Ethylenediaminetetraacetic Acid and ^{65}Zn Binding by Intestinal Digesta, Intestinal Mucosa and Blood Plasma[1] (35851)

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Kratzer *et al.* (1) reported that the chelating agent EDTA (ethylenediaminetetraacetic acid) was able to reduce the dietary zinc requirement of turkey poults fed rations containing soybean protein. Further studies (2) have shown that EDTA increased the absorption of ^{54}Mn and ^{65}Zn and decreased that of ^{60}Co and ^{59}Fe. Darwish and Kratzer (3) proposed that EDTA enhanced zinc absorption by forming a zinc–EDTA complex that rendered the element more absorbable. Although their hypothesis seems reasonable, there is no experimental evidence on the functions of EDTA on zinc absorption.

Recent findings by Starcher (4) and Suso and Edwards (5) have shown copper and zinc binding by proteins in the intestinal mucosa. These findings suggest possibilities of study of the mechanism of absorption whereby EDTA increases or decreases metal availability. The scope of this research was to investigate the mechanics involved in the EDTA enhancement of zinc absorption.

Materials and Methods. Experiments were conducted on two groups of five 4-week-old single comb white leghorn male chickens. They were fed a practical broiler type diet (6) supplemented with EDTA as needed, that contained 70 ppm zinc during a 2-week preexperimental period. The birds fasted overnight and then were allowed to eat freely their respective diets for 45 min before being dosed with the isotopes. Group 1 was fed the practical diet while group 2 received the practical diet supplemented with 0.125% EDTA. Both groups were dosed orally with 1 ml of a solution containing 50 μCi ^{65}Zn (4.9 mCi/mg of Zn). Group 2 was also dosed with 3×10^{-4} m*M* EDTA-^{14}C/bird (sp act 2.10 mCi/m*M*). Blood samples were obtained from the chickens by heart puncture 2 hr after dosing with the isotopes and blood plasma was prepared by centrifugation of the whole blood for 10 min at 10,000*g*. The birds were then sacrificed; and the small intestine and its contents were excised. Intestinal content was removed by washing the intestine with 10 ml of 0.25 *M* sucrose. The intestinal contents were homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000*g* for 20 min. The supernatant was centrifuged for 60 min at 60,000*g*. At the end of this centrifugation the supernatant was used as the intestinal digesta preparation. A mucosal soluble fraction was obtained by scraping the mucosa from the whole small intestine with a microscope slide and adding 2 vol. of 0.25 *M* sucrose/unit weight of tissue. The suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged by the same methods described for the intestinal digesta.

To determine if binding of ^{65}Zn and/or EDTA-^{14}C was taking place, 1-ml samples of intestinal digesta preparation, blood plasma, or mucosal soluble fraction were dialyzed with constant stirring against 4-liter solutions of distilled water at 5°. The presence of ^{65}Zn and EDTA-^{14}C in the samples dialyzed was determined by counting in a well-type scintillation counter and a liquid scintillation spectrometer, respectively. Counts were obtained at 0 and 150 hr of dialysis and were corrected for change in volume of the dialyzed solution. The results were expressed as percentage of the initial amount of ^{65}Zn and EDTA-^{14}C present in the samples.

To obtain information on the nature of the

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TABLE I. Retention of ^{65}Zn and EDTA-^{14}C by Plasma, Mucosal Soluble Fractions, and Intestinal Digesta Preparation Samples After 150 hr of Dialysis Against 4 Liters of Distilled Water.

Five 1-ml samples were dialyzed in each group at a temperature of 5°. EDTA-^{14}C and ^{65}Zn content of the samples were determined at the beginning and the end of the experiment in a liquid scintillation spectrometer and a well scintillation counter, respectively.

Diet	Sample	^{65}Zn (%)	EDTA-^{14}C (%)
Basal	Plasma	96.9	
Basal + EDTA	Plasma	99.2	95.5
Basal	Mucosa soluble fraction	70.2	
Basal + EDTA	Mucosa soluble fraction	68.7	64.3
Basal	Intestinal digesta preparation	12.5	
Basal + EDTA	Intestinal digesta preparation	15.1	14.1

different fractions that were binding zinc in the intestinal digesta preparation, mucosal soluble fraction and blood plasma samples were chromatographed by gel filtration. Optical density at 280 mμ was used to estimate protein. The presence of ^{65}Zn and EDTA-^{14}C was determined as described before in the dialysis experiment. The ratio of cpm EDTA-^{14}C:cpm ^{65}Zn was determined by adding individual counts of ^{65}Zn and EDTA-^{14}C for all the samples in each of the major peaks of ^{65}Zn and EDTA-^{14}C, and dividing the total cpm EDTA-^{14}C by the total cpm ^{65}Zn. An estimate of the molecular weight of the protein fractions from blood plasma and intestinal mucosa which contained EDTA-^{14}C and ^{65}Zn was obtained by comparing the elution volume of a standard solution containing dextran blue, plasma albumin, and myoglobin with the volume of elution of the EDTA-^{14}C and ^{65}Zn carrying proteins (6). The elution of an amino acid standard[2] was compared with that of the intestinal digesta preparation. The intestinal digesta preparation was subjected to gel filtration using a 2 column system where the effluent of the first column (1.5 × 30 cm) packed with Sephadex G-50 entered into a second column (1 × 15 cm) that contained Sephadex G-10.

Results. The dialytic pattern of ^{65}Zn in all the samples was not altered by the presence of EDTA in the diet of the chickens (Table I). Dialysis removed less than 5% of the ^{65}Zn and EDTA-^{14}C present in blood plasma and approximately 30% of ^{65}Zn and EDTA-^{14}C from the mucosal soluble fraction (Table I); however, 85% of the ^{65}Zn and EDTA-^{14}C present in the intestinal digesta preparation was removed during dialysis thus indicating that no large molecule was binding the element in the intestinal digesta.

Optical density readings at 280 mμ of the elute from gel filtration of the intestinal digesta preparation revealed two major peaks (Fig. 1B). The first one appeared at approximately the same elution volume as dextran blue (Fig. 1A). This seemed to indicate a high molecular weight polypeptide. The second peak that had a low molecular weight was very closely preceded by the ^{65}Zn peak and appeared to contain free amino acids, as evidenced by its elution volume when compared to an amino acid standard (Fig. 1A). When EDTA-^{14}C was dosed to chickens fed a diet containing 0.125% EDTA, ^{65}Zn in the intestinal digesta preparation was displaced from its position next to the free amino acids and was present together with the EDTA-^{14}C (Fig. 1C), indicating binding of ^{65}Zn with the ^{14}C-EDTA. The total ratio for the main peak of cpm EDTA-^{14}C:cpm ^{65}Zn was 1.10. Intestinal mucosa soluble fraction was chromatographed in a 2.5 × 45-cm Sephadex G-150 column. EDTA-^{14}C also appeared binding with ^{65}Zn to the mucosal soluble fraction as shown by gel filtration chromatography (Fig. 2C). In this case EDTA-^{14}C and ^{65}Zn appeared together in superimposed peaks

[2] Beckman, Amino Acid Calibration Mixture, Type I, containing a mixture of 18 amino acids.

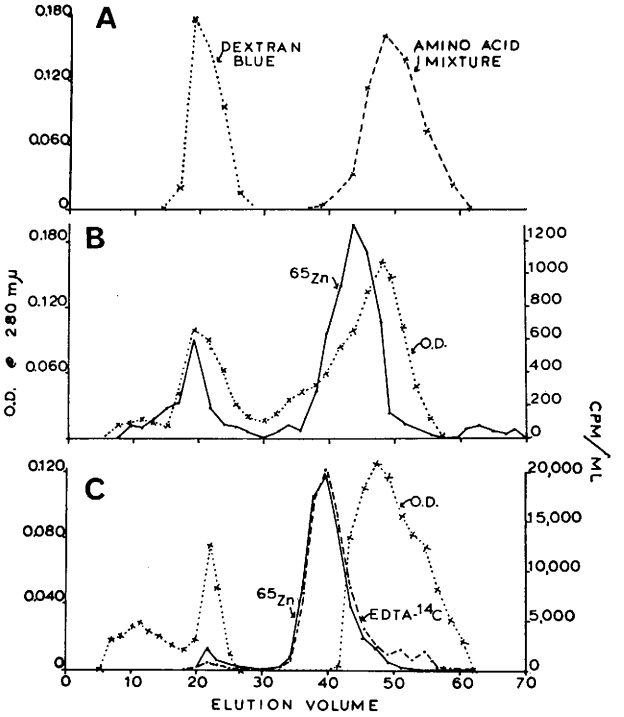

FIG. 1. Elution pattern obtained by a two column system of Sephadex G-50 (1.5 × 30 cm) and Sephadex G-10 (1 × 15 cm) using distilled water at neutral pH as eluent. Protein was estimated by reading OD at 280 mμ and ^{65}Zn and EDTA-^{14}C contents were determined by counting in a well scintillation counter and a liquid scintillation spectrometer, respectively. Graphs (A) and (C) were obtained from one bird representative of the five birds in its group. (A) A standard solution containing dextran blue and a mixture of 22 amino acids. (B) Intestinal digesta preparation from chickens fed the control diet and dosed ^{65}Zn orally. (C) Intestinal digesta preparation from chickens fed the control diet supplemented with 0.125% EDTA, dosed ^{65}Zn and EDTA-^{14}C orally.

that had a ratio of 1.26 cpm EDTA-^{14}C:cpm ^{65}Zn. The ^{65}Zn was bound by the same protein fraction regardless of the presence of EDTA in the diet (Fig. 2B). The molecular weight of the zinc binding fraction was estimated by gel filtration to range from 14,000 to 18,000 in different runs as evidenced by its elution volume being the same as the standard myoglobin (Fig. 2A). The blood plasma samples were chromatographed by gel filtration following the same procedure used for the mucosal soluble fraction. The elution pattern followed by EDTA-^{14}C and ^{65}Zn in blood plasma was the same as the one followed by the ^{65}Zn alone (Fig. 3). In both cases, elution volume of ^{65}Zn and of EDTA-^{14}C were the same as the one observed for standard plasma albumin. The ratio obtained for EDTA-^{14}C:^{65}Zn when eluted from blood plasma was 1.26.

Discussion. Dialysis results indicated that ^{65}Zn attached to zinc binding proteins in the mucosal soluble fraction and in the blood plasma. The fact that during dialysis a larger amount of ^{65}Zn was removed from the mucosal soluble fraction than from blood plasma suggests that plasma binds zinc with greater strength than the mucosal fractions. Previous findings on the dialytic pattern of mucosal preparations (5) together with those reported in this communication seem to indicate that there are two types of zinc in the intestinal mucosa, one loosely bound (30%) that was removed during dialysis and a tightly bound zinc capable of withstanding dialysis. The results indicated that the ^{65}Zn peak slightly preceded one of the free amino acids in intestinal digesta preparation. When using

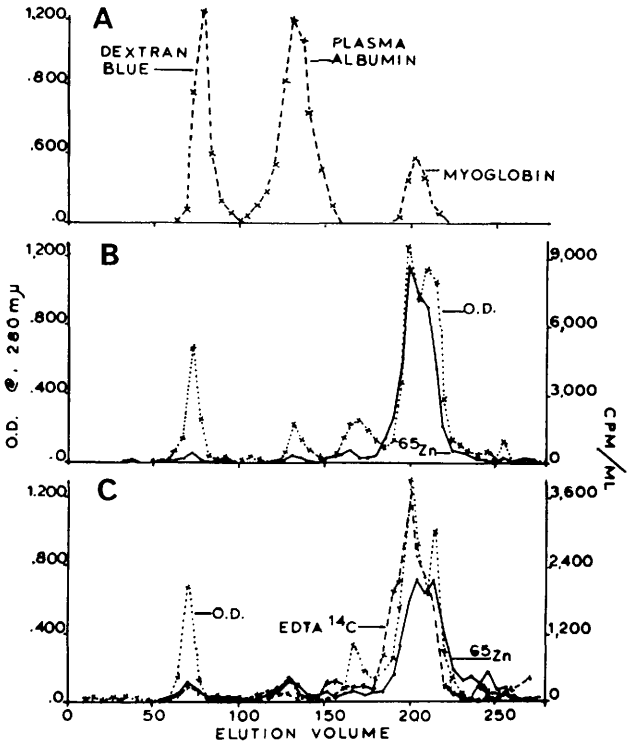

FIG. 2. Gel filtration by Sephadex G-150 (2.5 × 4.5 cm) eluting with neutral pH distilled water. Protein and radioactivities were measured as described in Fig. 1. Graphs (B) and (C) were obtained from one bird representative of the five birds in its group. (A) Standard solution containing dextran blue, plasma albumin, and myoglobin. (B) Mucosal soluble fraction from chickens fed the control diet, ^{65}Zn was dosed orally. (C) Blood plasma from chickens fed the control diet supplemented with 0.125% EDTA, ^{65}Zn and EDTA-^{14}C dosed orally.

Sephadex G-50 and G-10 columns, it is not possible for an element (^{65}Zn with a smaller mol wt) to be eluted before larger molecules (7). This raises the possibility that the ^{65}Zn was bound by some of the free amino acids present in the intestinal digesta preparation thus forming an amino acid ^{65}Zn complex which would be of a greater molecular weight than that of the free amino acids. The intestinal digesta from chickens fed the EDTA-supplemented diet, when separated by gel filtration chromatography, showed that the ^{65}Zn was present together with the EDTA-^{14}C. This may be due to a binding of the element by the chelating agent and a removal of ^{65}Zn from its amino acid binding. The protein binding the radioactive zinc in mucosal soluble fractions appears to have a molecular weight ranging from 14,000 to 18,000. Starcher (4) reported a copper and zinc binding protein with molecular weight of about 10,000. The present work seems to be in agreement with his findings since gel filtration techniques were used in both cases to estimate the molecular weights.

When blood plasma containing ^{65}Zn was chromatographed by gel filtration the zinc binding component was found to have an elution volume similar to that of the plasma albumin standard. Suso and Edwards (5) observed that the zinc binding protein as blood was eluted in the same fraction as blood plasma albumin. The present results would further suggest that under normal conditions the binding of zinc is carried out by a protein similar to plasma albumin.

The similarity observed in the EDTA-^{14}C:^{65}Zn ratios in column fractions from digesta, mucosa, and blood plasma give strong support to the idea brought forth by Koike *et*

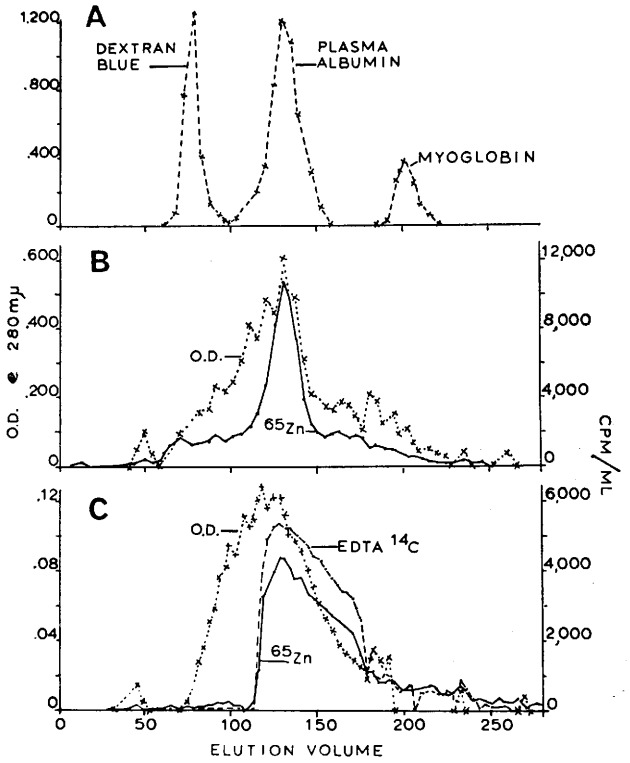

FIG. 3. Gel filtration chromatogram obtained when using G-150 in a 2.5 × 4.5 cm column eluted with distilled water at neutral pH. Protein and radioactivities were measured as described in Fig. 1. Graphs (B) and (C) were obtained from one bird representative of the five birds in its group. (A) Standard solution containing dextran blue, plasma albumin, and myoglobin. (B) Blood plasma from chickens fed the control diet ^{65}Zn was dosed orally. (C) Blood plasma from chickens fed the control diet supplemented with 0.125% EDTA and dosed ^{65}Zn and EDTA-^{14}C orally.

al. (8) and Darwish and Kratzer (3) that when EDTA is present in the diet, zinc is absorbed from the intestine in the EDTA zinc complex form. Darwish and Kratzer (3) using labeled EDTA ^{65}Zn suggested that EDTA had a great affinity for ^{65}Zn and that it would remove the element from any dietary complex and form an EDTA–Zn complex. The EDTA–zinc complex being smaller than the protein complexes of zinc could then be absorbed easily into the blood stream. This simple hypothesis still appears to be valid although the results of the present study indicate that the zinc transfers as a complex and not as a simple ion. The fact that EDTA-^{14}C binds to ^{65}Zn in the intestinal digesta and to the same proteins as ^{65}Zn in mucosal soluble fraction, and blood plasma in the absence of the chelating agent, seems to indicate that zinc is bound by EDTA in the intestinal lumen, forming the EDTA–zinc complex. When the ratios of EDTA-^{14}C:^{65}Zn for digesta mucosal soluble fraction and blood plasma are taken into consideration, it would seem that it is the EDTA–zinc complex that binds to the zinc binding protein present in the intestinal mucosa and in the blood plasma, perhaps through zinc. The dialysis experiments (Table I) support the hypothesis that the intestinal digesta preparation had lesser affinity for the complex than the zinc binding protein in blood plasma and subsequently the zinc EDTA complex was transferred from the mucosal protein to the blood protein. In view of the fact that the EDTA is still part of the zinc protein complex in the plasma, it undoubtedly will have still further influence on the metabolism of zinc. This may explain why EDTA and other antiarthritic compounds in the report of

Nielsen *et al.* (9) were capable of influencing bone abnormalities while they had little influence on growth.

Studies are in progress to determine the role of several antiarthritic drugs on zinc metabolism and to determine the effect of EDTA and other chelating agents on the release of zinc to various sites and enzyme systems in the animal.

Summary. The mechanisms of zinc absorption and the effect of EDTA (ethylenediaminetetraacetic acid) on zinc absorption were studied at the intestinal digesta, intestinal mucosa, and blood plasma levels. Dialytic and gel filtration studies showed complexing of ^{65}Zn with free amino acids or EDTA-^{14}C in the intestinal digesta. The EDTA-^{14}C:^{65}Zn complex was bound to a zinc binding protein in the intestinal mucosa (mol wt 14,000–18,000) and to a plasma protein (mol wt 65,000–70,000), that had a greater affinity for zinc than the mucosal protein. The ratios obtained for the cpm EDTA-^{14}C:cpm ^{65}Zn in the main peaks eluted from gel filtration columns (1.10 in intestinal digesta preparation, 1.26 in mucosa soluble fractions, and 1.26 in blood plasma) indicated strong binding of the EDTA–zinc complex. When EDTA was not present in the diet, ^{65}Zn complexed with amino acids in the intestinal digesta and with the same mucosal and blood proteins.

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