Interaction of Liposomes with Human Erythrocytes

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Interaction of liposomes (phospholipid vesicles) with human erythrocytes was studied by means of a spectroscopic method. Transfer of hemoglobin between liposomes and erythrocytes was observed. This transfer was mediated by a migration of band 3 proteins. In this case, a transfer of band 4.5 also was observed by means of electrophoresis. An interaction of lipid monomers from the liposomes with the erythrocyte membranes seemd to be closely correlated to the transfer of these proteins. It was presumed that this interaction induced some changes in the molecular organization of the cell membranes around band 3, resulting in release of the proteins from the erythrocyte membranes.

Keywords protein transfer; erythrocyte; liposome; band 3; hemoglobin; hemolysis

Introduction

It has been shown that several intramembrane proteins can transfer between artificial phospholipid vesicles (liposomes) and biological membranes.[1] This protein transfer is reversible and occurs without a membrane fusion. The extent of the transfer depends on the type of cells, the lipid composition of the liposomes, the exposure time and temperature.[1a,b] Newton *et al.*[2] found that in the system of erythrocyte membranes and dimyristoylphosphatidylcholine vesicles, four major proteins, *i.e.*, bands 3, 4.2 and 7, and acetylcholinesterase, transfer from the cells to the phosphatidylcholine liposomes.

On the other hand, it was suggested that hemoglobin molecules are bound to the integral components of the erythrocyte membrane, especially to band 3 proteins.[3] Band 3 is a major integral protein and responsible for the anion exchange in the erythrocyte. Concerning the hemoglobin binding to the erythrocyte membranes, two classes of binding sites were identified.[3a,c] A high-affinity class of sites is on the cytoplasmic portion of band 3, and a low-affinity class is located on the inner surface of the membranes. A difficulty in removing hemoglobin from the erythrocyte membrane is attributed to the specific high-affinity binding with band 3.

As a consequence of this evidence, it seems nautral that the results lead us to inquire as to whether band 3 can be a carrier or mediates hemoglobin from the cells to liposomes.[3c] In this report, we describe the transfer of hemoglobin from human erythrocytes to phosphatidylcholine liposomes.[4]

Experimental

Materials Dimyristoylphosphatidylcholine (DMPC) was a product of Nippon Oil & Fats Co., Ltd. Lysomyristoylphosphatidylcholine was from Sigma Chemical Co. They were used without further purification.

Preparation of Liposomes Small unilamellar liposomes were prepared by sonicating a suspension of the lipid (20 mg/ml) in a glass precipitation tube (10 ml) by using a bath type sonicator (Sono Cleaner Mini, 45 W, 38 KHz; Kaijo Electric Co., Ltd.). The size of the vesicles was determined by negative-stain electron microscopy and by a Submicro Coulter N4 counter. The mean diameter of the liposomes used was 250 Å.

Incubation of Liposomes with Erythrocytes and Separation of Liposomal Fraction A suspension of the liposomes in isotonic sodium phosphate buffer (310 ideal miliosmolarity (mosM), pH 7.4) was added to an equal volume of the packed cells of human erythrocytes which had been washed several times with 0.9% NaCl and phosphate buffer.[5] The mixtures were incubated under various conditions, and then centrifuged at $1000 \times g$ for 10 min to separate the liposomes from the cells. The resultant supernate was further centrifuged at $22000 \times g$ for 20 min to remove contaminating cell fragments. Then the supernate was applied to a Sepharose 4B column equilibrated with the phosphate buffer. This column was eluted with the same buffer and the eluates were monitored by measuring the absorbance at 415 nm. The liposomal fraction obtained by gel chromatography was analyzed by centrifuging on a discontinuous gradient of sucrose (10—40% w/v in 20 mosM phosphate buffer, pH 7.4) at $22000 \times g$ for 30 min. After the centrifugation, the sample was separated into fractions by sucking up from the top of the tube using a Hitachi Fractionator Typer DGF-U.

Preparation of Erythrocyte Ghosts Erythrocyte ghosts were prepared by the standard method of Dodge *et al.*[5] In this study, two types of resealed ghosts including hemoglobin were prepared. The first type of ghosts (trypsin-treated ghosts) were prepared as follows.[6,7] The ghosts were diluted with 1 volume of phosphate buffer (pH 8.0) and digested with 1 μg/ml of trypsin at 0 °C for 1 h. The digestion was stopped with the addition of 1 mM diisopropylfluorophosphate, the mixture was centrifuged at $22000 \times g$ for 10 min to remove water soluble fragments. The sediment was washed twice with 5 volume of 20 mosM phosphate buffer (pH 7.4). These trypsin-treated ghosts were resealed with 310 mosM phosphate buffer containing highly concentrated hemoglobin (about 1.5×10^{-2} M, which is about five times as high as that of erythrocytes). The resealed ghosts were washed three times with the phosphate buffer. The second type of ghosts were prepared by resealing the intact ghosts in the presence of concentrated hemoglobin (non-treated ghosts).

Eosin-5-isothiocyanate (EITC)-labeled erythrocytes were also prepared by incubating the packed cells (5 ml) with 1 mg of EITC for 3 h at 37 °C. The cells were then washed twice more with the isotonic buffer to remove unreacted EITC molecules.

Analysis of Transferred Proteins Sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[8] with 7.5% acrylamide gel. Protein bands were stained with silver by the method of Morrissey.[9].

Analysis of Cholesterol in Liposomes Recovered Cholesterol analysis for the recovered liposomes was carried out by using the color development between cholesterol oxidase and color-producing reagents (*p*-chlorophenol and 4-amino antipyrine) from Wako Pure Chemical Industries, Ltd.

Spectral Measurements The absorption spectra were taken by a Hitachi 220 spectrometer. The circular dichroism (CD) spectra were measured by a Jasco J-400X spectropolarimeter equipped with a data processor. The cell used was of 2 mm length and the sensitivity was 0.2 m°/cm. The observations were expressed in terms of milli degree/cm.

Results

Preparation of Small Unilamellar Liposomes The suspension of the lipid in a precipitation tube was sonicated for about 1 h at 30 °C. Then, the liposome suspension was filtrated through a polycarbonate membrane of 0.2 μ pore (Nuclepore). The size of the liposomes was made uniform by the filtration. The data on the size distribution is presented in Fig. 1a and Table I. Figure 1b shows an electron micrograph of the liposomes. It is very interesting that the liposome size became uniform after the filtration even though the pore size of the membrane was somewhat larger than the liposome diameters. This may be due to a pressure effect at the time of filtration from using a syringe. Such

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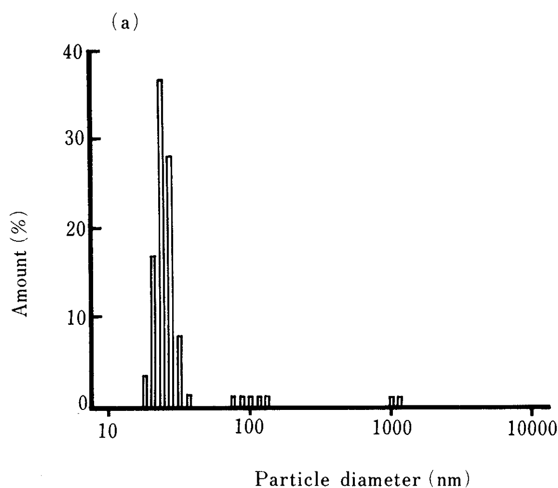

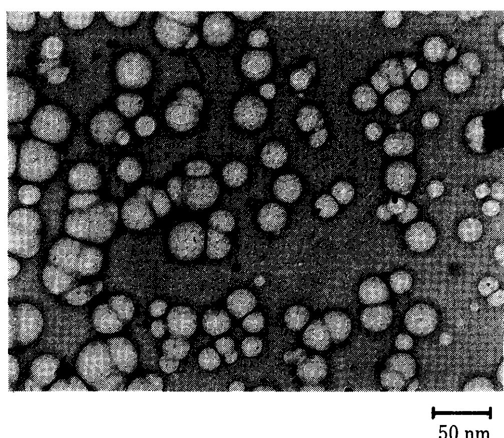

Fig. 1. (a) Size Distribution of Liposomes after Membrane Filtration. (b) Electron Micrograph of Liposomes

The procedure of the liposome preparation is described under Experiments and Results.

TABLE I. Size Distribution of Liposomes Estimated by Coulter N4 Counter

Size (nm)	S.D. (nm)	Amount (%)
24.5	4	92
99.9	25	5
1070	200	2

Liposomes were extruded through the polycarbonate membrane.

uniformity was not observed when the pore size was larger than 0.2 μ.

Interaction of Liposomes with Human Erythrocytes It is known that incubation of liposomes with human erythrocytes results in an exchange of phospholipids and/or certain proteins between the membranes.[1a,10] We first examined a separation of liposomes from erythrocytes after incubation of their mixture. The mixture was incubated for 1 h at 37 °C in 310 mosM phosphate buffer, pH 7.4. The supernate of the reaction mixture was obtained after centrifugation at $1000 \times g$ for 15 min and further at $22000 \times g$ for 20 min. Then, the supernate was passed through a column of Sepharose 4B. Although the liposomes and hemoglobin could be separated from each other,

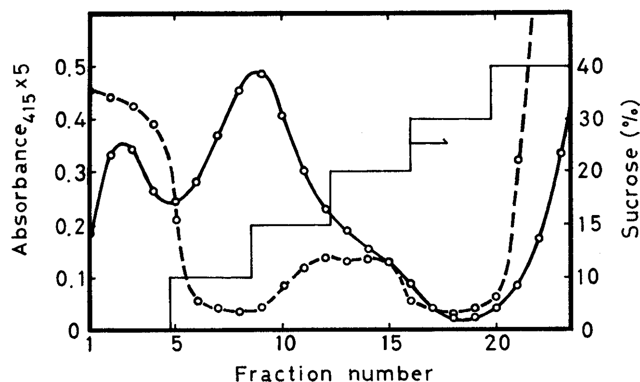

Fig. 2. Sucrose Density Gradient Centrifugation Pattern of the Fraction in the First Band in Fig. 1

The absorbance was measured using 2 mm cell. —○—, liposomes recovered after the gel-filtration; ---○---, erythrocytes hemolyzed by lysophosphatidylcholine; ——, concentration of sucrose.

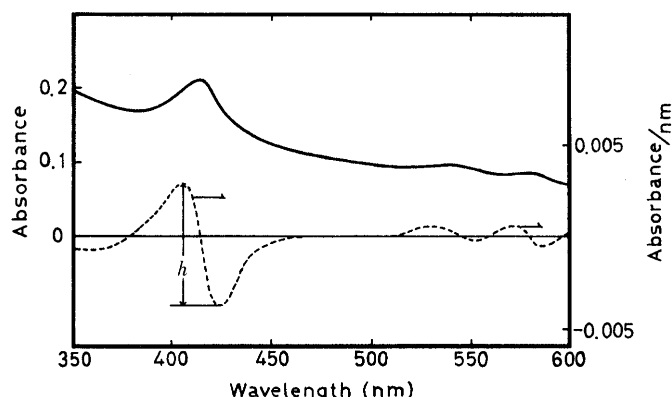

Fig. 3. Absorption Spectra of the Liposome Fraction in Fig. 2

They are shown as the absolute spectrum (solid line) and in first derivative mode (broken line).

membrane fragments or buds of erythrocytes could not be separated by this treatment. In order to recover the liposomes, further, the fractions containing liposomes from the gel-filtration were subjected to a sucrose density gradient centrifugation.[2]

Figure 2 shows the typical result of a separation of hemoglobin, liposomes and cell fragments or membrane buds in the fractions of the sucrose density gradient. By comparing a sedimentation pattern of the erythrocytes hemolyzed by lysomyristoylphosphatidylcholines (broken line) with that of the liposomal fraction from a Sepharose 4B column. It is obvious that hemoglobins (fraction Nos. 1—5), liposomes (Nos. 6—9), and cell fragments (Nos. 10—16) were separable in this order. No cholesterol was detected in the recovered liposomal fractions. Thus, the liposomes can be recovered without the membrane fragments of erythrocytes.

Hemoglobin Transfer Figure 3 shows the absolute absorption and its first derivative spectra of the liposomal fractions recovered by the above mentioned procedure. The background resulting from a light scattering by the liposomes was eliminated by recording the data as the first derivative (broken line). They exhibit characteristic bands of hemoglobin. In the present study, the amounts of hemoglobin transferred were determined from the value of peak to peak (h) in the first derivative spectrum. When the

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liposomes were incubated with the intact erythrocytes for 100 min at 37 °C, the amount of hemoglobin transferred reached about 2 μM/1 mM of DMPC.

In contrast with the liposome–erythrocyte system, when the liposomes were incubated in concentrated solution of free hemoglobin (*ca.* 1.5×10^{-2} M) under the same conditions, no hemoglobin was observed in the liposomal fractions. These results indicate that the hemoglobin observed in the liposomes come from erythrocytes, and that the hemoglobin transfer occurs between liposomes and erythrocytes.

Since hemoglobin is intracellular protein, the hemoglobin transfer should be migrated with a carrier protein, such as band 3.[2] In order to examine a transfer of band 3 accompanying hemoglobin molecules, the erythrocytes prelabeled with EITC were incubated with liposomes. The EITC molecule is known to bind predominantly to band 3 to inhibit the anion transport in erythrocytes and exhibit an induced CD spectrum.[11] The CD and absorption spectra of the recovered liposomal fraction are presented in Fig. 4.

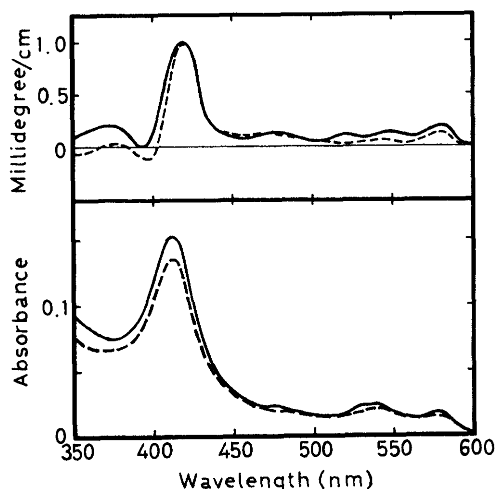

Fig. 4. CD and Absorption Spectra of the Liposomal Fraction in Fig. 2

——, CD and absorption spectra of liposomal fraction after the incubation with erythrocytes which were prelabeled with EITC in 310 mosM phosphate buffer, pH 7.4; ------, CD and absorption spectra of the liposomal fraction recovered after the incubation with intact erythrocytes. The absorption spectrum was illustrated as absolute one in such a way that the intensity at 600 nm was zero.

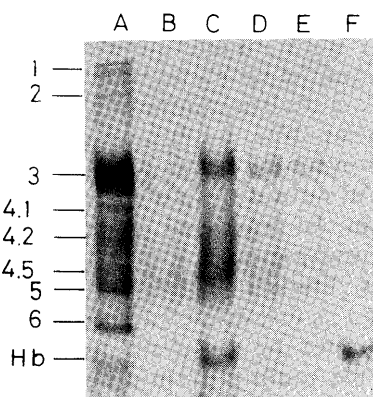

Fig. 5. SDS-PAGE of Liposomes Recovered

The photograph is of the wet gel stained with silver. The numbering of the ghost bands is according to Fairbanks *et al.*[13] Lane A is of intact erythrocytes; lanes B—E correspond to the liposomes obtained from Nos. 6, 9, 10, 11 fractions in Fig. 2, respectively; lane F is of hemoglobin.

Oxygenated hemoglobin shows CD extremes at 578, 546 and 418 nm with a shoulder at about 470 nm, and absorption maxima are observed at 578, 542 and 415 nm.[12] The liposomal fraction recovered from the incubated mixture of liposomes with the EITC-labeled erythrocytes showed the additional absorption bands at about 530 and 477 nm. Also in the CD spectrum, an additional positive CD band could be observed at about 520 nm. These additional bands originate from EITC molecules bound to band 3.[11b] Thus, although EITC molecules in solution were CD spectroscopically inactive, the induced CD of EITC was observed. This indicates that the conformation of the EITC binding site in band 3 does not change after the transfer from cells to liposomes.[11c]

The binding site of hemoglobin is coincident with the glyceraldehyde-3-phosphate dehydrogenase binding site on the cytoplasmic portion of band 3.[3b] This domain, 43-K fragment, can be removed from the cytoplasmic surface of the membrane by the trypsin-treatment.[6,7] When the liposomes were incubated with the trypsin-treated ghosts in which hemoglobin molecules had been included, no hemoglobin transfer was observed whereas in the non-treated ghost-liposome system the hemoglobin transfer was observed (data not shown). This suggests that band 3 comigrates with hemoglobin bound to the 43-K fragment. Thus, it was proposed that hemoglobin transferred together with band 3. In order to further confirm this event, the liposomes recovered were analyzed by SDS-PAGE. Figure 5 shows the protein pattern of the liposomes obtained after the sucrose density gradient centrifugation. From lane C, it is apparent that the liposomes contain band 3 and hemoglobin.[13] Further, band 4.5 was also observed in the lane, indicating that this protein transferred also from erythrocytes.

Effects of Incubation Time and Temperature on the Hemoglobin Transfer When erythrocytes were incubated with DMPC liposomes at 37 °C, a hemolysis was observed. In order to examine a correlation between the hemoglobin transfer and the lysis of the erythrocytes, the amount of transferred hemoglobin and percent of the hemolysis were plotted as a function of incubation time (Fig. 6). The time

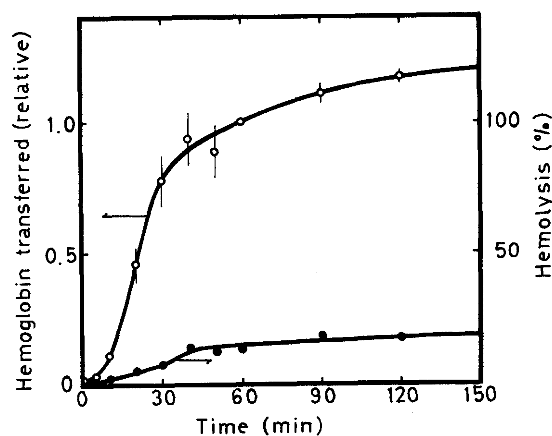

Fig. 6. Time Courses of the Hemoglobin Transfer to Liposomes and of the Hemolysis of Erythrocytes in the Presence of Liposomes

—○—, hemoglobin transfer; —●—, hemolysis. The amount of hemoglobin transferred to liposomes was estimated from the value peak to peak in the derivative spectrum (Fig. 3). Each data point is the mean of three determinations. The 100% hemolysis was determined using lysomyristoylphosphatidylcholine.

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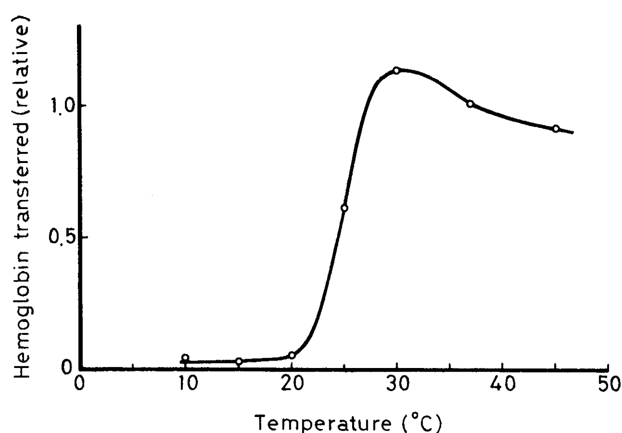

Fig. 7. Temperature Dependence of the Hemoglobin Transfer from Erythrocytes to Liposomes

The amount of hemoglobin transferred to liposomes was estimated from the value peak to peak in the first derivative spectrum after 1 h incubation with erythrocytes.

courses of the transfer and of the hemolysis seem to be parallel each other, indicating that the transfer is closely correlated to the extent of the hemolysis. As the process of the transfer is similar to that of an incorporation of phosphatidylcholines into erythrocytes,[1a] the incorporation of lipids should be responsible for the transfer.

Figure 7 represents a temperature dependence of the hemoglobin transfer. The amount of hemoglobin transferred to liposomes increases significantly with increasing temperature. The maximum of transfer was observed at about 30 °C. At higher temperatures, a hydrophobic interaction between phospholipids should increase, resulting in a decrease of the transfer. From this result, it seems that the membrane fluidity is closely correlated to the hemoglobin transfer.[1e]

Discussion

While there is considerable investigation concerning the transfer of phospholipid or cholesterol between biological membranes and liposomes, the transfer of proteins is not well understood.[14] The present series of experiments aims at investigating the hemoglobin transfer between cells and liposomes. Concerning the protein transfer between erythrocytes and liposomes, conflicting experimental results have been reported. Bouma *et al.*[1a] and Huestis[1b] reported that acetylcholinesterase can transfer from erythrocytes to liposomes. On the other hand, Ott *et al.*[15] proposed that the transfer of acetylcholinesterase should be due to a release of the vesicles from erythrocytes and that the vesicles contained hemoglobin. However, from the difference in their densities, the vesicles from erythrocytes can be separated from the liposomes by the sucrose gradient centrifugation.[2] Also in this study, when erythrocytes were incubated with a suspension of DMPC molecules, no hemoglobin could be detected in the liposomal fraction region of the sucrose gradient in Fig. 2 (data not shown). Further, cholesterol was not detected in the liposomes recovered. Thus, the vesicles from erythrocytes or fusion of the vesicles from erythrocytes with the liposomes are negligible. Bouma *et al.*[1a] said that detectable amounts of hemoglobin did not appear in the liposomes after incubation. However, several lines of evidence in this study suggest that hemoglobin can be transferred from erythrocytes to liposomes when erythrocytes are incubated with liposomes. This discrepancy may result from a difference in lipid monomer content in the liposome suspension, because hemolysis was observed during this experiment. In this connection, it is reported that the hemolysis is caused by the interaction of the lipid monomers with the erythrocyte membranes.[10]

From Figs. 4 and 5, it can be said that the transfer of hemoglobin is mediated by a migration of band 3. Therefore, the transfer of band 3 is essential for the hemoglobin transfer. It is well known that lipid molecules can be transferred from liposomes to erythrocytes by a mechanism which does not require cellular uptake of intact vesicles.[14a,b,f] It seems that there is a similar aspect between the protein transfer and lipid transfer. Enoch *et al.*[1c] reported two types of protein binding to membranes. One is designated as the tight binding form, in which the integral membrane protein in general does not readily undergo intermembrane transfer. The other is the loose binding form, designated by peripheral protein adsorbed on the membrane surface which can undergo intermembrane transfer. Band 3 corresponds to the former and acetylcholinesterase to the latter. According to Leto *et al.*,[16] a mechanism of intervesicle exchange of protein can be classified into two types. One is called an "aqueous transfer mechanism." In this route, the transfer of protein through the aqueous solution is accomplished as a result of reversible binding equilibrium. The other is a "collisional model," which requires the collision of donor and acceptor vesicles. It seems, however, that these two mechanisms can be applied only to loose binding proteins. Since band 3 is an integral protein and transverse through the membrane, of which the N terminus is on the inside surface, the transfer mechanism of band 3 should be different from the above mechanisms. The results in Figs. 6 and 7 imply that the hemolysis and/or vesiculation of erythrocytes, and membrane fluidity are closely associated with the band 3 transfer. Cook *et al.*[1e] said that the membrane fluidity is an important factor for the protein transfer. The membranes composed of egg phosphatidylcholine are most fluid among phosphatidylcholines used in this study. However, when the liposomes prepared from dipalmitoylphosphatidylcholine or egg yolk phosphatidylcholine were incubated with erythrocytes, no hemoglobin transfer was observed. Therefore, other factors as well as the membrane fluidity contribute to the hemoglobin transfer. Further, Mashino *et al.*[10] pointed out that short-chain phosphatidylcholines interact with membranes as monomers and cause a perturbation on the membranes, leading to hemolysis. Thus, it can be said that the chain-length of phospholipids is an important factor for the hemoglobin transfer. Since the hemoglobin transfer is coupled with the movement of band 3, the extent of the transfer depends also upon the association constant of lipid monomer with erythrocyte membrane around band 3. A motional restriction of band 3 may be perturbed by an insertion of phospholipid monomers from liposomes and the band 3 transfer should be correlated to the partial disruption of the membrane structure. The transfer of band 4.5 (Fig. 5) can be explained by the same mechanism as band 3, because this protein is transverse through the membrane.[17] On the other hand, although in this study bands 4.2 and 7 were not observed in the transferred

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liposomes by SDS-PAGE, the transfer of these proteins seems to be interpreted in the same way as hemoglobin, because they associate with the cytoplasmic portion of band 3.[18] Such consideration is also consistent with the results of Ferrel *et al.*[19] Further study of the mechanism of the hemoglobin transfer will be reported in the next paper.

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