Studies on Zinc Absorption: ^{65}Zn Binding by Homogenates of Rat Intestinal Mucosa (35250)

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(Introduced by J. K. Loosli)

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Little is known of the mechanism for the intestinal absorption of zinc. However, a number of studies have shown some fairly specific biological antagonisms between zinc and certain other cations (1–5). In previous experiments (6, 7), we found that zinc interfered with the intestinal absorption of copper and, conversely, copper interfered with the intestinal absorption of zinc. In either case, the interference was observed only when the two cations were administered together and implied that the elements were competing for a common absorption pathway. These observations, plus reports in the literature concerning metal-binding proteins for several elements (8–11), provided the impetus for the work reported here on the isolation and partial purification of zinc-binding substances from the intestinal mucosa of rats.

Materials and Methods. Animals. Male rats were of the Sprague-Dawley strain; were housed in stainless steel cages, and were fed a stock diet[1] containing approximately 50 ppm of zinc. They weighted between 250 and 400 g and were fasted overnight prior to the experiments.

Preparation of the mucosal supernatant. Rats were killed by decapitation and the small intestine was removed. After the contents had been removed by flushing with cold 0.9% saline, the intestine was split longitudinally and the mucosal tissue was scraped off with a microscope slide. This tissue was weighed and homogenized in 4 vol of 0.005 *M* Tris–HCl, pH 7.2, in a Potter-Elvejhem homogenizer. The homogenate was centrifuged for 30 min at 45,000*g*; the precipitate was discarded, and the 45,000*g* (45,000*g*) supernatant was saved for further fractionation.

^{65}Zn[2] labeling of the 45,000g supernatant. This supernatant was labeled in two different ways. One method was to give ^{65}Zn to rats by stomach tube, kill them at a predetermined time, and prepare a supernatant from the *in vivo* labeled mucosa. In the second method, the 45,000*g* supernatant was prepared first and the ^{65}Zn was added to the supernatant *in vitro*. Specific details of each experiment will be presented in conjunction with results of that experiment.

Gel filtration of the 45,000g supernatant. The supernatant was first fractionated with ammonium sulfate, and the fractions precipitating between 45 and 80% saturation were subjected to gel filtration through columns of Sephadex G-100.[3] The 45–80% precipitates were dissolved in 5 ml of 0.005 *M* Tris–HCl, pH 7.2, and put on a 2.5 × 40-cm column of Sephadex G-100 equilibrated with the same buffer. Three-ml fractions were collected and counted, and absorbance at 280 mμ was determined. The Sephadex columns were calibrated for molecular weight estimation with bovine serum albumin, hemoglobin, and pancreatic ribonuclease.

Electrophoresis. The peak radioactive fractions from the Sephadex columns were combined and lyophilized. Subsequently, the lyophilized samples were taken up in distilled water and subjected to disc electrophoresis on acrylamide gel. The gel columns consisted of

[1] Big Red Dog Chow, Agway, Inc., Syracuse, New York. Trade names and company names are included for the benefit of the reader and do not imply any endosement or preferential treatment of the product listed by the U.S. Department of Agriculture.

[2] Union Carbide Corporation, Tuxedo, New York.

[3] Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

1-cm diameter by 12-cm long columns of 5% gel topped by a 1-cm layer of a 3% "stacking" gel. A marker dye (bromothymol blue) and sufficient sucrose to increase the density of the sample so that it could be layered onto the gel were added to each sample. The buffer chambers contained 0.06 *M* Tris–boric acid buffer, pH 9.1, and 250 V were applied until the marker dye had migrated to within 0.5 cm of the end of the gel column. The gels were removed from the glass tubes and stained for 2 to 3 min in a 1% solution of amido black; unbound dye was removed with a destaining solution of acetic acid:methanol:water (2:7:11).

Results and Discussion. Fractionation of in vivo *labeled supernatant.* Three rats were given 1 ml each of a 0.001 *M* zinc solution containing 7.5 μCi of ^{65}Zn. After 2 hr, the rats were sacrificed, and homogenates of intestinal mucosa were prepared and fractionated as previously described. The results are presented in Fig. 1.

Three major peaks of radioactivity were observed. Peak 1 eluted with the void volume of the column, and represents materials having molecular weights in excess of the exclusion limit of Sephadex G-100 (ca. 150,000). Peak 2 generally eluted between tubes 50 and 55. This corresponds to the elution pattern of pancreatic ribonuclease and indicates

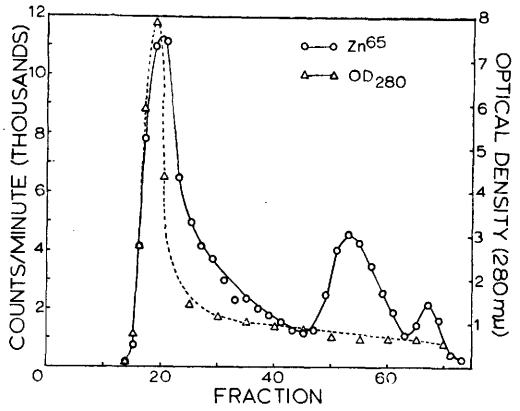

FIG. 1. Fractionation of ^{65}Zn bound *in vivo*. The supernatant fraction of the musosal homogenate was fractionated with ammonium sulfate, and the fraction precipitating between 45 and 80% saturation was applied to a 2.5 × 40-cm column of Sephadex G-100; 3-ml fractions were collected.

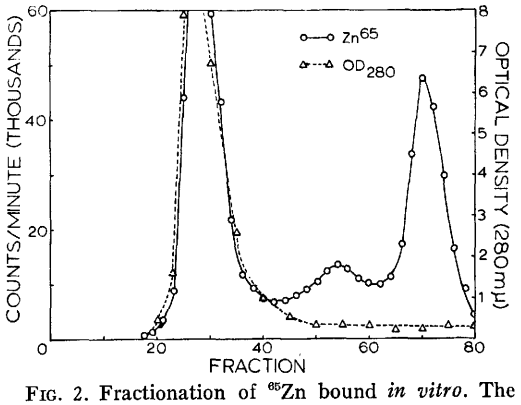

FIG. 2. Fractionation of ^{65}Zn bound *in vitro*. The supernatant fraction of the mucosal homogenate was fractionated with ammonium sulfate, and the fraction precipitating between 45 and 80% saturation was appiled to a 2.5 × 40-cm column of Sephadex G-100. Sample size was 5 ml, and 3-ml fractions were collected.

a molecular weight of the order of 12,500. Peak 3 occurred late in the elution pattern and corresponds to unbound ^{65}Zn.

Fractionation of in vitro *labeled supernatant.* Three rats were sacrificed and a 45,000*g* supernatant was prepared from the mucosal homogenate. Then, 1 ml of 0.001 *M* zinc solution containing 7.5 μCi of ^{65}Zn was added to this supernatant. After stirring for 15 min, the supernatant was fractionated as described previously. The results are given in Fig. 2.

The elution pattern was essentially the same as that obtained with the *in vivo* labeled preparations. One major difference was that the radioactivity of the fractions was somewhat higher in the *in vitro* preparations than in the *in vivo* preparations; this is not surprising since the ^{65}Zn was added directly to the supernatant rather than being given by stomach tube. A second quantitative difference was that the peak of unbound ^{65}Zn was relatively larger in the *in vitro* preparations. Again, this is not surprising in view of the difference in the two labeling techniques.

Qualitatively, the elution patterns were quite similar, regardless of how the ^{65}Zn was added. This indicates that, for the most part, the zinc is complexed or chelated rather than being incorporated into the molecules at the time of synthesis. This is further indicated by the fact that ^{65}Zn dissociates during electro-

phoresis and migrates to the cathode.

Electrophoresis. Fractions from each ^{65}Zn peak were pooled and lyophilized. The lyophilized samples were reconstituted with a minimum amount of distilled water and electrophoresed. No attempt was made to quantitate either the protein bands or the ^{65}Zn since the ^{65}Zn dissociated from the complexes during electrophoresis. Thus, after electrophoresis, there were a number of protein bands on the gels, but none of them contained any ^{65}Zn. The electrophoresis patterns are presented to indicate the number of proteins in each of the Sephadex peaks. Figure 3 is a typical illustration of the patterns that were obtained.

Peak 1 contained several proteins (at least 11 bands were visible), but since the ^{65}Zn had dissociated, it was not possible to tell which, if any, of the bands were originally labeled. Peak 2 was somewhat less complex in that it yielded only 3 bands; two rather narrow, fast moving bands, and a third rather diffuse band which migrated considerably slower than bovine serum albumin. In some experiments, a fourth band was observed which migrated just ahead of the large band in Fig. 3. Peak 3 fractions were also electrophoresed, but did not yield any visible protein bands.

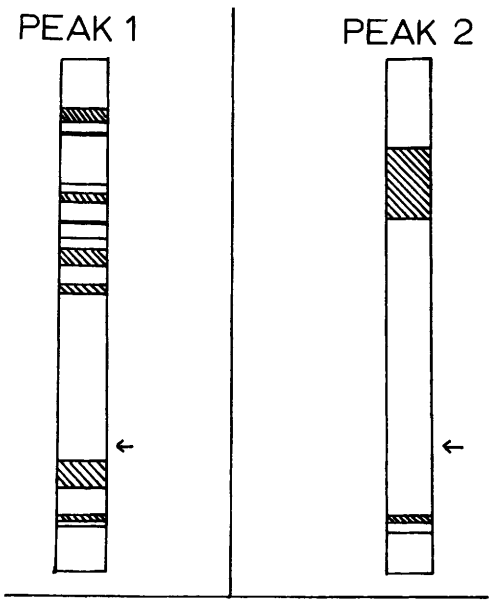

FIG. 3. Acrylamide disc gel electrophoresis of ^{65}Zn labeled material from Sephadex G-100 columns. Origin was at top (cathode) end of column. Arrows indicate the distance that bovine serum albumin migrated in this system.

The specific activity of the material that eluted from the Sephadex columns at Peak 2 was considerably higher than that of Peak 1. When expressed as cpm/OD_{280}, the most active fractions from Peak 2 yielded values 4 to 5 times higher than the most active fractions from Peak 1.

There is no evidence at present to indicate whether any of these compounds are involved in any way in zinc absorption. Of the metal-binding proteins that have been described previously (8–11), the calcium-binding protein is the only one for which there is good evidence linking it to a transport function.

The material in the second Sephadex peak of our experiments has an apparent molecular weight between 10,000 and 15,000. This is somewhat smaller than the 25,000 reported for calcium-binding proteins (12), but about the same magnitude as that reported for the copper-binding protein (9). The copper-binding protein is also capable of binding either zinc or cadmium, and the major differences between our results and Starcher's (9) are the relative sizes of Peaks 1 and 2 from the Sephadex columns. In his preparation, practically all of the ^{64}Cu and most of the ^{65}Zn was bound to a low-molecular weight material corresponding to Peak 2. In our experiments, the first peak accounted for much more of the total radioactivity, even though it was of somewhat lower specific activity. It is not possible to tell at present whether these differences are due to technique or species differences.

Summary. Approximately 60% of the ^{65}Zn in labeled homogenates of rat intestinal mucosa was recovered in a 45,000*g* supernatant, and about 50% of the counts in this supernatant could be recovered in a 45–80% ammonium sulfate precipitate. The bound ^{65}Zn in the ammonium sulfate precipitate could be resolved into two peaks by gel filtration on Sephadex G-100. The first radioactive fraction consisted of substances having molecular weights of 150,000 or more, and the second tion consisted of substances having molecular weights in the range of 10 to 15,000. Electro-

phoresis of the material from the first Sephadex peak always yielded 10 or more visible protein bands; whereas material from the second peak yielded either 3 or 4 visible bands. It was not possible to determine which, if any, of the protein bands were originally labeled, as the ^{65}Zn dissociated during electrophoresis and migrated to the cathode. To date, there is no evidence to link any of these compounds with intestinal absorption of zinc.

1. Smith, S. E., and Larson, E. J., J. Biol. Chem. **163**, 29 (1946).

2. Magee, A. C., and Matrone, G., J. Nutr. **72**, 233 (1960).

3. Hill, C. H., Matrone, G., Payne, W. L., and Barber, C. W., J. Nutr. **80**, 227 (1963).

4. Hill, C. H., Starcher, B., and Matrone, G., J. Nutr. **83**, 107 (1964).

5. Van Campen, D. R., J. Nutr. **88**, 125 (1966).

6. Van Campen, D. R., and Scaife, P. U., J. Nutr. **91**, 473 (1967).

7. Van Campen, D. R., J. Nutr. **97**, 104 (1969).

8. Wasserman, R. H., and Taylor, A. N., Science **152**, 791 (1966).

9. Starcher, B. C., J. Nutr. **97**, 321 (1969).

10. Suso, F. A., and Edwards, H. M., Fed. Proc., Fed. Amer. Soc. Exp. Biol **28**, 761 (1969).

11. Pearson, E. N., and Reich, M. B., J. Nutr. **99**, 137 (1969).

12. Wasserman, R. H., Calcif. Tissue Res. **2**, 301 (1968).

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