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# Release of Spectrin-Containing Vesicles from Human Erythrocyte Ghosts by Dimyristoylphosphatidylcholine[1]

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**Membrane vesicles, which were released from human erythrocyte ghosts by dimyristoylphosphatidylcholine (DMPC), showed a protein composition similar to that of the erythrocyte membrane, despite a reduction of in spectrin content. The spectrin content of vesicles decreased with increasing hemoglobin concentration within ghost membranes, but increased upon exposure of hemoglobin-free ghosts to a pressure of 100 MPa. The ESR spectra of spin-labeled membrane proteins showed that membrane proteins in ghosts became unfolded at high pressure. Furthermore, spectrin-poor and protein 4.1-rich vesicles were released by DMPC from diamide-treated ghosts in which spectrin was cross-linked and stabilized. Taking into account that the spectrin tetramer is stabilized by hemoglobin [Liu and Palek (1984) *J. Biol. Chem.* 259, 11556-11562], these results suggest that the spectrin content of DMPC-induced vesicles from erythrocyte ghosts increases with increasing instability of the cytoskeletal network in parent cells.**

**Key words: dimyristoylphosphatidylcholine, erythrocyte, hemoglobin, spectrin, vesiculation.**

The vesiculation of human erythrocytes is performed by various methods. The membrane protein composition of the released vesicles differs with the method of vesiculation. Spectrin-free and band 3-rich vesicles are released from erythrocytes on ATP depletion (*1*), $Ca^{2+}$-loading (*2*), and incubation with dimyristoylphosphatidylcholine (DMPC) liposomes (*3, 4*). On the other hand, upon the subjection of erythrocytes to high temperature (51-52°C) (*5*) or high pressure (200 MPa) (*6*), membrane vesicles containing cytoskeletal proteins such as spectrin and actin in addition to band 3 are released. In addition, the membrane protein composition of the released vesicles is modulated by cross-linking of membrane proteins in erythrocytes. In diamide-treated erythrocytes, in which transmembrane proteins are cross-linked with the cytoskeleton, protein 4.1-rich vesicles are released by high pressure (*7*) or DMPC treatment (*4*). Even when the membrane protein composition of the released vesicles is altered, the membrane phospholipid composition remains constant (*6, 7*). Thus, it seems likely that the membrane protein composition of vesicles is affected by the stability of the cytoskeleton in the erythrocyte membrane.

The stability of the erythrocyte membrane is controlled by the cytoskeleton (*8*). In particular, spectrin plays a crucial role in the maintenance of the membrane structure. Spectrin, the major protein of the erythrocyte membrane skeleton, comprises an $\alpha$ subunit (280 kDa) (*9*) and a $\beta$ subunit (246 kDa) (*10*). Spectrin $\alpha\beta$ heterodimers can associate head-to-head to form tetramers (*11*). The dimer-tetramer equilibrium of spectrin is affected by ionic strength and temperature (*12*). In the native membrane, spectrin exists as tetramers and/or higher oligomers (*13*). Many hereditary hemolytic anemias, such as hereditary elliptocytosis (*14*) and pyropoikilocytosis (*15*), are associated with defects of the cytoskeletal network, *i.e.*, diminished tetramer formation of spectrin. Thus, the dissociation of spectrin tetramers into dimers makes the membrane more fragile, so that membrane vesicles may be readily released under conditions of vesiculation.

The vesiculation of red cell membranes has been performed by using intact erythrocytes (*1-7*). However, ghost membranes are useful for examining the contributions of cytosol components to the membrane stability. In the present work, we first demonstrate that membrane vesicles containing cytoskeletal proteins such as spectrin and actin in addition to band 3 are released from erythrocyte ghosts by DMPC, and that this ghost-DMPC system provides valuable information on the membrane protein-protein interaction in the erythrocyte membrane.

## MATERIALS AND METHODS

*Materials*—The compounds used were obtained from the following sources: bis(*N,N'*-dimethylamide)diazinedicarboxylic acid (diamide), hemoglobin (Hb) from beef blood, and dimyristoylphosphatidylcholine (DMPC), Sigma; 4-maleimide-2,2,6,6-tetramethylpiperidinooxyl, Syva. All other chemicals were of reagent grade.

*Ghost Preparation*—Human blood was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged at 750×*g* for 10 min at 4°C. The plasma and buffy coat were removed carefully. The erythrocytes were washed three times with Tris buffer (10 mM Tris, 150 mM

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Abbreviations: diamide, bis(*N,N'*-dimethylamide)diazinedicarboxylic acid; DMPC, dimyristoylphosphatidylcholine; Hb, hemoglobin; Tris buffer, 10 mM Tris, 150 mM NaCl, pH 7.4.



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NaCl, pH 7.4). Hb-free or Hb-containing ghosts were prepared by changing the volume ratio of erythrocytes and lysis buffer (10 mM Tris, pH 7.4), according to the method of Dodge *et al.* (*16*). To prepare Hb-loaded ghosts, open white ghosts (about 3 ml) were added to 1 ml of a Hb solution (0.1 g/ml) prepared by dissolving purified bovine Hb in the lysis buffer and then incubated for 2 h at 0°C. To reseal the membrane, these ghost suspensions were brought to isotonic conditions by adding NaCl and then incubated for 1 h at 37°C. These sealed ghosts were washed three times with Tris buffer, and then used for vesiculation, diamide treatment, spin labeling, and protein extraction. The Hb content of the membrane was estimated by determining the concentrations of Hb and phospholipids (*6*) in red ghosts.

To cross-link membrane proteins, sealed ghosts, which were suspended at 20% hematocrit in Tris buffer, were incubated with 0.5 mM diamide for 30 min at 37°C and atmospheric pressure or 100 MPa. After incubation, the ghosts were washed twice in Tris buffer.

*Vesiculation*—Liposome suspensions were prepared by sonicating DMPC (0.75 mM) in Tris buffer. To prepare membrane vesicles, intact erythrocytes or Hb-free or Hb-containing ghosts were added to a liposome suspension at 10% hematocrit. These ghost suspensions were incubated for 7-10 h at 37°C, and then centrifuged at 4°C for 20 min each at $3,000\times g$ and then $10,000\times g$. The supernatants were filtered through a Millipore filter (pore size, 3.0 $\mu$M). The filtrates were centrifuged at about 20°C for 30 min at $56,000\times g$.

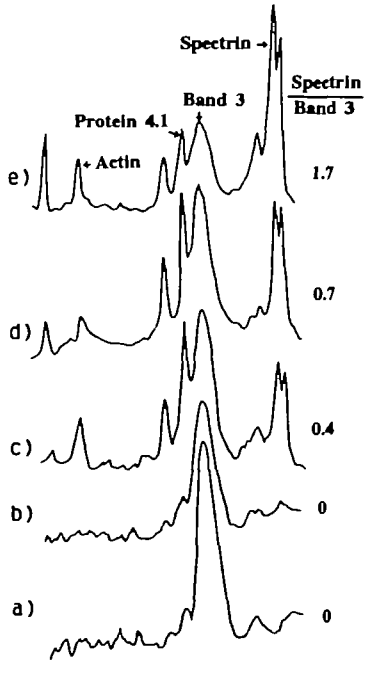


Fig. 1. **Analysis of membrane proteins in DMPC-induced vesicles.** Membrane vesicles were prepared from (a) intact erythrocytes (1.0), (b) red ghosts (0.6), (c) pink ghosts (0.1), and (d) white (Hb-free) ghosts (0) with DMPC. The numbers in parentheses are the relative Hb contents of the ghost membrane. Membrane proteins in vesicles and (e) control ghosts were analyzed by SDS-PAGE. Gels were stained with Coomassie Blue and scanned with a densitometer. On the basis of the peak area, the spectrin content of vesicles is given relative to the band 3 content.

*SDS-PAGE of Diamide-Treated Ghosts and Membrane Vesicles*—Membrane proteins in diamide-treated ghosts and released vesicles were analyzed by SDS-PAGE. Membrane proteins were separated on a slab gel containing 2.5% (stacking gel) and 8% (separation gel) acrylamide, according to the method of Laemmli (*17*). The gels were stained with Coomassie Blue and scanned with an Advantic DM-303 scanning densitometer.

*Protein Extraction*—To extract cytoskeletal proteins, ghosts were incubated at 10% hematocrit for 30 min at 37°C in 0.1 mM EDTA, pH 8.0. After incubation, the ghost suspension was centrifuged at $81,000\times g$ for 30 min at 4°C. The protein concentration of the supernatant was determined by the method of Lowry *et al.* (*18*).

*Spin Labeling*—Spin labeling of membrane proteins in ghosts was performed using a maleimide spin label, as previously described (*19*). Spin-labeled ghosts were suspended at 50% hematocrit in isotonic buffer and then subjected to a pressure of 100 MPa for 30 min at 37°C (*20-22*). After decompression, the ESR spectra of ghost suspensions were recorded on a JEOL JES-1X spectrometer at room temperature (23°C).

## RESULTS

*Vesiculation of Sealed Ghosts Containing Various Amounts of Hb by DMPC*—Vesiculation of the erythrocyte membrane by DMPC was examined by changing the Hb content of the membrane. The membrane protein composition of the released vesicles was analyzed by SDS-PAGE (Fig. 1). Spectrin-free and band 3-rich vesicles were released from intact erythrocytes (Fig. 1a) (*3, 4*). On the other hand, the membrane vesicles which were released from Hb-free ghosts showed a membrane protein composition similar to that of the erythrocyte membrane, although the spectrin content was decreased (Fig. 1, d and e). The spectrin content of the vesicles increased with decreasing Hb content of the ghost membrane (Fig. 1, b-d). No vesicles were released from these ghosts during incubation in the absence of DMPC (data not shown). In addition, we con-

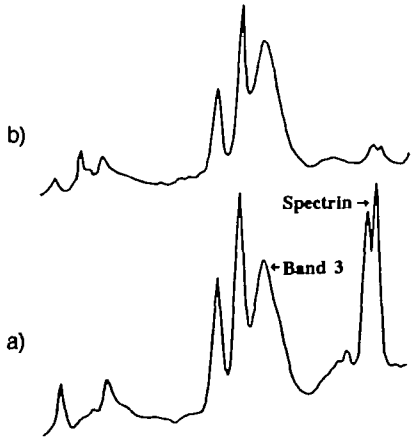


Fig. 2. **Analysis of membrane proteins in vesicles released from Hb-loaded ghosts by DMPC.** Open white ghosts were loaded with bovine Hb as described under "MATERIALS AND METHODS." Membrane proteins in DMPC-induced vesicles were analyzed by SDS-PAGE. a, vesicles from control ghosts; b, vesicles from Hb-loaded ghosts.

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firmed that there was no contamination by ghosts of the vesicle suspension by light microscopy (data not shown).

To confirm that membrane vesicles are released from right-side-out membranes (ghosts) and that the release of spectrin-free vesicles is associated with the Hb content of the ghost membrane, the purified Hb was loaded into open white ghosts. The spectrin content of the vesicles decreased with Hb loading (Fig. 2).

*Effect of Cross-Linking of Membrane Proteins on DMPC-Induced Vesiculation in Sealed Ghosts*— Previously, we showed that diamide treatment of erythrocytes under high pressure (100 MPa) results in unique cross-linking of membrane proteins (*7, 20, 21*). To cross-link membrane proteins, ghosts were treated with diamide at atmospheric pressure or 100 MPa. Prior to analyzing the cross-linking of membrane proteins, the pressure effect on the ghost membrane was examined. The electrophoretic pattern of pressure (100 MPa)-treated ghosts was similar to that of control ghosts, except in the cases of actin and protein 6 (Fig. 3, a and b). In ghosts treated with diamide at atmospheric pressure or 100 MPa, the bands of spectrin and band 3 decreased, and new bands due to the formation of high-molecular-weight aggregates appeared (Fig. 3, c and d). Vesiculation of such ghosts was carried out. Membrane vesicles which were released from pressure-treated ghosts by DMPC contained large amounts of spectrin, compared with those from untreated ghosts (Fig. 4, a and b). However, no vesicles were released from pressure-treated ghosts on similar incubation in the absence of DMPC (data not shown). Membrane vesicles containing band 3 and protein 4.1 as major membrane proteins were released by DMPC from ghosts treated with diamide at atmospheric pressure (Fig. 4c). On the other hand, protein 4.1-rich vesicles, in which less band 3 is present, were released by DMPC from ghosts treated with diamide at 100 MPa (Fig. 4d).

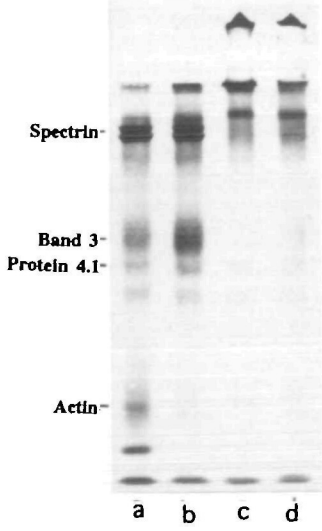


Fig. 3. **SDS-PAGE of pressure- or diamide-treated ghosts.** Ghosts were subjected to a pressure of 100 MPa for 30 min at 37°C or treated with 0.5 mM diamide for 30 min at 37°C under atmospheric pressure or 100 MPa. Gels were stained with Coomassie Blue. a, control ghosts; b, pressure-treated ghosts; c, ghosts treated with diamide at atmospheric pressure; d, ghosts treated with diamide at 100 MPa.

*Extraction of Cytoskeletal Proteins from Diamide-Treated Ghosts at Low Ionic Strength*—When the ghost membrane is exposed to low ionic strength medium (0.1 mM EDTA, pH 8.0), cytoskeletal proteins such as spectrin and actin are released from the membrane (*20*). So, similar experiments were performed on diamide-treated ghosts. The amounts of extracted cytoskeletal proteins decreased with the cross-linking of membrane proteins (Table I).

*Unfolding of Membrane Proteins Induced by High Pressure*—When the ghost membrane is treated with a maleimide spin label, its ESR spectrum is composed of two components, *i.e.*, a strongly immobilized component (s) and a weakly immobilized one (w) (Fig. 5). The amplitude ratio of the two components, Aw/As, sensitively reflects the conformational changes of membrane proteins. As the unfolding of membrane proteins occurs, the Aw/As value increases (*19, 22*). Spin-labeled ghosts were subjected to a pressure of 100 MPa and then the ESR spectrum was

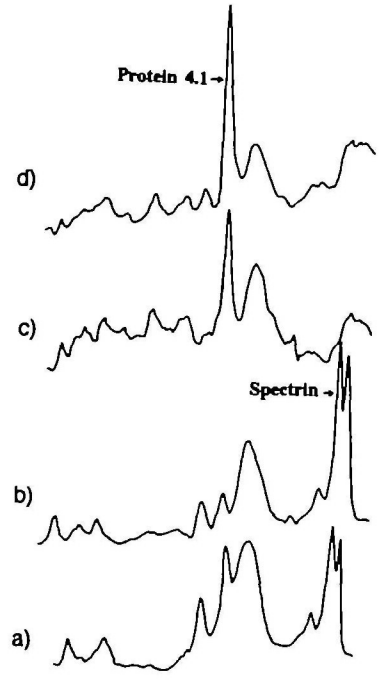


Fig. 4. **Effects of high pressure and cross-linking of membrane proteins on DMPC-induced vesiculation.** Membrane vesicles were prepared from pressure- or diamide-treated ghosts with DMPC. Membrane proteins in vesicles were analyzed by SDS-PAGE. a, vesicles from control ghosts; b, vesicles from pressure (100 MPa)-treated ghosts; c, vesicles from ghosts treated with 0.5 mM diamide at atmospheric pressure; d, vesicles from ghosts treated with 0.5 mM diamide at 100 MPa.

TABLE I. **Extraction of cytoskeletal proteins from diamide-treated ghosts at low ionic strength.** Ghosts (20% hematocrit) in Tris buffer were treated with 0.5 mM diamide for 30 min at 37°C under atmospheric pressure (0.1 MPa) or 100 MPa. Diamide-treated ghosts (10% hematocrit) in 0.1 mM EDTA, pH 8.0, were incubated for 30 min at 37°C, and then centrifuged at 81,000×*g* for 30 min. Values are means±SD for two experiments.

| | % Protein extracted |
|---|---|
| Control ghosts | 34.2±1.6 |
| Ghosts treated with diamide at 0.1 MPa | 16.6±0.7 |
| Ghosts treated with diamide at 100 MPa | 13.6±1.1 |



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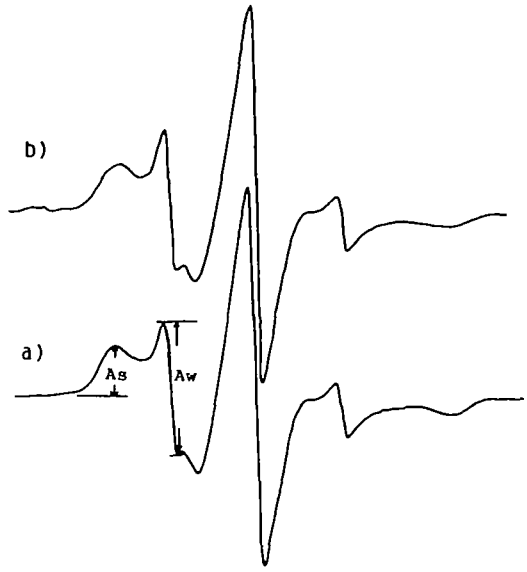


Fig. 5. **ESR spectra of spin-labeled ghosts.** Maleimide spin-labeled ghosts were incubated for 30 min at 37°C and atmospheric pressure (a) or 100 MPa (b). After decompression, the samples were allowed to come to room temperature (23°C). ESR spectra were recorded at room temperature. "As" and "Aw" denote the amplitudes of the strongly immobilized and weakly immobilized components, respectively.

measured at atmospheric pressure (Fig. 5). Upon exposure to a high pressure (100 MPa), the Aw/As value changed irreversibly from $1.48\pm0.02$ ($n=2$) to $1.63\pm0.01$ ($n=2$). On the other hand, no significant change in the Aw/As value was observed in ghosts treated at pressures lower (*e.g.*, 30 MPa) than 100 MPa.

## DISCUSSION

Among several methods of vesiculation, the DMPC method seems to be the most useful in that the interaction of transmembrane proteins with the cytoskeleton or the stability of the cytoskeleton can be monitored without perturbation of the cytoskeletal structure of the erythrocyte. Only vesiculation of erythrocyte membranes by DMPC using intact red cells has been reported to date. There are no data concerning the vesiculation of ghosts by DMPC. So, we have attempted to determine the contributions of cytosol components to the erythrocyte membrane stability through the vesiculation of intact erythrocytes or ghosts by DMPC.

The spectrin tetramer-dimer equilibrium is sensitive to temperature, and the equilibrium shifts toward the dimer at higher temperature (*12*). Such an equilibrium is also affected by heme-containing proteins and basic proteins such as cytochrome *c* and ribonuclease (*13*). These proteins enhance the self-association of spectrin heterodimers (*13*). In intact erythrocytes, spectrin tetramers or the cytoskeleton is stabilized by the large amount of Hb within the membrane. Thus, membrane vesicles released from intact erythrocytes by DMPC are spectrin-free and band 3-rich, although the vesiculation mechanism is not well known. On the other hand, DMPC-induced vesicles from ghosts contain cytoskeletal proteins such as spectrin and actin, in addition to band 3. The amount of cytoskeletal proteins within vesicles decreases with increasing Hb concentration within the ghost membrane. In addition, the results obtained for Hb-loaded ghosts indicate that spectrin-containing vesicles are not released from inside-out membranes (ghosts). These results suggest that the spectrin content of vesicles released from the erythrocyte membrane by DMPC decreases upon stabilization of the cytoskeleton. This idea has been confirmed by examining the protein composition of vesicles released from high pressure-treated ghosts or diamide-treated ones by DMPC.

High pressure induces the dissociation of oligomeric proteins (*23, 24*). Upon the subjection of intact erythrocytes to a high pressure, hemolysis starts to occur in the range of 110-130 MPa (*20*). In hemolyzed erythrocytes, the cytoskeletal network seems to be partially destroyed (*22*). Previously, we demonstrated that a high pressure effect on cross-linking of membrane proteins by diamide appears clearly at 100 MPa (*4, 7, 20, 21*). Thus, we chose 100 MPa as the pressure to be applied to the ghost membrane. Upon exposure of spin-labeled ghosts to a pressure of 100 MPa, the ESR spectra showed the irreversible unfolding of membrane proteins. Among spin-labeled membrane proteins, about 75% of the spin label is located in the spectrin-actin complex (*25*). Thus, we suggest that the cytoskeletal network in the ghost membrane is partially perturbed by high pressure, so the motional freedom of cytoskeletal proteins increases. As expected, membrane vesicles which were released from pressure-treated ghosts contained a large amount of spectrin, compared with vesicles from pressure-untreated ones.

Cytoskeletal proteins in the erythrocyte membrane are cross-linked by diamide (*20, 22*). In erythrocytes treated with diamide at atmospheric pressure, spectrin is mainly cross-linked, whereas high-molecular-weight aggregates due to cross-linking of transmembrane proteins with the cytoskeleton are formed on diamide treatment at 100 MPa (*20*). In the former, band 3-rich vesicles are released by DMPC (*4*), whereas in the latter, vesicles containing both band 3 and protein 4.1 are released (*4*). Protein 4.1 within vesicles is not cross-linked with other membrane proteins (*4, 7*). On the other hand, in ghosts treated with diamide at atmospheric pressure or 100 MPa, the bands corresponding to spectrin and band 3 decreased, and new bands due to the formation of high-molecular-weight aggregates appeared. Spectrin-poor and protein 4.1-rich vesicles, in which band 3 is also present, are released from such diamide-treated ghosts by DMPC. It is not clear how protein 4.1-rich vesicles are released from such erythrocytes or ghosts in which membrane proteins are cross-linked. The release of spectrin-poor vesicles suggests that the cytoskeleton in diamide-treated ghosts is stabilized through cross-linking of spectrin despite the long incubation of ghosts at 37°C. Such stabilization was shown by the effect of diamide on the extraction of cytoskeletal proteins from the membrane at low ionic strength.

In the present work, we have first demonstrated that spectrin-containing vesicles are released on the incubation of ghosts with DMPC. The effects of the Hb content (or Hb-loading), high pressure, and cross-linking of membrane proteins on the vesiculation of ghosts by DMPC revealed that the contents of cytoskeletal proteins in vesicles depend on the instability of the cytoskeletal network, mainly



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spectrin. Thus, analysis of membrane proteins which are involved in DMPC-induced vesicles provides useful information on membrane protein-protein interactions in the erythrocyte membrane.

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