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Identification and Molecular Size of Zinc-Binding Ligands in Pancreatic/Biliary Fluid of Rats

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Abstract

The exocrine pancreatic secretion is an important factor in the maintenance of zinc homeostasis. The daily pancreatic secretion of zinc into the gastrointestinal tract may be two or more times the daily dietary zinc intake. The objective of this study was to examine the distribution of proteins and zinc in pancreatic/biliary fluid following intraperitoneal ^{65}Zn injection into dietary prepared Sprague-Dawley rats. Distribution of zinc-binding protein in Sephadex G-75 subfractions showed a peak corresponding to the high molecular weight protein standard(<66kDa) in the pancreatic/biliary fluid. Zinc also was associated with the 29~35kDa molecular weight proteins. These are similar in size with zinc-containing enzymes, carboxypeptidase A and carboxypeptidase B. A more remarkable small molecular weight fraction eluted beyond the 6.5kDa standard protein peak. These results show the presence of small molecular weight compounds in pancreatic/biliary fluid associated with zinc. These small molecular weight compounds may serve as zinc-binding ligands for the secretion of enogenous zinc into the duodenum. These findings suggest that these lignads may dissociate zinc in the duodenum thus making it vulnerable to complexation with phytate in the upper gastrointestinal tract rendering the zinc unavailable for reabsorption.

Key words: pancreatic/biliary fluid, zinc-binding ligands, zinc homeostasis, phytate

INTRODUCTION

The major route of zinc secretion and excretion is via pancreatic/biliary duct into the gastrointestinal tract, presumably this is the normal excretory pathway(1-4). The injected ^{65}Zn accumalates in relatively high concentrations in the pancreas and the excretion of a major part was via the pancreatic secretion(5-7). In rat, bile serves as a route for the secretion of a minor portion of an injected dose of inorganic zinc(8,9).

The amount of zinc which is secreted into the duodenum through the pancreatic duct may be two or more times the daily dietary zinc intake(8,10,11). This large endogenous zinc secretion must be recycled via the gastrointestinal tract and becomes an important factor in the maintenance of zinc homeostasis.

Oberleas(12) found two pancreatic zinc pool. The first, a stable pool in which zinc forms stable complexes with zinc-dependent enzymes and other large molecular weight proteins. The second, a labile pool in which the zinc is loosely bound and is dissociated in the duodenum. The labile pool zinc, which in pancreatic fluid is at pH 8.0 vulnerable to complexes with phytate and other ligands. The labile zinc pool must be largely reabsorbed to sustain zinc homeostasis.

The objective of this study was to study the distribution of zinc-binding ligands associated with pancreatic/biliary fluid. Also to study these ligands in relationship to zinc homeostasis following intraperitoneal ^{65}Zn injection into a dietary prepared rat.

MATERIALS AND METHODS

Experimental design and diet

The experimental design was a 2×2 factorial arrangement of treatments to see the differences on zinc-binding ligands between the factors; calcium and phytate. The two variables were phytate levels(0 and 4.7g/kg, as sodium phytate for non-phytate and phytate group) and calcium levels(8 and 16g/kg diet as calcium carbonate for low and high Ca).

Forty-eight male Sprague-Dawley rats(Harlan Sprague-Dawley, Inc., Indianapolis, IN), weighing 97~105g initially, were used in this study. An initial 3 week growth period in which the rats were fed low calcium, non-

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phytate diet allowed the rats to achieve an average weight of 300g. Followed was 4 week of zinc-depletion period by feeding the rats with the phytate-containing diet in each calcium group. After zinc-depletion, 3 week of collection period was followed; initial(2wk) and crossover collection(1wk) period by diet. Supplemental calcium and phytate levels during the initial collection periods were: (1) 0g/kg of supplemental calcium carbonate and 4.7g/kg Na phytate(low Ca, phytate); (2) 0g/kg of calcium carbonate and 0g/kg Na phytate(low Ca, non-phytate); (3) 16g/kg of calcium carbonate and 4.7g/kg Na phytate(high Ca, phytate); (4) 16g/kg of calcium carbonate and 0g/kg Na phytate(high Ca, non-phytate). The diets were supplied in aluminum feed cups and the distilled water was supplied ad libitum from polyethylene water bottles with butyl rubber(neoprene) stoppers. Radioactive ^{65}Zn($ZnCl_2$, Dupont, Boston, MA; 10μCi in 0.1ml of 0.001M HCl in saline) was given by intraperitoneal injection to equilibrate the endogenous zinc pool. During the first 2 weeks of initial collection period, the rats were fed the phytate or non-phytate diet within each low or high calcium group. Following this initial collection period, each phytate or non-phytate diet was switched within each calcium level to complete the crossover portion of the collection period. The groups will subsequently be identified by the diet associated with their initial collection period.

Collection of pancreatic/biliary fluid

The average body weight of the rats at the terminus of the collection period was 341±7g. Pancreatic fluid was collected by the modified method of Rolf et al.(13) until the rat was exhausted. At the end of the entire 10 week experimental period, rats were anesthetized with a mixture of Ketaset(Ketamine HCl, Aveco Co. Inc, Fort Dodge, IA) and Xylazine(1ml/kg body weight), or Na pentobarbital(40mg/kg body weight, 10mg/ml). The rat abdomen was rinsed with 0.9% saline solution and cut with a mid-line incision to expose the abdominal cavity.

Because the rat does not have the gallbladder, pancreatic/biliary fluid was collected for the determination of zinc binding ligands. The surgery to collect the pancreatic/biliary fluid from the rat is shown on previously published paper(14). Non-radiopaque intramedic polyethylene tubing, i.d. 0.28mm, o.d. 0.61mm(PE-10, Clay Adams, Becton Dickerson & Co., Parsepsong, NJ 07054), was used for common bile duct cannulation. The tubing was cut with a blade held about 30° from the perpendicular until approximately 1/2 of the tube was incised. The blade angle was then increased to approximately 70° and the cut was extended to completely transect the tubing, resulting in a thin tapered bevel. The distal segment of the common bile duct was hemisected and the tubing placed into that distal segment of the duct. For the protein stimulation to stimulate pancreatic/biliary fluid flow, 5% of bovine serum albumin in 0.1M $NaHCO_3$, which was infused into the duodenum above the Sphincter of Oddi, was used.

Gel filtration column chromatography

Sephadex G-75 gel(Pharmacia Fine Chemicals, Inc., Piscataway, NJ) was used for the localization of the protein in pancreatic/biliary fluid(Pharmacia Fine Chemicals Inc., 1966). To prevent air bubbles which may be trapped in the gel slurry, the gel was swelled on a boiling water bath and gently stirred dath swelling time.

Packing of the gel:

The column(2.5cm×52cm, Kontes Corporation, Vineland, NJ) was mounted vertically, and the outlet was fitted with a narrow piece of tubing of approximately the length of the column. Air bubbles were removed by gently stirring the gel slurry with a glass rod. The swollen gel was mixed with sufficient buffer to allow air bubbles to rise from the slurry. To pack the gel bed necessary for the gel column, the remaining slurry was added as the buffer drained out of the column. The top surface of the gel in the column was adjusted to maintain even and uniform flow. Before the sample was run, the buffer was run through the whole column to stabilize the bed(two or three column volumes)(15).

Sample application:

A 1~2ml aliquot of each pooled sample of pancreatic/biliary fluid for each group was pipetted on top of the bed maintained at 22°C. After the sample was drained into the bed, the column was filled with buffer and connected to the buffer reservoir flask. The pump(Bio Chem Technology, Malbern, PA) was used for the pumping the buffer to maintain a consistent flow rate. The flow rate for elution was about 60ml per hour. Fractions, 2.5ml of the pancreatic/biliary fluid, were collected in the polyethylene tubes on the fraction collector(Retriever II, Isco Inc., Lincoln, NE). The void volume and the total volume of the clolumn were 82.47ml and 255.

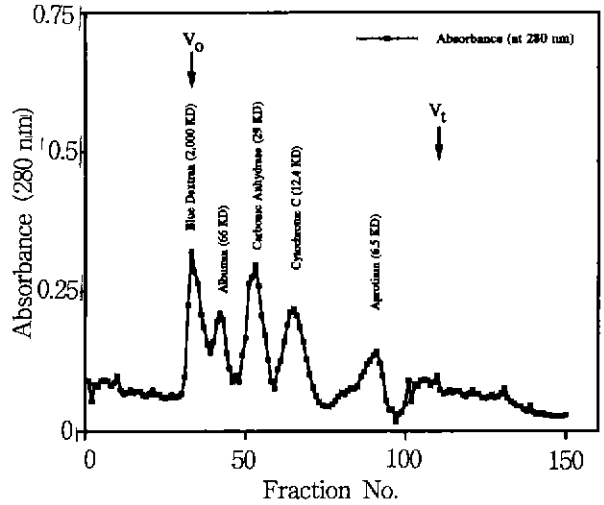

Fig. 1. Elution profile of the four standard proteins on Sephadex G-75 column.
Four standard proteins are albumin(66kDa), carbonic anhydrase(29kDa), cytochrome C(12.4kDa) and aprotinin(6.5kDa). Column size; 2.5×52cm, sample size; 1.5ml, fraction volume; 2.5ml, buffer; 0.01M Tris/HCl, pH 8.1, flow rate; 60ml/hr(Vo; void volume, 83ml, Vt; total volume, 255ml).

25ml, respectively. All columns were equilibrated with 0.01M Tris/HCl(pH 8.1) to maintain the original pH of pancreatic fluid at about 8.1~8.2. To prevent the bacterial and fungal growth, 0.02% sodium azide in the 0.01M Tris/HCl buffer was eluted through the column when not in use.

The column was calibrated with appropriate protein standards prior to use; albumin(66kDa), carbonic anhydrase(29kDa), cytochrome C(12.4kDa), aprotinin(6.5kDa) (Sigma Chemical Co., St. Louis, MO). The void volume of the column was determined with blue dextran(2,000 kDa). Elution protile of the four standard proteins is shown in Fig. 1.

Zn, protein and ^{65}Zn analyses

All fractions from the gel chromatography column were assayed for zinc with a flame atomic absorption spectrophotometer(Model 5000, Atomic Absorption Spectrometer, Perkin Elmer, Norwalk, CT).

For the detection of Zn-binding ligands, ultraviolet-absorbing material in each fraction was monitored at 280nm in a UV monitor(Model UV-260, UV-Visible Recording Spectrophotometer, Shimadzu, Kyoto, Japan). For ^{65}Zn radioactivity, the γ-Scintillation Counter(Cobra II, Auto-Gamma Counting System, Packard Instrument Co., Meriden, CT) was used.

RESULTS AND DISCUSSION

Analysis of pancreatic/biliary fluid

Protein concentration:

Protein concentration of the pancreatic/biliary fluid was 25.16±2.71mg/ml for the four dietary groups and there were no significant differences among groups. This agreed with the data of the Berger and Schneeman(16).

Zn concentration:

Total zinc in the pancreatic/biliary fluid was 3.97±0.76μg/ml for the low Ca group and 4.19±0.98μg/ml for the high Ca group. The zinc concentration in the pancreatic/biliary fluid in this study is similar with the data of Berger and Schneeman(16). Finley and Johnson(17) showed lower zinc concentration in the pancreatic/biliary fluid, 0.34μgZn/ml without protein stimulation and 2.22μgZn/ml with protein stimulation.

Radioactivity in the pancreatic/biliary fluid:

The ^{65}Zn radioactivity in the pancreatic/biliary fluid was the highest(75.5±0.98cpm/ml) in the low Ca, phytate(initial collection period) than any other groups, and was the lowest(16.6±5.3cpm/ml) in the high Ca, non-phytate(initial collection period)($p<0.05$).

Zn-Binding ligands in pancreatic/biliary fluid

Typical representative elution patterns by gel filtration on Sephadex G-75 of rat pancreatic/biliary fluids from each of the four diet groups are shown in Fig. 2 to 5. The pancreatic/biliary fluid from animals fed four diet regardless dietary variables had similar elution patterns. In most samples, a small amount of protein and zinc were present near the void volume and more zinc appeared near the peak of the small molecular weight(6.5kDa) standard protein. Zinc peaks were not always precisely associated with a major protein as measured by absorbance at 280nm. Zinc was eluted near the following areas; corresponding to the high molecular weight protein standard(>66 kDa), to the elution peak of carboxypeptidases A(molecular weight 35.3 kDa) and carboxypeptidase B(molecualr weight 34.3 kDa), and beyond the peak of the small molecular weight(6.5 kDa standard) of the column.

Radioactive ^{65}Zn was scattered across the whole elution without any prominent peaks for the four dietary groups. In the elution pattern, peaks of the ^{65}Zn were not sharp or prominent. It might be due to the dilution of ^{65}Zn radioactivity on the equilibrium of the body zinc

pool. Even the amount(10μCi) of injected ^{65}Zn was enough to equilibrate the body zinc pool, injected ^{65}Zn might be diluted in several minor endogenous zinc pools in the body. In this case, the pancreatic secretions may show weak radioactivity for the diluted ^{65}Zn radioactivity. Further studies will be necessary to define the ligands with this consideration.

Several fractions of eluate associated with low molecular weight peaks were pooled and lyophilized. The lyophilized samples were run on SDS-PAGE. The low level of radioactivity did not distinguish any bands by autoradiography.

Low Ca, phytate(initial collection period) and low Ca, non-phytate(crossover collection period) dietary group:

Among the 12 rats in this group, four rats were available for collection of pancreatic/biliary fluid. Pancreatic/biliary fluid from two rats were pooled to make one pooled sample for gel chromatography. The zinc eluted in three main pekas(Fig. 2). The first small zinc peak was associated with the proteins near the void volume, corresponding to compounds of molecular weight greater than 66kDa. The second zinc peak was shown between the void volume and the elution volume of the molecular weight 12.4kDa standard protein, cytochrome C. This second zinc peak showed a broader base than the first zinc peak. The third prominent zinc peak appeared beyond the low molecular weight standard, 6.5kDa. Zinc peaks were always eluted at the front of the protein peaks.

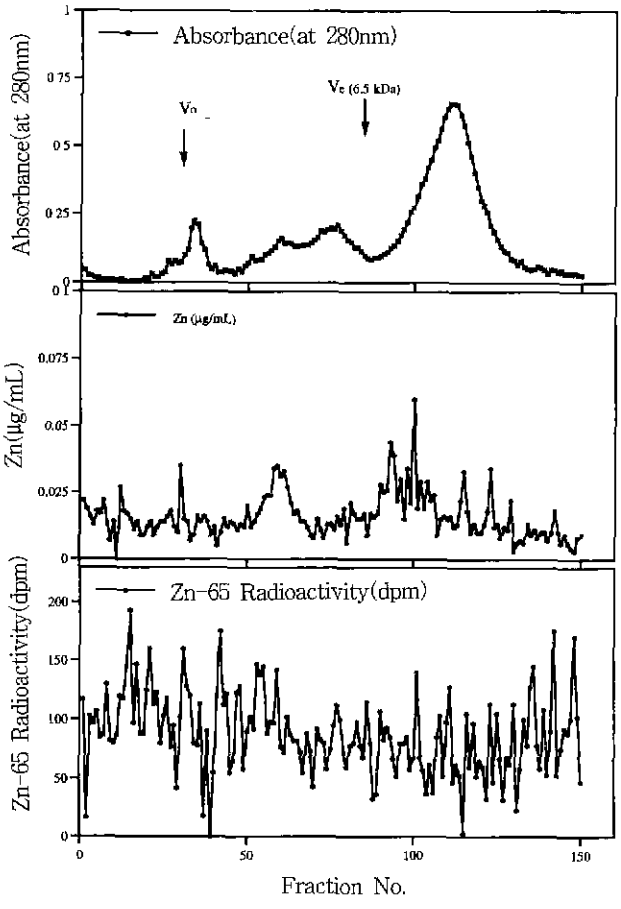

Fig. 2. Elution of rat pancreatic/biliary fluid of low Ca, phytate(initial collection period) and low Ca, non-phytate(crossover collection period) group on Sephadex G-75.
Sample size; 1.5ml, fraction volume; 2.5ml, buffer; 0.01M Tris/HCl, pH 8.1, flow rate; 60ml/hr(Vo; 83ml, Ve; elution volume of Aprotinin, 224ml).

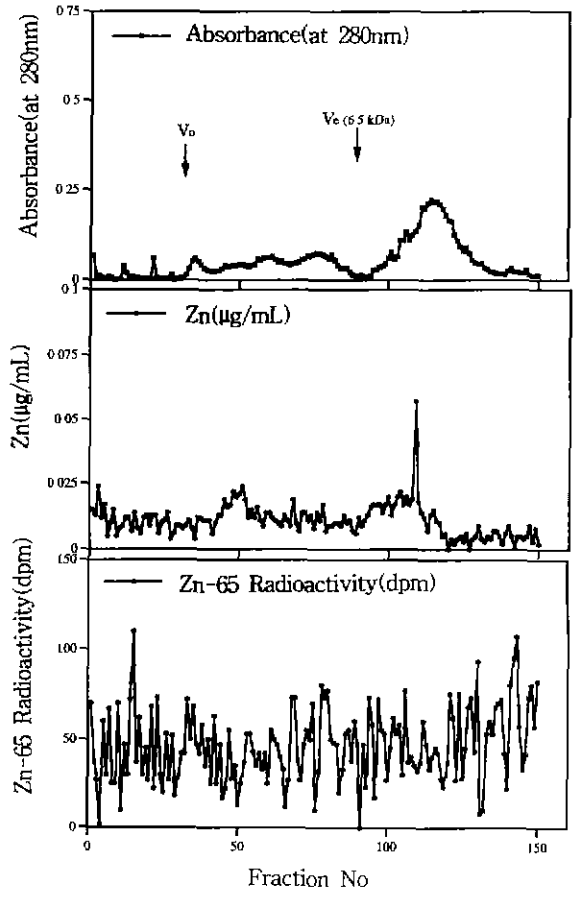

Fig. 3. Elution of rat pancreatic/biliary fluid of low Ca, non-phytate(initial collection period) and low Ca, phytate(crossover collection period) group on Sephadex G-75.
Sample size; 1.2ml. Any other conditions are same as in Fig. 2.

Low Ca, non-phytate(initial collection period) and low Ca, phytate(crossover collection period) dietary group:

Among the 12 rats in this group, pancreatic/biliary fluid was collected from seven rats. Pancreatic/biliary

fluid from 3 or 4 animlas were pooled to make each pooled sample for gel chromatography. In this dietary group, a zinc peak was not prominent. Only one zinc peak was shown associated with the low-molecular weight ligands which trailed the 6.5kDa standard protein(Fig. 3). The elution pattern confirms the presence of a low-molecular weight protein peak associated with zinc.

High Ca, phytate(initial collection period) and high Ca, non-phytate(crossover collection period) dietary group:

Among the 12 rats in this group, pancreatic/biliary fluid was collected from 10 rats. Pancreatic/biliary fluid samples were pooled from 2 rats to make one pooled sample for gel chromatography. Four prominent protein peaks are shown; one peak beyond the peak of the 6.5 kDa standard protein, and the other three peaks between the void volume and the elution volume of the 6.5kDa standard protein had an associated zinc peak(Fig. 4). However, unlike the elution pattern of the low calcium, phytate(initial collection period) gel chromatography, there was not a prominent zinc peak associated with the void volume or the molecualr weight more than 66kDa. A uniform distribution of zinc was associated with protein ligands across molecular weights from 66 kDa to <6.5kDa. The major zinc peak was at the elution volume in which molecualr weight is less than 6.5kDa.

High Ca, non-phytate(initial collection period) and high Ca, phytate(crossover collection period) dietary group:

Among the 12 rats in this group, pancreatic/biliary fluid was collected from 10 rats. Pancreatic/biliary fluid were pooled from 3 to 4 animals to make one pooled sample for gel chromatography. Two zinc peaks are

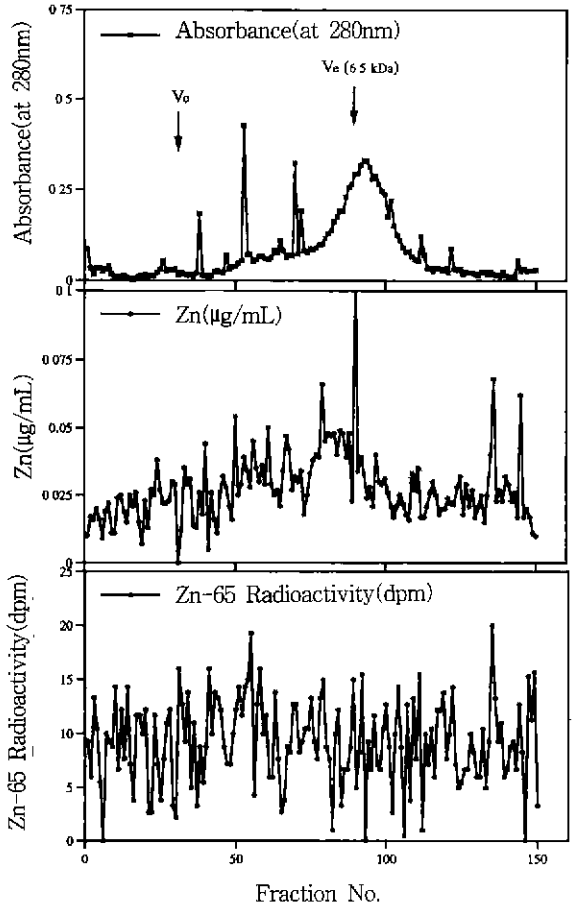

Fig. 4. Elution of rat pancreatic/biliary fluid of high Ca, phytate(initial collection period) and high, non-phytate(crossover collection period) group on Sephadex G-75.
Sample size; 1.5ml. Any other conditions are same as in Fig. 2.

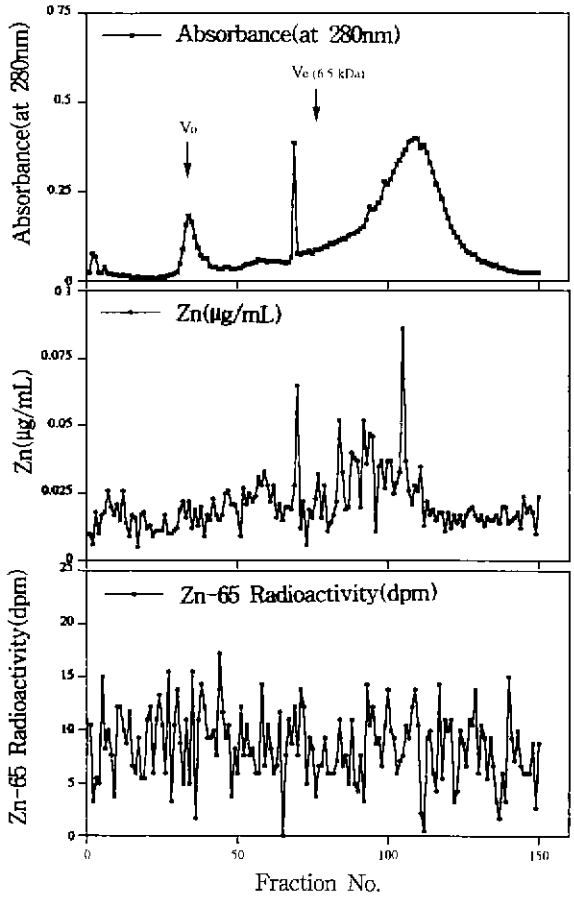

Fig. 5. Elution of rat pancreatic/biliary fluid of high Ca, non-phytate(initial collection period) and high Ca, phytate(crossover collection period) group on Sephadex G-75.
Sample size; 1.5ml. Any other conditions are same as in Fig. 2.

precisely associated with protein peaks(Fig. 5). The first major zinc peak was eluted between molecular weight 29kDa and 6.5kDa protein peak area. This peak corresponds to molecualr weight of similar to carboxypeptidase A or B. The second major zinc peak was eluted beyond the elution volume of 6.5kDa standard protein. Unlike the prominent elution patterns, no zinc peak was shown at the void volume of the colume of aprotinin standard protein of the column in which protein molecular weight was less than 6.5kDa.

Since most of the major zinc peaks from the pancreatic/biliary fluid were eluted beyond the elution volume of the smallest standard protein, aprotinin(6.5kDa), it may be considered that most of the zinc-binding ligands in the pancreatic/biliary fluid are low molecular weight peptides. Two zinc peaks beyond the elution pattern of the 6.5kDa standard protein in Fig. 4, near the end of gel chromatography may represent dissociated ionic zinc.

Lonnerdal et al.(18) reported that most of the zinc in bile was associated with a low molecualr weight fraction. In contrast, pancreatic secretions did not contain low molecular weight zinc complexes, but there were considerable amounts zinc bound to high molecualr weight compounds. They also reported that homogenates of pancreatic tissue, like pancreatic fluid, had no low molecular weight zinc complexes. They applied samples(2ml) to a Sephadex G-50 column(1.6×100cm) and calculated fraction volumes(3.8ml). One of the conditions of their experiment was the usage of 0.1M ammonium acetate buffer. pH 6.5, which was lower than the normal pH of pancreatic secretion(pH 8.0~8.3). They explained that with high levels of added zinc, the peak of low molecular weight was observed in pancreatic fluids. Therefore, their results should not be interpreted as pancreatic fluid being devoid of low molecular weight zinc-binding ligands. The results of the present study indicate that pancreatic/biliary fluid has low-molecular ligands which combine with the zinc, and transport it into the duodenum.

The results presented here agree with previous reports on rat pancreatic supernatant and pancreatic fluid in the dog on the size of zinc-binding ligand(19.20). Addition of ^{65}Zn to either the pancreatic fluid or rat pancreatic supernatant was eluted at the total volume of the column, near the area of low-moleular weight ligands. Evans et al.(19) also found that the body absorption of ^{65}Zn in rats in which the hepatic bile duct was ligated. This fact supports the concept that pancreatic secretions influence zinc absorption.

Reinstein et al.(21) reported that zinc secreted in pancreatic/biliary fluid did not play a major role in zinc homeostasis. Molecular localization of zinc in pancreatic/biliary fluid was associated with digestive zinc metalloenzymes, carboxypeptidase A or carboxypeptidase B known to be present in pancreatic fluid. However, they eluted only 250ml of buffer which is not sufficient to elute the small molecular weight fraction observed here. In the current study, more than 350ml buffer was used to form the elution pattern. The elution of the low molecular weight ligands was between 250~375ml of buffer. Reinstein et al.(20) used 0.1M ammonium acetate buffer with 0.5M sodium chloride, pH 6.5 to equilibrate the column before applying of the pancreatic/biliary fluid. The pH 6.5 does not represent a buffer appropriate for pancreatic secretion and is well below pancreatic fluid pH, 8.0~8.2.

In conclusion, distribution of zinc-binding protein through the four dietary groups in gel subfractions showed a peak corresponding to the high molecular weight protein standard(>66kDa) and with the molecular weight around 29~35kDa which were similar in size with zinc-containing enzymes in the pancreatic/biliary fluid. A more remarkable small molecular weight fraction was eluted near or after the 6.5kDa protein standard peak. The present study indicates that secretion of low-molecualr weight zinc-binding ligands smaller than the 6.5kDa in pancreatic fluid may represent loosely complexed zinc ligands. These ligands may dissociate zinc in the duodenum where pH is about 6. The dissociated zinc is easily to complexed with the phytate in the upper gastrointestinal tract where the pH is appropriate for maximal phytate-zinc complexation.

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