Zinc transport proteins in plasma

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1. Both albumin and transferrin have been suggested as carriers of zinc in plasma. Their relative importance in Zn transport was therefore investigated as a preliminary to a study of the rates of passage of Zn through plasma.

2. The apparent log stability constant for Zn binding to human apotransferrin at pH 7·4 was estimated to be approximately 5·9 which is substantially lower than previous reports of 7·0 for the corresponding value for Zn binding to albumin (Giroux & Henkin, 1972).

3. When the relative abilities of human albumin and apotransferrin to compete for Zn with low-molecular-weight chelators were compared at the same relative concentrations of these proteins as are found in plasma, albumin retained substantially more Zn than transferrin.

4. It seems likely that albumin acts as the major transport protein for Zn in plasma of most species, Zn also being present firmly bound to α_2-macroglobulin.

5. In porcine plasma or serum however, there were three major Zn-binding proteins, two of which were probably albumin and α_2-macroglobulin. The nature of the third component remains unknown but it appeared to have a molecular weight of between 100000–140000, it was precipitated by 2·2 M-ammonium sulphate and by 150 g polyethylene glycol/l.

6. There were no significant differences in Zn distribution in plasma of porcine blood obtained from the aorta, the posterior vena cava or from the hepatic portal vein but use of heparin as an anticoagulant altered the normal pattern of distribution of Zn in plasma.

In the plasma of most species approximately two thirds of the zinc present is bound to albumin and one third to α_2-macroglobulin (Himmelhoch *et al.* 1966; Parisi & Vallee, 1970; Giroux, 1975). In addition a small proportion is present in low-molecular-weight form. However, various reports had suggested that transferrin rather than albumin might be the physiologically-important Zn carrier (Boyett & Sullivan, 1970; Evans & Winter, 1975). In man, both the total concentration of Zn in plasma and its distribution between the major protein complexes have been shown to vary following a range of imposed stresses (Vikbladh, 1951; Hallbook & Hedelin, 1977). Similar changes have been observed in experimental animals (Pekarek & Beisel, 1969; Corrigall *et al.* 1976; Whitenack *et al.* 1978). The work reported here was part of a programme to study the stress-induced alterations in Zn flux which accompany these changes in plasma Zn concentration and to investigate their physiological significance (Chesters & Will, 1981). The present paper reports investigations of the nature of the transport form of Zn in plasma which were carried out before the studies of Zn flux mentioned previously.

EXPERIMENTAL

Materials

Human apotransferrin, human and porcine albumin (fraction V), N–(2–hydroxyethyl)-iminodiacetic acid, iminodiacetic acid, nitrilotriacetic acid and polyethylene glycol 6000 were obtained from Sigma Chemical Co., London; histidine from Cambrian Chemicals Ltd, Croydon, Surrey; and heparin from Evans Medical Ltd, Liverpool. All radioisotopes were supplied by the Radiochemical Centre, Amersham, Bucks. Cellulose acetate strips (Sephraphore III) were purchased from Gelman Hawksley Ltd, Northampton.

Porcine albumin was purified before use by continuous flow electrophoresis in 0·1 M-Tris-acetate buffer pH 8·3 using the apparatus described by Hannig (1961). The major

albumin containing fractions were pooled, dialysed three times against 10 vol. distilled water and freeze dried. Small samples of the purified albumin were then iodinated by the chloramine T method of Bolton (1977).

Blood samples

Porcine blood was obtained from gilts weighing 30–50 kg which had been fitted with catheters terminating in the abdominal aorta or in some instances in the posterior vena cava or in the hepatic portal vein (Scott & McIntosh, 1975). Other samples were obtained from the jugular of sheep, cattle and red deer, the heart of rats or from the veins of human volunteers.

Plasma was obtained by centrifugation of blood to which had been added heparin (100 U/ml final concentration) or sodium fluoride (0·24 M) as anticoagulants. Samples for serum were allowed to clot overnight at room temperature before centrifugation.

Estimation of the apparent stability constant of Zn-transferrin

Binding of Zn to human transferrin results in an alteration in the ultra-violet absorption of the protein. The difference spectrum of Zn-transferrin v. apo-transferrin showed absorption maxima at 245 and 295 nm and a minimum at 275 nm. These values are in close agreement with those found by Nagy & Lehrer (1972) and Tan & Woodworth (1969) and attributed by them to ionization of a tyrosine residue during binding of Zn. Changes in absorption at 245 nm were therefore used to monitor the competition for Zn between transferrin and a range of chelators of known affinity for Zn.

Human apotransferrin (4 mg) dissoved in 2·5 ml of 0·02 M-sodium bicarbonate containing 0·15 M-sodium chloride (buffer A) was placed in a 10 mm path-length cuvette and gassed with air–carbon dioxide (95:5, v/v) to pH 7·4. Serial additions of Zn (5 μl 3 mM-$ZnSO_4 \cdot 7H_2O$) were made to the solution which was re-gassed and the increase in absorbance at 245 nm ($+\Delta A_{245}$) was read against apo-transferrin after each addition. After five additions the transferrin approached saturation and serial additions (5 μl) were then made of a competing chelator and $-\Delta A_{245}$ was determined. All measurements were carried out at 37°.

Using the information obtained from the initial additions of Zn, ΔA_{245} was plotted against the total Zn present and this relationship was used to determine the ionic plus transferrin-bound Zn after each addition of the chelator. The amount of Zn in either mono- or di-complexes with the chelator was then obtained by difference. From a knowledge of the stability constants of these complexes and the equations below, the concentration of ionic Zn^{2+} was calculated for each concentration of the chelator (L).

$$[Zn^{2+}] = \frac{[ZnL]}{K_s[L]} = \frac{[ZnL_2]}{\beta[L]^2}$$
$$[L] = [\Sigma L] - [ZnL] - 2[ZnL_2]$$

$[ZnL] + [ZnL_2]$ is the total concentration of chelator-bound Zn, ΣL is the total amount of chelator added, L is the concentration of free chelator and K_s and β are apparent stability constants of the complexes at pH 7·4. Since the same concentration of ionic Zn^{2+} was in equilibrium with the Zn bound to transferrin the following relationships could be used to estimate the apparent stability constant of the Zn-binding sites of transferrin (K_{Tf}).

$$K_{Tf} = \frac{[ZnTf]}{[Zn^{2+}][Tf]} = \frac{[\Sigma Zn] - [Zn^{2+}]}{[Zn^{2+}]([\Sigma Tf] - [ZnTf])} = \frac{[\Sigma Zn] - [Zn^{2+}]}{[Zn^{2+}]([\Sigma Tf] - [\Sigma Zn] - [Zn^{2+}])}$$

where ΣZn is the total Zn either free or bound to transferrin and ΣTf is the total concentration of binding sites on transferrin either free or complexed with Zn. A mean ($\pm$ SE)

was then calculated from the individual estimates of K_{Tf} obtained after each addition of the chelator.

Where available, apparent stability constants for the chelators at pH 7·4 were taken from Giroux & Henkin (1972) to facilitate comparison of results. In other cases they were calculated from the values of log stability constants and pKa given by Sillen & Martell (1964, 1971). Human serum transferrin was assumed to have a molecular weight of 77000 (Mann *et al.* 1970) and to possess two identical Zn-binding sites per molecule (Nagy & Lehrer, 1972).

Relative Zn-binding affinities of albumin and transferrin

Human apotransferrin (4 mg) or human albumin (40 mg) was dissolved in 4 ml buffer A in a test tube. To this was added 30 μl ^{65}Zn containing 0·82 μg Zn (1·35 μCi/μg) and an appropriate amount of a chelator dissolved in less than 50 μl water. After gassing with air–carbon dioxide (95:5, v/v) to give a pH of 7·4 the mixture was left at 37° for 45 min to equilibrate and then ultrafiltered in situ at 37° by suction through a 'Pellicon' membrane with 10000 nominal-molecular-weight limit (Immersible Molecular Separator; Millipore UK Ltd, London). Since these filters were found to have a significant affinity for Zn the filtrate was returned to the protein residue and the mixture re-gassed and re-filtered until all components were in equilibrium with a common concentration of ionic Zn^{2+}. The relative affinities of the low-molecular-weight chelator and the protein for Zn were then assessed by expressing the filterable Zn as a proportion of the sum of the filterable and protein-bound Zn.

Gel filtration

Samples of serum or plasma (5 ml) were supplemented with 0·15 μCi ^{65}Zn (0·8–1·4 μCi/μg) and with approximately 0·03 μCi ^{125}I-labelled porcine albumin for subsequent identification of the distribution of albumin between fractions. The samples were left at room temperature for 5 h and were then loaded onto an 800 × 26 mm column of Sephadex G–100. The material was eluted with 0·02 M-Tris-HCl, pH 7·8, containing 0·15 M sodium chloride and collected in 5 ml fractions. These were counted for ^{125}I and ^{65}Zn in a gamma counter; their absorbance was measured at 280 nm and then after acidification with hydrochloric acid they were analysed for Zn and copper by atomic absorption (Chesters & Will, 1978). In a few instances, ^{59}Fe (0·02 μg and 0·25 μCi) was added to plasma along with the ^{65}Zn and ^{125}I-labelled albumin, the ^{59}Fe serving as a label for transferrin. Even in the presence of all three isotopes differential counting allowed satisfactory estimation of the quantities of each of the individual isotopes present. However Sephadex G-100 was found to have an appreciable affinity for Zn which resulted in substantial exchange between Zn bound to the column matrix and that in the sample. To minimize this problem, samples of radioactive plasma were initially passed through the column to waste until the over-all specific activity of the Zn in the eluate was within 5% of that in the plasma.

In certain other experiments, instead of using plasma, the initial samples were prepared by dissolving individual proteins, either albumin (40 mg/ml) or apotransferrin (4 mg/ml) or a mixture of the two, in buffer A. These were then supplemented with radioactive materials and chromatographed as described for plasma.

RESULTS

The binding of Zn to transferrin as assessed by ΔA_{245} indicated that the apparent log stability constant (log K_s) of the Zn-binding sites was approximately 5·9 (Table 1). When the balance between free and bound Zn was determined by ultrafiltration of a solution of transferrin and Zn, the calculated value of log K_s was 5·93. The Zn-binding affinities of albumin and transferrin were then compared at physiologically relevant concentrations and the proportions of Zn rendered ultrafiltrable by addition of chelator was always substantially higher with transferrin than with albumin (Table 2).

Table 1. *Apparent log stability constant of the zinc-binding sites of transferrin determined from changes in absorption at 245 nm when Zn was bound to transferrin in the presence of varying concentrations of competing chelators*

(Mean values with their standard errors of five estimates obtained on each of two occasions)

	Apparent log stability constant of Zn-transferrin	
Chelator	Mean	SE
Glycine	5·88	0·02
Histidine	5·85	0·03
Iminodiacetic acid	5·92	0·05

Table 2. *Effect of addition of a competing low-molecular-weight chelator on the percentage of Zn which was ultrafilterable in solutions of albumin or transferrin*

Percentage of Zn ultrafilterable (%)

Chelator	(μM)	Albumin (10 mg/ml)	Transferrin (1 mg/ml)
Histidine	200	5·2	39·4
N-(2-hydroxyethyl) iminodiacetic acid	10	17·1	76·1
Iminodiacetic acid	200	11·8	62·8
Nitrilotriacetic acid	5	18·5	85·6

Chromatography of mixed preparations of ^{65}Zn, ^{125}I-labelled albumin and transferrin bearing trace quantities of ^{59}Fe on Sephadex G-100 failed to separate the proteins sufficiently to allow estimation of the proportions of Zn bound to albumin and transferrin. When chromatographed individually ^{65}Zn-albumin produced coincident peaks of ^{65}Zn and absorbance at 280 nm whereas ^{65}Zn applied as Zn-transferrin became bound to the column and eluted only slowly. None of the ^{65}Zn eluted along with the transferrin.

Gel filtration of porcine plasma on Sephadex G-100 produced a Zn-distribution profile (Fig. 1a) which differed markedly from that previously reported for human serum and confirmed in the present study (Fig. 1b). The proportion of Zn present in the peak at fraction no. 31 was greatly reduced and a third Zn-binding component was present centred on fraction no. 26. The possible presence in other species of the Zn-binding component at fraction no. 26 was investigated but chromatography of serum or fluoride-treated plasma from sheep, cattle, deer and rats as well as man failed to demonstrate any significant concentration of Zn at fraction no. 26. Within the species investigated this component was therefore of major importance only in the pig. $(NH_4)_2SO_4$ fractionation of porcine serum followed by gel chromatography showed that the Zn-binding material at fraction no. 26 was precipitated by 2·2 M-$(NH_4)_2SO_4$ and was absent from the albumin fraction remaining soluble at this salt concentration. Similarly this unidentified Zn-binding compound was precipitated by treatment of serum with polyethylene glycol 6000 at 150 g/l (Chesters & Will, 1981) but was absent from the supernatant fraction which contained albumin. When the material found in fractions 25–39 was concentrated by ultra-filtration and re-applied to the column, there was a substantial loss of Zn and ^{65}Zn from fraction no. 26 and the only residual Zn in the high-molecular-weight region co-chromatographed with albumin.
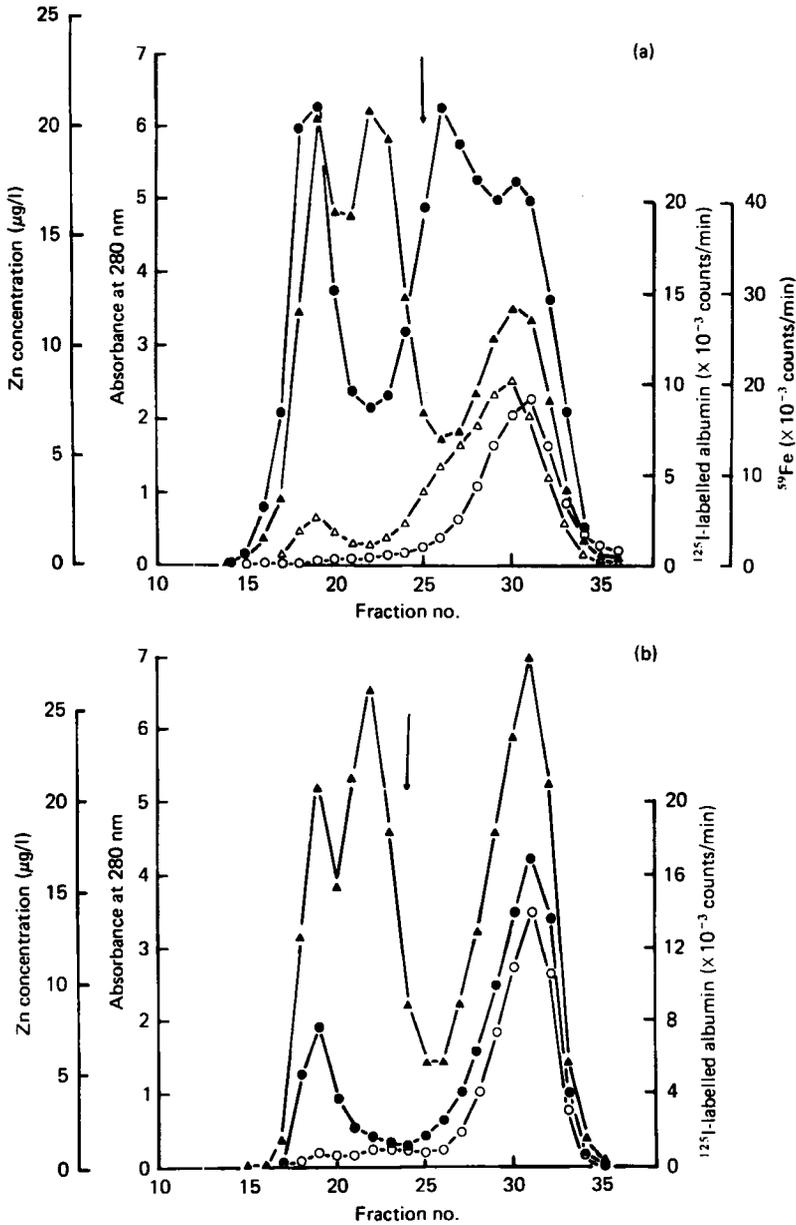
Fig. 1. Chromatography of (a) porcine plasma and (b) human serum on Sephadex G-100. Zn concentration (●——●), absorbance at 280 nm (▲——▲), ^{125}I-labelled albumin (○——○), ^{59}Fe (△——△). ↓, Position of the peak concentration of caeruloplasmin.

Plasma for the previous investigations was obtained from pigs with arterial catheters. However, comparable samples were also obtained from both venous and hepatic portal blood of pigs. When these samples were chromatographed on Sephadex G-100, no differences were found in the distribution of Zn in plasma from any of these sources. Furthermore, the pattern of distribution of Zn between fractions was similar when either porcine serum or fluoride-treated plasma was applied to the column. In contrast, with
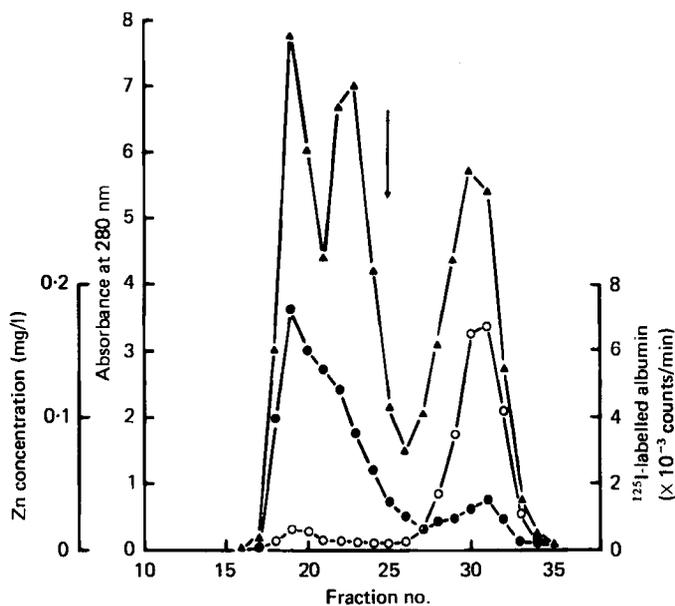
Fig. 2. Chromatography of heparinized porcine plasma on Sephadex G-100. Zn concentration (●——●), absorbance at 280 nm (▲——▲), ^{125}I-labelled albumin (○——○). ↓, Position of the peak concentration of caeruloplasmin. Heparin was present at 100 I.U./ml in the sample applied to the column.

plasma which had been heparinized the distribution of Zn was markedly altered by a major shift of Zn into the region of fraction nos. 21–22, reduction in Zn at fraction no. 31 and disappearance of that at fraction no. 26 (Fig. 2).

DISCUSSION

As early as 1951, Zn in human serum was shown to be largely bound to protein, two-thirds being loosely attached to one protein and one-third more strongly bound to another (Vikbladh, 1951). Subsequent studies have shown that these proteins were albumin and α_2-macroglobulin respectively (Himmelhoch et al. 1966; Parisi & Vallee, 1970; Giroux, 1975). However, Boyett & Sullivan (1970) reported Zn to be present also in a transferrin fraction following electrophoretic separation of plasma proteins. More recently, Evans & Winter (1975) reported that 70% of newly absorbed ^{65}Zn in portal plasma of rats was bound to transferrin and that even in peripheral plasma a substantial proportion was still present on this protein. Evans (1976) also showed that with a plasma membrane preparation from duodenal mucosa which had been labelled with ^{65}Zn in vivo, addition of apo-transferrin was more effective in removing ^{65}Zn than was albumin.

Taken together these previous studies suggested an important role for transferrin in Zn transport through plasma. There are however several reasons for questioning this suggestion. A re-examination of the paper of Boyett & Sullivan (1970) indicated that in contrast to the authors' summary, none of the four Zn-binding factors which they demonstrated co-electrophoresed with transferrin. Furthermore, in the investigations of Zn in portal blood by Evans & Winter (1975), heparinised plasma was used and the transferrin fraction was found to chromatograph on Sephadex G-150 well in advance of albumin. In contrast the present studies with both human and porcine proteins separated on Sephadex G-100 (Fig. 1) and studies of rat plasma by Smith et al. (1978) using Sephadex G-200 have indicated that transferrin and albumin do not separate under chromatographic conditions

similar to those used by Evans & Winter (1975). It is possibly significant that heparinized plasma was used for the later investigations since use of porcine plasma which had been heparinized (Fig. 2) resulted in artifactual binding of Zn to a component with an elution characteristic on Sephadex similar to the fraction described by Evans & Winter (1975) as Zn-transferrin. Also in contrast to the latter studies, no differences were detected in the pattern of Zn-binding by porcine plasma of arterial, venous or hepatic-portal origin.

The present studies have indicated a log K_s for the Zn-binding sites of transferrin of approximately 5·9. Under similar conditions, Giroux & Henkin (1972) found that the log K_s of the 1:1 Zn-albumin complex was approximately 7·0. Since albumin is generally present in plasma in tenfold molar excess over transferrin, it seems unlikely that an appreciable proportion of Zn could remain on transferrin in plasma. The chelator competition studies reported here and those published recently by Charlwood (1979) further confirm that the equilibrium of Zn between transferrin and albumin is such that very little Zn will be present on transferrin when the two proteins are present together in the proportions found in plasma. Finally perfusion studies with rat intestinal preparations have shown that addition of albumin to the vascular perfusion medium was highly effective in mediating the uptake of Zn into the medium and that saturation of serum in the perfusate with iron failed to inhibit Zn uptake (Smith *et al.* 1978). The present evidence suggests therefore that in most species albumin is the major transport protein for Zn in plasma, that one third of the Zn is bound to α_2-macroglobulin and that Zn-binding to transferrin is quantitatively insignificant.

The present studies have shown that porcine plasma in contrast to that of other species contains another component which binds about one-third of the Zn present, this change resulting almost entirely from a reduction in the proportion of Zn on albumin. The nature of this extra component (fraction no. 26) which so far appears to be restricted to porcine plasma remains unknown. Its behaviour on Sepahadex G-100 suggests a molecule of 100000–140000 molecular weight and it precipitated with the globulins when plasma was fractionated either with $(NH_4)_2SO_4$ or with polyethylene glycol. It eluted appreciably sooner than transferrin on Sephadex G-100 but attempts to purify the fraction obtained from gel chromatography by rechromatography even on the same column resulted in loss of both Zn and ^{65}Zn from the region of fraction no. 26. The same applied even when anaerobic conditions were used to avoid the possiblity of destruction of oxygen-sensitive ligand groups.

There have been two previous reports of metal-binding proteins from plasma with properties similar to the material in fraction no. 26. Himmelhoch *et al.* (1966) found a Zn-binding protein in human serum which was precipitated with 2·2 M $(NH_4)_2SO_4$, had a molecular weight of approximately 120000 and migrated with the albumins on electrophoresis. Furthermore, this protein was also unstable after separation by gel chromatography. Strangely no such protein has since been reported in human serum. More recently Nandedkar *et al.* (1974) isolated from rabbit plasma a Co-binding protein with a molecular weight of 120000. This was immunoelectrophoretically identical with albumin and was postulated to be a dimer of it but the authors were unable to generate the monomer from it by reduction. No such fraction has been seen in the present experiments when purified porcine albumin has been chromatographed on Sephadex G-100 in the presence of Zn. The nature of the material in fraction no. 26 requires further investigation.

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