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Transferrin Function in Zinc Absorption and Transport[1] (39305)

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In a recent publication, Evans *et al.* (1) proposed a hypothetical mechanism for zinc absorption based on experimental data obtained both *in vivo* and *in vitro*. In this hypothetical mechanism, the authors suggested that zinc is bound to a low molecular weight binding factor and transported through the intestinal cells to receptor sites on the basolateral plasma membrane where the zinc is subsequently transferred to albumin. Albumin was suggested as the zinc-transport protein in blood since: (a) the major fraction of zinc in venous plasma is bound to albumin and (b) metal-free albumin, obtained by treatment of the protein with ethylenediaminetetraacetic acid (EDTA), removed ^{65}Zn from labeled basolateral plasma membranes. Although this author still supports the general concepts of the original hypothesis, recent evidence obtained in our laboratory (2) indicates that transferrin, rather than albumin, transports zinc through the portal blood to the liver. The experiments outlined below describe a repetition of the previously reported *in vivo* data (2). In addition, *in vitro* experiments are described in which the results demonstrate that transferrin removes zinc from the intestinal basolateral plasma membrane more effectively than does albumin.

Materials and methods. Adult, male Sprague-Dawley rats were used for the experiments. To reexamine zinc binding in portal blood, the rats were anesthetized with sodium pentobarbital, a midline incision was made, and carrier-free ^{65}Zn (0.1 μCi in 1.0 ml 0.85% NaCl) was injected into the lumen of the duodenum. A 27-gauge needle was inserted into the portal vein, after which 3 ml of blood was drawn into a heparinized syringe over a period of approximately 15 min. The blood samples were then centrifuged in a table-top centrifuge; the plasma was removed and analyzed by gel filtration chromatography, ion-exchange chromatography, and electrophoresis as described previously (2).

To examine zinc removal from the basolateral plasma membrane, the rats that had been given ^{65}Zn as described above were decapitated and a 15-cm section of the small intestine was removed. The basolateral plasma membrane was isolated by the two-phase method described by Lesko *et al.* (3). The isolated membranes were incubated at 37° for 1 hr in 10 ml of 25×10^{-3} *M* KH_2PO_4 buffer, pH 7.4, that contained: (a) no additions; (b) 10 mg apotransferrin; (c) 10^{-3} *M* histidine; (d) 10 mg zinc-free rat albumin prepared with EDTA; or (e) 10 mg zinc-free rat albumin prepared with histidine (see below). Following incubation, the membranes and incubation medium were separated by centrifugation for 15 min at 2000*g*. The membranes and a 1-ml aliquot of the incubation medium were assayed for radioactivity in a gamma counter (Nuclear Chicago Model 4233).

Human apotransferrin was purchased from Sigma Chemical Company and was dissolved in the incubation buffer without further treatment. Rat albumin was purchased from Sigma Chemical Company, and zinc-free rat albumin was prepared by two separate methods: (a) albumin was mixed with EDTA after which the solution was dialyzed and chromatographed as described in a previous publication (1); (b) rat albumin was dissolved in a solution that contained 10^{-2} *M* histidine after which the solution was chromatographed on a 2.5×40 cm column of Sephadex G-25 (Pharmacia Fine Chemicals) that had been equilibrated with 10^{-2} *M* histidine. The latter technique was based on the experiments of Giroux and Henkin (4) who demonstrated that chroma-

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tography of albumin on columns equilibrated with 10^{-2} *M* histidine effectively removes zinc from the protein. To ensure adequate removal of zinc from albumin by this procedure, a solution of ^{65}Zn-albumin was prepared by mixing carrier-free ^{65}Zn with albumin and subsequently chromatographing the solution on a column of Sephadex G-25 that had been equilibrated with 20 × 10^{-3} *M* Tris-5.0 × 10^{-3} *M* HCl, pH 8.6. When the ^{65}Zn-albumin preparation was chromatographed on a column equilibrated with 10^{-2} *M* histidine as described above, 100% of the ^{65}Zn was removed from the albumin and the isotope was recovered at the total volume of the column. This observation substantiates the experiments of Giroux and Henkin (4) and demonstrates that histidine effectively removes zinc from albumin.

To test the effect of the histidine treatment on subsequent zinc binding to albumin, a fraction of the histidine-treated protein was chromatographed on a Sephadex G-25 column equilibrated with water. Thereafter, the histidine-treated albumin and labeled zinc were mixed in a 1:1 molar ratio and the preparation was chromatographed on a Sephadex G-25 column. Following chromatography, 98% of the ^{65}Zn was recovered with the albumin, which indicates that the histidine treatment did not impair zinc binding to albumin.

Results and discussion. As illustrated in Fig. 1A, when rat portal blood plasma was chromatographed on Sephadex G-150, the major fraction of the isotope was recovered in a fraction that eluted near the void volume of the column. This elution volume corresponds with that of proteins with a molecular weight of approximately 90,000 daltons (2). Following gel filtration chromatography the tubes that contained the major portion of ^{65}Zn (70–80 ml) were applied to a DEAE-Sephadex column and the sample was eluted with a linear gradient. As shown in Fig. 1B, a small fraction of ^{65}Zn was eluted with the starting buffer while the major portion of the recovered isotope was eluted about three-fourths of the way through the gradient. When the fractions that contained ^{65}Zn eluted from DEAE were analyzed by electrophoresis, the fractions that eluted with the starting buffer contained no stainable protein, while the fractions that contained the major fraction of the eluted isotope consisted of a single band that migrated with the β-globulins and transferrin (Fig. 2). These results substantiate the observations of Evans and Winter (2) and demonstrate that zinc bound to transferrin is transported from the intestine through the portal plasma of the rat.

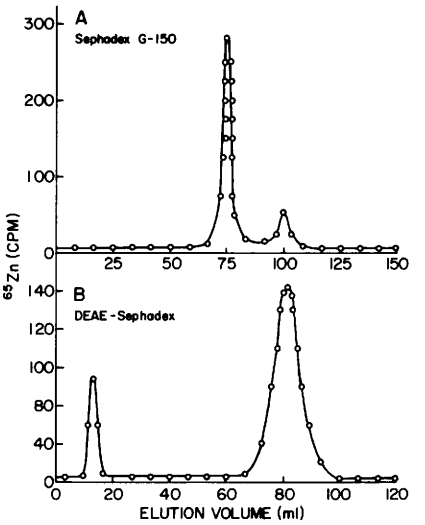

FIG. 1. Elution of ^{65}Zn in rat portal blood plasma from Sephadex G-150 and DEAE-Sephadex. (A) Samples were applied to a 1.5 × 90-cm column of Sephadex G-150 that had been equilibrated with 2 × 10^{-2} *M* Tris-5 × 10^{-3} *M* HCl, pH 8.6. The elution volume of three standard proteins was as follows: human transferrin, 70–80 ml; rat albumin, 95–105 ml; rat hemoglobin, 105–115 ml. (B) Fractions 70–80 ml from Sephadex G-150 were pooled and applied to a 0.9 × 15-cm column packed with DEAE-Sephadex A50 that had been equilibrated with 2 × 10^{-2} *M* Tris-5 × 10^{-3} *M* HCl, pH 8.6. After sample application, 25 ml of starting buffer was passed through the column followed by a 100-ml linear gradient (limit buffer, 2 *M* Tris-0.5 *M* HCl, pH 8.6).

The observations described above prompted us to examine the effect of both transferrin and albumin on zinc removal from the intestinal basolateral plasma membrane. When ^{65}Zn-labeled plasma membranes were incubated in the buffer medium that contained apotransferrin approximately 60% of the isotope was recovered in the incubation medium (Table I). However, less than 7% of the ^{65}Zn was recovered in the incuba-

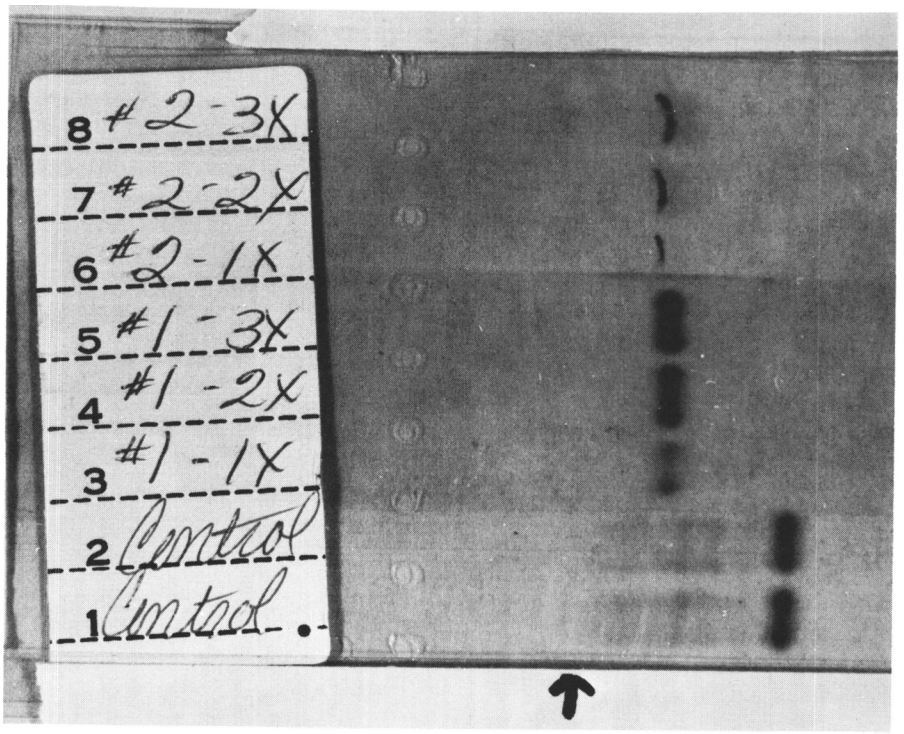

FIG. 2. Electrophoresis of the zinc-binding protein from portal plasma, apotransferrin, and human serum. Samples were applied to Beckman cellulose acetate membranes in barbital buffer, pH 8.6, and run for 18 min at 250 V. The arrow indicates the point of application. Samples 1 and 2, 0.25 μl human serum; samples 3, 4, 5, 0.25 μl, 0.50 μl, and 0.75 μl of a solution that contained 10 mg apotransferrin/ml; samples 6, 7, 8, 0.25 μl, 0.50 μl, and 0.75 μl of pooled and freeze-dried fractions 75–85 ml obtained after chromatography on DEAE-Sephadex (Fig. 1).

TABLE I. EFFECT OF APOTRANSFERRIN, HISTIDINE, AND ZINC-FREE ALBUMIN ON ^{65}Zn RELEASE FROM LABELED BASOLATERAL PLASMA MEMBRANES.

Additions to buffer medium	Membrane (cpm)	Medium (cpm)	Medium (% total)
None	22,145 ± 5,130	1,500 ± 200	6.4
Apotransferrin	7,330 ± 2,220	10,949 ± 4,286	59.7
Histidine	19,683 ± 4,218	1,452 ± 196	6.7
Zinc-free albumin[a]	14,050 ± 710	4,700 ± 1,300	25.1

[a] Prepared by chromatography on a Sephadex G-25 column equilibrated with 0.01 *M* histidine.

tion media that contained either no additions or histidine. These observations obtained *in vitro* coincided with the results obtained *in vivo* (see above), but the specificity of the interaction was questionable in view of the results we reported previously (1). Therefore, we reexamined the effect of albumin on zinc removal from the basolateral plasma membrane.

When zinc-free albumin prepared with EDTA was added to the buffer medium, 95% of the isotope was recovered in the incubation medium. These results are similar to those reported previously (1). However, as shown in Table I, zinc-free albumin prepared with histidine was much less effective in removing ^{65}Zn from the basolateral plasma membrane. These observations raised the question of whether or not the zinc-free albumin prepared with EDTA was actually free of this potent chelator. To test this, each of the incubation media was freeze-dried and subsequently chromatographed on a Sephadex G-25 column. In the medium that contained zinc-free albumin prepared with EDTA, 100% of the isotope was recovered near the total volume of the column at an elution volume corresponding

with that of ^{65}Zn–EDTA. In contrast, in the media that contained either apotransferrin or zinc-free albumin prepared with histidine, 95% of the applied isotope was recovered at the void volume of the column and was bound to transferrin or albumin respectively. A second indication of EDTA contamination was obtained in an experiment utilizing labeled EDTA. When 0.1 *M* [^{14}C]EDTA (Mallinckrodt, St. Louis, Mo.) was dialyzed against 1 g rat albumin and the albumin was subsequently dialyzed against several changes of distilled water, 1.0% of the labeled chelator was recovered with the albumin. Following chromatography on Bio-Gel P-10 (1), 30% of the applied [^{14}C]EDTA was recovered in the albumin fraction. Similar results have been reported by Levi *et al.* (5). Thus, in spite of prolonged dialysis and subsequent chromatography, EDTA remained associated with the albumin preparations, and as a result, erroneous data were obtained from the *in vitro* incubation experiments (1).

In the past, this author, and probably others, had assumed that zinc was transported from the intestine to the liver bound to albumin. This supposition was based on the fact that the major portion of zinc in venous plasma or serum is bound to albumin (6, 7). The data obtained when zinc-free albumin prepared from EDTA was incubated with ^{65}Zn-labeled plasma membranes (1) compounded this erroneous supposition. The experiments described in this paper indicate that apotransferrin interacts with the intestinal basolateral plasma membrane and removes zinc more effectively than zinc-free albumin (Table I). We cannot be certain that the experiments with the isolated membranes reflect the mechanisms operating *in vivo*. However, previous investigations (8) have demonstrated that apotransferrin binds to epithelial cells but, to date, we have been unsuccessful in our attempts to demonstrate albumin binding to epithelial cells (unpublished observations). Thus, apotransferrin apparently contains groups which combine with specific sites on the plasma membrane and enable the protein to remove zinc and/or iron. Moreover, as described above and in a previous publication (2), the major portion of recently absorbed zinc in the portal plasma of the rat is bound to transferrin. This observation is particularly impressive in view of the fact that the concentration of transferrin in plasma, 200–320 mg/100 ml, is approximately 1/15 that of albumin, 3500–4500 mg/100 ml.

In view of the recent observations obtained in this laboratory, the previously proposed (1) hypothetical mechanism for zinc absorption must be revised as follows: (a) The pancreas secretes a zinc-binding ligand into the intestinal lumen where the metal binds to the ligand and is subsequently transported into the intestinal cell; (b) in the cell, zinc is transferred to binding sites on the basolateral plasma membrane; (c) apotransferrin interacts with the plasma membrane and removes zinc from the receptor sites; and (d) transferrin transports zinc through the portal blood to the liver where the metal is incorporated into albumin and other zinc-binding proteins.

Summary. Blood was drawn from the portal vein of rats immediately after the insertion of ^{65}Zn into the duodenal lumen. Analysis of the plasma from the portal blood demonstrated that the major portion of absorbed ^{65}Zn was bound to transferrin. When apotransferrin was incubated with ^{65}Zn-labeled basolateral plasma membranes, 60% of the isotope was removed from the membrane. Zinc-free albumin prepared with histidine removed 25% of the ^{65}Zn from labeled membranes and histidine had no effect. These results demonstrate that transferrin functions in the absorption and transport of zinc.

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