References
Blake, R. D., & Fresco, J. R. (1973) *Biopolymers 12*, 775.
Burgers, P. M. J., & Eckstein, F. (1979) *Biochemistry 18*, 450.
Craine, J. E., & Klee, C. B. (1976) *Nucleic Acids Res. 3*, 2923.
Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685.
Glonek, T., Lunde, M., Mudgett, M., & Myers, T. (1971) *Arch. Biochem. Biophys. 142*, 508.
Godefroy-Colburn, T., & Grunberg-Manago, M. (1972) *Enzymes, 3rd Ed. 7*, 533.
Harvey, R. A., & Grunberg-Manago, M. (1970) *Biochem. Biophys. Res. Commun. 23*, 448.
Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652.
Kulaev, I. (1979) *The Biochemistry of Inorganic Polyphosphates*, Wiley, New York.
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95.
Marlier, J. F., Bryant, F. R., & Benkovic, S. J. (1981) *Biochemistry 20*, 2212.
McMaster, G. K., & Carmichael, G. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835.
Moses, R. E., & Singer, M. F. (1970) *J. Biol. Chem. 245*, 2414.
Murray, A. W., & Atkinson, M. R. (1968) *Biochemistry 7*, 4023.
Salhany, J. M., Yamane, T., Shulman, R. G., & Ogawa, S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4966.
Sheu, K., & Frey, P. F. (1977) *J. Biol. Chem. 252*, 4445.

# Proteoliposome Interaction with Human Erythrocyte Membranes. Functional Implantation of γ-Glutamyl Transpeptidase†

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ABSTRACT: The transfer of detergent solubilized and purified γ-glutamyl transpeptidase (γ-GTase), of hog kidney cortex, from proteoliposomes into human erythrocyte ghost membranes has been studied. The transfer of γ-glutamyl transpeptidase was observed upon incubation of γ-GTase incorporated dipalmitoylphosphatidylcholine vesicles with erythrocyte ghost membranes at 37 °C for 12 h. The extent of transfer was dependent upon the fluidity of donor proteoliposomes, being more when dipalmitoylphosphatidylcholine proteoliposomes were used compared to dimyristoylphosphatidylcholine, and intermediate values were observed when binary mixtures of DMPC and DPPC were used. Moreover, the transfer of γ-GTase was facilitated when rigid basic phospholipid proteoliposomes were used as donor. The transfer of γ-GTase has been observed to be associated with the removal of intrinsic membrane proteins and lipids from erythrocytes, mainly acetylcholinesterase, sphingomyelin, and cholesterol. An enhancement in the fluorescence due to resonance energy transfer was observed when ghost membranes containing fluorescent donor probe were incubated with proteoliposomes containing fluorescent acceptor probe, indicating that fusion but not adsorption of vesicles occurs during the transfer process. However, the inability of entrapped [$^{14}$C]-sucrose delivery from proteoliposomes into ghost membrane vesicles suggests that fusion per se is not primarily involved in the transfer process. It appears that the transfer of γ-glutamyl transpeptidase occurs by a collisional transfer process resulting in intermembrane protein transfer. The γ-glutamyl transpeptidase implanted ghost membranes exhibited the uptake of L-glutamate which was inhibited by serine–borate, an inhibitor of transpeptidase activity. In addition, the uptake of L-glutamate was inhibited by the dipeptide γ-glutamyl-L-glutamate, thus supporting the proposed role of γ-glutamyl transpeptidase in the uptake of amino acids in biological membranes.

The interaction of phospholipid vesicles with cells for the introduction of new materials, e.g., drugs and enzymes into mammalian cells, has received considerable attention in recent years (Pagano & Weinstein, 1978; Kimelberg & Mayhew, 1978). Studies have shown that several membrane-associated proteins can undergo transfer spontaneously between artificial phospholipid vesicles (Roseman et al., 1977; Enoch et al., 1977) and between cells and phospholipid vesicles (Bouma et al., 1977; Cook et al., 1980). Roseman et al. (1977) showed that cytochrome $b_5$ bound to egg phosphatidylcholine vesicles when incubated with protein-free phospholipid vesicles underwent exchange between lipid bilayers. Subsequently Enoch et al. (1977, 1979) demonstrated that cytochrome $b_5$ reductase bound to dimyristoylphosphatidylcholine vesicles could be transferred into another artificial membrane, and this exchange did not involve vesicle fusion. Bouma et al. (1977) have shown that several membrane-associated proteins from intact human erythrocyte membrane were transferred to dimyristoylphosphatidylcholine vesicles when erythrocytes were incubated with liposomes. It was further shown by Cook et al. (1980) that transfer of protein from erythrocyte membrane to phospholipid vesicles was facilitated when the recipient phospholipid vesicles were more fluid than the donor erythrocyte membranes.

Recent studies from our laboratory (Kalra et al., 1981) have shown that detergent solubilized γ-glutamyl transpeptidase when incorporated into dipalmitoylphosphatidylcholine vesicles followed by incubation with erythrocytes resulted in the transfer of γ-glutamyl transpeptidase into erythrocyte membrane. The extent of transfer of γ-glutamyl transpeptidase into erythrocytes was relatively low when dimyristoylphosphatidylcholine vesicles were used as compared to DPPC[1]

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vesicles. The mechanism of transfer of γ-glutamyl transpeptidase from liposomes into erythrocyte membrane is, however, not clearly understood.

In an attempt to shed light on the mechanism of transfer of γ-glutamyl transpeptidase into erythrocyte membrane, we have varied the fluidity and charge of the phospholipid vesicles and analyzed the effect of these factors on the orientation and functional incorporation of γ-glutamyl transpeptidase into erythrocyte ghost membranes. We have used a fluorescence resonance energy-transfer assay system to show that association/implantation of γ-glutamyl transpeptidase into erythrocyte membrane does not occur as a result of strong adsorption. The transfer of γ-glutamyl transpeptidase from proteoliposomes into ghost membrane presumably occurs as a result of intermembrane protein transfer. Further, our studies demonstrate that γ-glutamyl transpeptidase implanted erythrocyte ghost membrane exhibits uptake of L-glutamate and L-alanine but not of L-glutamine. Moreover, this uptake of amino acids by implanted ghost membranes was inhibited by γ-glutamyl transpeptidase inhibitors. In addition, the uptake of glutamate by γ-glutamyl transpeptidase implanted erythrocyte membrane was inhibited by γ-glutamylglutamate, a dipeptide. These results support the proposal (Meister & Tate, 1976) that γ-glutamyl transpeptidase plays a functional role in the transport of amino acids and dipeptides in biological membranes.

## Materials and Methods

Dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, phosphatidylserine, dicetyl phosphate, cholesterol, phosphatidylcholine, stearylamine, L-γ-glutamyl-*p*-nitroanilide, glycylglycine, glutathione, L-amino acids, papain, Triton X-100, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5,5′-dithiobis(2-nitrobenzoic acid), acetylthiocholine iodide, and cytochalasin B were purchased from Sigma; $^{14}$C-labeled L-amino acids, [$^{14}$C]sucrose, 3-*O*-[$^{14}$C]methyl-D-glucose, [$^{14}$C]inulin, and 2-deoxy-D-[G-$^{3}$H]-glucose were from New England Nuclear; Sephadex G-25, Sephacryl S-200, Sepharose 4B, dextran T-70, and Ficoll were from Pharmacia Fine Chemicals; fluorescent probes octadecylrhodamine B and 5-(dodecanoylamino)fluorescein were from Molecular Probes Inc.; $^{125}$I was from Amersham Corp. The reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. γ-Glutamyl-L-glutamate and γ-glutamyl-L-alanine were purchased from Vega Chemicals. All other chemicals were of reagent grade purity.

*Isolation and Purification of γ-Glutamyl Transpeptidase.* γ-Glutamyl transpeptidase was isolated from fresh hog kidney cortex following solubilization with deoxycholate and purified essentially according to the procedure of Orlowski & Meister (1965) and modified as described earlier (Kalra et al., 1981). The enzyme exhibited a specific activity of 50–60 units when assayed at 37 °C in a reaction mixture containing 5 mM L-γ-glutamyl-*p*-nitroanilide, 40 mM glycylglycine, 150 mM sodium chloride, 11 mM magnesium chloride, and 100 mM Tris-HCl, pH 8.6. One unit of enzyme activity refers to 1 μmol of *p*-nitroaniline liberated min$^{-1}$ (mg of protein)$^{-1}$.

*Preparation of Proteoliposomes.* Purified γ-glutamyl transpeptidase (γ-GTase) was incorporated into phospholipid vesicles which were prepared by a freeze–thaw and sonication procedure (Kalra et al., 1981). In the initial studies the purification of these proteoliposomes to remove the unincorporated enzyme was carried out on a Sepharose 4B column (350 × 13 mm). However, no advantage in doing so was observed as incubation of free enzyme with erythrocyte ghost membrane did not result in any significant incorporation of the enzyme into the erythrocyte membrane. Determination of γ-GTase activity in proteoliposomes showed that 0.5–1.0 unit of γ-GTase was incorporated into liposomes, and the enzyme exhibits right side out orientation to the extent of 95% for DPPC proteoliposomes and 85% for DMPC proteoliposomes.

*Preparation of Erythrocyte Ghosts.* Fresh heparinized blood from normal volunteers was passed through a cellulose column to remove leukocytes and platelets, and erythrocytes were washed with phosphate-buffered saline before use (Kalra et al., 1981). Ghosts were prepared by hemolysis of erythrocytes with 40 volumes of 5 mM phosphate buffer, pH 8.0, further washed to remove hemoglobin, and resealed by incubating at 25 °C for 20 min in 5 mM phosphate buffer, pH 8.0, containing 2 mM magnesium sulfate (Kant & Steck, 1973; Steck, 1974). Any solute to be entrapped was added to the ghost suspension in 5 mM phosphate buffer, pH 8.0, and kept at 37 °C for 30 min before the resealing. About 95% of the ghost membranes thus prepared were observed to be sealed as ascertained by centrifugation on 4% dextran T-70 gradient (Steck, 1974). Determination of acetylcholinesterase activity in these sealed ghost membranes in the absence and presence of Triton X-100 (0.2%) further revealed that 90–95% of erythrocyte ghost membranes are oriented right side out.

*Interaction of Proteoliposomes with Erythrocyte Ghost Membranes.* A 1.0-mL sealed ghost suspension (2–3 mg of protein) in 20 mM Tris–acetate buffer, pH 8.0, containing 140 mM NaCl was incubated with 1 mL of transpeptidase-incorporated liposomes (4–8 μmol of phospholipid and about 25–30 μg of protein). The suspension was gently agitated (40 oscillations/min) on a shaking water bath at 37 °C for a period of 12–15 h unless otherwise indicated. At the end of the incubation, separation of erythrocyte ghost membranes and vesicles was accomplished by centrifugation at 13000*g* for 20 min. By this procedure ghost membranes were pelleted, with most of the liposome vesicles remaining in the supernatant. The supernatant was removed and either processed immediately or stored at 4 °C for analysis. Further removal of loosely adsorbed liposomes from incubated erythrocyte ghost membranes was carried out by centrifugation of the ghost pellet on 1% Ficoll gradient at 13000*g* for 60 min. The ghost membrane pellet was washed twice with buffer to remove any traces of Ficoll. For transport studies, sealed ghost membrane vesicles were further separated from unsealed vesicles by centrifugation at 60000*g* for 60 min on 4% dextran T-70 gradient using polyallomer tubes (89 × 14 mm) and a Beckman SW-41 rotor. Subsequent washings were carried out with buffer to remove any traces of dextran from the γ-glutamyl transpeptidase implanted sealed ghost membrane vesicles.

*Assay of Transport.* γ-Glutamyl transpeptidase implanted erythrocyte ghost membranes (0.6–0.8 mg of protein) containing 1.0–1.5 μmol of glutathione (mg of ghost protein)$^{-1}$ were suspended in 0.6 mL of 20 mM Tris–acetate buffer, pH 8.0, with 140 mM NaCl in a test tube (75 × 10 mm) having a micro stir bar (Nalgene). After 5 min of preincubation at 30 °C, the reaction was initiated by the addition of 0.1 mL of radioactive amino acid (0.5 μCi). At the indicated time intervals, 0.1-mL aliquots were removed and immediately diluted in 1 mL of chilled buffer. The suspension was rapidly filtered on a Millipore membrane filter (0.45-μm pore size, 25-mm diameter) and washed twice with 1 mL of the same

[1] Abbreviations: γ-GTase, γ-glutamyl transpeptidase; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)-aminomethane; DON, 6-diazo-5-oxo-L-norleucine.

buffer. The filters were removed and dried under a 600-W lamp. The radioactivity retained on the filters was counted after the dried filter was immersed in 6 mL of Econofluor (New England Nuclear) scintillation fluid and counted in a Beckman L S-8000 counter. Where indicated, an inhibitor or dipeptide was added to the reaction mixture and equilibrated for 15 min prior to initiating uptake studies with radioactive solute.

*Analysis of Supernatant.* Supernatant, which was collected after incubation of γ-GTase proteoliposomes or liposomes with erythrocyte ghost membrane, was used to assay the activities of enzymes, γ-glutamyl transpeptidase and acetylcholinesterase, and for monitoring the exchange of lipids and proteins during incubation. For protein-exchange studies, proteoliposomes present in supernatant were separated from other soluble proteins by gel filtration through a Sepharose 4B column (350 × 13 mm). Protein-containing liposomes were collected at the void volume. These proteoliposomes along with total supernatant sample (20–30 μg of protein) were further subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (5.6% gels) according to the procedure of Fairbanks et al. (1971). For studies on the exchange of various phospholipids and cholesterol during incubation, lipids were extracted from the supernatant and also from the erythrocyte ghost membrane by the modified Bligh and Dyer's procedure (Kates, 1975). The lipid extract was then analyzed by thin-layer chromatography using silica gel G plates and chloroform–methanol–water (65:25:4 v/v) as the solvent system.

*Radioiodination of γ-Glutamyl Transpeptidase.* γ-Glutamyl transpeptidase was iodinated with $^{125}I$ by the Chloramine T method of Hunter & Greenwood (1962). $^{125}I$ (20 μL) (10 mCi/mL) was added to the reaction vial containing 50 μL of phosphate buffer (100 mM, pH 7.5). To this was added 200 μL of purified γ-glutamyl transpeptidase (40 μg of protein) and 50 μL of Chloramine T (5 mg/mL) in quick succession while mixing the content by using a small magnetic stirrer bar, and after 30 s, 50 μL of sodium metabisulfite (10 mg/mL) was added to terminate the reaction. The contents were immediately transferred to a Sephadex G-25 (medium) column (20 × 1.8 cm) and eluted with phosphate buffer, and 1.0-mL fractions were collected. A 10-μL sample of each fraction was counted for radioactivity in a Beckman γ-4000 counter and a corresponding sample taken for determination of γ-glutamyl transpeptidase activity. In most of the assays, fractions 14–15 were the peak fractions which contained radiolabeled γ-glutamyl transpeptidase protein.

*Fluorescence–Polarization Measurements.* 1,6-Diphenyl-1,3,5-hexatriene has been used as a fluorescent probe to measure the fluidity of lipid bilayers in both liposomes and biological membranes (Shinitzky & Inbar, 1974). Liposomes or proteoliposomes (2 mL) were incubated with diphenylhexatriene (2 μM) for 45 min at room temperature in the dark. Where indicated erythrocyte ghost membranes were incubated with 2 μM diphenylhexatriene in 5 mM phosphate buffer, pH 8.0, at 37 °C for 60 min. Fluorescence polarization was measured with a Perkin-Elmer MPF-4 spectrophotometer equipped with polarizers and a thermostatically controlled cuvette holder. The excitation wavelength was 360 nm, and the emission was measured at 426 nm as described previously (Haggerty et al., 1978). The fluorescence polarization *P* value was calculated from the fluorescence intensity measurements with excitation and analyzer polarizers in vertical and horizontal orientations (Haggerty et al., 1978).

*Methods for Monitoring Protein Transfer.* Two methods were followed to study the mode of transfer of liposome contents to the erythrocyte ghosts. (a) DPPC proteoliposomes were prepared by sonication of phospholipid (10 μmol) in 1 mL of Tris–acetate buffer containing 50 μmol of [$^{14}C$]sucrose (4 μCi) (Bouma et al., 1977). The sample was immediately loaded on a Sepharose 4B column (300 × 13 mm) and eluted with 20 mM Tris–acetate buffer containing unlabeled sucrose (50 mM). Erythrocyte ghosts were then incubated at 37 °C for 6 h with these phospholipid vesicles having internal [$^{14}C$]sucrose (Bouma et al., 1977). A parallel vesicle sample was prepared by sonication of phospholipid in Tris–acetate buffer, and [$^{14}C$]sucrose was added externally during incubation with erythrocyte ghosts. Incorporation of $^{14}C$ label into the 3 times washed ghost cells was determined by liquid scintillation counting. (b) In the second method (fluorescence resonance energy-transfer assay) two fluorescent probes, octadecylrhodamine B chloride (R-18) and 5-(dodecanoylamino)fluorescein (F-12), were used (Keller et al., 1977). The rhodamine probe (1.7 μmol) and phospholipid (10 μmol) were dissolved in chloroform. The contents were dried to thin film and proteoliposomes prepared as described above. Erythrocyte ghost membranes were incubated with the fluorescein probe (2.4 μmol) for 30 min at 37 °C before they were sealed. Proteoliposomes with the R-18 probe were incubated for 12 h at 37 °C with erythrocyte ghosts having the F-12 probe. After incubation the ghost membrane fraction was washed 3–4 times with buffer and used for resonance energy-transfer measurements in a Perkin-Elmer MPF-4 fluorescence spectrophotometer. Excitation of the diluted sample was done at 460 nm, and emission spectra were observed from 480 to 620 nm. The results were compared with a preparation of ghost membrane in which both the probes were added into the same membrane preparation before they were sealed ("mock fusion") and also in the sample where the two probe membranes were simply mixed at the time of taking spectral measurements ("mixing").

*Determination of Intravesicular Volume.* The intravesicular volume of the sealed ghost membrane vesicles was determined with 3-*O*-[$^{14}C$]methyl-D-glucose and [$^{14}C$]inulin, as described by Hissin & Hilf (1978). Erythrocyte ghost membrane vesicles under these conditions had 60 ± 14 μL/mg of protein as the total internal volume.

*Analytical Methods.* Acetylcholinesterase activity in erythrocyte ghosts and supernatants obtained after incubation was assayed essentially by the method of Steck (1974) using acetylthiocholine iodide as substrate. For this procedure, 2–10 μg of sample protein was added to 1 mL of reaction mixture containing 0.5 μmol of DTNB, 100 μmol of phosphate buffer, pH 7.0, and 0.625 μmol of substrate, and the rate of hydrolysis of substrate was followed at 412 nm by using a Perkin-Elmer Model 552 spectrophotometer recorder attachment. Phospholipid phosphorus was estimated by the colorimetric procedure after perchloric acid digestion (Wagner et al., 1962), and cholesterol was determined by the procedure of Zlatkis et al. (1953). The methods for determining reduced glutathione content in erythrocyte ghost and estimation of protein in proteoliposomes and other samples were the same as those employed in our previous study (Kalra et al., 1981).

## Results

*Effect of Fluidity and Charge of Proteoliposomes on γ-Glutamyl Transpeptidase Transfer to Erythrocyte Ghost Membranes.* Previous studies have shown that detergent-solubilized γ-glutamyl transpeptidase can be incorporated into egg lecithin, dipalmitoylphosphatidylcholine, and dimyristoylphosphatidylcholine vesicles (Sikka & Kalra, 1980; Kalra et al., 1981). The extent of incorporation of γ-GTase

Table I: Effect of Fluidity and Charge of Donor Proteoliposomes on the Incorporation of γ-Glutamyl Transpeptidase into Erythrocyte Ghost Membranes[a]

| phospholipid composition of proteolipsomes | charge | P value[b] | γ-glutamyl transpeptidase act. [nmol min$^{-1}$ (mg of ghost protein)$^{-1}$] in erythrocyte membrane | rel extent of transfer (%) |
|---|---|---|---|---|
| DPPC | neutral | 0.330 ± 0.01 | 84.8 ± 8.8 | 100 |
| DPPC/DMPC (7:3) | neutral | 0.315 ± 0.01 | 74.8 ± 5.1 | 88 |
| DPPC/DMPC (5:5) | neutral | 0.144 ± 0.03 | 64.4 ± 7.2 | 76 |
| DPPC/DMPC (3:7) | neutral | 0.120 ± 0.02 | 57.7 ± 6.8 | 68 |
| DPPC/stearylamine (8:2) | positive | ND | 129.5 ± 11.1 | 153 |
| DPPC/cholesterol/stearylamine (7:1:2) | positive | ND | 80.3 ± 16.0 | 95 |
| DPPC/dicetyl phosphate (8:2) | negative | ND | 61.8 ± 10.8 | 73 |
| DPPC/phosphatidylserine (9:1) | negative | ND | 45.9 ± 3.0 | 54 |
| DMPC | neutral | 0.114 ± 0.01 | 53.7 ± 8.5 | 63 |
| DMPC/stearylamine (8:2) | positive | ND | 62.0 ± 8.9 | 73 |
| DMPC/phosphatidylserine (9:1) | negative | ND | 19.0 ± 2.2 | 22 |
| DMPC/cholesterol/stearylamine (7:1:2) | positive | ND | 48.3 ± 6.2 | 57 |
| egg lecithin | neutral | ND | 4.8 ± 1.5 | 6 |

[a] γ-Glutamyl transpeptidase was incorporated into the indicated phospholipid vesicles as described under Materials and Methods. These proteoliposomes (1 mL) were incubated with sealed erythrocyte ghosts (1 mL, 2–3 mg of protein) at 37 °C for 15 h. Ghost membranes were separated, washed, and centrifuged at 13000*g* for 60 min over 1% Ficoll gradient. The pellet was washed twice with PBS before assay of γ-glutamyl transpeptidase activity. Data are shown as mean ± SEM of three to six determinations. PBS, phosphate-buffered saline. [b] Fluorescence polarization *P* value of recipient ghost membranes at 37 °C was 0.245 ± 0.01. ND, not determined.

into various phospholipid vesicles has been further shown to be dependent upon the fatty acyl chain length and unsaturation. The incorporation of γ-GTase into dilauroylphosphatidylcholine liposomes was 2.2 times more when compared to dimyristoylphosphatidylcholine liposomes. Among the phosphatidylcholine liposomes the extent of incorporation was in the following order: $C_{12} > C_{14} > C_{16} > C_{18}$ of fatty acyl chain length. When dioleoylphosphatidylcholine, containing unsaturated $C_{18}$ fatty acyl chain, was used, the extent of incorporation of γ-GTase was similar to that observed with DMPC vesicles. These results indicate that the fluid environment of liposomes enhances the incorporation of γ-GTase into the lipid bilayer.

Incubation of γ-glutamyl transpeptidase incorporated DPPC or DMPC liposomes with erythrocyte ghost membranes at 37 °C resulted in the transfer of protein into ghost membrane as a function of incubation time (Figure 1). The extent of transfer of γ-glutamyl transpeptidase from proteoliposomes into erythrocyte membrane was relatively high when DPPC proteoliposomes were used as compared to DMPC proteoliposomes. Studies were undertaken to delineate whether fluidity and charge of liposomes affected the protein transfer into ghost membranes. As shown in Table I, binary mixtures of DMPC and DPPC, whose phase transition temperatures are intermediate between those of pure lipid components, were used for studying transfer of γ-glutamyl transpeptidase from proteoliposomes into ghost membrane. As the mole fraction of DMPC in DPPC increases in proteoliposomes the fluorescence polarization *P* value of incorporated diphenylhexatriene at 37 °C decreases. In proteoliposomes, containing 70% DPPC and 30% DMPC, the extent of protein transfer in ghost membranes increased by 1.4-fold as compared to proteoliposomes containing 100% DMPC. As shown, the transfer of γ-GTase into erythrocyte membranes did not occur to a significant extent when proteoliposomes were prepared from egg lecithin. Moreover, incubation of γ-GTase incorporated DPPC proteoliposomes with erythrocyte ghost membranes at 4 °C did not result in the transfer of γ-GTase into erythrocyte membranes (data not shown).

Studies related to the role of liposome surface charge on the extent of transfer of γ-glutamyl transpeptidase into

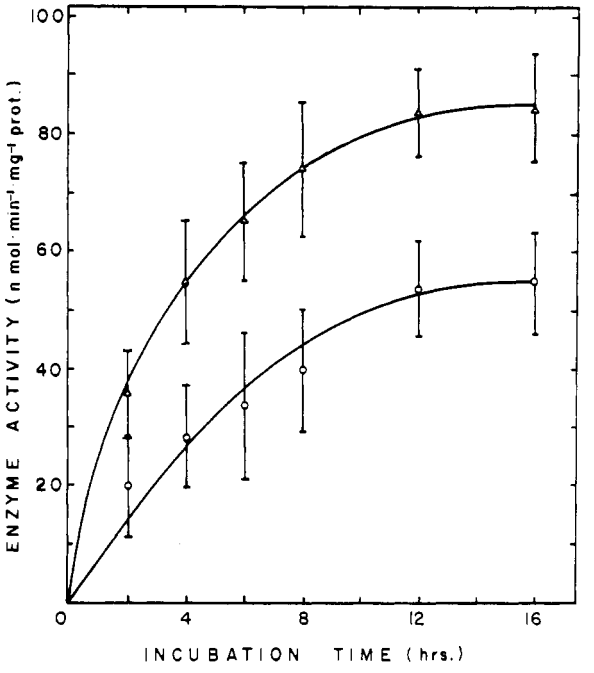


FIGURE 1: Time course of γ-glutamyl transpeptidase transfer from proteoliposomes into erythrocyte membrane. Three milliliters of sealed ghost suspension (6–8 mg of protein) was incubated with 3 mL of γ-glutamyl transpeptidase incorporated liposomes (4–8 μmol/mL phospholipid) at 37 °C as described under Materials and Methods. At indicated time intervals, 1 mL of sample was removed, mixed with 9 mL of chilled buffer, and centrifuged at 13000*g* for 20 min. The ghost pellet was subjected to density gradient centrifugatin by using 1% Ficoll and washed twice with buffer before assaying for γ-glutamyl transpeptidase activity. (△) Incubation with DPPC proteoliposomes and (○) incubation with DMPC proteoliposomes. Each point is the mean ± SEM of three experiments.

erythrocyte membrane were ascertained by characterizing the interaction of proteoliposomes in which the liposome composition was varied as follows: (a) neutral liposomes containing only DPPC or DMPC; (b) acidic phospholipids containing DPPC and dicetyl phosphate or DPPC/DMPC containing phosphatidylserine; (c) basic liposomes containing DPPC/DMPC and stearylamine. As shown in Table I negatively

Table II: Effect of Chemical Fusogens on the Incorporation of γ-GTase into Erythrocyte Membranes[a]

| proteoliposomes | fusogen | γ-glutamyl transpeptidase act. [nmol $min^{-1}$ (mg of protein)$^{-1}$] in erythrocyte membrane ($n = 3$) | rel extent of transfer (%) |
|---|---|---|---|
| (a) DPPC | none | 84.8 ± 8.8 | 100 |
| | 2 mM $Ca^{2+}$ | 68.3 ± 8.9 | 81 |
| | 5 mM $Ca^{2+}$ | 103.0 ± 8.8 | 121 |
| | 10 mM $Ca^{2+}$ | 85.6 ± 9.9 | 101 |
| | 20 mM $Ca^{2+}$ | 90.3 ± 10.1 | 106 |
| | 2 mM EGTA | 93.8 ± 8.8 | 111 |
| | 5 mM EGTA | 101.8 ± 11.5 | 120 |
| (b) DMPC/cholesterol/stearylamine (7:1:2) | none | 48.3 ± 6.2 | 100 |
| | 0.1 mM $La^{3+}$ | 62.2 ± 10.8 | 129 |
| | 0.2 mM $La^{3+}$ | 59.3 ± 7.1 | 123 |
| | 0.5 mM $La^{3+}$ | 61.0 ± 6.0 | 126 |
| | 1.0 mM $La^{3+}$ | 57.4 ± 6.2 | 119 |
| | 1 mM $Yb^{3+}$ | 49.5 ± 5.2 | 102 |
| | 5 mM $Ca^{2+}$ | 59.0 ± 8.9 | 122 |

[a] γ-Glutamyl transpeptidase incorporated proteoliposomes were incubated with erythrocyte ghosts at 37 °C for 15 h in the presence of various chemical agents. Enzyme activity was assayed after washing the ghosts as explained in Table I.

Table III: Effect of Ratio of Proteoliposome to Ghost Membrane on the Extent of γ-Glutamyl Transpeptidase and Acetylcholinesterase Transfer[a]

| ratio of proteoliposomes to ghost membrane | | γ-glutamyl transpeptidase act. [nmol $min^{-1}$ (mg of protein)$^{-1}$] | | | acetylcholinesterase act.[b] [μmol $min^{-1}$ (mg of protein)$^{-1}$] | |
|---|---|---|---|---|---|---|
| v/v | μmol of PL/mg of protein | in incubation mixture at zero time | in ghost membranes after incubation | rel extent of transfer (%) | in ghost membrane after incubation | release from membranes (%) |
| 0.25:1 | 0.66 | 128.6 ± 11 | 26.56 ± 3.9 | 20.6 | 1.27 ± 0.32 | 21.1 |
| 0.33:1 | 0.88 | 168.2 ± 12 | 39.31 ± 7.8 | 23.4 | 1.16 ± 0.30 | 27.9 |
| 0.5:1 | 1.33 | 250.1 ± 18 | 53.12 ± 8.7 | 21.2 | 1.14 ± 0.30 | 29.2 |
| 1:1 | 2.66 | 490.0 ± 27 | 99.86 ± 7.9 | 20.4 | 1.10 ± 0.24 | 31.7 |
| 1.5:1 | 4.00 | 750.4 ± 40 | 105.17 ± 10.5 | 14.0 | 0.89 ± 0.20 | 44.7 |
| 2:1 | 5.33 | 996.8 ± 34 | 106.23 ± 11.2 | 10.6 | 0.88 ± 0.32 | 45.3 |
| purified γ-GTase | | 985.3 ± 14 | 2.90 ± 1.5 | 0.3 | 1.44 ± 0.18 | 10.5 |

[a] Purified γ-glutamyl transpeptidase was incorporated into DPPC liposomes as described under Materials and Methods. Varying amounts of proteoliposomes (8 μmol of phospholipid/mL) were incubated with 1 mL of ghost membranes (3 mg of protein) at 37 °C for 15 h. Ghost membranes were separated from supernatant and washed as described in Table I, and assays of γ-glutamyl transpeptidase and acetylcholinesterase activities were carried out as indicated under Materials and Methods. Values are given as mean ± SEM of four determinations. [b] Acetylcholinesterase activity in ghost membranes before incubation (1.61 ± 0.38 units). PL, phospholipid.

charged phospholipids, i.e., DPPC/dicetyl phosphate and DPPC/phosphatidylserine, exhibited inhibition in the transfer of γ-glutamyl transpeptidase into erythrocyte membranes. However, positively charged liposomes, i.e., DPPC/stearylamine as compared to DPPC, enhanced the transfer of protein from proteoliposomes into the membrane. A moderate increase (15%) in the amount of γ-GTase transferred to erythrocyte membrane was observed when proteoliposomes of DMPC/stearylamine were used as compared to DMPC alone. This enhanced transfer of γ-GTase from positively charged proteoliposomes into erythrocyte membrane was not due to increased adsorption resulting from electrostatic interaction with negatively charged cell membrane since washing with high ionic strength buffer (0.5 M KCl) did not diminish γ-GTase activity associated with erythrocyte membranes. However, the possibility of strong adsorption contributing to the apparent increase in enzyme activity in membranes could not be ruled out by this approach.

*Effect of Chemical Fusogen on the Transfer of γ-Glutamyl Transpeptidase.* Various chemical agents known to have fusogenic activity (Lucy, 1977; Baker & Kalra, 1979) were used to investigate their effect on the transfer of γ-glutamyl transpeptidase from proteoliposomes into erythrocyte membranes. As shown in Table II, approximately 20% increase in γ-GTase activity in erythrocyte membrane was observed upon incubation of ghost membranes with DPPC proteoliposomes in the presence of $Ca^{2+}$ (5 mM). The transfer of γ-GTase from proteoliposomes was not due to endogenously membrane bound $Ca^{2+}$ since EGTA (2–5 mM) did not affect the transfer process. When DMPC/cholesterol/stearylamine (7:1:2) proteoliposomes were used, $La^{3+}$ (1 mM) enhanced (20%) the transfer of enzyme. The presence of $Yb^{3+}$ (1 mM) did not alter the extent of incorporation. It is pertinent to mention that $La^{3+}$ and $Yb^{3+}$ have been observed to enhance the fusion of erythrocytes to polyhomokaryons (Majumdar et al., 1980).

*Effect of Protein and Lipid Content of Proteoliposomes on Enzyme Transfer.* Studies were carried out to determine whether the extent of transfer of γ-GTase from proteoliposomes to erythrocyte ghosts was dependent upon the amount of phospholipid and of enzyme present in the incubation mixture. As shown in Table III, the total enzyme activity implanted in the erythrocyte membrane increases initially with the increase in the amount of proteoliposomes present in the incubation mixture for a constant amount of erythrocyte membranes (3 mg of protein). With a 4-fold increase in the ratio (v/v) of proteoliposome to ghosts (from 0.25:1 to 1:1) the amount of enzyme transferred also showed about a 4-fold enhancement. On further increasing the ratio to 2:1, there was no more increase in the transfer of enzyme activity into membranes. When the amount of proteoliposomes in the

Table IV: Exchange of Lipids upon Incubation of Liposomes with Erythrocyte Ghost Membranes[a]

| phospholipids | erythrocyte ghost membrane (μmol of lipid/mg of protein) | | | supernatant (μmol of lipid/mg of protein) after incubation of ghost membranes with | |
|---|---|---|---|---|---|
| | before incubation | after incubation with | | DPPC liposomes | DPPC proteoliposomes |
| | | liposomes | proteoliposomes | | |
| total phospholipids | 2.99 ± 0.12 | 3.96 ± 0.12 (32) | 3.62 ± 0.10 (21) | 1.88 ± 0.11 | 2.10 ± 0.19 |
| phosphatidylserine (PS) | 0.86 ± 0.10 | 0.73 ± 0.07 (15) | 0.79 ± 0.08 (8) | 9.21 ± 0.03 | 0.16 ± 0.04 |
| sphingomyelin | 0.76 ± 0.10 | 0.63 ± 0.08 (17) | 0.63 ± 0.09 (17) | 0.23 ± 0.04 | 0.19 ± 0.01 |
| phosphatidylcholine (PC) | 0.81 ± 0.09 | 2.01 ± 0.16 (148)[b] | 1.62 ± 0.13 (100)[b] | 1.44 ± 0.14 | 1.75 ± 0.16 |
| phosphatidylethanolamine (PE) | 0.56 ± 0.08 | 0.59 ± 0.11 (5) | 0.58 ± 0.07 (4) | 0.00 | 0.00 |
| cholesterol | 0.82 ± 0.14 | 0.54 ± 0.10 (34) | 0.51 ± 0.11 (38) | 0.32 ± 0.11 | 0.36 ± 0.13 |

[a] DPPC liposomes were prepared with and without γ-glutamyl transpeptidase (γ-GTase) and incubated with sealed ghosts as described in footnotes to Table I. Lipids were extracted from the supernatant and from washed ghost membranes and analyzed by TLC as described under Materials and Methods. Each value represents mean ± SEM of four determinations. Values in parentheses denote percent change. [b] $P < 0.05$.

incubation mixture is increased, more of the enzyme becomes available for transfer. However, this increased enzyme availability does not affect the extent of enzyme transferred into ghost membrane but shows a decreasing trend beyond a 1:1 ratio (v/v) of proteoliposome to ghost membrane (micromoles of proteoliposomal PL to milligrams of ghost protein ratio being 2.7). In the absence of liposomes, the enzyme is not transferred to ghost membranes. These results show that an optimal ratio of donor proteoliposomes to acceptor ghost membrane during incubation is required for any significant amount of intermembrane protein transfer. Moreover, acetylcholinesterase enzyme, which is associated with the external surface of erythrocyte membrane, is removed into the supernatant during incubation. The extent of removal of this enzyme is also in response to the amount of proteoliposomes present in the incubation mixture (Table III). The removal of acetylcholinesterase from ghost membranes was more (15–20%) in the presence of DMPC proteoliposomes than with DPPC proteoliposomes in the incubation mixture (data not shown).

*Exchange of Lipids and Proteins upon Incubation of Proteoliposomes with Erythrocyte Ghost Membranes.* Incubation of erythrocyte ghost membranes, with γ-GTase-incorporated DPPC liposomes, resulted in the removal of phospholipids and cholesterol from the membrane. Thin-layer chromatographic analysis of the lipid extract of the supernatant obtained after incubation of erythrocyte ghosts with proteoliposomes showed that 17% of sphingomyelin and 8–15% of phosphatidylserine present in the ghost membrane were removed (Table IV). Phosphatidylethanolamine was not detected in the supernatant. It is pertinent to mention that ghost membranes used for transfer studies were 95% right side out, as determined by accessibility of the marker enzyme acetylcholinesterase. The remaining 5% of the inside out membrane may have contributed to the removal of a small percentage of PS noted in these studies. Erythrocyte ghost membranes upon incubation with proteoliposomes resulted in release of 34–38% of membrane cholesterol in the supernatant. Phosphatidylcholine (PC) content showed a 2–3-fold increase in the implanted ghost membranes compared to control. This increase in phosphatidylcholine content was significantly more ($P < 0.05$) when incubation of erythrocyte membranes was carried out in the presence of protein-free liposomes as compared to proteoliposomes (Table IV).

Analysis of the supernatant, obtained after incubation of erythrocyte ghosts with proteoliposomes, revealed the presence of γ-GTase and acetylcholinesterase enzyme activities and an increased content of protein (data not shown). Studies were undertaken to determine whether the acetylcholinesterase enzyme and other protein components were associated with the phospholipid vesicles or remained free in the supernatant. For this determination, the supernatant was passed through a Sepharose 4B column to separate protein-containing liposomes from soluble proteins as well as from pure lipid vesicles. γ-Glutamyl transpeptidase and acetylcholinesterase activities were observed in the void volume fraction (proteoliposomal fraction) of the supernatant.

$NaDodSO_4$ gel electrophoretic pattern (Figure 2A) of the proteoliposomal fraction (void volume), obtained by incubation of γ-GTase liposomes with erythrocyte ghosts, showed three major protein bands. Two of these polypeptide species with apparent molecular weights 100 000–105 000 (component a) and 59 000–63 000 (component d) correspond to γ-GTase (Kalra et al., 1981) and the third with molecular weight of approximately 90 000 corresponds to acetylcholinesterase (Bouma et al., 1977). The $M_r$ 90 000 polypeptide component (band b in Figure 2) was observed to be present in the proteoliposomal (void volume) fraction (Figure 2A,B) as well as in the total supernatant (Figure 2C,D), which was obtained after incubation of erythrocyte ghosts with either γ-GTase containing proteoliposomes or protein-free liposomes (no γ-GTase). In addition to this $M_r$ 90 000 polypeptide species, three other polypeptide components with apparent molecular weights 42 000–47 000 (band e), 30 000–35 000 (band f), and 20 000–23 000 (band g) were observed in the proteoliposomal fraction (Figure 2B) and in the total supernatant (Figure 2D) obtained from incubation of ghost membranes with protein-free liposomes. Of the three polypeptide components, the 42 000–47 000 components (band e) were not observed in the proteoliposomal fraction (Figure 2A) or in the total supernatant sample (Figure 2C) obtained from incubation of erythrocyte ghosts with γ-GTase-incorporated proteoliposomes. The two extracted species (components b, i.e., 90 000, and e, i.e., 42 000–47 000) apparently correspond to the previously identified protein components of erythrocyte membranes ($M_r$ 90 000–93 000, either acetylcholinesterase or band 3, and $M_r$ 45 000 comigrating with band 5) while the identities of the other extracted components are not yet known. Since acetylcholinesterase activity is observed in supernatant following incubation of ghost membrane with either γ-GTase-containing proteoliposomes or protein-free liposomes, the 90 K polypeptide is presumably acetylcholinesterase.

These results show both qualitative and quantitative differences in the erythrocyte membrane proteins extracted as well as association with phospholipid vesicles when proteoliposomes compared to liposomes are incubated with ghost membranes. This indicates that the presence of protein in liposomes affects the extraction of membrane proteins from

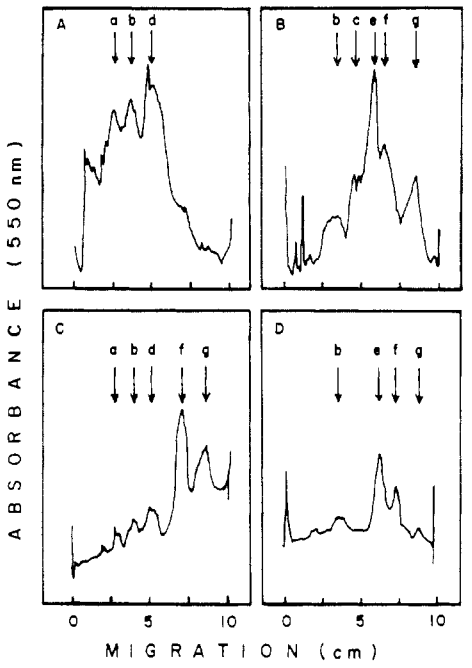


FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of proteins in supernatant fraction. DPPC liposomes or proteoliposomes (1 mL, 4–8 μmol of phospholipid) were incubated with erythrocyte ghost suspension (2 mL, 3 mg of protein) at 37 °C for 12 h. After incubation, supernatant was separated from ghost membranes, and a 2-mL sample was passed through a Sepharose 4B column to obtain the proteoliposomal fraction at the void volume, as described under Materials and Methods. $NaDodSO_4$ gel electrophoresis of total supernatant and proteoliposomal fractions was carried out by using 5.6% polyacrylamide gels. Gels were stained with Coomassie blue and scanned at 552 nm. $NaDodSO_4$–polyacrylamide gel electrophoresis standard high molecular weight markers from Bio-Rad were run simultaneously. Densitometer scans of $NaDodSO_4$–polyacrylamide gels of (A) proteoliposome (void volume) fraction of supernatant sample obtained from incubation of ghosts with γ-GTase incorporated liposomes, (B) proteoliposome (void volume) fraction after ghosts were incubated with liposomes without γ-GTase, (C) total supernatant sample obtained after incubation of ghost membranes with γ-GTase liposomes, and (D) total supernatant sample after incubation of ghost membranes with liposomes without γ-GTase. Arrows indicate positions of major protein species with molecular weights (a) 100 000–105 000, (b) 90 000–93 000, (c) 80 000–83 000, (d) 59 000–63 000, (e) 42 000–47 000, (f) 30 000–35 000, (g) 20 000–23 000.

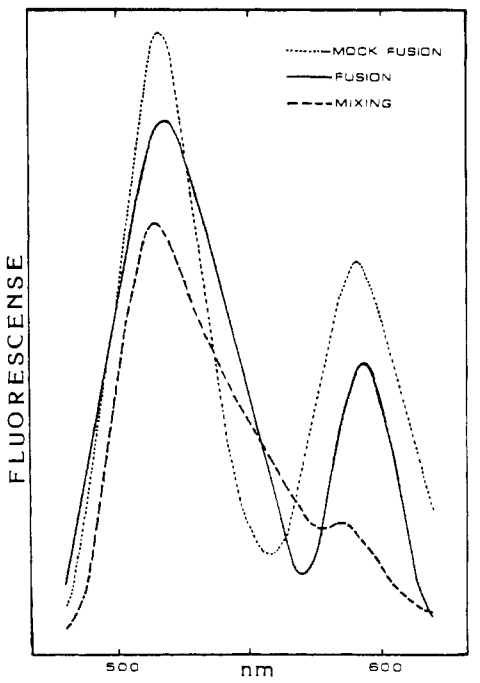


FIGURE 3: Fluorescence emission spectra in ghost membranes. DPPC proteoliposomes containing rhodamine probe (1.7 μmol) and erythrocyte ghost membranes containing fluorescein probe (2.4 μmol) were prepared as described under Materials and Methods. These probe-containing proteoliposomes and ghost membrane were mixed and incubated for 12 h at 37 °C and washed with buffer, and the ghost pellet thus obtained was applied on 8 mL of 4% dextran T-70 gradient to separate sealed from unsealed vesicles. Sealed vesicles were washed twice and used for measurement of emission spectrum as indicated under Materials and Methods. In the mock fusion experiment rhodamine probe and fluorescein probes were incorporated into ghost membrane. Excitation was at 460 nm.

the erythrocyte membranes.

*Orientation of γ-Glutamyl Transpeptidase Transferred into Erythrocyte Membranes.* The orientation of γ-glutamyl transpeptidase in implanted erythrocyte membrane was ascertained by using $^{125}I$-labeled γ-glutamyl transpeptidase. Incubation of DPPC liposomes containing $^{125}I$-labeled γ-GTase with erythrocyte ghost membrane resulted in the transfer of radiolabel into the erythrocyte membrane. Treatment of these membranes with papain resulted in 46–50% decrease in membrane-associated radioactivity (data not shown). Addition of 0.05–0.1% Triton X-100, which presumably makes the membrane leaky, in the presence of papain did not enhance the cleavage of radioactivity associated with the erythrocyte membrane. Therefore, the remaining radioactivity associated with membrane could be due to $^{125}I$-labeled tyrosine residues buried in the lipid bilayer which are not accessible to cleavage by papain treatment. These results indicate that the majority of the enzyme is oriented right side out since there was no difference in the release of radioactivity in the presence and absence of Triton X-100 upon papain treatment. Hughey et al. (1979) have shown that rat kidney Triton purified γ-glutamyl transpeptidase when incorporated into lecithin vesicles is 95% oriented right side out. Electron microscopic observations of the γ-GTase implanted ghosts revealed the absence of vesicles adhering on the surface of the ghost membrane (data not shown). In our earlier studies the association/implantation of γ-glutamyl transpeptidase into the erythrocyte membrane was demonstrated by the addition of antibody to γ-GTase followed by incubation with $^{125}I$-labeled protein A (Kalra et al., 1981).

*Monitoring the Proteoliposome–Erythrocyte Ghost Interaction Using Fluorescence Energy-Transfer Assay.* Studies were undertaken to determine whether transfer of γ-glutamyl transpeptidase from DPPC liposomes to erythrocyte membrane upon incubation at 37 °C occurred as a result of adsorption, endocytosis, fusion, or some other mechanism. For this purpose octadecylrhodamine B, a hydrophobic probe, was incorporated into γ-glutamyl transpeptidase liposomes while the (dodecanoylamino)fluorescein probe was inserted into erythrocyte ghost membranes. The hydrocarbon chain of these molecules causes the probes to partition into membranes (Keller et al., 1977). As shown in Figure 3, when rhodamine-containing liposomes were mixed with erythrocyte ghosts containing fluorescein probe (mixing) and excited at 460 nm, a peak at 515 nm corresponding to the excitation peak of the fluorescein probe was observed. However, when both of these probes were incorporated into erythrocyte membranes (mock fusion), there was a marked enhancement in emission at 585 nm due to resonance energy transfer between the two neighboring probe molecules in the same membranes. When DPPC proteoliposomes containing rhodamine probe were incubated at 37 °C for 12 h with erythrocyte ghosts containing fluorescein probe, an enhancement of the rhodamine emission (585 nm) in the resulting spectrum was observed upon excitation at 460 nm,

the excitation peak of the fluorescein probe. Since the amount of resonance energy transfer is approximately proportional to the extent of probe intermixing, comparison of the ratio of fluorescence intensity at 585 to 515 nm in mock fusion and control suggests that 80–90% of proteoliposomal content was transferred into erythrocyte membrane interstices. This enhancement in fluorescence at 585 nm indicates that the two probes must be close together, within a distance of 50 Å. Since biological membranes are 60–90 Å in thickness, the possibility of adsorption of proteoliposomes to erythrocyte membranes contributing to enhancement of fluorescence is ruled out. These results indicate that the close proximity of probes occurs as a result of either fusion or intermixing of the lipid bilayers of erythrocyte membranes and proteoliposomes.

*Transfer of Proteoliposome Content into Erythrocyte Membranes.* Studies were carried out to determine whether transfer of γ-GTase from proteoliposomes into erythrocyte membrane occurred as a result of fusion or endocytosis. Sealed erythrocyte ghost membrane vesicles were incubated with [$^{14}$C]sucrose either entrapped in γ-GTase proteoliposomes or external to the vesicles. Incorporation of [$^{14}$C]sucrose into erythrocyte membrane was not enhanced when sucrose-containing proteoliposomes were used, as has been observed by Bouma et al. (1977) upon incubation of liposomes with erythrocytes. Thus fusion per se with mixing of proteoliposome contents with those of erythrocyte ghosts presumably may not have occurred.

*Uptake of Glucose and Amino Acids in the γ-Glutamyl Transpeptidase Implanted Erythrocyte Ghost Membranes.* Studies were carried out to determine whether transfer/implantation of γ-glutamyl transpeptidase protein in erythrocyte membrane caused the membranes to become leaky and thus affected the uptake of neutral small molecules. For this purpose γ-glutamyl transpeptidase implanted erythrocyte membranes were applied on a 4% dextran T-70 gradient. It was observed that about 60–70% of the membranes remained sealed when either proteoliposomes or protein-free liposomes were incubated with erythrocyte membranes. The remaining 30–40% of the ghost membranes became leaky during the incubation period and formed a pellet at the bottom of the gradient.

As shown in Figure 4, the sealed γ-GTase implanted membranes obtained from the dextran gradient exhibited the uptake of 2-deoxy-D-[$^{3}$H]glucose. The uptake of glucose in this system was sensitive to cytochalasin B (50 μM) as has been observed in control ghost membranes. Thus according to this criterion the γ-GTase implanted ghost membrane vesicles were not leaky and appeared to be sealed.

In our previous studies (Kalra et al., 1981) we observed that γ-glutamyl transpeptidase incorporated into erythrocytes exhibited the uptake of L-glutamate and L-alanine which were inhibited by transpeptidase inhibitors. We determined whether γ-GTase implanted ghost membranes which contained entrapped glutathione (1600 ± 180 nmol/mg of ghost protein) exhibited the uptake of these amino acids. The γ-glutamyl transpeptidase implanted erythrocyte ghost membrane vesicles showed the uptake of L-glutamate. This uptake of L-glutamate was not inhibited by L-serine (20 mM) alone but could be inhibited (60–70%) by L-serine (20 mM) in the presence of borate (20 mM), an inhibitor of γ-glutamyl transpeptidase activity as was previously observed in implanted erythrocytes (Kalra et al., 1981). The ghost membranes which were incubated with protein-free liposomes (not containing γ-glutamyl transpeptidase) showed approximately 40–50% of the uptake of L-glutamate observed with γ-glutamyl transpeptidase implanted ghost membranes. However, the uptake of L-glutamate in control ghosts was not inhibited by serine–borate to a significant extent (20–25%) (data not shown). Uptake of L-alanine in these γ-GTase implanted erythrocyte membrane vesicles was also inhibited (35–40%) by serine–borate (data not shown).

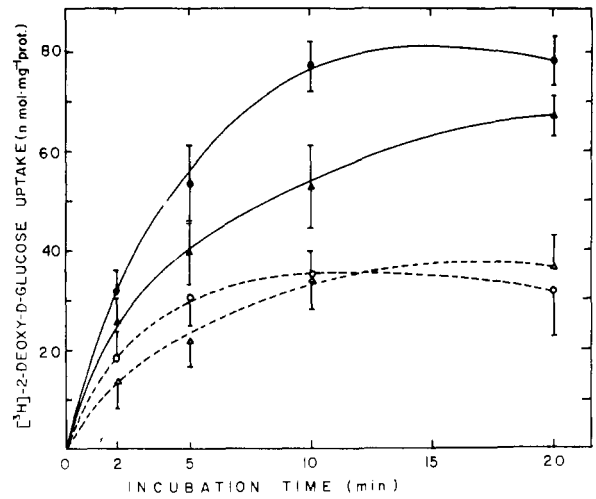


FIGURE 4: Uptake of 2-deoxy-D-[$^{3}$H]glucose in γ-glutamyl transpeptidase implanted erythrocyte ghosts. To 300 μL of sealed ghosts (300–400 μg of protein) obtained after dextran gradient centrifugation, 0.1 mL of 20 mM Tris–acetate buffer (pH 8.1) containing 140 mM NaCl and 100 μL of 2-deoxy-D-[$^{3}$H]glucose (40 mM, 1 μCi) were added and incubated at 30 °C. At the indicated time intervals, 100-μL aliquots were removed into 1 mL of ice-cold buffer, filtered rapidly on 0.45-μm membrane filter, and washed twice with 1 mL of cold buffer. Where indicated, cytochalasin B (50 μM) was preincubated at 30 °C with the ghost preparation for 20 min prior to the addition of radioactive solute. Data represent mean ± SEM for three separate determinations. Control sealed ghosts (●) without cytochalasin B and (○) with cytochalasin B; γ-GTase implanted sealed ghosts (▲) without cytochalasin B and (△) with cytochalasin B.

Since γ-glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl moiety of glutathione to the amino acid to yield γ-glutamyl amino acid (Meister & Tate, 1976), it was necessary to determine the formation of such a product during the uptake of amino acid in implanted ghost membranes. TLC analysis of the radioactive product of L-[$^{14}$C]glutamate uptake in γ-glutamyl transpeptidase implanted ghost membranes and control membranes in the presence and absence of serine–borate by using authentic standards. Most of the radioactivity comigrated with the γ-glutamylglutamate standard in γ-GTase implanted ghost membranes as compared to control membranes (incubated with protein-free liposomes). Moreover, in enzyme-implanted membranes, the radioactivity which comigrated with the γ-glutamylglutamate spot was reduced by 65–70% when serine–borate was added prior to the initiation of the uptake. These results demonstrate that the formation of γ-glutamylglutamate occurs during the transport of L-glutamate in γ-glutamyl transpeptidase implanted ghost membranes containing entrapped glutathione. The possibility that some glutathione may have leaked out during uptake studies and thus formed γ-glutamylglutamate outside the cell membrane prior to being transported as the γ-glutamylglutamate moiety cannot be ruled out by these studies.

It should be pointed out that the addition of the dipeptide, γ-glutamylglutamate (20 mM), in the incubation mixture prior to initiation of uptake of L-[$^{14}$C]glutamate in implanted erythrocyte membranes caused inhibition (55–65%) in the uptake of glutamate, while no inhibition in the uptake of glutamate was observed in the presence of another dipeptide, γ-glutamyl-L-leucine (data not shown). These results suggest

that the dipeptide γ-glutamylglutamate and glutamate share a common binding site in the γ-glutamyl transpeptidase moiety in the implanted erythrocyte membranes.

## Discussion

The present study shows that the detergent-solubilized and purified γ-glutamyl transpeptidase, from hog kidney cortex, could be functionally incorporated into human erythrocyte membranes by incubating ghosts with γ-glutamyl transpeptidase proteoliposomes. The extent of transfer of γ-glutamyl transpeptidase from proteoliposomes into erythrocyte membrane was dependent upon the fluidity of the donor proteoliposomes. A relatively greater amount of γ-GTase protein was transferred to erythrocyte ghost membranes when proteoliposomes were prepared from dipalmitoylphosphatidylcholine as compared to dimyristoylphsophatidylcholine, with intermediate values when binary mixtures of DPPC and DMPC were used. Proteoliposomes prepared from DPPC are less fluid at 37 °C as compared to erythrocyte ghost membranes as determined by the fluorescence polarization of the probe, diphenylhexatriene. Thus the extent of transfer of γ-GTase into ghost membranes was facilitated when the recipient erythrocyte membranes were in a more fluid state than the donor proteoliposomes at the temperature of incubation at 37 °C. These results are consistent with the finding of Cook et al. (1980), who observed that the transfer of intrinsic membrane proteins, specifically acetylcholinesterase, from erythrocytes to liposomes was enhanced severalfold when the recipient phospholipid vesicles were in an increased fluid state as compared to the fluidity of the lipid bilayer of donor erythrocyte membranes. Our studies further show that the nature of proteins extracted from the membranes varies depending upon whether the liposomes or the proteoliposomes are incubated with erythrocytes. In addition to selective transfer of proteins, Bouma et al. (1977) observed the removal of cholesterol and some phospholipids from erythrocyte membranes on incubation of human erythrocytes with DMPC vesicles. We also observed the removal of phospholipids, cholesterol, and some membrane proteins, mainly acetylcholinesterase, on incubation of erythrocyte ghosts with DPPC proteoliposomes. In these studies we observed the removal of 30–40% acetylcholinesterase, 35% cholesterol, 17% sphingomyelin, and 8–15% phosphatidylserine from ghost membranes. It appears that incubation of proteoliposomes with ghost membrane causes the selective removal of proteins and most of the lipids from the outer membrane monolayer. Studies of Verkleij et al. (1973) showed that most sphingomyelin and phosphatidylcholine are localized in the outer monolayer while phosphatidylserine and phosphatidylethanolamine are present on the inner membrane monolayer of erythrocyte ghosts. The removal of small amounts (8–15%) of phosphatidylserine from the inner monolayer may be because of the presence of inside out ghost membranes (5%) in our preparation as well as the generation of a small percentage (15–20%) of unsealed ghosts during long (12–15 h) incubation of sealed ghosts with proteoliposomes, thus exposing the inner monolayer to interactions with liposomes. Studies of Sandra & Pagano (1979) have also shown that interaction of liposomes with cells results in the transfer of vesicle lipids preferentially into the outer leaflet of the cell plasma membrane by one-for-one exchange mechanism.

In addition to fluidity, a number of other membrane properties, particularly net surface charge, may also function in bringing the two membranes in close juxtaposition, leading to protein transfer. In the present studies we observed that the extent of transfer of γ-GTase was enhanced when the proteoliposomes used were prepared from basic phospholipids as compared to neutral and acidic phospholipids. As expected, the positive charge of lipids will tend to bring the proteoliposomes in contact with negatively charged erythrocyte membranes. Thus the apparent transfer of γ-GTase into membranes, when basic phospholipids are used, may be simply due to electrostatic interactions leading to adsorption. It is pertinent to mention that Szoka et al. (1980), from their studies utilizing fluorescence recovery after the photobleaching technique, demonstrated that interaction of liposomes with cells, in the majority of cases, results in association of lipids with membrane as a result of adsorption. The possibility of adsorption contributing to the apparent transfer/implantation of γ-GTase observed in ghost membranes was ruled out as the major contributing factor since (a) implanted ghost membranes when repeatedly washed with high ionic strength buffer (0.5 M KCl) retained γ-GTase activity, (b) the transfer did not occur when the experiment was conducted at 4 °C, and (c) fluorescence resonance energy transfer was observed when ghost membranes containing donor fluorescent probe and proteoliposomes containing acceptor fluorescent probe molecules were incubated for 12 h.

The transfer of γ-GTase protein from proteoliposome into erythrocyte membrane observed in the present studies may thus occur as a result of vesicle–cell fusion, endocytosis, or collisional transfer mechanism. A fluorescence resonance energy-transfer assay was used to define and characterize further the mechanism of transfer of γ-GTase into erythrocyte membranes. An acceptor fluorescent probe, octadecylrhodamine B, was incorporated into γ-GTase proteoliposomes while a donor fluorescent probe, (dodecanoylamino)fluorescein, was incorporated into sealed erythrocyte vesicles. Because of the hydrophobic nature of probes, these molecules are partitioned into membranes (Keller et al., 1977). When these labeled proteoliposomes and erythrocyte ghost membranes were incubated at 37 °C for 12 h and washed to remove unbound probe, the ghost membranes exhibited an enhancement in the emission at 585 nm, due to the resonance energy transfer between two neighboring probe molecules (Haugland et al., 1969; Keller et al. 1977). This enhancement in fluorescence could only occur when the donor and acceptor probe molecules' proximity is less than 50 Å (Stryer & Haugland, 1967); thus intermixing or fusion of proteoliposomes with membrane vesicles must have occurred. However, in studies in which delivery of [$^{14}$C]sucrose, entrapped in proteoliposomes, into erythrocyte ghosts was quantified, the data revealed that lipid and γ-GTase transfer occurred without the mixing of cell and vesicle contents, as has previously been demonstrated for the interaction of liposomes with intact erythrocytes (Bouma et al., 1977). Thus fusion per se or endocytosis (Korn et al., 1974; Martin & MacDonald, 1976; Papahadjopoulos et al., 1979; Pagano & Weinstein, 1978; Deamer & Uster, 1980) as the mechanism for the transfer of γ-GTase protein seems unlikely.

The transfer of γ-GTase into ghost membrane did not require chemical fusogens, such as $Ca^{2+}$ or rare earth metals which have been shown to induce fusion of human erythrocytes (Baker & Kalra, 1979; Majumdar et al., 1980). In contrast the incorporation of cytochrome oxidase (Gad et al., 1979) and $Ca^{2+}$-ATPase (Eytan & Eytan, 1980) from lecithin-containing proteoliposomes into erythrocyte membranes has been shown to require the presence of high concentration of $Ca^{2+}$ (20 mM). Since the transfer of γ-GTase from proteoliposomes into erythrocyte membrane is a time-dependent process, not stimulated by the presence of chemical fusogen and facilitated by the fluid bilayer of the recipient membrane,

the transfer probably occurs by collisional transfer process (Enoch et al., 1977). Enoch et al. (1979) showed that cytochrome $b_5$ could be transferred between large and small single bilayer vesicles, but the transfer of cytochrome $b_5$ from microsomes to egg lecithin vesicles did not occur. It has been proposed (Enoch et al., 1979) that transfer of protein from one membrane to another will transpire only when protein is extrinsically or partially buried in the bilayer and not when it spans the bilayer.

It is not clear how general this type of intermembrane transfer of protein may be since γ-glutamyl transpeptidase which is transferred into recipient erythrocyte membrane has been observed to mediate the translocation of certain amino acids, e.g., L-glutamate and L-alanine, indicating that this enzyme must span the bilayer. The uptake of these amino acids into γ-glutamyl transpeptidase implanted erythrocyte ghost membrane vesicles was not inhibited by L-serine (20 mM) alone but could be inhibited by serine–borate, a specific inhibitor of transpeptidase activity (Revel & Ball, 1959; Meister & Tate, 1976). Furthermore, the accumulated product in the intravesicular volume of implanted ghosts during the uptake of L-glutamate was characterized as γ-glutamylglutamate, the product of transpeptidation reaction (Meister & Tate, 1976). If one considers that 40% of transpeptidase is oriented on the cytoplasmic side in implanted membranes and participates in the formation of γ-glutamylglutamate intravesicularly, then it is difficult to envision why L-glutamate and L-alanine uptake is specifically blocked by serine–borate, azaserine, and DON–maleate (Sikka & Kalra, 1980), the γ-glutamyl transpeptidase inhibitors (Tate & Meister, 1977). Results presented in the present studies thus show that γ-glutamyl transpeptidase can mediate the translocation of certain amino acids, as has been observed previously in proteoliposomal system (Sikka & Kalra, 1980) and in intact erythrocytes (Kalra et al., 1981). Moreover it was observed that dipeptide γ-glutamyl-L-glutmate but not γ-glutamyl-L-leucine inhibited the uptake of glutamate in γ-GTase implanted erythrocyte membranes. It thus appears that dipeptide γ-glutamyl-L-glutamate binds to the amino acid binding site on the transpeptidase moiety and thus may prevent the translocation of L-glutamate in these membranes.

It is pertinent to mention that recently, Ormstad et al. (1980) studied the uptake of methionine, glutamate, cysteine, and cystine in rat kidney cells and observed that uptake of these amino acids was inhibited 50% by serine–borate, the transpeptidase inhibitor. Moreover, a concomitant decrease in the levels of intracellular glutathione was observed under conditions of amino acid uptake, indicating that γ-glutamyl transpeptidase plays a role in glutathione-linked amino acid translocation. Our studies lend further support to the proposal (Meister & Tate, 1976) that γ-glutamyl transpeptidase plays a functional role in the translocation of amino acids and dipeptides. Further studies are being carried out to determine the specificity of uptake of amino acids and dipeptides which presumably utilize the γ-glutamyl transpeptidase pathway for uptake in artificial and natural membranes.

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References

Baker, R. F., & Kalra, V. K. (1979) *Biochem. Biophys. Res. Commun. 86*, 920–928.

Bouma, S. R., Drislane, F. W., & Huestis, W. H. (1977) *J. Biol. Chem. 252*, 6759–6763.

Cook, S. L., Bouma, S. R., & Huestis, W. H. (1980) *Biochemistry 19*, 4601–4607.

Deamer, D. W., & Uster, P. S. (1980) in *Introduction of Macromolecules into Viable Mammalian Cells* (Beserga, R., Croce, C., & Rovera, G., Eds.) pp 205–220, Alan R. Liss, Inc., New York.

Enoch, H. G., Fleming, P. J., & Strittmatter, P. (1977) *J. Biol. Chem. 252*, 5656–5660.

Enoch, H. G., Fleming, P. J., & Strittmatter, P. (1979) *J. Biol. Chem. 254*, 6483–6488.

Eytan, G. D., & Eytan, E. (1980) *J. Biol. Chem. 255*, 4992–4995.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Gad, A. E., Broza, R., & Eytan, G. D. (1979) *FEBS Lett. 102*, 230–234.

Haggerty, D. F., Kalra, V. K., Popjak, G., Reynolds, E. E., & Chiappelli, F. (1978) *Arch. Biochem. Biophys. 189*, 51–62.

Haugland, R. P., Yguerabide, J., & Stryer, L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 23–30.

Hissin, P. J., & Hilf, R. (1978) *Biochim. Biophys. Acta 508*, 401–412.

Hughey, R. P., Coyle, P. J., & Curthoys, N. P. (1979) *J. Biol. Chem. 254*, 1124–1128.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Kalra, V. K., Sikka, S. C., & Sethi, G. S. (1981) *J. Biol. Chem. 256*, 5567–5571.

Kant, J. A., & Steck, T. L. (1973) *J. Biol. Chem. 248*, 8457–8464.

Kates, M. (1975) in *Laboratory Techniques in Biochemistry and Molecular Biology-Techniques of Lipidology* (Work, T. S., & Work, E., Eds.) pp 347–392, American Elsevier, New York.

Keller, P. M., Person, S., & Snipes, W. (1977) *J. Cell Sci. 28*, 167–177.

Kimelberg, H. K., & Mayhew, E. (1978) *CRC Crit. Rev. Toxicol. 6*, 25–79.

Korn, E. D., Bowers, B., Batzri, S., Simmons, S. R., & Victoria, E. J. (1974) *J. Supramol. Struct. 2*, 517–528.

Lucy, J. A. (1977) *Trends Biochem. Sci. (Pers. Ed.) 2*, 17–20.

Majumdar, S., Baker, R. F., & Kalra, V. K. (1980) *Biochim. Biophys. Acta 598*, 411–416.

Martin, F. J., & MacDonald, R. C. (1976) *J. Cell Biol. 70*, 506–526.

Meister, A., & Tate, S. S. (1976) *Annu. Rev. Biochem. 45*, 559–604.

Orlowski, M., & Meister, A. (1965) *J. Biol. Chem. 240*, 338–347.

Ormstad, K., Jones, D. P., & Orrenius, S. (1980) *J. Biol. Chem. 255*, 175–181.

Pagano, R. E., & Weinstein, J. N. (1979) *Annu. Rev. Biophys. Bioeng. 7*, 435–468.

Papahadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 10*, 1–121.

Revel, J. P., & Ball, E. G. (1959) *J. Biol. Chem. 234*, 577–582.

Roseman, M. A., Holloway, P. W., Calabro, M. A., & Thompson, T. E. (1977) *J. Biol. Chem. 252*, 4842–4849.

Sandra, A., & Pagano, R. E. (1979) *J. Biol. Chem. 254*, 2244–2249.

Shinitzky, M., & Inbar, M. (1974) *J. Mol. Biol. 85*, 603–615.

Sikka, S. C., & Kalra, V. K. (1980) *J. Biol. Chem. 255*, 4399–4402.

Steck, T. L. (1974) *Methods Membr. Biol. 2*, 245–281.
Stryer, L., & Haugland, R. A. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 719–726.
Szoka, F., Jacobson, K., Derzko, Z., & Papahadjopoulos, D. (1980) *Biochim. Biophys. Acta 600*, 1–18.
Tate, S. S., & Meister, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 931–935.
Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., & Van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.
Wagner, H., Lissau, A., Holzl, J., & Horhammer, L. (1962) *J. Lipid Res. 3*, 177–180.
Zlatkis, A., Zak, B., & Boyle, A. J. (1953) *J. Lab. Clin. Med. 41*, 486–492.

# Rotational Mobility of an Erythrocyte Membrane Integral Protein Band 3 in Dimyristoylphosphatidylcholine Reconstituted Vesicles and Effect of Binding of Cytoskeletal Peripheral Proteins†

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ABSTRACT: Band 3 protein was isolated from human erythrocyte membranes, purified, and reconstituted into a well-defined phospholipid bilayer matrix (dimyristoylphosphatidylcholine). The preparation yielded uniform single-bilayered vesicles of the diameter 40–80 nm. The rotational motion of band 3 was studied by saturation transfer electron spin resonance (ESR) spectroscopy of covalently attached maleimide spin-labels. The rotational mobility changed in response to the host lipid phase transition. The rotational correlation time was in a range from 73 (37 °C) to 94 μs (26 °C) in the fluid phase and from 240 (15 °C) to 420 μs (5 °C) in the solid phase. The motion was analyzed based on the anisotropic rotation of band 3 in the reconstituted vesicles. To obtain information on the rotational diffusion constant around the axis parallel to the membrane normal, we made an attempt to measure the angle between the spin-label magnetic axis and the membrane normal. The result gave $3.9 \times 10^4$ $s^{-1}$ at 37 °C as a rough estimate for the diffusion constant. This is compatible to anisotropic rotation of a cylinder of radius 3.3 nm in a two-dimensional matrix with inner viscosity 2 P and inner thickness 4 nm. The cytoskeletal peripheral proteins caused a definite increase in the rotational correlation time (from 73 to 180 μs at 37 °C, for example). The restriction of the rotational mobility was shown to be due to the ankyrin-linked interaction between band 3 and spectrin–actin–band 4.1 proteins in the reconstituted membranes.

Transmembrane control is a key mechanism in cellular response to outer signals. The molecular tool for the control must include interactions of the cell membrane integral protein with the peripheral proteins and the cytoplasmic fibrous structures. Human erythrocytes provide a simple model system for studying the interactions between integral and peripheral proteins since they lack cytoplasmic fibrous structures. Another advantage in using the erythrocytes is that the molecular components are exceptionally well characterized (Steck, 1974). The anchoring role of band 2.1 has recently been elucidated by Bennett & Stenbuck (1979, 1980) and Tyler et al. (1979, 1980). This protein anchors the integral protein band 3 and also binds to spectrin on the other hand, thus playing a key role in connecting the integral protein to the cytoskeletal network structure consisting of spectrin, actin, and band 4.1 (Lux, 1979).

Morphological studies of erythrocytes have been carried out that give evidence for linking of the integral proteins to the peripheral cytoskeletal structure (Nicolson & Painter, 1973; Pinto da Silva & Nicolson, 1974; Elgsaeter & Branton, 1974). We are studying this subject by measuring dynamic properties of the integral protein, rotational mobility of band 3 with saturation transfer ESR[1] spectroscopy of covalently attached spin-labels (Thomas et al., 1976), and lateral mobility with photobleaching recovery measurements of covalently attached fluorescent probes (Koppel et al., 1976). We are studying the protein–protein interactions by measuring the effect of binding of the cytoskeletal peripheral proteins on mobility. In this paper, we describe the results of a rotational study on a reconstituted membrane system, purified band 3 in dimyristoylphosphatidylcholine (DMPC). The results of a lateral study appear in an accompanying paper (Chang et al., 1981). In this series of papers, we have been able to make a detailed analysis of the rotational and lateral motions of an integral protein in a well-defined pure lipid matrix and also able to show a definite interaction between the integral protein and the cytoskeletal component proteins for the first time using such a simplified system.

Similar studies have been carried out with erythrocyte ghost membranes. Cherry and his collaborators investigated the rotational motion of band 3 by using flash-induced dichroism of covalently attached eosin probes. They concluded from the spectrin-depletion experiments that the spectrin–actin network has little or no effect on band 3 rotation in the ghost membranes, throwing doubt on the possibility of direct physical linking between them (Cherry et al., 1976; Nigg & Cherry, 1979). In a recent paper, however, they have reported restriction of the rotational mobility by the cytoskeletal components including band 2.1 does occur (Nigg & Cherry, 1980). Lateral diffusion of band 3 in erythrocyte membranes has been studied, and restriction of the mobility by the spectrin–actin

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DTAC, dodecyltrimethylammonium chloride; EDTA, ethylenediaminetetraacetic acid; MSL, a short-chain maleimide spin-label, 3-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy; (1,14)MSL, a long-chain maleimide spin-label, ester of 16-doxylstearic acid and *N*-(2-hydroxyethyl)maleimide; 5-doxylstearic acid, *N*-oxy-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; ESR, electron spin resonance.

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