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

J. Mario WOLOSIN*

Biophysics Section, Institute of Life Sciences, Hebrew University, Jerusalem, Israel

(Received 2 January 1980)

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 $\mathrm{SO}_4^{2-}$ 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

$^{35}\mathrm{SO}_4^{2-}$ (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

* Present address: Department of Physiology and Anatomy, University of California, Berkeley, CA 94720, U.S.A.

Abbreviation used: SDS, sodium dodecyl sulphate.
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-isothio-
cyan-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, 20mM-
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 cen-
trifuged at 40000 g 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
20000 g 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 50000); 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-Na2P, 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 2000 g
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 100000 g,
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 M-anilinonaphthalene-8-sul-
phonic acid as very small fluorescent spots undetect-
able by phase-contrast microscopy. The presence of
amorphous material in the preparation was moni-
tored 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 eluant,
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 radioac-
tivity 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 eluant
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 (t½) was obtained.
Throughout the text the apparent rate constant of
exchange (kapp.) refers to (ln2)/t½. No statistical
analysis of results was attempted, but the uncer-
tainty in t½ was usually not more than ±10% and
ever exceeded ±20%. Treatments with 4,4'-di-
isothiocyano-2,2'-stilbenedisulphonate of either
erthrocytes or reconstituted vesicles were
performed according to Cabantchik & Rothstein
(1974). 4

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 279 nm 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 560 nm 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 (10–15 nmol/mg of protein) compared with the content in the 0.5% extract (60–80 nmol/mg of protein), and no periodate/Schiff-base-positive bands could be detected.
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 D,L-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
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 Mg²⁺ (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-isothiocyanato-2,2'-stilbenedisulphonate were performed. Treatment with 4,4'-di-isothiocyanato-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 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-isothiocyanato-2,2'-stilbenedisulphonate applied at a high

![Graph showing time course of 35SO42− influx under exchange conditions for vesicles obtained from normal and 4,4'-di-isothiocyanato-2,2'-stilbenedisulphonate-pretreated cells.](image)

**Fig. 3. Time course of 35SO42− influx under exchange conditions for vesicles obtained from normal and 4,4'-di-isothiocyanato-2,2'-stilbenedisulphonate-pretreated cells.** The vesicles have 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-NaNO3/0.1 mM-EDTA/10 mM-sodium phosphate, pH 6.3, and sampled (50 μl) at different intervals. Protein concentration was 50 μg/ml. Radioactivity of the incubation solutions was 2.16 x 10⁶ c.p.m./ml; ○, vesicles from control cells (time in min); ●, vesicles from cells pretreated with 20 μM-4,4'-di-isothiocyanato-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'.

Vol. 189
Fig. 4. Temperature-dependence of \( ^{35}SO_4^{2-} \) exchange

Vesicles loaded with \( ^{35}SO_4^{2-} \) 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.

![Graph showing temperature-dependence of \( ^{35}SO_4^{2-} \) exchange](image)

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\(_2\)SO\(_4\), 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 \( ^{35}SO_4^{2-} \). (b) The vesicles were prepared in 300 mM mannitol/75 mM-sodium N-2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethane-sulphonic acid. To three equal portions of the suspension, enough Na\(_2\)SO\(_4\) 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.

<table>
<thead>
<tr>
<th></th>
<th>Medium complement</th>
<th>pH</th>
<th>( t_1 ) complement/</th>
<th>( t_1 ) mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>6.3</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>6.3</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>6.3</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>6.3</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodide</td>
<td>6.3</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>6.3</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>8.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>8.0</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Graph showing pH profile for \( SO_4^{2-} \) exchange in the reconstituted system](image)

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.

![Graph showing pH profile for \( SO_4^{2-} \) exchange in the reconstituted system](image)
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 48h 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 (\( \mu l \) of intravesicular volume/\( \mu g \) of protein) was obtained. \( k_{rel} = k_{app} \times n \), where \( n \) is the ratio of specific trapped volumes between each preparation and the preparation taken as reference (the 1:50 phosphatidylcholine/cholesterol preparation).

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

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 (113 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-isothiocyanato-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-isothiocyanato-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 persantin 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 steep 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.


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 \( Q = k_{app,1}/k_{app,2} \) 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 \(Q_n = N \times (k_{\text{app}}^1/k_{\text{app}}^2)\). 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\(_2\)SO\(_4\), pH 6.3, 25°C) a \(k_{\text{app}}\) 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 \(\mu\)m (Seeman, 1968), and for the vesicles a radius of between 0.05 and 0.1 \(\mu\)m (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.

I am grateful to Professor Wilfred Stein, Dr. H. Ginsburg, Dr. Z. I. Cabanchik, Dr. A. Lydiatt and Dr. R. Venn for valuable discussion and advice. This work was supported in part by a grant from the Branch for Basic Research of the Israeli Academy of Science to Drs. H. Ginsburg and Z. I. Cabanchik.

References


Vol. 189