Effect of Zinc Deficiency on Enzyme Activities in Rat and Pig Erythrocyte Membranes[1] (43139)

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Abstract. **There is need for a reliable index of zinc status in humans. Considering the importance of zinc in membrane function, activities of erythrocyte membrane enzymes have been measured in animals of low and normal zinc status as possible indices. Immature rats and neonatal pigs were fed low and adequate zinc diets; the latter was fed both *ad libitum* and restricted so as to control for food intake effects. Low rates of gain and plasma zinc concentrations demonstrated that animals fed the low zinc diets were of low zinc status. Erythrocyte membranes were prepared and assayed for Na,K-ATPase, 5′-nucleotidase, and calcium-ATPase activities. Na,K-ATPase activity was not affected by zinc status, but 5′-nucleotidase was significantly lower in deficient animals of both species than in controls, whose food intake was restricted to maintain comparable weight (2.76 vs 3.94 nmol/hr/mg of protein in rats and 60.5 vs 119 in pigs). The basal calcium-ATPase activities were also decreased by low zinc status in both species. Addition of calmodulin *in vitro* stimulated activity two-fold to four-fold and resulted in the same maximal activities for all treatments. The results show that erythrocyte membrane 5′-nucleotidase activity is an index of zinc status in these species. It is suggested that the decreased membrane calcium-ATPase activity in zinc deficiency is caused by a defect in calmodulin metabolism.** [P.S.E.B.M. 1990, Vol 195]

The assessment of zinc status in humans is difficult because conditions other than dietary status may affect plasma zinc concentration, the commonly used index. Plasma zinc is decreased not only by zinc deficiency but also by infection, pregnancy, endotoxins, and other physiologic stresses (see (1) for a review). In the absence of such stress, plasma zinc promptly reflects dietary zinc deprivation but does not necessarily reflect long-term zinc status. With the exception of bone, the zinc concentration in most tissues is not changed appreciably, even in severely zinc-deficient animals (2, 3). In spite of extensive research, there are few reliable indices now available for clinical evaluation of long-term zinc status.

Various enzyme activities have been used to measure zinc status in animals. The activity of alkaline phosphatase in the serum of rats is decreased by 50% after 4 days of zinc depletion and returns to near control levels within 3 days after repletion (4). Angiotensin converting enzyme activity is rapidly depressed in rat plasma by zinc deprivation (5, 6). However, its activity is readily restored by zinc *in vitro* so that it is no more valuable as a status indicator than is plasma zinc. The metallothionein level in serum and erythrocytes is a promising but unproven index (7). In studies in humans, there are reports of decreased purine nucleoside phosphorylase and 5′-nucleotidase activities in lymphocytes of mildly zinc-deficient patients (8). Thymulin activity was also found to be low in persons of low zinc status (9).

There is ever-increasing evidence that zinc plays a specific role in the structure and function of plasma membranes (10). Thus, one might expect enzymes, receptors, and ion channels associated with the plasma membrane to be indicators of zinc status. The goal of this study was to determine the effect of zinc deprivation on the activities of enzymes found in erythrocyte membranes of rats and pigs.

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Materials and Methods

Animals and Diets. Groups of 10 immature rats were treated as described previously (11). The males analyzed in the first experiment were the same as those studied earlier for another purpose (11). Females were used in the second experiment. In each experiment, one group was fed a low zinc diet *ad libitum* (−zinc, AL), one was fed an adequate zinc diet *ad libitum* (+zinc, AL), and one was fed the adequate zinc diet restricted (+zinc, PF) to the intake of pair-mates fed the low zinc diet. The low zinc diet contained EDTA-treated soy protein and, by analysis, 0.52 ± 0.06 mg of zinc/kg; the control diet was the same except it was supplemented with 100 mg of zinc/kg as $ZnCO_3$. Deionized water and diets were supplied during a 28-day experimental period. The rats were weighed weekly; blood was collected at the end of the period for preparation of plasma and red blood cells as described previously (11).

Three groups of six pigs were used in a similar experiment. Cross-bred (Yorkshire × Landrace × Duroc) male pigs were weaned at 14 days of age and housed individually in stainless steel cages in a room with a 16.5:7.5-hr light:dark cycle and a temperature of 27–32°C. A 7-day adaptation period preceded the 21-day experimental period. During the adaptation period, all pigs consumed the basal diet (0.8 mg of zinc/kg) described in Table I; deionized water was supplied *ad libitum*. The pigs were then assigned to three groups. One group was fed a low zinc diet (basal supplemented with 4 mg of zinc/kg) *ad libitum* (−zinc, AL), one was fed the zinc-adequate diet (100 mg of zinc/kg) control diet *ad libitum* (+zinc, AL), and one was fed the control diet restricted (+zinc, RF) to a level that allowed weight gain at the same rate as those fed the low zinc diet. Weights were recorded daily. Blood was collected at the end of the trial, as described previously (12), for preparation of erythrocytes and plasma.

Composition of the basal diet for pigs is shown in Table I. It was based on egg white protein which had been denatured by autoclaving (12). Since young pigs will not survive on this diet because of its low zinc content, the low zinc diet used in this study was the basal diet supplemented with 4 mg of zinc/kg of diet. By atomic absorption spectrophotometric analysis, this diet contained 4.6 ± 0.2 mg of zinc/kg. The control diet was supplemented with 100 mg of zinc/kg and, by analysis, contained 103 ± 1.3 mg of zinc/kg. The diets were fed dry.

Preparation of Erythrocyte Plasma Membranes. Pig erythrocyte membranes were prepared according to the method of Steck *et al.* (13). Briefly, red blood cells were washed three times with phosphate-buffered saline (145 m*M* NaCl in 5 m*M* sodium phosphate, pH 8.0). They were lysed in 5 m*M* sodium phosphate (pH 8.0) and washed three times in the lysis buffer. The membranes (ghosts) were then washed twice with 36 m*M* NaCl to remove phosphate, which interfered in the ATPase assay. The preparation was diluted to a protein concentration of 1–3 mg/ml with 36 m*M* NaCl and stored at −80°C after being quick frozen in liquid nitrogen. The ghosts were thawed and kept on ice in preparation for enzyme assays.

Rat erythrocyte membranes were prepared and stored similarly, as described earlier (11). The protein content of the membrane preparations was determined according to the method of Lowry *et al.* (14) using bovine serum albumin as the standard.

Table I. Composition of Basal Diet For Pigs

Ingredient	Concentration (g/kg)
Autoclaved egg white[a]	260
Glucose hydrate	299
Corn syrup solids[b]	289
Corn oil[c]	60
Mineral premix[d]	50
Cellulose[e]	30
Vitamin premix[f]	10
Choline premix[g]	2

[a] Spray-dried egg white, obtained from Kraft Foods, Inc., Marshall, MO, was reconstituted with deionized water (1:1.2) and autoclaved at 121°C for 30 min (12).
[b] Star-Dri 24R, A. E. Staley Manufacturing Co., Decatur, IL.
[c] Mazola; Best Foods CPC International, Englewood Cliffs, NJ.
[d] Supplied (mg/kg of diet): calcium, 9000; phosphorus, 7000; magnesium, 400; iron, 100; copper, 6; manganese, 4; selenium, 0.3; iodine, 0.14. These elements were supplied by the following salts: $CaHPO_4 \cdot H_2O$, $CaCO_3$, $MgSO_4$, Ferric citrate (17% iron), $CuSO_4$, $MnSO_4$, Na_2SeO_3, KIO_3. Egg white supplied adequate potassium, sodium, and chlorine; by analysis, the diet contained 0.35% potassium, 0.45% sodium, and 0.65% chlorine.
[e] Solka Floc R, James River Corp., Berlin, NH.
[f] Supplied the following per kilogram of diet: vitamin A, 2200 IU; vitamin D, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; riboflavin, 4.0 mg; niacin, 20.0 mg; vitamin B12, 0.02 mg; calcium pantothenate, 12.0 mg; thiamin HCl, 1.5 mg; pyridoxine HCl, 2.0 mg; biotin, 0.08 mg; folic acid, 0.3 mg; ascorbic acid, 250 mg; neomycin, 154 mg; oxytetracycline, 165 mg.
[g] Supplied 0.6 g of choline chloride/kg of diet.

Zinc and Enzyme Assays. Plasma was analyzed for zinc according to the method of Parker *et al.* (15). Diets were dry ashed, dissolved in diluted HCl, and aspirated into the flame of an atomic absorption spectrophotometer.

Na,K-ATPase activities were assayed according to the method of Das *et al.* (16), and 5′-nucleotidase activities were assayed by an adaptation of the method of Gentry and Olsson (17). In the 5′-nucleotidase assay, 0.1 ml of the ghost preparation was added to 60 m*M* Tris-HCl buffer (pH 7.4) containing 5 m*M* $MgCl_2$ (total volume, 1.0 ml). The reaction was started by the addition of 150 μM AMP and 0.010–0.015 μCi of [^{14}C]

AMP. After being incubated for 15 min at 37°C, 200 μl each of 5% $ZnSO_4$ and 0.3 *N* $Ba(OH)_2$ was added to stop the reaction and remove unreacted AMP from the product, [^{14}C]adenosine, which remained in solution. The tubes were centrifuged at 1000*g* for 10 min, and 0.9 ml of the supernatant solution was added to the scintillation cocktail for estimation of radioactivity. A tube containing the nucleotidase inhibitor, α,β-methyleneadenosine-5′-diphosphate, sodium salt, was used to correct for interfering activity; 5′-nucleotidase activity was measured as the difference between total phosphatase and inhibitor-insensitive activity.

Calcium-ATPase activity was assayed according to the method of Vincenzi *et al.* (18). Basal ATPase activity was that observed in the absence of added Ca^{2+}. To calculate calcium-ATPase activity, this value was subtracted from the total activity obtained in the presence of 2 m*M* Ca^{2+}. Calmodulin-stimulated activity was obtained by subtracting the basal activity from that obtained with added Ca^{2+} and calmodulin (2 μg/ml; Bovine brain preparation (Sigma) containing >40,000 units/mg). Phosphate released in the assay was determined by reaction with ammonium molybdate and extraction of the complex into an equal volume of 1:1 isobutanol to benzene (19). The absorbance of the complex was measured spectrophotometrically at 410 nm. A freeze-thaw cycle was essential for optimal stimulation of calcium-ATPase by calmodulin, presumably to allow calmodulin access to the enzyme binding site.

Data were analyzed by the analysis of variance followed by the post hoc *t* test (Interactive Statistics Package; Crunch Software, San Francisco, CA).

Results

As shown in Table II, the rates of gain and plasma zinc concentrations were decreased in both rats and pigs fed the low zinc diets. These indicators provide good evidence that the animals were zinc deficient. Pair-feeding of the rats restricted their gain to a level that was not significantly different from the −zinc, AL group, but their plasma zinc levels were not different from those of the *ad libitum* controls (+zinc, AL). Restricted intake of the control diet by pigs also maintained a rate of gain comparable to that of the −zinc, AL group. However, the restricted pigs had lower zinc levels in plasma than *ad libitum*-fed controls.

Erythrocyte membrane Na,K-ATPase activity was not affected by zinc status in either species (Table III) and thus served as baseline activity that is unaffected by nutritional status. This makes the observed decrease in 5′-nucleotidase activity even more significant. Relative to pair-fed controls, membranes from both rats and pigs fed the low zinc diets had lower 5′-nucleotidase activity. The *ad libitum*-fed control pigs were not statistically different from either the deficient or restricted fed control pigs. The restricted fed group in both species is considered the more valid control because of the dramatic effect of zinc deficiency on food intake and growth rate.

As shown in Table IV, the calcium-ATPase activity of erythrocyte membranes from both rats and pigs fed the low zinc diet was decreased compared with that in pair-fed controls. Addition of excess calmodulin (2 μg/ml) to the assay medium stimulated all activities nearly four-fold and produced maxima which were not different among dietary treatments. For an unknown reason, the membranes from restricted fed control pigs had higher activity than those from *ad libitum*-fed controls without added calmodulin. A similar relationship was observed in the 5′-nucleotidase assay and possibly reflects the stress of restricted food intake. In any case, the restricted fed group is the better control and the results show that zinc deficiency has an effect beyond decreased food intake. On the basis of the data, it appears that low-zinc status in both species decreases the quantity of calmodulin associated with the isolated membranes.

Discussion

Considering the evidence that zinc is concerned with plasma membrane function (20, 21), it is pertinent to examine the activity of membrane-bound enzymes

Table II. Zinc Status of Rats and Pigs[a]

Zinc status indices	Dietary zinc level[b]		
	−Zinc, AL	+Zinc, RF	+Zinc, AL
Rat			
Weight gain (g/day)	0.29 ± 0.05c[c]	0.73 ± 0.14c	5.04 ± 0.19d
Plasma zinc (μg/ml)	0.26 ± 0.03c	1.13 ± 0.04d	1.21 ± 0.04d
Pig			
Weight gain (g/day)	63.7 ± 12.1c	60.2 ± 11.8c	243 ± 13.7d
Plasma zinc (μg/ml)	0.33 ± 0.03c	0.85 ± 0.07d	1.15 ± 0.04e

[a] Mean ± SE for at least nine male rats and five male pigs per group.
[b] The low zinc rat diet contained <1 mg of zinc/kg; the low-zinc pig diet contained approximately 5 mg of zinc/kg; and the adequate-zinc diets contained approximately 100 mg of zinc/kg.
[c] Means within rows with different letters are statistically different; $P < 0.05$.

Table III. Effect of Zinc Status on Na,K-ATPase, and 5′-Nucleotidase Activities of Erthrocyte Membranes[a]

Dietary treatment	Na,K-ATPase activity (μmol/hr/mg of protein)	5′-Nucleotidase activity (nmol/hr/mg of protein)
Rat		
−Zinc, AL	1.21 ± 0.12	2.76 ± 0.29b[b]
+Zinc, PF	1.21 ± 0.11	3.94 ± 0.33c
Pig		
−Zinc, AL	0.36 ± 0.05	60.5 ± 9.2b
+Zinc, RF	0.42 ± 0.04	119 ± 24.7c
+Zinc, AL	0.44 ± 0.10	79.9 ± 9.4b,c

[a] See footnotes to Table II. Membranes from *ad libitum*-fed rats (+zinc, AL) were not analyzed.
[b] For each species, means within each column with different letters are statistically different; $P < 0.05$.

Table IV. Effect of Zinc Status on Calcium-ATPase Activity in Rat and Pig Erythrocyte Membranes[a]

Dietary treatment	μmol/hr/mg of protein	
	Calcium-ATPase activity	Calmodulin-stimulated Calcium-ATPase activity
Rat		
−Zinc, AL	1.59 ± 0.12b[b]	4.83 ± 0.29
+Zinc, PF	1.95 ± 0.06c	4.87 ± 0.23
+Zinc, AL	1.88 ± 0.07c	5.07 ± 0.20
Pig		
−Zinc, AL	1.90 ± 0.13b	8.25 ± 0.53
+Zinc, RF	2.31 ± 0.12c	8.14 ± 0.67
+Zinc, AL	1.81 ± 0.16b	7.06 ± 0.44

[a] Means ± SE for at least eight female rats per group whose plasma zinc concentrations for the −zinc, AL, +zinc, PF, and +zinc, AL groups were 0.30b, 0.84c and 0.92d μg/ml, respectively, and for five male pigs.
[b] For each species, means within a column with different letters are statistically different; $P < 0.05$.

as indices of zinc status. Well-recognized plasma membrane marker enzymes include 5′-nucleotidase, alkaline phosphatase, Na,K-ATPase, and calcium-ATPase. Two of these, alkaline phosphatase (22) and 5′-nucleotidase (23) are recognized zinc metalloenzymes. As it occurs in lymphoblasts, activity of the latter is regulated by the concentration of zinc in the medium (24). Na,K-ATPase and calcium-ATPase are ion pumps which span the membrane and play key roles in the function of most cells. 5′-Nucleotidase is an ectoenzyme that hydrolyzes nucleotides to form nucleosides. Nucleosides move through the plasma membrane readily while nucleotides do not. In this regard, it is of interest that the erythrocytes of adult pigs, unlike other mammals, are unable to take up and metabolize glucose (25). It is now clear that nucleosides, particularly inosine, are used by pig red blood cells for ATP production (26). This may explain why the pig erythrocyte membrane contains 20-fold higher 5′-nucleotidase activity than that of the rat.

In regard to zinc status, it is notable that there is decreased activity of 5′-nucleotidase activity in the plasma membranes of two species which have widely different inherent activities. It is particularly important because the difference occurs in a tissue that can be obtained readily from humans for clinical evaluation. A decrease in the plasma level of this enzyme has been observed previously (27), and there is a report of decreased liver plasma membrane activity (28). Presumably, the red blood cell membrane activity reflects longer term zinc status than does plasma activity but the relationship between the two is presently unknown. In any case, it is probable that the concentration of membrane apoenzyme is determined at the time of cell development and that it changes little during the life span of the red blood cell. Whether or not the plasma zinc concentration affects membrane enzyme activity directly remains to be determined. It should be noted that the decreased 5′-nucleotidase activity reported here was measured in the same rats that showed decreased erythrocyte membrane zinc concentration and increased red blood cell fragility (11).

Internal free Ca^{2+} and its flux play important regulatory roles in cell function. As a calcium pump, calcium-ATPase is a key component of the plasma membrane in the maintenance of the normal low concentration of internal cell calcium. A defect in this calmodulin-stimulated enzyme might account for the cellular malfunction observed in zinc deficiency, such as occurs in platelets (29) and red blood cells (21). Decreased calcium-ATPase activity might well result in increased, even toxic, levels of cell calcium and lead to membrane damage of the type described by Vincenzi *et al.* (18). They found that calcium loading of red blood cells led to abnormal polyacrylamide gel electrophoresis patterns of the membrane proteins.

The decreased level of unstimulated (no added calmodulin) calcium-ATPase activity observed in the red blood cell membranes of rats and pigs fed low zinc diets is significant for two reasons. In the first place, it may provide a useful index of zinc status in animals

and humans. In the second, it gives insight into the mechanism by which zinc functions at the plasma membrane level. In the presence of excess calmodulin, membrane calcium-ATPase activity was stimulated to the same maximal activity in all dietary treatments. The data support the concept that zinc-deficient membranes lose calmodulin more readily than do normal ones, i.e., they bind calmodulin less avidly. Another possibility is that the calmodulin concentration in the zinc-deficient cell is lower than normal, resulting in less calmodulin bound to the plasma membrane. Preliminary kinetic studies in our laboratory show no evidence that zinc deficiency alters the K_m of the membrane enzyme for calmodulin. Law *et al.* (30) showed that rats fed a low-zinc diet had lower concentrations of zinc and calmodulin in the testes than did controls. There is no information relative to the calmodulin content of red blood cells in animals of low-zinc status, but such information would help explain current findings.

Clearly, the activities of the red blood cell plasma membrane enzymes, 5′-nucleotidase and calcium-ATPase, are decreased in animals of low-zinc status. They are potential indicators of zinc status in the human population, particularly in as much as red blood cells are readily accessible for assay. Obviously, their value as indices of long-term zinc status in humans must be assessed in the human, preferably in combination with plasma zinc. Low plasma zinc always occurs in zinc deficiency, but alone it does not establish low zinc status. In small animals, plasma zinc concentration drops within hours after zinc deprivation but this does not reflect body zinc stores, e.g., metabolizable bone zinc. Furthermore, plasma zinc rises during an overnight fast, even in animals and humans of low-zinc status (31, 32). For these reasons, plasma zinc should be coupled with another index of status that reflects long-term status. Possible indices include membrane zinc concentration, red blood cell fragility, and 5′-nucleotidase and calcium-ATPase activities. In the pigs studied here, food deprivation per se increased these enzyme activities so that food intake must be considered. Since zinc deficiency tends to decrease food intake, a decrease in these enzyme activities in an animal or patient with low plasma zinc would provide strong evidence for low-zinc status.

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