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Identification of Albumin as the Plasma Carrier for Zinc Absorption by Perfused Rat Intestine

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The isolated vascularly perfused rat intestine exhibits an obligatory need for a protein carrier in order to absorb zinc. Therefore this system is ideal for use as a model to identify the plasma carrier during zinc absorption. Affinity chromatography on Blue Sepharose CL-6B was employed to separate the major serum zinc-binding proteins in the portal effluent of the perfused intestine. It was found that 94% of newly absorbed ^{65}Zn was transported in the portal serum-containing perfusate as an albumin–^{65}Zn complex. The identity of albumin as the plasma carrier was confirmed by polyacrylamide-slab-gel electrophoresis. This evidence suggests that albumin is the plasma protein that is involved in removal of zinc from intestinal-mucosal cells and subsequent transport of the metal in portal blood to the liver.

The mechanism of the zinc-absorption process has not been fully elucidated. However, it is clear that once dietary zinc is taken up from the lumen into intestinal-mucosal cells it is either integrated into intracellular zinc pool(s) or is rapidly (within minutes) transported across the basolateral membrane to the plasma compartment (Davies, 1973; Smith *et al.*, 1978; Cousins, 1979*a*,*b*).

In plasma, zinc has been found to be associated with albumin, α_2-macroglobulin, transferrin and amino acids (Vikbladh, 1951; Parisi & Vallee, 1970; Boyett & Sullivan, 1970; Prasad & Oberleas, 1970). However, it has repeatedly been shown that albumin is the major plasma protein with which zinc is associated during systemic transport. In contrast, it has been suggested that newly absorbed zinc in portal plasma is transported from the intestine as a zinc–transferrin complex (Evans & Winter, 1975; Evans, 1976). Recently we have been using an intestinal-perfusion technique, in which the lumen and vascular supply of the rat small intestine are simultaneously perfused, to examine the characteristics and regulatory aspects of zinc absorption (Smith *et al.*, 1978). This isolated system offers the unique opportunity to examine the vascular carrier in greater detail than is possible in intact-animal experiments. Moreover, we have utilized affinity chromatography on Blue Sepharose CL-6B (Travis & Pannell, 1973; Wille, 1976) to fractionate the serum proteins in the vascular effluent of the perfused intestine. This method of serum-protein fractionation has eliminated the need for ion-exchange-chromatographic procedures, which may not be compatible with the metal-binding properties of the carrier protein(s) involved. In the present paper, we demonstrate that newly absorbed zinc in the portal effluent of the isolated perfused rat intestine is transported by albumin.

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Materials and Methods

Animals

Male rats (Sprague–Dawley strain, 150–200g; Charles River, Wilmington, MA, U.S.A.) were fed on a standard commercial diet (Ralston Purina Co., St. Louis, MO, U.S.A.) and tap water *ad libitum*.

Reagents and materials

All chemicals were reagent grade. Buffers and reagents were prepared in water that was glass-distilled and then deionized (Ultrapure-Barnstead Co., Framingham, MA, U.S.A.). ^{65}Zn (carrier-free) and $^{59}FeCl_3$ (20mCi/mg) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. M-199 tissue-culture medium and high-molecular-weight dextran (mol.wt. 70000–90000) were from GIBCO, Grand Island, NY, U.S.A. Blue Sepharose CL-6B, Sephadex G-25 and G-200 and DEAE-Sephadex (A-50) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Dexamethasone and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was obtained from Research Organics Inc., Cleveland, OH, U.S.A. Ultrafiltration membranes

(UM-20) were obtained from Amicon Corp., Lexington, MA, U.S.A.

Intestinal perfusion

All rats were deprived of food for 18–24h before surgery. The intestinal isolation and perfusion procedure is described in detail elsewhere (Smith *et al.*, 1978). The lumen perfusate was 10ml of modified M-199 tissue-culture medium. The zinc content of this medium was elevated to 0.75mM with $ZnSO_4$; 25μCi of carrier-free ^{65}Zn was added to each 10ml portion 1h before use. In some perfusions 25μCi of $^{59}FeCl_3$ was added to the perfusate in place of ^{65}Zn.

The standard vascular perfusate was composed of Krebs–Ringer bicarbonate buffer containing 5% rat serum, 4.7% high-molecular-weight dextran, 0.1% glucose and dexamethasone (25μg/100ml) (Smith *et al.*, 1978). After the 1h perfusion period, samples of the vascular perfusate, which were collected as fractions (7.0ml), were counted for ^{65}Zn or ^{59}Fe radioactivity with a Beckman 4000 gamma spectrometer [7.6cm (3in) crystal; Beckman Instruments, Fullerton, CA, U.S.A.].

The composition of the vascular perfusate was modified to examine the influence of specific components on zinc absorption. In some experiments normal rat serum was replaced with serum that had first been dialysed and then equilibrated with excess iron by the method of Ramsay (1953), in order to saturate transferrin with iron. In other experiments, serum was first dialysed against 0.9% NaCl in 50mM-Hepes buffer (pH7.4) for 48h before the vascular perfusate was prepared. Alternatively, rat serum albumin, which was prepared by Sephadex G-200 gel-filtration chromatography followed by DEAE-Sephadex ion-exchange chromatography (Killander, 1964), was substituted for serum in the perfusate in an amount (2.5mg/ml) equivalent to that found in perfusate containing 5% serum. A final series of perfusions was conducted with serum-free perfusate made iso-osmotic by adjusting the amount of high-molecular-weight dextran initially added to the perfusate solution.

Analysis of the vascular perfusate by Blue Sepharose chromatography

Portions (4ml) of those fractions of vascular effluent with the most radioactivity, thus indicating maximum absorption, were desalted by passage through a Sephadex G-25 column (1.5cm×30cm), equilibrated with 50mM-Tris/HCl/50mM-KCl, pH7.0 (buffer A). Fractions containing material excluded by the gel were analysed for ^{65}Zn or ^{59}Fe content, pooled and applied to a column (1.5cm×30cm) of Blue Sepharose CL-6B that was prepared and equilibrated with buffer A. Blue Sepharose is an affinity gel that selectively removes albumin, while transferrin and α_2-macroglobulin pass through the column. The column was washed with 50ml of buffer A. Next, bound albumin was eluted by passing 50ml of 50mM-Tris/HCl/1.5M-KCl, pH7.0 (buffer B) through the column. The ^{65}Zn or ^{59}Fe content of all fractions (2.5ml each) was measured as described above.

To verify the identity of albumin as the ^{65}Zn-binding species eluted with buffer B, the ^{65}Zn-containing fractions were pooled, concentrated by ultrafiltration and subjected to electrophoresis on a 10% polyacrylamide slab gel (6% stacking gel), at 120V for 3h, by the method of Laemmli (1970). Coomassie Brilliant Blue-stained bands were then scanned at 563nm (Beckman model 35 spectrophotometer). Commercially prepared bovine serum albumin was used as the standard protein marker.

DEAE-Sephadex chromatography of serum, albumin and vascular perfusate

Rat serum (1.0ml) was mixed with 1μCi of $^{59}FeCl_3$, and left at 4°C for 4h. The mixture was then passed through a column (2.5cm×60cm) of Sephadex G-200 equilibrated at 4°C with 100mM-Tris/HCl 1.0M-NaCl, pH7.4. Albumin and transferrin are not resolved by this chromatographic step (Killander, 1964). Fractions containing these proteins were pooled and dialysed overnight at 4°C against 50mM-Tris/HCl, pH7.0. The dialysed sample was applied to a column (1.5cm×30cm) of DEAE-Sephadex A-50. The proteins were eluted with a linear gradient (250ml) of 50–500mM-Tris/HCl, pH7.4.

In another experiment 10mg of rat albumin (prepared as described above) was dissolved in 50mM-Tris/HCl/50mM-NaCl (pH7.4) and incubated with 1μCi of ^{65}Zn for 2h at 4°C. The mixture was passed through a column (1.5cm×30cm) of Sephadex G-25 (in the same buffer) and the void-volume fractions were collected. These fractions were pooled and subjected to ion-exchange chromatography on DEAE-Sephadex as described above.

In some experiments the portal vein of rats was cannulated 15min after 50μCi of ^{65}Zn was placed in the intestinal lumen. A total of 3.0ml of blood was collected, allowed to clot and then centrifuged at 12000*g* (r_{av}. 8.0cm, 10min) to obtain the serum. The serum was then sequentially fractionated on Sephadex G-200 and DEAE-Sephadex as described above. These fractionation procedures were also used to fractionate the vascular perfusate in one series of experiments. The perfusion was carried out as described in the preceding section, except that 50μCi of ^{65}Zn was used in the lumen perfusate.

Results

The effect on the ^{65}Zn-absorption process in the perfused rat intestine, when modifications were made in the composition of the vascular perfusate, is

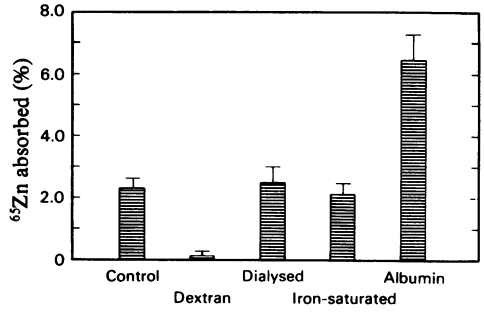

Fig. 1. *Influence of certain modifications to the vascular perfusate on the ^{65}Zn-absorption process*
Vascular perfusions were carried out as described in the Materials and Methods section. Perfusates contained 5% normal rat serum, no serum, 5% dialysed rat serum, 5% dialysed iron-saturated rat serum or rat albumin (2.5mg/ml). Results are cumulative absorption expressed as percentage of dose absorbed during a 1h perfusion period.

illustrated in Fig. 1. Approx. 2% of the available ^{65}Zn in the lumen was transferred to the vascular perfusate when the latter contained 5% rat serum. However, no transfer was observed when serum was not present in the vascular perfusate. These data demonstrate the need for a serum carrier in the vascular supply in order to transport newly absorbed zinc from intestinal-mucosal cells. On the other hand, when purified rat serum albumin alone was added to the perfusate, there was an increase in ^{65}Zn absorption which greatly exceeded the value observed with serum containing the same quantity of albumin. This increase might be explained in part by the fact that during the preparation of albumin by DEAE-Sephadex chromatography zinc is removed from the protein (discussed below).

Dialysed serum had no significant effect on zinc absorption by the perfused rat intestine. Similarly, when the vascular perfusate contained serum to which excess iron had been added in order to saturate the iron-transport protein transferrin, no effect on ^{65}Zn absorption was observed. These perfusion data indirectly suggest that albumin is the carrier protein involved in the transport of newly absorbed zinc.

Affinity chromatography on Blue Sepharose CL-6B was used for the selective removal of albumin from the vascular effluent of the isolated intestine. Perfusates containing ^{59}Fe or ^{65}Zn were taken directly from the portal-vein cannulation and passed through a Sephadex G-25 column for removal of low-molecular-weight substances. The recovery of radioisotope from the column was routinely greater than 95%. The fractions representing the void volume of this column were pooled and placed on a

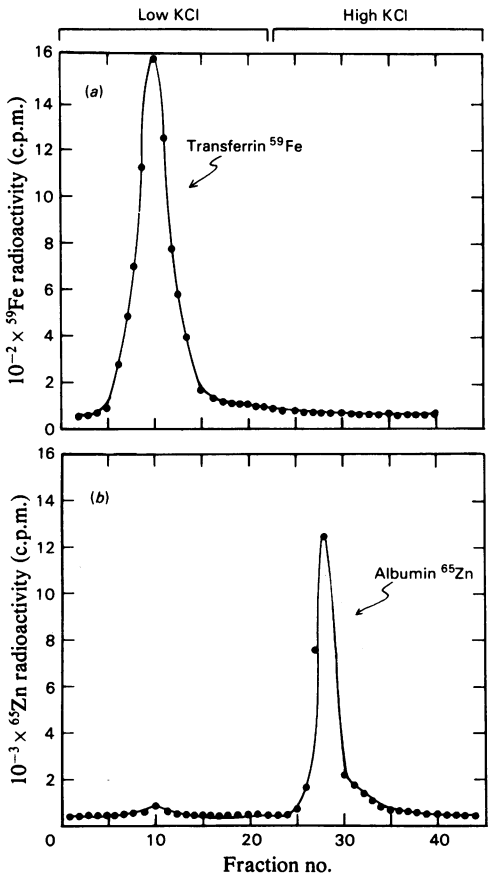

Fig. 2. *Blue Sepharose CL-6B chromatography of vascular perfusates during (a) ^{59}Fe or (b) ^{65}Zn absorption*
Vascular perfusions were carried out as described in the Materials and Methods section. Perfusates containing ^{65}Zn or ^{59}Fe were first desalted by passage through a Sephadex G-25 column (1.5cm×30cm) eluted with 50mM-Tris/HCl, pH7.0. The fractions excluded from this gel were pooled and applied to a column (1.5cm×30cm) of Blue Sepharose CL-6B. After a 50ml wash with 50mM-Tris/HCl/50mM-KCl, pH7.0, the bound material was eluted with 50ml of 50mM-Tris/HCl/1.5M-KCl, pH7.0. Fractions (2.5ml) were collected.

column (1.5cm×30cm) of Blue Sepharose CL-6B, equilibrated with low-KCl buffer (buffer A). Albumin binds to the immobilized dye on the gel matrix, whereas transferrin does not bind and passes through the column. The column was subsequently eluted with high-KCl buffer (buffer B), which removes albumin from binding sites on the gel (Fig. 2). When intestinal perfusions were performed with $^{59}FeCl_3$ in the lumen, virtually all of the ^{59}Fe which appeared

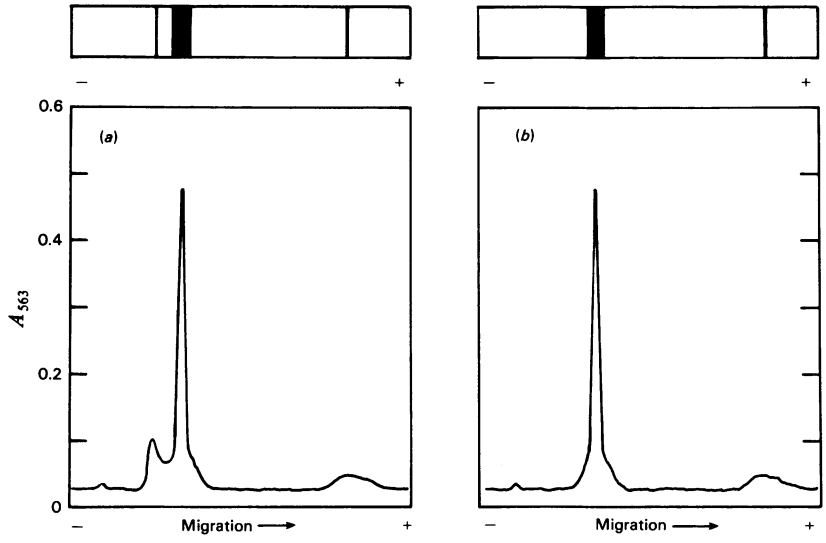

Fig. 3. *Polyacrylamide-slab-gel electrophoresis of ^{65}Zn-containing peak from Blue Sepharose CL-6B*
Electrophoresis of the ^{65}Zn-containing peak was carried out as described in the Materials and Methods section. Coomassie Brilliant Blue-stained gel slabs were scanned at 563nm. (*a*) Vascular perfusate from Blue Sepharose CL-6B chromatography; (*b*) commercially prepared bovine serum albumin.

in the vascular perfusate was eluted with transferrin in the low-KCl buffer. In contrast, when ^{65}Zn was added to the lumen perfusate, 94% of the ^{65}Zn in the vascular perfusate which was applied to the column was eluted from the affinity column with the high-KCl buffer, i.e. with albumin (^{65}Zn recovery > 90%). These data demonstrate the resolving power of the vascular perfusion procedure, since it clearly is able to discriminate between the different carriers for iron and zinc. Polyacrylamide-slab-gel electrophoresis of the ^{65}Zn–albumin peak from Blue Sepharose chromatography yielded one major band, which exhibited the same mobility as commercially prepared albumin (Fig. 3).

A markedly different distribution of ^{65}Zn among the serum proteins was observed when they were fractionated by DEAE-Sephadex ion-exchange chromatography. When serum was removed from the portal vein of intact rats, in which ^{65}Zn was placed in the intestinal lumen and subsequently chromatographed on Sephadex G-200, 95% of the ^{65}Zn applied to the column was recovered with the albumin–transferrin fractions. However, when those fractions were placed on a DEAE-Sephadex column, ^{65}Zn was eluted at the same concentration of chloride, as is transferrin (Fig. 4*a*). Moreover, only 18% of the ^{65}Zn applied to this column was recovered; 70% of this was as transferrin.

Similarly when ^{65}Zn-labelled serum proteins in the vascular perfusate were subjected to the combination of gel filtration and ion-exchange chromatography substantial losses were noted during the latter (Fig. 4*b*). In this case total recovery from the DEAE-Sephadex column was 30%, of which 81% was recovered with the transferrin fractions.

The migration of ^{65}Zn-labelled albumin and ^{59}Fe-labelled serum is shown in Fig. 5. It is clear that ^{65}Zn does not migrate to any appreciable amount with albumin during DEAE-Sephadex chromatography. Since the ^{65}Zn–albumin complex was first passed through Sephadex G-25 to remove free ^{65}Zn, it appears that DEAE-Sephadex chromatography alters the conditions necessary to maintain zinc and albumin as a complex. ^{59}Fe-labelled serum proteins were eluted to show the position of transferrin (Fig. 5*a*). A comparison of the two profiles shows that a large portion of the ^{65}Zn from the ^{65}Zn–albumin complex is eluted at a concentration similar to that required for transferrin elution (Fig. 5*b*).

Discussion

The experiments described here provide direct evidence that zinc is transported from the intestine as a ^{65}Zn–albumin complex. These results are in agreement with the widely held hypothesis that circulating zinc is transported mainly by albumin (Boyett & Sullivan, 1970; Prasad & Oberleas, 1970). However, our observations are in direct contrast with other data (Evans & Winter, 1975; Evans, 1976), which suggest

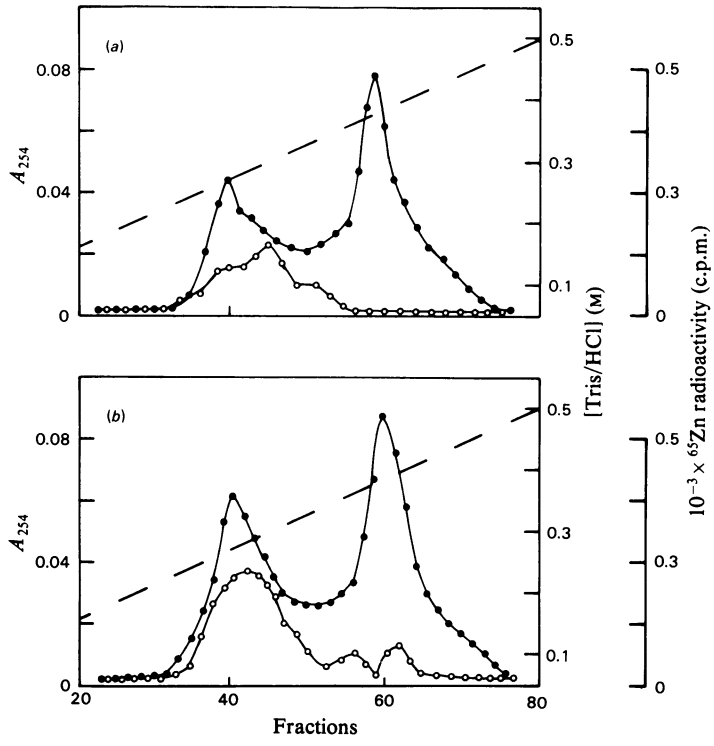

Fig. 4. *Ion-exchange chromatography of portal rat serum and vascular perfusate*

Portal serum (*a*) and vascular perfusate (*b*) were collected as described in the Materials and Methods section. Samples of either serum or perfusate were first fractionated on Sephadex G-200. Transferrin and albumin were eluted as a single protein peak. These fractions were pooled and then ion-exchange chromatography was carried out on a column of DEAE-Sephadex (1.5cm×30cm), with a linear gradient (— —; 50–500mM-Tris/HCl, pH7.0). Fractions were analysed for A_{254} (●) and ^{65}Zn (○). Fraction size was 3.0ml.

that transferrin is the carrier of newly absorbed zinc in the portal plasma. The reason for this discrepancy may lie in the nature of zinc–albumin binding and the chromatographic methodology used. The loose association between zinc and albumin does not appear to withstand DEAE-Sephadex ion-exchange chromatography, the standard technique used for separation of transferrin and albumin. We found that losses of zinc from albumin were extensive (>80%) when ^{65}Zn–albumin complexes were subjected to DEAE-Sephadex chromatography (Fig. 4). These observations may explain the results obtained by Evans (1976), which were interpreted as evidence for an association of ^{65}Zn with transferrin in the portal plasma.

In the present experiments 90% of the ^{65}Zn applied to the Blue Sepharose columns was recovered in the high-KCl-buffer fractions as an albumin–^{65}Zn complex. This affinity-chromatography technique appears to provide a separation method that is applicable to detailed investigations on the role of albumin in zinc transport. It is a rapid quantitative alternative to DEAE-Sephadex ion-exchange chromatography.

The role of albumin in zinc transport in portal plasma is supported on the basis of the relative concentrations of albumin and transferrin in plasma. These proteins account for approx. 55 and 3% of the total protein content of plasma respectively (Boyett & Sullivan, 1970; Prasad & Oberleas, 1970). In this regard Evans (1976) has shown *in vitro* that apotransferrin would bind twice as much ^{65}Zn from labelled basolateral membranes of intestinal cells as would metal-free albumin. Nevertheless, considering the 18-fold higher concentration of albumin relative to transferrin in plasma, more than 85% of the plasma zinc would be predicted to be carried by albumin, although zinc is more tightly bound to transferrin.

Our observation that purified albumin can transport more zinc from the perfused intestine, compared with serum with a comparable amount of albumin, supports a zinc-transport role for this plasma protein.

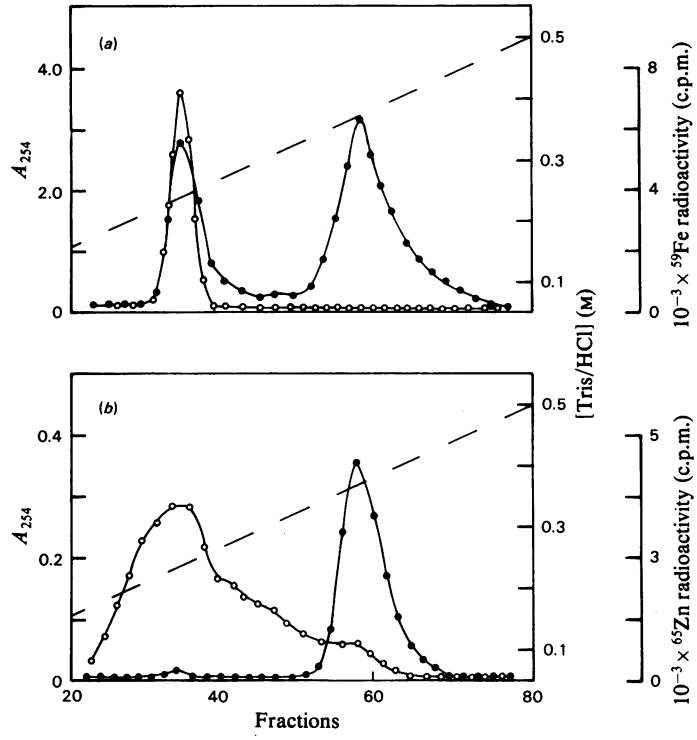

Fig. 5. *Ion-exchange chromatography of rat serum and purified rat albumin*

Rat serum was labelled with ^{59}Fe and a purified rat albumin–^{65}Zn complex was prepared as described in the Materials and Methods section. (*a*) The ^{59}Fe-labelled serum was first passed through a Sephadex G-200 column. ^{59}Fe-labelled transferrin and albumin were eluted as a single peak. Those fractions were pooled and dialysed overnight against 50mM-Tris/HCl, pH7.0. (*b*) The ^{65}Zn–albumin complex was first passed through a column of Sephadex G-25. DEAE-Sephadex ion-exchange chromatography was carried out on both samples on a 1.5cm×30cm column with a linear gradient — —; 50–500mM-Tris/HCl, pH7.0). All fractions were analysed for A_{254} (●) and ^{59}Fe or ^{65}Zn (○). Fraction size was 3.0ml.

Since this rat albumin sample was prepared by DEAE-Sephadex chromatography, appreciable losses of zinc would be expected. This in turn would increase the number of zinc-binding sites available in the vascular perfusate. The relationship of these zinc-binding sites to absorption has important clinical implications. For example, the decrease in zinc status associated with liver dysfunction and hypoalbuminaemia (Underwood, 1977) could be explained in part on the basis of a reduction in absorption. In addition, the data presented above demonstrating the lack of absorption when albumin is deleted from the vascular perfusate suggests that absorption may be diminished in patients maintained on extensively diluted blood plasma.

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