥70000) and the second one of molecular weight 27000–30000 (from protein calibration of the column used). By using ^{67}Cu-albumin solutions the first peak is established as the copper–albumin complex (curve A, Fig 2); there is a slight peak behind the major one, probably owing to some impurity in the albumin used, but nothing corresponding to a molecular weight of 27000–30000. The amount of radio-

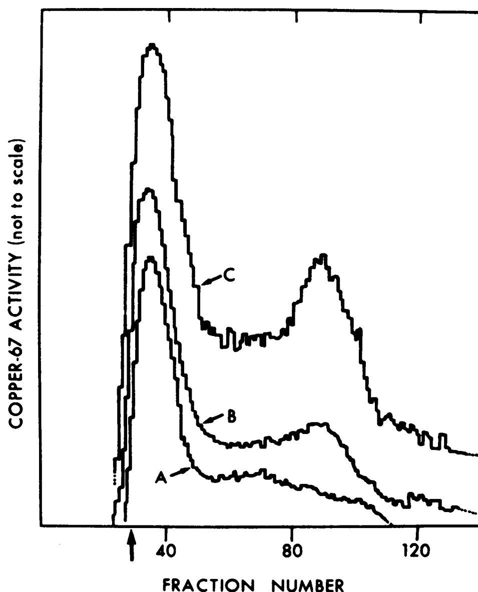

Fig 2 *High molecular weight species in the system albumin-^{67}Cu-D-penicillamine from Sephadex G-75 fractionation of the protein cell after equilibrium dialysis. Column eluted with 0.1 M N-ethylmorpholine-HCl buffer, pH 7.50, [Cu-albumin]: [D-penicillamine], 1:0* (A); *1:4* (B); *1:10* (C)

activity in the second fraction increases as the concentration of pen is raised (curves B and C, Fig 2) and therefore suggests this to be a copper-pen polymer. Further evidence for this was obtained by using L-cysteine instead of pen. L-cysteine and D-pen behave similarly towards Cu(II)-albumin in terms of low molecular weight species and polymer formation (Fig 1). By using double labelling, i.e. ^{67}Cu and ^{14}C, ^{14}C activity was assayed six weeks after measuring the ^{67}Cu activity and then remeasured until the activity reached a constant level. Assay was with a Nuclear Chicago Mark I three-channel liquid scintillation counter (calibration and corrections being made in the normal way) and we observe that the second peak contains both L-cysteine and copper (Fig 3). Further work is required to elucidate the nature of this polymer and its dependence on chloride ion. To date, we have evidence that the same polymer is formed in aqueous solutions os copper salts with pen and that its formation if very complex (dependent on Cl^-, O_2, time, and copper:pen ratio), it may also be heterogeneous.

Of most significance here is the fact that even at very high pen concentrations, most of the activity is still in the copper-albumin fraction, e.g. at copper-albumin:pen ratios of 1:4 and 1:10, the amount of copper–albumin complex remaining is 74% and 55% respectively. Presumably, the polymer formation prevents any further removal of copper from the protein.

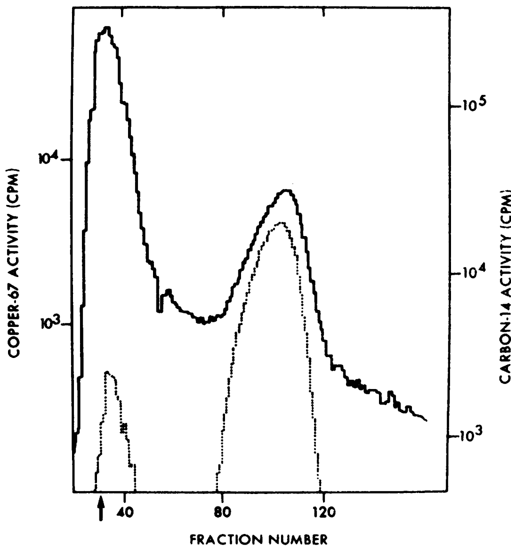

Fig 3 *High molecular weight species in the system albumin-^{67}Cu-^{14}C-L-cysteine from Sephadex G-75 fractionation, elution with 0.1* M *N-ethylmorpholine-HCl buffer, pH 7.50 [Cu-albumin]: [L-Cys] 1:40. Upper curve, ^{67}Cu activity. Lower curves, ^{14}C activity*

Table 1

Formation of low molecular weight species (LMW) from equibrium dialysis experiments (concentrations 10^{-5}M)●

Serum			*Percentage of LMW species*	
Albumin protein	*Cu(II)*	*Ligand*	*D-pen*	*Trien*
58.04	0.12	0	0	0
58.04	0.12	2.12	0	71.1±0.2
58.04	0.12	5.31	0.5	92.1
58.04	0.12	8.49	0.5	95.5
58.04	0.12	12.73	4.8	95.3
29.6	0.60	2.2	1.4	—
29.6	0.60	5.5	3.1	—
29.6	0.60	10.9	0.4	—
29.6	0.60	12.1	0	—

● At 6°, μ = 0.15M, pH 7.47 buffer

Although these studies are of chemical interest, they do not reflect the clinical conditions where, for example, the albumin:copper ratio is very much higher in favour of albumin. The penicillamine concentration found in blood serum is also very much lower than that used in the above experiments.

We therefore set up the dialysis experiment to reflect more closely the clinical conditions. Thus, on one side of the membrane clinical concentrations of albumin and copper were added together with the appropriate amount of D-pen, this was then dialysed against a buffered saline solution. The amounts of pen used are based on levels of 0.02–0.100 μmol/ml human plasma observed 1–3 h after a 0.15–1.0 g pen dose. As can be seen from Table 1, pen has a negligible effect in terms of copper mobilization even when the albumin to copper ratio is very much reduced to the levels typical of those observed in the plasma of Wilson's disease patients (i.e. 29.60:.60, alb:Cu(II) shown in lower half of Table 1). Trien, on the other hand, is very effective even at the high albumin:copper ratios. These results are consistent with those observed with whole human blood serum presented in the next section.

Effect of Pen and Trien on the Mobilization of Copper in Human Blood Serum

Ultracentrifugation experiments were carried out to determine the mobilization of copper by trien and pen when added to serum. Pooled normal human serum of known albumin concentration was used. Cupric chloride labelled with trace amounts of ^{67}Cu was dissolved in physiological saline and added to the serum to give a copper: albumin molar ratio of 0.25. The pH of this was adjusted to 7.4.

The trien or pen was prepared in a concentrated form in physiological saline; the pH was adjusted to 7.4 and small volumes of these solutions were added to aliquots of the labelled

Table 2

Copper-binding capacity of trien and pen in the presence of human serum and copper (0.25M concentration of albumin)

Ratio agent/albumin in native serum	*Percentage ^{67}Cu in supernatants*		
	Pen●	*Trien*●	*Control*
0	—	—	0.6
0.1	—	31.5	
0.25	—	57.3	
0.5	0.8	65.3	
1.0	0.9	71.7	
2.0	0.9	69.8	
4.0	1.4	72.9	
8.0	50.8	76.5	
12.0	58.2	66.8	

● Agent

copper-containing serum to achieve various molar ratios of agent:albumin, from 0 to 12. Physiological saline, pH 7.4, was used to equalize the total volume of each aliquot, resulting in a small dilution of the original human serum. The final concentration of albumin varied between 4.0 and 4.35×10^{-4} M. For separation into low and high molecular weight components, these samples were subjected to a centrifugal force of 183000 *g* at 4° for 19 hours in a Beckman Model L-350 Preparative Ultracentrifuge. An aliquot of the supernatant removed from the upper fourth of the tube was counted for ^{67}Cu activity as previously described. This was compared to a similar sample taken before centrifugation. The activity in the supernatant is expressed as the percentage of the activity in the sample before centrifugation (Table 2).

The results show that trien is capable of mobilizing copper at a low trien:albumin ratio. Pen, on the other hand, requires a much higher pen:albumin ratio before any copper appears in the small molecular weight fraction of serum. These results are consistent with those from the equilibrium dialysis experiments.

Organ Distribution of Copper Following Administration of Pen and Trien

The introduction of chelating agents into the body of a rat must affect the distribution of copper. Experiments were devised to determine the distribution of 'mobilizable' copper (copper capable of equilibrating with radioactive copper within three minutes of injection) in serum, liver and kidney of rats. Trien and pen (40 mg/kg body weight) were introduced into the body and their effect on mobilizable copper in the body was monitored over a period of time.

The techniques used in these experiments were similar to those developed previously in our laboratory (Harris & Sass-Kortsak 1967). Male Wistar rats, weighing 250–350 g, were used. The animals were starved overnight before the experiment. Ether was used as an anæsthetic for all injections and samplings.

A tracer dose of ^{67}Cu carried by 10% rat serum and physiological saline was injected into the inferior vena cava of the experimental animal 3 min before samples were taken. All measurements for copper are based on the equilibration of the radioactive copper with the copper available in the animal. This copper we designate 'mobilizable', so results ignore all non-dialysable copper. The results as given in Table 3 are expressed as a percentage of the total dose of ^{67}Cu injected. Total dose was determined from the total counts in the syringe before injection, less the counts remaining in the syringe after injection. Corrections were made for the decay factor of ^{67}Cu when calculating the percentage of the dose.

The equivalent of 40 mg of trien or pen per kg body weight of the rat was injected into the tail vein. The pH of the injected solution was adjusted

Table 3

Distribution of copper in rats after injection of chelating agents

	Percentage dose of ^{67}Cu 3 min after introduction								
		Time after injecting trien				*Time after injecting pen*			
	Control	*15 min*	*30 min*	*60 min*	*120 min*	*15 min*	*30 min*	*60 min*	*120 min*
Serum:									
Total	39.03 ±9.1(9)	34.9 ±7.2(6)	34.1 ±12.6(6)	35.3 ±10.5(6)	38.5 ±10.0(7)	24.6 ±6.4(7)	20.0 ±2.9(6)	23.3 ±8.1(6)	29.0 ±8.6(6)
Small molecules	1.4 ±0.3(8)	20.8 ±4.5(6)	19.8 ±7.9(6)	20.1 ±5.4(6)	22.9 ±7.7(7)	1.8 ±0.9(7)	1.2 ±0.25(6)	0.83 ±0.25(6)	0.70 ±0.1(6)
Liver:									
Total	17.59 ±1.4(9)	14.3 ±3.0(6)	9.8 ±9.9(6)	9.1 ±0.8(6)	8.6 ±1.0(7)	10.4 ±1.6(7)	20.9 ±0.6(6)	24.8 ±0.6(6)	15.5 ±2.0(6)
Soluble fraction	9.1 ±1.0(9)	6.1 ±0.6(6)	5.7 ±0.9(6)	5.0 ±0.7(6)	4.3 ±0.5(7)	5.7 ±0.8(7)	5.0 ±0.6(6)	5.8 ±0.9(5)	7.3 ±1.2(6)
Small molecules	2.51 ±0.4(9)	0.6 ±0.2(6)	1.1 ±0.4(6)	1.2 ±0.3(6)	1.2 ±0.2(7)	1.6 ±0.2(7)	0.8 ±0.2(6)	1.2 ±0.2(5)	1.8 ±0.2(6)
Kidney:									
Total	6.9 ±1.0(8)	6.1 ±0.9(6)	10.9 ±1.4(6)	11.8 ±2.1(6)	9.1 ±1.8(7)	9.0 ±1.6(6)	9.6 ±2.0(6)	9.5 ±2.4(6)	11.5 ±1.5(6)

Numbers of samples shown in parentheses

to 7.4. The final volume injected did not exceed 0.6 ml. All solutions of the chelating agents were prepared immediately before injection.

Blood serum: Blood was removed from the inferior vena cava close to the point of injection of the ^{67}Cu. The withdrawal was started 3 min after the introduction of radioactive copper. The blood was allowed to clot, then spun, and an aliquot of the serum removed for ^{67}Cu assay. The total counts in serum were calculated on the basis of 40.4 ml of serum per kg body weight of a rat. Two ml of the serum were centrifuged at a high centrifugal force to remove large protein molecules. Tests in this laboratory show that protein in the supernatant is less than 0.3% of the total protein, and that albumin in the supernatant is less than 0.15 mg/ml. An aliquot of the supernatant was removed from the upper quarter of the centrifuge tube. A measured volume was counted for radioactivity and this was compared with the serum sample to determine the percentage of ^{67}Cu bound to small molecules.

As was expected from our previous results, the presence of trien significantly increased the amount of copper bound to small molecules, with no decrease even after 2 h (Table 3). Penicillamine had no visible effect on the amount of low molecular weight species.

Liver: After removing the blood from the animal, the liver was taken out, placed in ice-cold 0.25 M sucrose solution, trimmed, blotted and weighed. A portion (1–2.5 g) was homogenized in 0.25 M sucrose. An aliquot was assayed for radioactivity and the rest was centrifuged at 36000 rpm for 90 min. The soluble fraction was decanted and an aliquot counted for radioactivity. Proteins were removed from the soluble fraction by centrifuging the sample.

The liver shows a significant change in the distribution of copper. The amount of copper entering the soluble fraction of the liver is decreased compared to normal, indicating that the normal pathways for uptake of copper have been affected. Trien itself appears unable to enter the liver, as the amount of copper bound in the soluble fraction is actually significantly decreased, and it is unlikely that a protein more efficient than trien would be in the soluble fraction. Pen tends to increase the amount of copper in the whole liver, but this effect is transitory, and is accompanied by a decrease in copper levels in the soluble fraction.

Kidney: Both kidneys were removed, trimmed of vessels and clots, rinsed and counted in a deepwell gamma counter. Total counts are expressed as a total of the ^{67}Cu dose injected.

An increase in copper is seen after the injection of pen and trien. The increase with pen is slower; only after 2 h is it significant. The trien shows a marked increase in the level of copper in the kidney after 15 min, and the increase is returning to normal after 2 h.

Excretion of Copper Following Administration of Pen and Trien

The measurement of the amount of copper being excreted through the kidney is a more effective indicator of the importance of these chelating agents in the removal of copper from a physiological point of view.

Male Wistar rats, 300–400 g in weight, were used for injection studies. Tail cups were in place to prevent contamination from fæces. The rats were caged in copper-free plastic cages and dextrose in deionized water was the only sustenance given during the experiment.

The agent to be tested was dissolved in saline, the pH adjusted to 7.4, and not more than 0.8 ml was injected immediately into the tail vein of the rat. The amount injected was the molar equivalent of 1, 20, 40 or 80 mg of pen per kg body weight of the rat.

Table 4

Copper excretion in urine above base line in 24 h (expressed in μg)

	Amount injected (mg/kg body wt)			
Agent	*10 mg*	*20 mg*	*40 mg*	*80 mg*
Pen	16.4±7.8(4)	20.2±4.6(5)	32.9±4.0(8)	42.0±2.1(5)
Trien	16.7±3.7(8)	19.7±2.6(10)	32.8±3.9(8)	32.1±2.1(9)

Numbers of samples shown in parentheses

The urine was collected in copper-free containers kept on ice for 24 h. Urine samples were collected from the same rat under the same conditions, both before and after injection. Copper analysis was done on each urine sample. The results as given in Table 4 are expressed as μg of copper excreted in excess of the base line. The average base line excretion for these rats was $8.37 \pm 1.46\ \mu g$/day.

Parallel experiments are presently being conducted with copper-loaded rats (one ml of a 1% copper sulphate solution per kg body weight being injected per day for a five-day period). To date, the results show both pen and trien to be equally effective in removing the copper.

Conclusions

Copper-trien is a small molecular complex, whereas the copper-pen complex is polymeric in nature. Trien effectively competes for copper bound to albumin, whereas pen performs relatively poorly. Similar results were observed in the whole human serum. Pen mobilizes copper in the liver, while trien appears to be unable to

enter the liver. Both agents mobilize copper in kidney and both effectively remove copper through urinary excretion. Our results are in accord with the conclusions of Walshe (1973) that these two ligands act on different pools of copper in patients with Wilson's disease. However, our results are in variance with the conclusion that trien mobilizes copper from tissue while pen binds plasma copper rendering it available for filtration at the glomerulus. The possible causes for this discrepancy may be that pure trien could not be obtained by the previous method (Dixon *et al.* 1972), and that Walshe used very much higher concentrations of pen and trien in his plasma filtration studies (molar ratios of ligand:copper–albumin of several thousand, explain his observation of a 72.9% mobilization of copper). However, the different mode of action of these two agents in mobilizing copper as originally proposed by Walshe concurs with our conclusion.

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DISCUSSION

Dr H E Amos (*Carshalton*): Is it established that trien works only in the serum compartment and nowhere else; or does it have any effect in other places?

Dr Sarkar: Perhaps I might summarize our experiments with trien in human serum. When the copper to albumin ratio was 0.25:1, at a very low trien concentration we found that most of the copper was in the copper-trien, small molecular weight form. In other words, the trien was mobilizing copper in the human serum situation. We did not find that with penicillamine.

Dr Amos: I think I missed the significance of that in the slide in which the difference was shown between penicillamine and trien in organs. There were essentially different results in the liver, but about the same in urinary excretion.

Dr Sarkar: Yes, that is correct.

Dr Amos: How sure is Dr Sarkar of his molecular weight data which are all based on Sephadex elutions?

Dr Sarkar: The polymeric form of the copper penicillamine complex has a molecular weight between 27 000 and 30 000. This value is an approximate one since it is obtained by Sephadex gel filtration technique using proteins of different molecular weights, e.g. albumin, ribonuclease, insulin, &c, and then obtaining a plot of their molecular weight versus elution volume. The copper penicillamine complex appears in an elution volume that falls in the molecular weight range of 27 000–30 000.