PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 480–484 (1979)

Zinc in Erythrocyte Ghosts (40708)

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In our previous paper we demonstrated that a dietary supplementation of a high dose of zinc to rats renders red blood cells more resistant to osmotic hemolysis while an addition of Zn^{2+} to washed erythrocytes does not affect their osmotic fragility (1). On the other hand, erythrocytes from zinc-deficient rats which have low plasma zinc are more fragile under osmotic stress than those of controls (2). Bettger *et al.* (2) also showed that erythrocyte membranes are stabilized by zinc, while copper ions have a labilizing effect. Avigad and Bernheimer (3) demonstrated that hemolysis induced by bacterial toxin or other lytic agents is inhibited by Zn^{2+}. Montgomery *et al.* (4) showed similar zinc stabilizing effects using complement-mediated hemolysis.

Zinc was found to stabilize the integrity of lysosomes in *in vitro* experiments (5). As this effect was also produced by zinc–8-hydroxyquinoline unsaturated complexes (1:1, 1:2) which are impermeable to the membrane, we assumed that the effect of zinc is confined to the surface of the membrane (6). There are several possibilities to explain the stabilizing effect of zinc. Supplemental zinc may interact with intrinsic as well as extrinsic macromolecules of the membrane structure, thus contributing to their integrity (7). Yamamoto and Takahashi (8) found that Zn^{2+} suppressed complement-induced lysis of erythrocytes by acting directly on the erythrocyte membrane. In fact, zinc, as well as other inorganic ions, may be an integral part of various membranes, performing the same function as has been shown for calcium. As far as the effect on mechanical properties of membranes is concerned, a hardening effect of Ca ions on cytoplasmic surfaces or model membranes has been repeatedly demonstrated. Recent evidence suggests that zinc also increases the stability of various membranes. Zinc has long been used by biologists in the isolation of intact cell plasma membranes from animal cells (9). Zinc and cadmium contribute to the assembly and the integrity of brain microtubules (10). Direct evidence of the membrane stabilizing effect of zinc and its presence in biomembranes is still lacking. The only reference indicating that zinc is tightly bound to cell membranes refers to the leaves of *Silene cucubatus* (11), the membranes of which contain about 10% of the total zinc in the plant.

It is the aim of this study to present the evidence of the presence of zinc in erythrocyte ghosts and to demonstrate some implications of the role of this metal in the structure of the membrane.

Materials and methods. Isolation of red blood cells. Whole blood from normal dogs was collected in heparinized containers. Fresh blood was centrifuged at 1000*g* and the supernate and buffy coat were aspirated. The packed cells were then washed three times with 15 vol of phosphate buffer (pH 7.6, 210 mOsm) at 1000*g*.

Ghost preparation. Red cell membranes were prepared by the procedure of Dodge *et al.* (12) by washing three times with 30 vol of ice-cold hypotonic (20 mOsm) 5 m*M* phosphate buffer, pH 7.4. In order to study a possible sequestration of metals from the ghost constituents by the complexing effect of phosphate, in some experiments we substituted phosphate with hypotonic barbital (20 mOsm, 13.3 m*M*, pH 7.4) which does not form a complex with zinc ions. Centrifugation of the washed ghosts was done at 20,000*g* in an angle head for 40 min. A thorough aspiration of the supernate at the surface of the pellet was done after each spin. This step was repeated four to five times as needed until the final pellet was white and the supernatant visibly free of hemoglobins.

Solubilization of ghost proteins. Ghost protein solubilization was achieved with sodium

0037-9727/79/110480-05$01.00/0
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dodecyl sulfate (SDS), 1% in 0.01 *M* Tris buffer, pH 8.5, containing 0.05 *M* NaCl. Approximately 1 mg of ghost protein was dissolved in 100 μl of the above solution and incubated at room temperature in the presence of 0.05 m*M* sodium azide. The proteins in the supernate were separated by spinning at 45,000*g* and subjected to further analysis.

Protein separation. Protein separation was performed at room temperature in a Sephadex G-200 (Pharmacia) column 30 cm long and 1.5 cm in diameter, eluted at 8 ml/hr with 0.01 *M* Tris buffer (pH 8.5) and 0.1% SDS. The proteins in the eluate were recorded on a DB-Beckman spectrophotometer at 280 nm. For rechromatography the pooled samples were lyophilized without dialysis and run under identical conditions (see Table I).

Extraction of ghost lipids. Total lipids of the ghosts were extracted by the procedure of Folch *et al.* (13) and determined by weighing in aluminum weigh boats in a microbalance. In order to obtain separation of major classes of membrane lipids we used chromatography on silicic acid columns as described by Rouser *et al.* (14). Aliquots of ghost lipids chromatographed on silicic acid columns were eluted first with chloroform, then with methanol, and finally with acetone. In individual fractions the content of cholesterol, phosphorus, zinc, and copper was determined.

TABLE I. CONTENT OF ZINC IN MACROMOLECULAR COMPONENTS OF ERYTHROCYTE MEMBRANE.[a]

Fraction	Protein[b] (mg/fraction)	Zinc (μg/fraction)	Zn/protein (μg/mg)
1 I	0.35	0.29	0.83
2 II	0.64	1.00	1.56
3 III	0.41	0.17	0.41
4 IV	0.30	0.08	0.28
5 I-1	0.16	0.13	0.81
6 I-2	0.12	0.10	0.83
7 I-3	0.07	0.06	0.83
8 II-1	0.30	0.47	1.70
9 II-2	0.19	0.30	1.58
10 II-3	0.15	0.23	1.53
11 III-1	0.20	0.08	0.40
12 III-2	0.21	0.09	0.43

[a] The table presents data of one representative experiment from a total of five similar fractionation studies.

[b] Protein, 1.8 mg, was put on the column; 94% protein was recovered. Proteins solubilized from the dog ghost were fractionated on a Sephadex G-200 column. Four fractions (I–IV) were obtained. Fractions I, II, and III were rechromatographed and eight additional fractions were obtained.

Assays. Protein was measured by the method of Lowry *et al.* (15). Phospholipids were determined by means of phosphate analysis according to Bartlett (16). The amount of phospholipids was calculated from total phosphorus content assuming a phospholipid/phosphorus ratio of 25. Cholesterol was measured by the procedure of Zak *et al.* (17). Total lipids were determined gravimetrically following the procedure of Folch *et al.* (14).

The content of zinc was measured by atomic absorption spectroscopy, using a Perkin-Elmer ASS, Model 305, with a Zn-selective lamp. The metal was determined either directly in solubilized samples or after nitric acid–hydrogen peroxide digestion of ghost samples.

Lipid peroxidation of the ghost was ascertained by measuring malondialdehyde and fluorescent products as described elsewhere (1).

Results. Zinc in the ghosts subjected to peroxidation. In our previous work we showed that in the presence of zinc ions the lipid peroxidation of biological structures was decreased (1). Although the mechanism of this effect of zinc is unknown, we speculated that one of the possible modes of Zn^{2+} action may be a direct interaction with either polyunsaturated fatty acids, mainly of phospholipid moiety, or reactive groups of membrane proteins, thus protecting these membrane components against peroxidative deterioration. An experiment was designed to test the function of zinc in relation to membrane constituents under the effect of peroxidative changes.

Hemoglobin-free ghosts, prepared from dog erythrocytes by the procedure of Dodge and co-workers (12), were incubated in the presence of ferrous ions and ascorbate, inducing lipid peroxidative changes in the membrane constituents (Fig. 1). The magnitude of lipid peroxidation was evidenced by measuring the formation of malondialdehyde and fluorescent product (top, Fig. 1). Parallel with the continuous increase in these parameters, both protein and phospholipids were

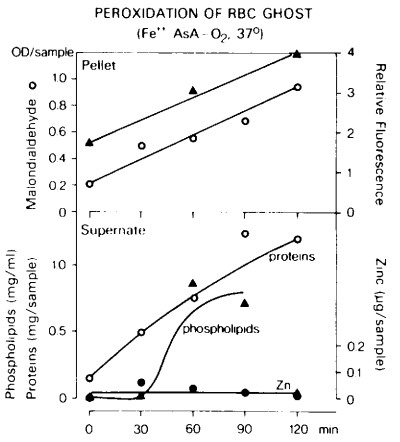

FIG. 1. Hemoglobin-free ghosts isolated from dog erythrocytes were incubated in 0.05 *M* Tris–0.15 *M* NaCl (pH 7.4) at 37°C in the presence of 200 μM L-ascorbic acid and ferrous sulfate. The medium was aerated for 15 sec with oxygen and incubated for given time periods under gentle shaking. Some biochemical indicators were determined in the pellet or in the supernate. The data of one representative experiment from a total of three similar experiments are presented. The variability for either parameter studied did not exceed 15% of the mean value.

released from the ghosts and leaked into the medium. No release of zinc from the ghost structure was observed during the 2 hr of incubation (bottom, Fig. 1).

Zinc and protein components of ghosts. Pure, hemoglobin-free ghosts were treated with 1% SDS in 0.01 *M* Tris buffer, pH 7.4, with the hope of solubilizing most of the membrane proteins (18). Chromatography on Sephadex G-200 column equilibrated with 0.1% SDS was further used to separate and rechromatograph the proteins.

A total of 12 fractions were obtained and analyzed for the content of protein and Zn (Table I). The results showed that zinc was present in every protein fraction obtained by this separation procedure. Proteins constituting peak 2 of the original Sephadex G-200 eluant contained three to five times more zinc per fraction than the other three original fractions.

Under rechromatography of the original four peaks of proteins (fractions 1–4, see Table I), peak 2 separated into three more fractions (8–10, see Table I), each of which contained a higher amount of zinc than the other proteins, irrespective of whether the data were expressed as zinc/fraction or zinc/milligram protein.

Effect of the method of ghost preparation on zinc content. Phosphate is known to form strong complexes with zinc, the logarithm of the complexity constant being 10.4 (19). As the most widely used method of preparing ghosts (12) uses hemolysis and washing with hypotonic phosphate buffer, we studied the possible sequestration of zinc by phosphate as compared to isomotic barbital buffer, which does not form complexes with zinc. The results (Table II) showed the erythrocyte ghost content of zinc to be three times lower in phosphate buffer. Another striking difference was that significantly less zinc linked to proteins in specimens prepared with phosphate buffer. It is interesting that in the course of extraction of erythrocyte ghosts by water, no release of zinc from the membrane was observed, while at the same time distinct amounts of calcium leaked out into the incubation medium. Still, the values reported here for the erythrocyte ghosts do not necessarily reflect the status of zinc in the membrane of intact erythrocytes.

Based on this experiment we learned that the method of preparation of ghosts is essential for the total content of zinc in the sample and that phosphate buffer displaced zinc preferentially bound to protein and minimally affected zinc bound to lipids.

Zinc content in lipid fractions of ghosts. We already indicated that in barbital-purified ghosts almost 69% of the zinc is linked to

TABLE II. EFFECT OF THE ISOLATION PROCEDURE ON THE GHOST ZINC CONTENT IN PURIFIED ERYTHROCYTES.[a]

	Barbital buffer	Phosphate buffer
Protein (mg/mg dry substrance)[b]	0.33	0.38
Lipid (mg/mg dry substance)[b]	0.67	0.58
Zinc (μg/mg dry substance)[b, c]	0.22 ±0.037	0.062 ±0.022
Zinc bound to proteins (% of total)	32.2	8.4
Chloroform–methanol (2:1) extractable (% of total)	68.8	91.6

[a] Refers to dog erythrocytes.
[b] Dry substance corrected for salts.
[c] Variability for zinc content is given by SD, based on determination of three to five independent ghost preparations.

lipids (Table II). The consecutive extractions of ghosts from rabbit erythrocytes by chloroform, acetone, or methanol, as described under Materials and Methods, were performed and the content of cholesterol and phospholipids was determined in each fraction together with the content of zinc and copper (Table III). As expected, most of the cholesterol was extracted into chloroform and most of the phospholipids were extracted into methanol. The data show that phospholipid-rich fractions contain more zinc per unit of weight of lipid than the other two fractions. Copper content in either fraction is similar to zinc content.

Discussion. As already indicated by Reynolds (18) there is an increasing body of experimental data, which suggests that in at least the erythrocyte membrane system inorganic cations are an important structural component. The same author showed that virtually all of the protein from human ghosts is soluble in aqueous media when inorganic cations are removed by chelating agents. The study by Burger *et al.* (20) also indicates that the differing stability of bovine vs human erythrocytes correlates with the actual concentration of divalent cations in the membrane. The results of this study show that zinc is a component of the membrane of red blood cells. The findings that zinc is not released when erythrocyte ghosts undergo peroxidative deterioration or partial solubilization with water indicate that this metal possibly stabilizes a certain framework of ghost structure by linking to a component of the membrane. We speculate that this rather insoluble structural component is not extractable because of its association with zinc. Based on these speculations we suggest that zinc contributes to the integrity of the erythrocyte membrane. More experimental evidence is needed to support this view.

There is a relative increase in binding of zinc to lipid phase. This linkage seems to be even more stable than zinc–protein linkage as phosphate buffer does not sequester the metal from the lipid moiety and releases zinc only from the protein. The finding that zinc remains within the ghost structure while oxidized lipids (measured as phospholipids) are released during the peroxidation cleavage of the fatty acids possibly indicates that those lipid components linked to zinc are not affected and disrupted by the oxidative process.

Recent findings on the correcting effect of zinc on sickle cell anemia brought several interesting observations, implicating zinc as a calcium antagonist in the red cell membrane (21, 22). Calcium incorporation into red blood cell ghosts was reduced by 50 to 80% in the presence of zinc in the medium (23). These studies indicate that metals in the ghosts exist in a state of dynamic equilibrium, reflecting the actual content of these metals in the whole blood. This would explain why dietary supplementation of zinc or zinc deficiency states affect the fragility of erythrocytes (1, 2). As shown by the study of Sastry *et al.* (24), the content of zinc in erythrocyte ghosts is negligible as compared to its content in the whole cell, where it is linked mostly to proteins. Our data show, however, that zinc is an integral part of the erythrocyte membrane.

Summary. Proteins and phospholipids but not zinc were released into the medium containing hemoglobin-free ghosts from dog erythrocytes incubated in peroxidation-inducing agents (ascorbic acid, Fe^{2+}, O_2). The

TABLE III. METAL CONTENT IN LIPID FRACTIONS FROM RBC GHOSTS.[a]

	Fraction[b] content	Cholesterol content (% of fraction)	Phospholipids[c] by weight (% of fraction)	Metal content (μg/g lipid)	
				Zn	Cu
Total lipid content (% of dry weight)	54.0	31 ± 4.5	51.2 ± 6.2	24.2 ± 4.5	64.0 ± 7.0
Chloroform fraction (% of total lipid)	44.0	60 ± 8.5	3.2 ± 0.4	72.0 ± 10.6	72.0 ± 9.6
Acetone fraction (% of total lipid)	45.0	10 ± 0.8	10.9 ± 1.7	82.7 ± 11.2	110.3 ± 12.1
Methanol fraction (% of total lipid)	50.3	3 ± 0.2	78.5 ± 9.6	115.0 ± 9.7	128.0

[a] Refers to rabbit erythrocytes, variability given as $\bar{X} \pm$ SE.
[b] Refers to prercentage of total dry weight of RBC ghost.
[c] Phospholipid content calculated as $P_i \times 25$.

content of zinc in the pure ghost depends on the purification procedure; using phosphate buffer it amounted to 62 ± 22 μg/g protein, and using barbital buffer-extracted ghost it was three times higher.

Although most of the zinc was linked to the lipid phase of the membrane, there is a certain protein moiety of membrane proteins binding more zinc than the other protein fractions. Among various fractions of lipids, zinc is linked mainly to phospholipids and this linkage is not broken by phosphate buffer.

It is concluded that zinc is part of the erythrocyte ghost, linked to protein as well as lipid phase.

This work is supported in part by NIH Research Grants AM 16489, ES 00790, and ES 01570. The professional assistance of Mrs. Waltraud W. Nichols is highly appreciated.

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Received March 8, 1979. P.S.E.B.M. 1979, Vol. 162.