A Procedure for Membrane–Protein Reconstitution and the Functional Reconstitution of the Anion Transport System of the Human-Erythrocyte Membrane

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A short procedure for the isolation of band-3 protein, the protein responsible for anion exchange in erythrocytes, in a reasonable degree of purity was developed. Using this protein preparation and a novel procedure for membrane–protein reconstitution, vesicles displaying the basic features of the anion-exchange system of the erythrocyte were obtained. The reconstitution procedure is based on slow direct removal of Triton X-100 from aqueous lipid/detergent solutions. According to the composition of the reconstitution medium, either small single-walled or large multi-walled vesicles are obtained. The procedure conserves protein properties well, as is revealed by the similarity of the rates of SO_4^{2-} exchange in erythrocytes and reconstituted vesicles when corrected for the relevant volumes. A number of functional features of the exchange system were studied and compared with those of the native membrane.

Incorporation of isolated cell membrane components into lipid bilayers of controlled chemical composition is a rapidly developing tool in membrane research (Hilden *et al.*, 1974; Segrest *et al.*, 1974; Warren *et al.*, 1974; Triss *et al.*, 1977).

Intrinsic proteins, some of the most important membrane components, can be extracted by the use of non-ionic detergents (Yu *et al.*, 1973; Clarke, 1975; Helenius & Simons, 1975). Specific proteins can sometimes be extracted (Yu *et al.*, 1973), but when this is not possible, a further purification step is needed. Most of the current purification techniques (Vesterberg, 1971; Yu & Steck, 1975) require the presence of a non-ionic detergent throughout the procedure to keep the intrinsic hydrophobic proteins in solution. Thus the final state of a purified intrinsic membrane protein would frequently be in a non-ionic detergent solution. Reconstitution of such a protein into a lipid bilayer by the well-established cholate–dialysis technique requires additional steps of detergent exchange (Yu & Branton, 1976). The present report describes the reconstitution of closed phospholipid vesicles by direct removal of Triton X-100 from lipid/detergent solutions and the functional incorporation of the 95 000-mol.wt. protein

(band 3) of the human erythrocyte membrane into these vesicles.

In the last few years, several attempts to identify the membrane components responsible for anion exchange have led to the suggestion that the 95 000-mol.wt. protein, the major polypeptide component of the erythrocyte membrane, known also as band 3, is involved in anion permeability (Cabantchik & Rothstein, 1972, 1974; Rothstein *et al.*, 1975). Studies with vesicles isolated from the native membrane bearing the 95 000-mol.wt. protein as the only relevant component (Wolosin *et al.*, 1977) showed that these vesicles display various characteristic features of anion translocation resembling those of the native system. On the basis of these characteristics, the anion-transport system of the erythrocyte has been attributed to this protein. In the present work, the preservation of anion-transport capacity and certain characteristic features of the native system in reconstituted vesicles carrying the 95 000-mol.wt. protein are shown.

Materials and Methods

Materials

$^{35}\text{SO}_4^{2-}$ (carrier-free) was purchased from the Nuclear Research Centre, Negev, Israel. Triton X-100 (purified) was from Koch-Light. Bio-Beads SM-2 were from Bio-Rad and Sephadex G-75 was

Abbreviation used: SDS, sodium dodecyl sulphate.

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from Pharmacia. CF-50 ultrafiltration cones were from Amicon. DL-Phosphatidylcholine-(1-octadec-9-enyl-2-hexadecyl ether) [2-O-hexadecyl-1(3)-O-(9-octadecenyl)-sn-glycero-3(1)-phosphocholine] was from Calbiochem, and egg lecithin, cholesterol and phosphatidylserine (bovine brain) were from Sigma. Persantine was a gift from Geigy. 4,4'-Di-isothiocyano-2,2'-stilbenedisulphonate was a gift from Dr. Z. I. Cabantchik, Hebrew University of Jerusalem. All other reagents were of analytical grade. Cholesterol was recrystallized twice from boiling methanol before use.

Methods

Protein isolation. Slightly pink erythrocyte 'ghosts' were prepared from outdated blood as described previously (Wolosin *et al.*, 1977). The 'ghosts' were resuspended in 5 vol. of iso-osmotic buffered saline solution (0.89% NaCl, 20 mM-sodium phosphate, pH 8.0), spun down, extracted for 20 min with 6 vol. of 0.05% Triton X-100 in 1:5 water-diluted buffered saline solution and washed once more with 5 vol. of the diluted buffered saline solution to remove free protein and detergent. The washed membranes were extracted for 20 min with 5 vol. of 0.5% Triton X-100 in 1:5 diluted buffered saline solution; the residual membranes were centrifuged at 40 000g for 30 min in order to obtain a hard pellet, and the supernatant, containing the band-3 protein, was collected. All steps were carried out at 0°C in solutions de-oxygenated by bubbling N₂ prior to use. The extraction solutions contained 0.2 mM-dithiothreitol. Standard centrifugations were done at 20 000g for 8 min at 0–2°C. The extraction solution was exchanged for the reconstitution solution by using ultrafiltration membrane cones CF-50 (mol. wt. cut-off 50 000); the Triton X-100 content was then adjusted to 0.5% and the extract was frozen in liquid N₂ and stored at –20°C.

Vesicle reconstitution. Phospholipid (20 mg) was dissolved in 2 ml of chloroform in a vacuum flask. The solvent was removed under vacuum and the lipid was resuspended in 12 ml of reconstitution solution (100 mM-mannitol, 60 mM-Na₂SO₄, 0.1 mM-EDTA, 0.2 mM-Na₂N₃, 0.5% Triton X-100 and up to 2 mg of protein). The cloudy solution was stirred at room temperature until clear. Then 300 mg (dry wt.) of Bio-Beads SM-2, prepared as described by Holloway (1973), and 0.01% dithiothreitol were added, the container flask was closed under N₂ and vigorously shaken overnight at 4°C. The beads were removed by filtration in a glass sinter; a second batch of 300 mg of Bio-Beads was added, which was again replaced after 8 h by 1 g of Bio-Beads which was left for a further 24 h. The beads were filtered off and the vesicle suspension was centrifuged at 2000g for 20 min in order to remove any impurities present (aggregated material or small residues of the beads).

Finally the vesicles were spun down at 100 000g, resuspended in reconstitution solution or a solution iso-osmotic to it, frozen in liquid N₂ and stored at –20°C.

Electron microscopy. Negative staining with phosphotungstic acid was performed as described by Munn (1974). Freeze-fracture of samples was performed in a Balzers instrument (Fisher & Branton, 1974). Grids were examined in a Philips 300 electron microscope at 60 or 80 kV.

Light microscopy. Reconstituted vesicles were made visible in 0.1 mM-1-anilinonaphthalene-8-sulphonic acid as very small fluorescent spots undetectable by phase-contrast microscopy. The presence of amorphous material in the preparation was monitored by phase-contrast microscopy.

Flux measurements. For each experiment a convenient amount of vesicles was thawed and incubated for 1 h at 37°C. All fluxes were measured under equilibrium exchange conditions by gel filtration on Sephadex G-75 columns (2 ml). A detailed description of this method has been given elsewhere (Cabantchik *et al.*, 1977; Wolosin *et al.*, 1977). Vesicles were eluted from the columns by addition of a precalibrated volume of eluent, normally 620 µl, directly into scintillation vials. Column pre-equilibration and elution were done with the incubation solution of the particular assay. To each eluted sample, 4 ml of scintillant [666 ml of toluene, 333 ml of Triton X-100, 0.1 g of 1,4-bis-(4-methyl-5 phenyloxazol-2-yl)benzene and 5.5 g of 2,5-diphenyloxazole] was added and the radioactivity was counted in a Packard Tri-Carb liquid-scintillation spectrometer. Quenching was monitored using the spectrometer external source. As the volume of aqueous solution of the samples was constant in a given experiment (the sample plus eluent volumes), no differences in quenching were observed and the c.p.m. values were thus used as a direct measure of the radioactivity in the vesicles. For each determination, 10–16 portions were sampled at convenient times and the results were plotted as radioactivity (c.p.m.) versus time. The best line was fitted visually and from it, the half time for radioactivity equilibration (t_4) was obtained. Throughout the text the apparent rate constant of exchange (k_{app}) refers to $(\ln 2)/t_4$. No statistical analysis of results was attempted, but the uncertainty in t_4 was usually not more than $\pm 10\%$ and never exceeded $\pm 20\%$. Treatments with 4,4'-diisothiocyano-2,2'-stilbenedisulphonate of either erythrocytes or reconstituted vesicles were performed according to Cabantchik & Rothstein (1974).

Analytical methods. Lipids were extracted by the method of Folch *et al.* (1957). Protein (Wang & Smith, 1975), sialic acid (Warren, 1959) and lipid phosphorus (Chen *et al.*, 1965) were assayed as described. Triton X-100 was measured spectro-

scopically at 279nm after dilution in 1% SDS and the protein contribution was subtracted (Clarke, 1975). Polyacrylamide-gel electrophoresis in 5% acrylamide/0.2% SDS was performed as described by Fairbanks *et al.* (1971). Densitometric scans were performed at 560nm in a Gilford 240 apparatus.

Results

Protein composition

Extraction with low concentrations of Triton X-100 removes from the membrane most of the sialoglycoproteins (Yu *et al.*, 1973), but no appreciable amounts of band 3 are extracted (Yu *et al.*, 1973; Wolosin *et al.*, 1977). Extraction with 0.5% detergent solubilized band 3, small amounts of other proteins stained by Coomassie Blue and previously unextracted sialoglycoproteins (Fig. 1). Densitometric scans of SDS/polyacrylamide gels of the membranes before and after extraction with 0.5%

Triton X-100, with the use of band 5 as an internal standard, showed that the extract contains 70% of the total band 3 in the membrane. In order to determine the percentage of sialoglycoproteins present in both extracts (0.05% and 0.5% Triton X-100), the sialic acid content of the membrane residues before and after each extraction was assayed. Before the assay, the membranes were extracted twice with 2 vol. of chloroform/methanol (2:1, v/v) and thus only protein-associated sialic acid was measured. The 0.05% Triton X-100 extract contained 80% of the sialoglycoproteins and the remaining 20% was present in the 0.5% extract. The band-3/sialoglycoprotein molar ratio in the extract is therefore 3.5 times higher than the molar ratio in the erythrocyte membrane, in which it is 2:1 (Steck, 1974). The reconstituted vesicles had, however, a very low sialic acid content (10–15nmol/mg of protein) compared with the content in the 0.5% extract (60–80nmol/mg of protein), and no periodate/Schiff-base-positive bands could be detected

Fig. 1. SDS/polyacrylamide-gel electrophoresis of material obtained at various steps in the purification of band 3. Materials were solubilized, electrophoresed and stained with Coomassie Blue as described by Fairbanks *et al.* (1971). From left to right: (i) pale-pink 'ghosts'; (ii) membranes treated with iso-osmotic salt solution; (iii) membrane residue after extraction with 0.5% Triton X-100 solution; (iv) membrane residue after extraction with 0.5% Triton X-100 solution; (v) 0.05% Triton X-100 extract; (vi) 0.5% X-100 extract; (vii) 0.5% extract containing considerable amounts of band 4.2; (viii) reconstituted membranes derived from this extraction. The amount of protein loaded on gels (i)–(iv) was derived from 25 μ l of initial pink 'ghosts', and that loaded on gels (v)–(viii) was from 12 μ l of initial pink 'ghosts'.

after SDS/polyacrylamide-gel electrophoresis of the vesicle material. These two results indicate that the sialoglycoproteins were not incorporated into the reconstituted membrane and therefore the 0.5% Triton extract was considered satisfactory for studies of band-3 reconstitution and was used without further purification. The presence of dithiothreitol throughout the extraction steps seemed to be essential in order to avoid considerable co-extraction of band 4.2. When co-extracted, gel electrophoresis of the material after reconstitution showed that band-4.2 protein was incorporated into the membrane (Fig. 1). The extract had a 1:1.2 (w/w) protein/phospholipid ratio (assuming an average mol.wt. of 800 for the phospholipids).

Reconstitution step

Fig. 2 shows the changes in phospholipid, protein and detergent content during the reconstitution procedure and after the final centrifugation of the vesicles. The continuous changes in absorbance of the reconstitution solution are also shown. The S-shaped increase in turbidity, as seen by inspection of the reconstitution solution by fluorescence light microscopy, represents the transition from the micellar to the vesicular state. The low bead/detergent ratio used in the first step is essential both to

avoid phospholipid losses by adsorption to the beads during the micellar stage and to allow slow detergent removal during the transition from this stage to the lamellar stage. The use of higher bead/detergent ratios induced the appearance of amorphous material, easily observed with the phase-contrast microscope, probably as a result of fast detergent removal and/or interaction of lipids with the surface of the beads.

To select the proper initial bead/detergent ratio, binding studies were performed. In the absence of lipid and protein and at 1% detergent, 1 mg of dry beads binds up to 0.35 mg of detergent. Under the conditions of reconstitution, 1 mg of beads removes 0.2–0.25 mg of detergent, provided that the detergent/phospholipid ratio is greater than 1 (in this experiment, the detergent concentration remaining in solution was measured after 24, 48 and 72 h, the maximal binding being achieved in the period 24–48 h).

Thus a 5:1 bead/detergent ratio in the first step provides a low removal rate, while, at the same time, very few hydrophobic sites in the beads remain free to interact with non-polar domains of phospholipids or protein. Once vesicles are formed, the remaining detergent is removed by partition between the vesicles and freshly added beads.

When protein was omitted from the reconstitution mixture, lower amounts of beads were needed at the final stage to obtain almost complete removal of the detergent. Such a result may indicate that the strongest binding sites for the detergent in the vesicles reside in the protein. Studies on the binding of Triton X-100 to a number of proteins (Clarke, 1975) have, in fact, shown a remarkably high affinity between this detergent and the band-3 protein.

The structural and most of the functional studies were performed by using a 24:1 (w/w) mixture of DL-phosphatidylcholine-(1-octadec-9-enyl-2-hexadecyl ether) [2-O-hexadecyl-1(3)-O-(9-octadecenyl)-sn-glycero-3(1)-phosphocholine] and phosphatidylserine. This structural analogue of phosphatidylcholine in which the ester linkages are replaced by ethers overcomes the hazard of lysophosphatidylcholine formation during the slow vesicle-reconstitution process. Functional similarities and differences between ether and ester phosphatidylcholine have been studied and discussed by Schwarz & Paltauf (1977). The similarity of behaviour between the two phosphatidylcholines throughout the reconstitution steps was determined by measuring phospholipid and detergent content and the turbidity of the suspension by using either the synthetic ether phosphatidylcholine or egg lecithin. The only difference found was a 10% higher value for the turbidity of the egg lecithin suspension once the vesicles were formed.

Nevertheless, the ether phospholipid could not be

Fig. 2. Changes in lipid phosphorus, protein and Triton X-100 at times of bead replacement and the continuous change in the absorbance of the reconstitution solution for a representative reconstitution experiment in the presence of EDTA (0.1 mM)

To monitor the absorbance changes, the reconstitution solution was pumped through a flow cell in a closed circuit. The abscissa outlines the different treatments. Details are given in the text. The ordinate gives the percentage of the maximal recorded value, which for lipid phosphorus (▲) was 22 mg, for protein (●) was 2 mg, for Triton X-100 (○) was 60 mg and for absorbance (---) was 1.1.

EXPLANATION OF PLATE 1

Structural features of reconstituted vesicles

Negative stainings (*b* and *d*) and freeze-fracture replicas (*a* and *c*) of vesicles obtained in the presence of EDTA (0.1 mM) or Mg^{2+} (2 mM) respectively. The bars denote 0.2 μm . The freeze fractures were etched for 1 min. Vesicles were reconstituted at a 10:1 phospholipid/protein ratio. (*e*) is a 5-fold magnification of (*c*). Intramembranous particles are visible in the convex fractured face as well as in the concentrically arranged membranes. In the latter case, the mild etching performed allows a cross sectional view of the membrane.

used in experiments that included cholesterol. This phospholipid interacts only weakly with cholesterol (Schwarz & Paltauf, 1977), and milky suspensions of phospholipid/cholesterol mixtures were formed in Triton X-100. The suspensions did not clarify even if the detergent concentration was raised to 5%. When egg lecithin was used, clear solutions were readily obtained at the normal detergent concentration provided that the molar phospholipid/cholesterol ratio was greater than 2.

Structural characterization of reconstituted vesicles

As revealed by negative staining (Plate 1b) the reconstituted vesicles are more or less uniform in diameter (0.1–0.2 μm).

Replacement of the EDTA by 2 mM-Mg²⁺ or -Mn²⁺ in the reconstitution solution resulted in the formation of large and multi-walled structures (Plate 1c and d), especially in the case of Mn²⁺. Some of these multi-walled structures were cut (Plate 1c) rather than fractured during the freeze-fracture procedure. Sometimes two superimposed membrane fracture faces were visible (Plate 1c upper side). Freeze fractures of the vesicles (Plate 1a and c) show the classical 8.5-nm intramembrane particles observed in the native membrane (Pinto da Silva *et al.*, 1971). The presence of cholesterol in the reconstitution medium resulted in an increase in the trapped volume per mg of protein (see Table 2) for vesicles bearing the same protein/phospholipid ratio.

Functional assay

Functional studies were performed by using single-walled vesicles. At first, parallel reconstitutions using extracts from either untreated cells or cells that had been pretreated with 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate were performed. Treatment with 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate, an irreversible specific inhibitor of anion transport (Cabantchik & Rothstein, 1974), had no influence upon the amounts and pattern of protein extraction (Rothstein *et al.*, 1975), so that comparison of anion flux in both preparations provides an accurate assessment of the specific permeability properties conferred by the anion-transport system to the reconstituted vesicles.

The transport system provides a common pathway for both uni- and bi-valent anions (Jennings, 1976), but owing to its high turnover capacity for small univalent anions (Brahm, 1976), only species slowly translocated, such as sulphate, are useful for kinetic studies in small vesicles. A comparative plot of the radioactivity (³⁵SO₄²⁻) uptake under equilibrium exchange conditions for the parallel reconstitutions (Fig. 3) shows that the preparation derived from untreated erythrocytes

Fig. 3. Time course of ³⁵SO₄²⁻ influx under exchange conditions for vesicles obtained from normal and 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate-pretreated cells

The vesicles has a 1:10 (w/w) protein/phospholipid ratio. The suspension was incubated at 25°C in 100 mM-mannitol/60 mM-Na₂SO₄/0.2 mM-NaN₃/0.1 mM-EDTA/10 mM-sodium phosphate, pH 6.3, and sampled (50 μl) at different intervals. Protein concentration was 50 $\mu\text{g}/\text{ml}$. Radioactivity of the incubation solutions was 2.16×10^8 c.p.m./ml; ●, vesicles from control cells (time in min); ○, vesicles from cells pretreated with 20 μM -4,4'-di-isothiocyano-2,2'-stilbenedisulphonate at 0°C during 20 min in buffered saline soln., pH 7.0 (time in h). Complete inhibition of flux in the treated erythrocytes was verified as described (Cabantchik & Rothstein, 1972) before proceeding to the preparation of 'ghosts'.

reached equilibrium within minutes, whereas the preparation derived from pretreated erythrocytes required hours. The temperature-dependence of sulphate exchange was studied in efflux experiments on two independent preparations (Fig. 4). The preparations differed 3-fold in absolute transport rates, but showed similar activation energies of about 84 kJ/mol (20 kcal/mol). Fig. 4 exemplifies, also, the reproducibility of the method of assay: the same values for k_{app} were obtained for the same vesicle preparation in independent assays.

Univalent-anion translocation is too fast to be measured in the vesicles, but their competitive-inhibition effect on sulphate flux (Gruber & Deuticke, 1973; Schnell *et al.*, 1977) can be used to investigate the affinity properties of the reconstituted transport system. Therefore the influence of univalent anions or sulphate itself in the rate of equilibration of ³⁵SO₄²⁻ across the membrane was assessed. Results of those experiments are presented in Table 1. The effect of non-competitive inhibitors was also tested in two preparations. 4,4'-Di-isothiocyano-2,2'-stilbenedisulphonate applied at a high

Fig. 4. Temperature-dependence of $^{35}\text{SO}_4^{2-}$ exchange efflux

Vesicles loaded with $^{35}\text{SO}_4^{2-}$ by preincubation for 1 h at 25°C were freed of extracellular medium by gel filtration and collected at 0°C. Batches from this vesicle suspension were brought quickly to the desired temperature by admixing with a fixed volume of solution; then portions were sampled at convenient time intervals. The same buffer as in Fig. 3 was used throughout the experiment. The logarithm of the rate constant ($k_{\text{app.}}$) for every temperature is plotted versus the reciprocal of the absolute temperature. The lower line represents two independent experiments (●, ○) for the same preparation. The upper line represents a third experiment with a different preparation. The lines were fitted visually. The activation energies corresponding to their slopes are indicated.

Fig. 5. pH profile for SO_4^{2-} exchange in the reconstituted system

Efflux and influx experiments were performed on various preparations. Unbuffered vesicle suspensions (solution as described in the Materials and Methods section) were brought to the desired pH by addition of a concentrated sodium phosphate buffer to a final concentration of 10 mM. After 1 h of incubation fluxes were assayed. An assay at pH 6.3 was always performed and the results are presented as the percentage of the rate at this pH. For comparative purposes a pH profile obtained with erythrocytes is superimposed (----; Schowch *et al.*, 1974). ●, ○, ▲, Influx experiments; △, efflux experiments.

Table 1. Rates of SO_4^{2-} exchange (a) in the presence of univalent anions and (b) as a function of SO_4^{2-} concentration

(a) The vesicle suspensions were kept overnight at 0°C in a medium composed of 80 mM-mannitol, 60 mM- Na_2SO_4 , 10 mM-sodium phosphate and either 20 mM of the sodium salt of the anion studied or 40 mM-mannitol. The suspensions were brought to 25°C and flux measurements were started by addition of $^{35}\text{SO}_4^{2-}$. (b) The vesicles were prepared in 300 mM-mannitol/75 mM-sodium *N*-2-[1,2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethane-sulphonic acid. To three equal portions of the suspension, enough Na_2SO_4 was added to reach the desired concentration of the salt. After incubation of the vesicles for 48 h at 4°C, the rate of equilibration of each batch was measured in an influx experiment. Specific trapped volumes in all three preparations (see legend to Table 2) were very similar.

(a) Medium complement	pH	$t_{\frac{1}{2}}$ complement/ $t_{\frac{1}{2}}$ mannitol
Mannitol	6.3	1.0
Acetate	6.3	0.83
Chloride	6.3	0.83
Nitrate	6.3	0.76
Iodide	6.3	0.6
Thiocyanate	6.3	0.42
Mannitol	8.0	1.0
Thiocyanate	8.0	0.30

(b)	$[\text{SO}_4^{2-}]$ (mM)	$k_{\text{app.}}$
	2.5	0.72
	30	0.42
	100	0.12

concentration (100 μM) caused a 50% irreversible inhibition of the flux. When the concentration of the reagent was doubled, no further inhibition was obtained. Complete irreversible inhibition is obtained in erythrocytes at these concentrations (Cabantchick & Rothstein, 1972). Persantine, one of the most effective non-competitive inhibitors (Schnell, 1972), did not inhibit the flux at a concentration of 0.1 mM.

In Fig. 5, the pH profile for sulphate-equilibrium exchange is presented. Results were obtained from a number of independent preparations either by influx or efflux assays. The characteristic pattern obtained in erythrocytes (Fig. 5, broken line) is superimposed on the experimental results.

Finally, to assess the influence of the lipid environment on the transport capacity of the protein, the half-times of equilibration of $^{35}\text{SO}_4^{2-}$ for different phosphatidylcholine preparations or preparations with different cholesterol concentrations were com-

Table 2. Influence of cholesterol and phospholipid concentration of the trapped volume and on the rate of sulphate exchange

The cholesterol/phosphatidylcholine ratios quoted are those of the starting reconstitution solutions. Vesicles were reconstituted at a protein/phospholipid ratio of 1:20. To measure the intravesicular volume, samples of vesicle suspensions containing the same amount of protein were incubated for 48 h in a solution of $^{35}\text{SO}_4^{2-}$ (long enough to achieve equilibrium), and the vesicles were freed of extracellular medium by gel filtration at 0°C. From the specific radioactivity of the incubation solution and the radioactivity trapped in the vesicles, the specific trapped volume (μl of intravesicular volume/ μg of protein) was obtained. $k_{\text{rel.}} = k_{\text{app.}} \times n$, where n is the ratio of specific trapped volumes between each preparation and the preparation taken as reference (the 1:50 phosphatidylcholine/cholesterol preparation).

Experiment	Phosphatidylcholine	Specific trapped volume ($\mu\text{l}/\text{mg}$ of protein)	Molar ratio (cholesterol/phosphatidylcholine)	$k_{\text{rel.}}$
1	Egg	105	1:2	19
1	Egg	80	1:5	11
1	Egg	55	1:12.5	6
1	Egg	55	1:50	3
2	Synthetic		No cholesterol	3.5
2	Egg		No cholesterol	1.5

pared. Rates of equilibrium are inversely proportional to the trapped volume per mol of units of transport (specific trapped volume). Table 2 shows that specific trapped volumes were different for each lipid composition, so that for the purposes of comparison, the experimental rates had to be corrected accordingly. The correction for each lipid composition was done by multiplying its experimental t_1 by its specific trapped volume and dividing by the specific trapped volume of the preparation with the lower cholesterol concentration (1:50). These normalized results are also included in Table 2.

Discussion

In the present work, a procedure yielding small single-walled or large multi-walled vesicles with incorporated membrane protein has been presented. This procedure is highly effective in incorporating the band-3 protein into the reconstituted membrane. Freeze-fracture replicas of the vesicles display intramembrane particles similar to those observed in the erythrocyte membrane.

The results from the freeze-fracture treatment on the vesicles obtained from bivalent-cation medium deserves special consideration. In the cut multi-walled vesicles, etching exposed the membrane to a depth that allowed its cross-sectional examination. The same intramembrane particles present at the convex fracture face are seen in the cut membranes as particles that span the membrane and protrude into the aqueous solution (Plate 1e). Extensive biochemical work (Steck *et al.*, 1976; Jenkins & Tanner, 1977; Cabantchik *et al.*, 1978) based mainly on partial proteolytic digestion studies have already predicted such a structure for band 3.

However, the possibility that plastic deformation (Sleyter & Robards, 1977) or water condensation (Staehelin & Bertaud, 1971) gave rise to apparent structures cannot be discarded.

Functional properties of reconstituted vesicles incorporating band-3 protein

The presence of non-transport protein in the bilayer structure (Papahadjopoulos *et al.*, 1975) or low concentrations of Triton X-100 (Schlieper & De Robertis, 1977) increases non-specific ion fluxes through membranes. The results presented in Fig. 3 show that the non-specific flux of sulphate through the vesicles is very small and can be neglected in the subsequent transport studies.

To evaluate the reconstitution effectiveness, a number of characteristic features of sulphate exchange in the native system have been studied.

(1) *Temperature-dependence.* The activation energy displayed by the reconstituted vesicles (84 kJ/mol) was lower than that found in intact cells [138 kJ/mol (Schnell, 1972; Schowch *et al.*, 1974)] or in isolated vesicles (119 kJ/mol) of similar protein composition (Wolosin *et al.*, 1977), but was still significant. A considerable decrease in inter-facial negative charge in the vesicles, as compared with cells (as a result of dilution of negatively charged protein and lipid), and the change in lipid environment in going from the cholesterol-rich viscous erythrocyte membrane (Cherry *et al.*, 1976) to a fluid phosphatidylcholine bilayer may account, wholly or in part, for the observed decrease in activation energy. Ross & McConnell (1978) have found virtually no activation energy for the sulphate exchange flux in band-3-containing vesicles reconstituted by dialysis of the detergent dodecyl-

trimethylammonium chloride. Various reasons may account for the lack of temperature dependence in the Ross & McConnell system. However, the considerable activation energy found in the present report suggests that the cationic detergent used by Ross & McConnell may not be suitable for the functional reconstitution of transport systems.

(2) *Intrinsic affinity of the transport system.* Sulphate and phosphate self-exchange rates decrease in the presence of univalent anions (Gruber & Deuticke, 1973; Schnell *et al.*, 1977), and at low concentrations of the anions the effect seems to be competitive. Schnell *et al.* (1977) have been able to obtain the apparent inhibition constants (K_i) of a number of univalent anions for sulphate transport on erythrocytes. In the same work, the dependence of sulphate-exchange rates on the substrate concentration was studied. The Michaelis-Menten (K_m) constant for sulphate exchange in the erythrocyte changes with pH, but at the optimum pH it is approx. 30 mM (Schnell *et al.*, 1977).

Although the data obtained are insufficient to determine an accurate K_m , it can be established that the intrinsic affinity towards sulphate does not differ significantly from that of the native system: at 30 mM-sulphate the apparent rate constant of exchange was about half of that at very low (2.5 mM) sulphate concentration.

The inhibitory effect of the univalent anions followed the general trend known for the native system (Schnell *et al.*, 1977; Gruber & Deuticke, 1973), but, although in the native system nitrate is as effective as thiocyanate (Schnell *et al.*, 1977), its effect in the reconstituted vesicles was much weaker.

(3) *Effect of non-competitive inhibitors in the reconstituted system.* In experiments with leaky 'ghosts', Cabantchik & Rothstein (1974) observed that 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate was unable to react with the anion-transport system at the inside face of the membrane. Being a highly charged molecule, 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate is unlikely to penetrate the vesicles, and its failure to produce more than 50% inhibition is an expected result if half of the protein molecules are directed right-side-out in the reconstituted membrane. The implications of the lack of influence of persantine on the reconstituted system cannot be worked out, as the mode of action of the drug is not yet understood.

(4) *pH profile of sulphate exchange.* The anion-exchange capacity of the erythrocyte membrane has been shown to be strongly pH-dependent in the case of both uni- and bi-valent anions (Gunn *et al.*, 1973; Schowch *et al.*, 1974; Dalmark, 1975; Schnell *et al.*, 1977). Three features stand out in a comparative analysis of the pH profiles for the native and reconstituted systems (Fig. 5): (a) in both cases the flux decreases as pH increases, but tends to a

baseline of about 10% of the maximal value; (b) in both cases an inflexion point is observed at pH 6.9; (c) in the native membrane a maximum is reached at pH just over 6.0. Then, for more acidic pH, a sharp fall in the rate occurs (Schowch *et al.*, 1974; Schnell *et al.*, 1977). In the reconstituted system, this latter fall is absent, and a slight decrease is observed only at the most acidic pH.

Yu & Branton (1976) presented evidence that the major cytoplasmic membrane-bound protein induces aggregation of intramembrane particles composed of band-3 at pH 5.5 in reconstituted membranes. The observed inhibition of flux at this pH in the native membrane might arise from a specific interaction between band 3 and spectrin, resulting in the blocking of the anion pathway.

(5) *Dependence of transport activity on the lipid composition of the membrane.* The presence of cholesterol in membranes results in an increase in the density and viscosity of the lipid region of membranes found in the liquid crystalline state (Oldfield & Chapman, 1972; Demel & De Kruijff, 1976). Changes in membrane fluidity influence the transport of metabolites and ions and the activity of membrane proteins (Rottem *et al.*, 1973; Kimelberg & Papahadjopoulos, 1974).

Although the native erythrocyte membrane is very rich in cholesterol, uncertainty about its distribution in the membrane (Murphy, 1962) and the failure to attain a high cholesterol depletion (Gottlieb, 1976) hinders an assessment of the influence of cholesterol upon anion transport. Grunze & Deuticke (1974) have shown that a 30% depletion in cholesterol of the membrane does not affect the anion exchange. However, such limited depletion has only a minor influence on other permeability properties of the erythrocyte membrane (Deuticke & Ruska, 1976). The reconstitution procedure as described above makes possible the variation in cholesterol concentration over a wider range. The drastic effect of cholesterol on the flux of sulphate suggests that the transport activity of the system is strongly dependent upon the physical state of the membrane.

(6) *Turnover capacity.* The most relevant test for the evaluation of reconstitution effectiveness is probably the comparison of absolute rates of sulphate exchange with those of the native system under similar conditions.

The apparent rate constant of exchange, k_{app} , is directly affected by the surface (A) to volume (V) quotient of the membrane-enclosed compartment in question ($k \propto A/V$).

For two membrane-enclosed compartments (1 and 2) having the same membrane-surface concentration of transport units (band-3), the ratio Q ($Q = k_{app}^{-1}/k_{app}^2$) will equal the ratio T , of the two surface to volume quotients [$T = (V/A)_1/(V/A)_2$], assuming that the transport capacity of the transport units in

both systems is the same. If the surface density of the transport units differs, the density ratio, N , between systems 2 and 1 should be introduced to obtain a normalized $Q_n = N \times (k_{app}^1/k_{app}^2)$. If the transport capacity of the units in system 2 is lower than those of system 1 (either as a result of lower capacity of each individual unit or as a result of the presence of inactive units), the T/Q_n ratio will indicate the overall fractional capacity of the units in system 2 compared with those in system 1.

As the surface area in erythrocyte or vesicle membrane is largely determined by the lipids, assuming that the mean area per lipid molecule does not differ significantly in both cases, the surface density can be represented by the band-3/lipid (w/w) ratio. In the reconstituted system (Fig. 3) this ratio is 1:10, whereas in the erythrocyte membrane it is approx. 1:2 (Steck, 1974); thus N is approx. 5. Under similar physiological conditions (60 mM- Na_2SO_4 , pH 6.3, 25°C) a k_{app} of 0.35 min⁻¹ is obtained in the vesicles (Fig. 3) and of 0.035 min⁻¹ in resealed 'ghosts' (Schnell *et al.*, 1977), and a Q_n value of about 50 can be calculated.

The compartments in question are both spherical and T reduces to the radius ratio T_1/T_2 . For resealed 'ghosts', the radius is 3.1 μm (Seeman, 1968), and for the vesicles a radius of between 0.05 and 0.1 μm (Plate 1b) can be assumed; therefore T will range between 30 and 60 and $Q_n \approx T$. The comparison will improve or deteriorate depending upon whether it is performed at lower or higher temperatures, as the activation energies differ significantly. It should also be remembered that the reconstituted system used for the comparison is cholesterol-free. On using the results obtained for the preparations containing cholesterol the comparison will deteriorate.

Nevertheless, the similarity between Q_n and T values indicates a high preservation of the turnover capacity of the whole population of reconstituted transport units.

To conclude, the functional studies show that the method presented allows the reconstitution of the anion-transport protein in tightly closed vesicles, preserving some of the basic properties of the system, namely affinity towards substrates and overall turnover capacity. Some differences with results obtained in the native membrane have been observed. Whether those differences arise from modification of the protein during the process of reconstitution or reflect different environments remains to be clarified.

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