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Molecular Size and Distribution of Zinc-binding Ligands in Rat Pancreatic Tissue

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Abstract

The pancreas is an important organ in the maintenance of zinc homeostasis. The pancreatic tissue used in this study was obtained from rats fed varying levels of dietary Ca and phytate followed by intraperitoneal ^{65}Zn injection. The objective of this study was to determine the molecular size and distribution of compounds that may represent zinc-binding complexes in pancreatic tissue homogenates. The supernatant of the homogenized pancreatic tissue was separated using a Sephadex G-75 column with Tris buffer at pH 8.1. All subfractions were assayed for zinc, protein and ^{65}Zn activity. The elution of subfractions from pancreatic tissue homogenates showed a prominent peak corresponding to the high molecular weight protein standard (>66kd). A small molecular weigth protein(<6.5kd), that was absorbed at 280nm, was also present; prominently in low Ca group, however not much as in high Ca group. These small compounds may combine weakly with zinc in pancreatic tissue and serve as zinc-binding ligands in pancreatic/biliary fluid. In the duodenum, these ligands dissociate zinc into an ionic form which becomes vulnerable to phytate complexation.

Key words: rat pancreatic tissue homogenates, zinc-binding ligands, endogenous zinc, zinc homeostasis

INTRODUCTION

Most of the endogenous zinc is secreted from the exocrine pancreas, and then enters into the duodenum where it can be combined with other zinc ligands. This endogenous zinc should be reabsorbed to maintain zinc homeostasis. Since the pancreas is the main source for the endogenous zinc secretion, it is an important organ in zinc homeostasis. The pancreas maintains higher zinc concentrations than other tissues, which also suggests an important metabolic role for zinc(1). Injected radioactive ^{65}Zn accumulates in relatively high concentrations in the pancreas, and the excretion of a major part of the injected radioactive ^{65}Zn was via the pancreatic secretion (2-5). During periods of zinc deficiency, pancreatic zinc concentration is decreased and pancreatic concentration of zinc has increased during zinc excess, which implies the exocrine function of the pancreas in zinc absorption/reabsorption(6-8).

Zinc secretion from the pancreas is closely associated with the exocrine secretion of proteins(4). Low-molecular weight ligands were reported previously in pancreatic secretions(9-12) and both pancreas and liver(13-15). Oberleas(16) suggested that there are two zinc pools in the pancreas, depending on the status of zinc-binding to the ligands; one, a stable pool in which zinc is bound with zinc-dependent enzymes and other large molecular weight ligands. Zinc in the stable zinc pool which is tightly bound, is not affected by phytate and is always excreted in the feces. The zinc in the labile pool is dissociated in the duodenum and is subject to complexation with phytate and other ligands but must be largely reabsorbed to sustain zinc homeostasis. The identification of the zinc-binding ligands in the labile pool would help to understand the mechanism of zinc homeostasis.

Ca and phytate have been regarded as the antagonists for zinc absorption/reabsorption. It would be also helpful to clarify whether the various levels of Ca and phytate, which can decrease the zinc absorption, affects on the size and the amount of Zn-binding ligands in pancreatic tissue for the zinc secretion. The objective of this study was to identify the molecular size and distribution of zinc-binding ligands in rats fed different Ca and phytate level diets.

MATERIALS AND METHODS

Experimental design, animal care and diets

The two variables in the present study were two levels of phytate(0 and 4.7g/kg as sodium phytate for non-

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Table 1. Experimental diets composition

Ingredient	Low Ca phytate	Low Ca non-phytate	High Ca phytate (g/kg diet)	High Ca non-phytate
Glucose[1)]	665.3	670	645.3	650
Vegetable oil	40	40	40	40
Fat-soluble Vit premix[2)]	10	10	10	10
Cellulose	30	30	30	30
Casein	200	200	200	200
Mineral premix[3)]	30	30	30	30
Calcium carbonate	0	0	20	20
Trace element premix[4)]	10	10	10	10
B-complex premix[5)]	10	10	10	10
Na phytate	4.7	0	4.7	0

[1)]Any substitutions were made at the expense of glucose
[2)]Fat-soluble vitamin premix(g/kg diet): Vitamin A acetate, 0.00069; Vitamin D, 0.0000075; Menadione, 1; Alpha tocopherol acetate, 3; Butylate hydroxy anisole(antioxidant), 10; Vagetable oil, 985.3
[3)]Mineral premix(g/kg diet): Calcium carbonate, 210; Calcium phosphate(2 waters). 330; Magnesium oxide, 15; Potassium chloride, 10; Sodium chloride, 30; Ferrous sulfate(7 waters), 4; Potassium acid phosphate, 120; Manganese carbonate, 2; Cupric sulfate(5 waters), 0.8; Potassium iodate, 0.1; Sodium fluoride, 0.4; Glucose, 277.7
[4)]Trace element premix(g/kg diet): Sodium molybdate(2 waters), 0.00126; Chromium III acetate(1 water), 2.4; Sodium selenite(5 waters), 0.0167; Glucose, 997.5
[5)]B-complex vitamin premix(g/kg diet): Thiamine HCl, 1.6; Riboflavin, 1.6; Nicotinic acid, 17.32; Pyridoxine HCl, 1.6; Calcium pantothenate, 4; Biotin, 0.02; Folic acid, 0.5; Cyanocobalamin(0.1% in manitol), 5.0; DL-Methionine, 200; Choline bitartrate, 300; Glucose, 468.36

phytate and phytate group) and two levels of calcium(8 and 16g/kg as calcium carbonate for low Ca and high Ca group). Diet composition is shown in Table 1. A randomized complete block design with a 2×2 factorial arrangement of treatments was used, which gave four experimental dietary groups; low Ca and phytate, low Ca and non-phytate, high Ca and phytate group, and high Ca and non-phytate group.

Each twenty four male Sprague-Dawley rats(Harlan Sprague-Dawley, Inc., Indianapolis, IN) were Zn-depleted by feeding the phytate containing diet in each low or high Ca dietary group for 4 weeks. After 4 weeks of body zinc depletion, just before being reassigned into each dietary group, all of rats in low or high Ca group were injected with 10μCi of radioactive $^{65}Zn(ZnCl_2$, Dupont, Boston, MA) in 0.001M HCl diluted with saline. Radioactive ^{65}Zn was given by intraperitoneal injection to label the endogenous zinc which would be reabsorbed. Each tweleve rats, after then, were reassigned by weight into each phytate or non-phytate group within each low Ca or high Ca group. The experimental diets were fed for 2 weeks. The diets were supplied in aluminum feed cups and distilled water was supplied ad-libitum from polyethylene water bottles with butyl rubber(neoprene) stoppers.

Collection of pancreatic tissue

The average body weight of the rats at the time of surgery was 341±7g. At the end of experimental period, rats were anesthetized with a mixture of Ketaset(Ketamine HCl, Aveco Co. Inc. Fort Dodge, IA) and Xylzine (1ml/kg body weight), or Na Pentobarbital(40mg/kg body weight, 10mg/ml). The rat abdomen was rinsed with 0.9% saline and a mid-line incision was made to expose the pancreas. The pancreas was removed and rinsed with saline. The pancreatic tissues from 4 rats were pooled and homogenized at 1°C with 2.5vol.(w/v) of 0.01mol/ L Tris/ HCl buffer(pH 8.1). Homogenates were centrifuged immediately at 2500~2600rpm for 15 minutes. The cytoplasmic supernatants were removed and frozen for further analysis.

Sephadex G-75 gel chromatography

Sephadex G-75(Pharmacia Fine Chemicals, Inc., Piscataway, NJ) gel chromatography(17) was used for identification of the moelcular size of Zn-binding protein in pancreatic tissue homogenates.

Sephadex G-75 particle diameter, 40-120μm; water regain, 7.5±0.5ml/g dry Sephadex; bed volume, 12~15ml/g dry Sephadex; fractionation range for peptides and globular proteins, 3,000~70,000 and for dextrans, 1,000~50,000) was allowed to swell in excess 0.01M Tris/HCl buffered saline solution(pH 8.1). The gel was left to stand for 24 hours at room temperature or 3 hours on boiling water bath. To prevent of the bacterial and fungal gr-

owth, 0.02% sodium azide in the 0.01M Tris/HCl buffer was eluted through the column when not in use.

A 2ml aliquot of each pooled sample of from the supernatant of the homogenized pancreatic tissue was used for gel filtration. For use of Sephadex G-75 gel chromatography, the previous paper showed in detail(13). The flow rate was about 60ml/hr. Fractions(3.0ml) was collected in the polyethylene tubes on the fraction collector (Isco Inc., Retriever II, Lincoln, NE). The void volume and the total volume of the column were 82.5ml and 255.3ml, respectively. All columns were equilibrated with 0.01mol/L Tris/HCl(pH 7.9~8.2) to maintain the original pH of pancreatic tissue, pH 8.2.

The column(2.5cm×52cm, Kontes Coporate, Vineland, NJ) was calibrated with appropriate protein standards; albumin(66kd), carbonic anhydrase(29kd), cytocyrome C (12.4kd), and aprotinin(6.5kd)(Sigma Chemical Co., St. Louis, MO). The concentration of each of the four standard proteins was 1mg/ml. The void volume of the column was determined with blue dextran(2,000kd)(Sigma Chemical Co., St. Louis, MO). Standard calibration curve for the standard proteins is shown in Fig. 1.

Fraction measurement

Ultraviolet-absorbing material, representing the protein compound in each fraction from the gel chromatography column, was monitored at 280nm with a UV-visible recording spectrophotometer(Model UV-269, Shimadzu, Kyoto, Japan). Zinc concentration was analyzed with a flame atomic absorption spectrophotomer(Model 5000, Perkin Elmer, Norwalk, CT). The accuracy of the atomic absorption analysis was verified by using the National Institute of Standards and Technology(NIST) peach leaves standard(Standard Reference Material 1547). The analyzed zinc value found for the standard peach leaves, 17.9μg Zn/g, sample compared with standard 18.3μg Zn/g sample reported by NIST which gave the recovery rate, 97.8%. For ^{65}Zn radioactivity for measurement of the endogenous zinc, an Auto γ-Scintillation Counter(Packard Instrument Co., Meriden, CT) was used.

	Ve/Vo	Molecular weight(daltons)
Aprotinin	2.51	6500
Cytochrome C	1.97	12400
Carbonic anhydrase	1.62	29000
Albumin	1.33	66000

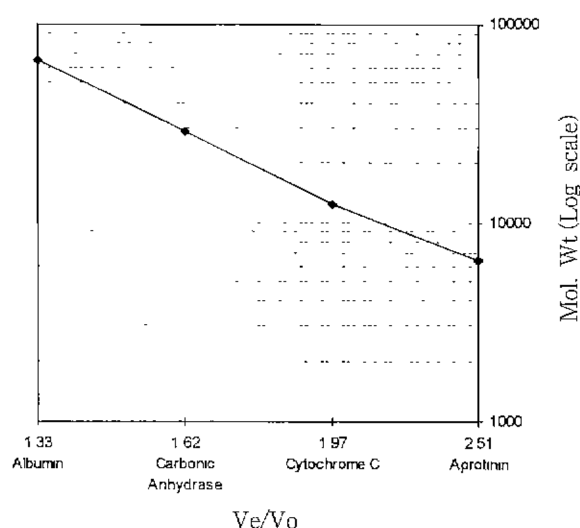

Fig. 1. Calibration Curve obtained with four standard proteins as run on Sephadex G-75 column(2.5cm ×52cm).(Ve: elution volume, Vo: void volume)

RESULTS AND DISCUSSION

Pancreatic tissue homogenates from animals fed the four different diets showed almost identical elution patterns in protein and zinc distribution. Total zinc was eluted near the void volume associated with large molecular weight proteins(>66kd). Less prominent zinc peaks were associated with the small molecular weight proteins (6.5kd) through for the four dietary groups. Small zinc peak for the Zn-containing enzyme, carboxypeptidase A or B, also appeared near the large molecular weight protein(29kd). The profile of the radioactive ^{65}Zn showed scattered profile without distinct peaks due to the weak radioactivity.

Two prominent zinc peaks were present beyond the elution volume of 6.5kd standard protein(Fig. 2). The elution curve of the pancreatic cytosol showed the most prominent zinc peaks were associated with the small molecular weight ligands less than 6.5kd. The broad and less prominent zinc peaks appeared near the void volume, which was the large molecular(>66kd) weight compounds. Between the void volume and the elution volume of aprotinin(6.5kd), near the elution volume of carbonic anhydrase (29kd), several zinc peaks appeared. These zinc peaks might be the elution of the zinc-binding enzymes, such carboxypeptidases(34~35kd) which is secreted from the pancreas.

Most of the zinc was associated with the large molecular weight ligands in this dietary group. One prominent radioactive ^{65}Zn peak corresponded with the large molecular weight protein. Both of the analyzed zinc peaks and radioactive ^{65}Zn peaks were eluted with the void vol-

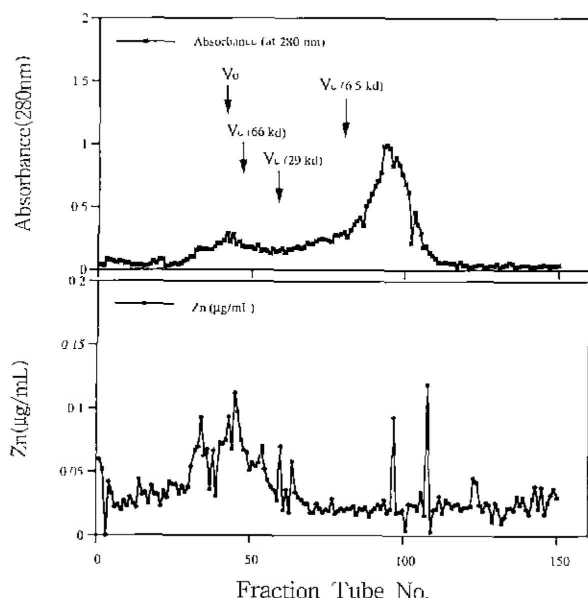

Fig. 2. Elution of the supernatant from rat pancreatic tissue of low Ca, phytate dietary group on Sephadex G-75 column.
Sample size: 2.0ml, fraction volume: 3.0ml, buffer: 0.01M Tris/HCl, pH 8.1, flow rate: 60ml/h. Vo: Void volume, Ve: Elution volume of albumin(66kd), carbonic anhydrase(29kd), and aprotinin(6.5kd).

ume at the same large molecular weight compound in the elution profile. One broad zinc peak was shown just behind the void volume. Part of that zinc peak might be related to carboxypeptidase A or B of which the molecular weight is 34~35 kd. The less prominent zinc peaks were eluted in the area of elution volume of 6.5kd standard protein(Fig. 3).

The elution of the pancreatic tissue homogenate from this dietary group showed that most of the zinc was associated with the large molecular weight ligands which were eluted near the void volume, presumably, a molecular weight more than 66kd(Fig. 4). Secondly prominent zinc peaks followed beyond the void volume. These peaks might be the zinc-binding enzymes elution area. A very tiny zinc peak was eluted near the elution volume of 6.5kd standard protein. The amount of eluted zinc was the highest in this dietary group among the four dietary groups, probably due to the Ca and phytate effect on the zinc secretion from pancreas.

The elution of the pancreatic tissue homogenate in this dietary group showed almost identical pattern with that of high Ca, phytate group. The elution of the pancreatic tissue homogenate in this dietary group showed most of the zinc was associated with the large molecular weight ligands which were eluted near the void volume (Fig. 5). The second zinc peaks which were considered as the elution of the Zn-binding enzymes, appeared just followed the elution of large molecular weight compound.

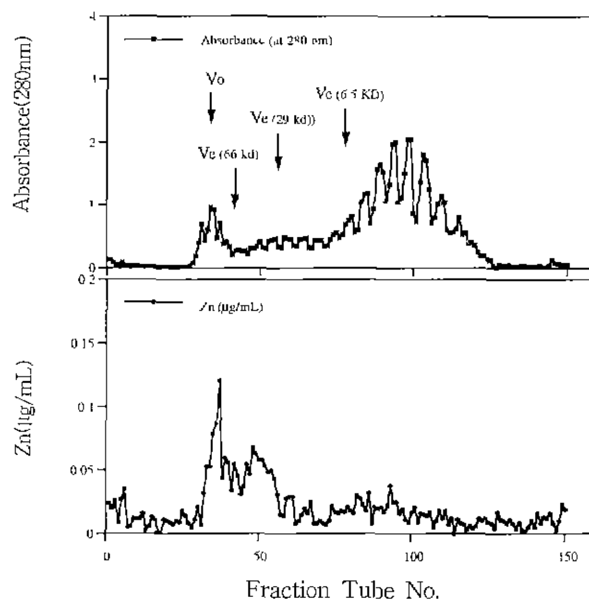

Fig. 3. Elution of the supernatant from rat pancreatic tissue of low Ca, non-phytate dietary group on Sephadex G-75 column.
Sample size: 2.0ml, fraction volume: 3.0ml, buffer: 0.01M Tris/HCl, pH 8.1, flow rate: 60ml/h. Vo: Void volume, Ve: Elution volume of albumin(66kd), carbonic anhydrase(29kd), and aprotinin(6.5kd).

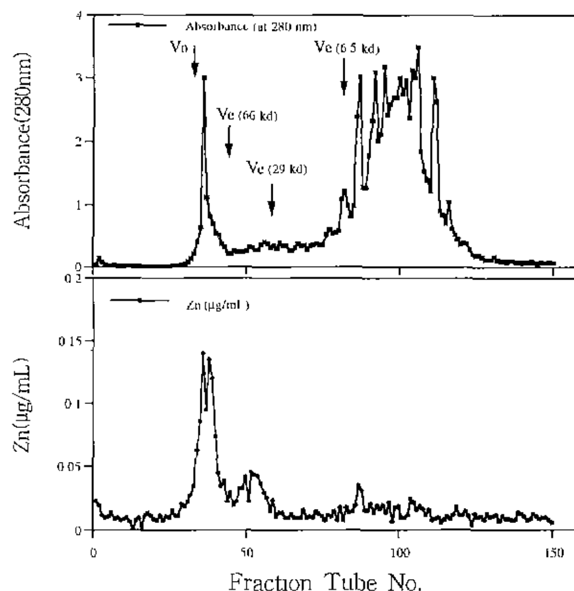

Fig. 4. Elution of the supernatant from rat pancreatic tissue of high Ca, phytate dietary group on Sephadex G-75 column.
Sample size: 2.0ml, fraction volume: 3.0ml, buffer: 0.01M Tris/HCl, pH 8.1, flow rate: 60ml/h. Vo: Void volume, Ve: Elution volume of albumin(66kd), carbonic anhydrase(29kd), and aprotinin(6.5kd).

These second peaks were much less prominent and broader ones comparing to those of the high Ca, phytate group (Fig. 4). A very small zinc peak was eluted near the elution volume of 6.5kd standard protein.

The elution pattern of rat pancreatic tissue in the present study showed that more prominent zinc peaks appeared near the large molecular weight protein(<66kd), comparing to the elution profile of pancreatic/biliary fluid in which more zinc was associated with the small molecular weight ligands(<6.5kd)(12).

In both low and high Ca groups, more zinc was eluted in phytate-containing group. Low Ca, phytate group showed higher zinc elution peaks(Fig. 2), comparing to low Ca, non-phytate group(Fig. 3), which implies more zinc occured with the presence of phytate intake. High Ca, phytate group also showed higher and bigger zinc elution peaks(Fig. 4), comparing to those of high Ca, non-phytate group(Fig. 5); however, the difference was not that big as much as it was in low Ca group. Among four dietary groups, the highest and the most prominent zinc peaks appeared in the high Ca, phytate group in which zinc was synergistically complexed to phytate with the presence of the high Ca level in the digestive tract(Fig. 4). Depending on the Ca level, low Ca groups showed more scattered zinc elution peaks(Fig. 2, 3), while high Ca group showed more prominent zinc peaks(Fig. 4, 5). Further study would be needed for the effect of phytate, Ca intake on the pancreatic zinc secretion; pattern, contents, volume, etc.

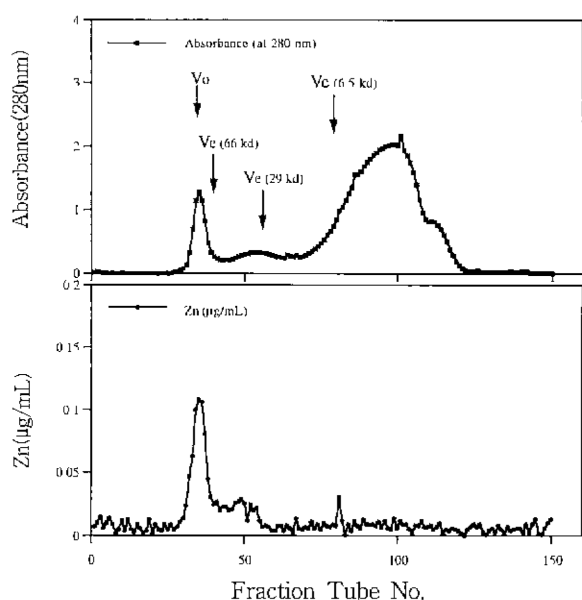

Fig. 5. Elution of the supernatant from rat pancreatic tissue of high Ca, non-phytate dietary group on Sephadex G-75 column.
Sample size: 2.0ml, fraction volume: 3.0ml, buffer: 0.01M Tris/HCl, pH 8.1, flow rate: 60ml/h. Vo: Void volume, Ve: Elution volume of albumin(66kd), carbonic anhydrase(29kd), and aprotinin(6.5kd).

On the elution of ^{65}Zn-labeled endogenous zinc, due to the weak radioactivity, prominent zinc peaks were not shown through the four dietary groups except that low Ca, non-phytate group showed one ^{65}Zn peak just beyond the large molecular weight protein(>66kd) where ^{65}Zn peak was almost coupled with the peaks of the total zinc elution peak(not shown). In the present study, rats were Zn-depleted for four weeks with phytate-containing diet. Therefore, it might be considered that there are small molecular weight zinc-binding peptides which are less than 6.5kd, and large molecular weight zinc-binding ligands more than 66kd, mainly, in rat pancreatic tissue. The results agree with previous reports on zinc-binding ligands in pancreatic tissue supernatant and pancreatic fluid. Cikrt et al.(9) reported that zinc has been found to occur especially in two fractions in rats pancreatic tissue, one with low molecular weight and another with high molecular weight. Casey et al.(10), on the other hands, reported that a low molecular weight zinc binding ligand was present in duodenal secretions in which most components come from the pancreatic secretions in AE(Acrodermatitis Enteropathica) patients and from healthy adults. Bremner et al.(13) fractionated the tissue supernatant on Sephadex G-75 with the 0.01mol/L Tris-acetate buffer, pH 8.2 and reported that a low molecular weight(about 12kd) zinc-binding protein might be synthesized in response to an increase in pancretic zinc content. Kincaid et al.(14) reported that the elution profile of the soluble fraction of both the pancreas and liver of calves revealed a 10kd molecular-weight protein associated with the elevated zinc. Alexander et al.(15) isolated the low-molecular weight zinc-binding complex from homogenized pancreas and reported zinc was mainly bound to low molecular weight compounds eluted, corresponding to the zinc-glutathione complexes in rat bile injected with $^{65}ZnCl_2$.

Since most of large molecular weight of Zn-binding ligands, including Zn-containing enzymes, are tightly combined with zinc as a stable zinc pool type, it wouldn't be dissociated easily. However, small molecular weight Zn-ligands in labile zinc pool are much easier to be dissociated in pancreatic secretion. Therefore, the small molecular weight zinc-binding ligands may assist in the regulation of zinc homeostasis through the secretion with

zinc into the duodenum. The ligands which is under pH 8.2 in pancreatic secretion may dissociate zinc in the duodenum where pH is about 6. The dissociated zinc is easily complexed with the phytate in that upper gastrointestinal tract where zinc-phytate complexing can be the maximum under the condition of pH 6.0. It is suggested that the affinity of zinc to the zinc-binding ligands is interfered by phytate which decreases the dietary and endogenous zinc absorption(16).

In this study of pancreatic tissue homogenates, the distribution of zinc-binding ligands in the subfractions were associated with prominently high molecular weight proteins(<66kd) and small molecular weight compounds(6.5 kd) through the various levels of Ca and phytate. The presence of small molecular weight compounds associated with zinc in pancreatic tissue homogenates may serve as ligands for the secretion of endogenous zinc to the pancreas and further more into the duodenum. In the duodenum, these ligands dissociate zinc to make it vulnerable to phytate complexation. It would be the next research topic to identify these small molecular weight Zn-ligands more specifically.

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