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Hemoglobin-Depleted Human Erythrocyte Ghosts: Characterization of Morphology and Transport Functions

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Summary. A method of preparing hemoglobin-depleted resealed ghosts with an extremely low hemoglobin content is described. The membrane morphology, the crossed immunoelectrophoresis pattern of the membrane proteins, and the transport function of these ghosts have been examined.

Electron microscopic examination of the ghosts on hydrophilic as well as hydrophobic grid surfaces revealed a faint filamentous network (spectrin) associated with the membrane. The ghosts were found to have permeabilities towards small polar molecules (water and mannitol) and ions (chloride, sodium, and potassium) which are quantitatively very close to those of intact red cells.

It is concluded that white ghosts prepared by the present method are well suited for simultaneous studies of morphology, membrane biochemistry, and membrane transport functions.

The ease with which erythrocyte membranes can be isolated for biochemical and transport studies have made them an object of choice for studying the correlation between protein chemistry, membrane morphology, and membrane transport function. However, all biochemical studies have been performed on hemoglobin depleted ghosts, prepared by the method of Dodge, Mitchell and Hanahan (1963). The latter membrane preparation is unsuited to studies of transport processes because the ghosts are "leaky" even towards macromolecules. During recent years methods of preparing red cell membranes (pink ghosts) for transport studies have improved very much, largely due to the work of Passow and collaborators (Schwoch & Passow, 1974). The latter type of ghost is not well suited to biochemical studies since it retains a considerable amount of the original hemoglobin of the red cell. Attempts to prepare hemoglobin-depleted ghosts for transport studies have met with some success (Jung, 1971), but the results have shown that these ghosts are considerably more leaky than the red cells from which they were prepared.

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The mannitol permeability of white ghosts was, for instance, reported by Jung (1971) to be 5×10^{-8} cm sec^{-1}, considerably higher than the normal permeability of erythrocytes (5×10^{-10} cm sec^{-1}).

The object of the present study has therefore been to develop a method for the preparation of hemoglobin-depleted white ghosts, which are well suited to biochemical and morphological studies and which at the same time have a membrane permeability as close as possible to that of the intact membrane. Some of the results obtained have been reported previously in a short communication (Bjerrum & Tranum-Jensen, 1977).

Materials and Methods

All media used were prepared from reagent grade chemicals.

Media for Preparation of Ghosts (mM)

Hemolysis medium (*A*): 4 $MgSO_4$, 3.8 CH_3COOH, (pH 3.6–3.8).
Washing medium (*B*): 4 $MgSO_4$, 1.2 CH_3COONa, 2 NaH_2PO_4/Na_2HPO_4 (pH 7.0).

Reversal solutions, medium (C_I), (C_{II}) and (C_{III}): (I) 2000 KCl; (II) 2000 KCl, 25 tris/HCl (pH 7.2); (III) 2000 KCl, 25 tris (pH 10.5).

Suspension Medium (D) for Transport Studies (mM)

165 KCl, 2 tris/HCl (pH 7.2).

Suspension Medium (E) for Morphological Studies (mM)

20 KCl, 2 NaH_2PO_4/Na_2HPO_4, 4 $MgSO_4$ (pH 7.2).

Radioactive Isotopes

The radiochemicals used in the transport studies, *viz.* ^{14}C-mannitol (sp act 60 mCi mmol^{-1}), ^{36}Cl as NaCl (sp act 500 μCi mmol^{-1}), ^{3}H-inulin (sp act 900 mCi mmol^{-1}), and ^{22}Na as NaCl (sp act 1700 mCi mmol^{-1}) were purchased from The Radiochemical Centre, Amersham, England; $^{3}H_2O$ (sp act 180 μCi mmol^{-1}) and ^{42}K as KCl (sp act 6.3 mCi mol^{-1}) were purchased from AEK, Risø, Denmark.

Preparation of Resealed Ghosts

The technique for preparing a uniform population of resealed white ghosts was developed from the method for the preparation of pink ghosts described by Funder and Wieth, (1976).

Freshly drawn heparinized human blood was washed thrice in isotonic KCl at room temperature. The buffy coat was carefully removed after each centrifugation.

I. Lysis. The cells were maintained strictly at 0 °C during the ghost preparation procedure. The cells were lyzed by injecting 20 ml 50% hematocrit from a syringe into an ice-cooled centrifuge tube containing 200 ml hemolyzing medium *A*. After 1 min the cell suspension (pH 5.8–6.2) was centrifuged at 13,000 rpm ($23{,}000 \times g$) for 12 min (Sorvall RC-5 superspeed refrigerated centrifuge, Du Pont Instruments, Sorvall Operations, Newton, Conn.), and the supernatant was removed as completely as possible. By this procedure the hemoglobin content of the ghosts was reduced to approximately 3% (wt/wt) as determined spectrophotometrically at the Soret band (411 nm). The ghosts can at this stage be transformed into resealed pink ghosts for flux experiments after reversal of the KCl concentration and resealing (*see* below).

II. Removal of hemoglobin from open ghosts. The tonicity of the 3% (wt/wt) hemoglobin-containing "packed ghosts" was increased by adding 100 µl of medium C_I per ml of ghost suspension. The increase in tonicity causes the ghosts to shrink, thereby, concentrating the hemoglobin in the unsealed ghosts and favoring its efflux (*cf.* Funder & Wieth, 1976, Fig. 5). It was very difficult to obtain white ghosts if this step was omitted. The ghosts were washed (rehemolyzed) after 2 min with 220 ml medium *B* (pH 7.0), and the membranes were again centrifuged at 13,000 rpm for 12 min. This washing procedure was repeated 3–4 times until the ghosts had a greyish white color.

III. Reversal of tonicity and resealing. The KCl concentration of the ghost suspension (white or pink ghosts) was increased to 180 mM by adding 0.9 ml reversal solution C_{II} or C_{III} per 10 ml ghost suspension. To obtain a final pH of 7.1–7.3, medium C_{II} was used for white ghosts and medium C_{III} for pink ghosts. The ghost suspension was left at 0 °C for 10 min after addition of reversal solution and the ghosts were finally resealed by incubation at 38 °C for 45 min.

The ghosts were then washed twice in the electrolyte medium (medium *D*; 0 °C) used for efflux experiments. Ghost suspensions with other pH values (*see* Fig. 7) were obtained by titration with 0.1-M solutions of KOH or HCl in 165 mM KCl. After titration, these ghosts were washed in electrolyte medium *D* at the pH to be used in the efflux experiment.

IV. Labeling and packing of resealed ghosts for experiments. While the ghosts were still unsealed at low KCl concentration, radioactive tracers with a low permeability (^{14}C-mannitol, ^{22}Na, and ^{42}K) were added to the ghost suspension (80% cytocrit) to a final activity of 0.2–0.6 µCi ml^{-1}. After resealing, the ghosts were quickly washed twice at 0 °C and used immediately afterwards for the efflux experiments. By this washing the ghosts lose 10–40% of the radioactive tracers ^{14}C-mannitol, ^{42}K, and ^{22}Na. This loss was not found for ^{3}H-inulin. As described later (*cf.* Fig. 5, 8 and 9) the release is due to incomplete resealing of a fraction of the ghosts. Tracers with a high permeability (^{36}Cl and $^{3}H_2O$) were first added after resealing and washing the ghosts, by adding 0.2–0.6 µCi per ml ghost suspension (50%). The ghosts were incubated for at least 6 half-periods of tracer exchange to ensure tracer equilibrium.

A sample of suspension was retained for ghost counting (Coulter Counter Model DN) and the remaining portion was centrifuged for 15 min at 20,000 rpm ($44{,}000 \times g$) in nylon tubes (0.8 ml, i.d. 3 mm) (Funder & Wieth, 1966). The cytocrit of the packed ghosts was measured and the packed ghosts and the supernatant were isolated by cutting the tube just below the interface. The mean ghost cellular volume, MCV, was determined from the hematocrit, the ghost count, the water content of the packed ghosts and the fraction of extracellular ^{3}H-inulin trapped between the packed ghosts after centrifugation

at $44{,}000 \times g$ for 15 min (Sorvall RC-5). The hemoglobin content of the white ghosts was measured by determining the iron content by means of atomic absorption spectroscopy. The relative experimental SD of a single measurement in a series of double and triple determinations was found to be 13.2%.

Determination of the Efflux Rate

All experiments were performed by measuring the rate of efflux of radioactive isotopes from packed, labeled human erythrocyte ghosts under steady-state conditions. Packed ghosts in nylon tubes were used directly for transport studies by injecting the preloaded ghosts into a well-stirred isotope-free, thermostated electrolyte medium (KCl 165 mM, tris 2 mM). When measuring mannitol or sodium permeabilities, efflux medium and ghosts containing 2 mM concentrations of each reagent were used. After addition of the ghosts to the suspension medium (0.5–1% cytocrit), ghost-free samples were collected serially by use of the millipore Swinnex filtering technique as described by Dalmark and Wieth (1972). The efflux of tracers with a low permeability (5×10^{-10} cm sec^{-1}) were followed over at least an 18 hr period. The measured rate coefficients were corrected for the spontaneous rupture of ghosts and for the rupture of ghosts caused by stirring. This rupturing was measured by determining the accompanying release of ^{3}H-inulin (final activity 0.4–1.0 μCi ml^{-1}). The use of inulin release as a measure of ghost rupture was justified by the following experiments. Inulin release was found to depend on the stirring rate. In unstirred samples the release rate was about 5×10^{-7} sec^{-1} at 0 °C and about 11×10^{-7} sec^{-1} at 38 °C. Identical results were obtained with ^{3}H-inulin (mol wt 5,000) and with the much larger ^{3}H-dextran molecules (mol wt 70,000), making it likely that the tracer release is caused by membrane rupture rather than by increased permeabilities to the intracellular markers. The water permeability of the white ghosts was measured by Dr. J. Brahm by means of the flow tube technique (Brahm, 1977). Duplicate samples of blanks, ghost-free supension, and equilibrium samples were counted in a liquid scintillation counter.

Calculations

The experiments were all carried out at a hematocrit below 1%, and the tracer efflux was found to follow the first order kinetics of a closed two-compartment system with the following relation between activity, a_t, in the medium and time, t.

$$a_t = (a_\infty - a_0)\,(1 - e^{-kt}) + a_0 \tag{1}$$

where a_∞ is the concentration after isotope equilibrium has been achieved, k the rate coefficient of tracer efflux ($time^{-1}$) and a_0 is the activity at $t=0$, caused by the introduction of extracellular medium trapped between the packed cells injected.

The rate coefficient (k) was determined by linear regression analysis of a plot of $\ln(1 - a_t/a_\infty)$ *vs.* time and was converted to the apparent permeability P_{app} (in cm sec^{-1}) or the efflux J (in mol cm^{-2} sec^{-1}) related as expressed in Eq. (2).

$$J = P_{app} \times C_i = k \times C_i \times V_i / A \tag{2}$$

where C_i is the intracellular concentration of the labeled molecule (in mol cm^{-3}), k is the rate coefficient of tracer efflux (in sec^{-1}), V_i the intracellular volume (in cm^3), and A the membrane area (in cm^{-2}).

The calculation assumed a surface area of 142 μm^2 per ghost (Westermann, Pierce & Jensen, 1961). This value corresponds within 15% to the membrane area of the hemoglobulin-depleted resealed ghosts calculated from the hemolytic volume of the ghosts.

Transport Inhibitors

The amino-reagent DIDS is an irreversible inhibitor of chloride transport in human red cells (Cabantchik & Rothstein, 1974). 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) was synthesized from 4,4′-diaminostilbene-2,2′-disulfonic acid (DADS) by M.P. Hancock, Chemical Laboratory II, University of Copenhagen. The methods employed for preparation and purification of DIDS have been reported by Funder, Tosteson and Wieth (1978). Resealed white ghosts were incubated for at least 2 min with ^{36}Cl (0.2–0.4 μCi per ml ghost supension) at room temperature. The ghosts were then treated with DIDS for 45 min at 38 °C by adding 100 μl of 10^{-2} M DIDS to 10 ml of ghost suspension (50%). After cooling to 0 °C and centrifugation, the ghosts were resuspended in a DIDS-free medium, transferred immediately afterwards to nylon tubes, and the ^{36}Cl exchange rate was determined.

The inhibition of water transport by *p*-chloromercuribenzoic acid (*p*CMB) was investigated after the ghosts (cytocrit 5%) had been incubated for 90 min at 25 °C in a medium containing *p*CMB (1 mM), KCl (165 mM), and phosphate buffer (1 mM), pH 7.25. The ghosts were isolated by centrifugation and ^3H_2O exchange was determined at 25 °C by the flow-tube technique (Brahm, 1977).

Solubilization of Ghost Membranes and Crossed Immunoelectrophoresis

Two preparations of erythrocyte ghosts were compared. One was prepared according to Dodge *et al.* (1963) and the other as described above under the ghost preparation procedure. The resealed ghosts in KCl (165 mM) and tris (2 mM) were washed twice in KCl (10 mM) and tris (2 mM) at 0 °C before solubilization. Both types of ghosts were solubilized to a final concentration of approximately 2×10^9 ghost membranes per ml (about 1 mg protein per ml) at 0 °C in a medium containing glycine (10 mM), tris (3.8 mM, pH 9.2), and 1% wt/vol of the nonionic detergent Berol EMU-043 (MoDokemi, Stenungsund, Sweden) by sonication on ice for 2×5–10 sec at 16,000 Hz with a MSE 100-W ultrasonic disintegrator. The supernatants obtained after centrifugation at 0 °C for 60 min at $30{,}000 \times g$ (Sorwall RC-5) were used immediately for crossed immunoelectrophoresis.

The electrophoresis was performed as described by Bjerrum and Lundahl (1974). The agarose employed (HSA 364) was purchased from Litex A/S, Gostrup, Denmark. 30 μl of solubilized material were subjected to electrophoresis at 10 V/cm for 60 min in the first dimension and at 2 V/cm for 18 hr in the second dimension gel (16–18 °C). Rabbit antibodies against human erythrocyte membrane material (A 104, from Dakopatts A/S, Copenhagen, Denmark) were added to the second dimension gel to a concentration of 5.4 μl/cm^2. Staining of the electrophoresis plates was performed with Coomassie Brilliant Blue R (Gurr, Searle) as described by Weeke (1973).

Osmium Tetroxide Fixation of Hemoglobin-Depleted Resealed White Ghosts

White ghosts at the hemolytic volume (before KCl concentration reversal and resealing) were suspended in the medium *E* (50% cytocrit) and were then incubated at 38 °C for 45 min to reseal them.

The ghosts were fixed in 0.25% osmium tetroxide prepared from a stock solution of 0.5% osmium tetroxide in a phosphate buffer solution (25 ideal milliosmole), pH 7.2. The fixed ghosts were kept on ice for 1 hr and were then washed 3 times in a 20 mM KCl solution.

Preparation of Negatively Stained and Shadowed Ghost Membranes

Carbon coated Fromvar films on copper grids were used as supports. Grids with hydrophobic surface properties were produced by leaving the grids for about 15 min under high vacuum in the evaporator after the carbon coating in order to deposit a trace of diffusion pump oil on the grid surface. Hydrophilic grid surfaces were obtained by glow discharge of carbon coated grids for 1 min at 0.06 torr in an Edwards "plasma-glo" apparatus operating at 1.5 A. Ghost membranes were examined on each of the grid-types after the following preparation procedures:

1) Freshly prepared, unfixed white ghosts were applied to the grid as a drop of a 25% suspension and washed on the grid with a few drops of distilled water to remove phosphate before staining with 1% aqueous uranyl acetate. After staining for 30 sec, grids were blotted by touching the drop with a piece of filter paper and then left for evaporation of residual water.

For shadowing, the grids were dried after the distilled water washing. Shadowing was performed at low angle (10–20°) in an Edwards evaporator at 10^{-5} torr using a pointed carbon rod wound with platinum wire.

2) The same procedure was applied to ghosts fixed in 0.5% glutaraldehyde in 10 mM phosphate buffer, pH 7.2, for 3–4 hr at 0 °C.

3) Ghosts fixed in OsO_4 were drawn a few times through a 24-gauge hypodermic needle whereby the rigidly-fixed ghosts are broken into flakes by shear forces, allowing observation of single layers of membrane. Such suspensions of ghost-flakes were treated as in 1.

Specimens were examined at 60 kV in a Phillips 300 electron microscope equipped with a cold trap. Magnifications were calibrated using a Fulham grating replica N. 1002.

Preparation for Scanning Electron Microscopy

Ghosts were OsO_4-fixed, and transferred as a 10% suspension to dehydration capsules closed by nylon filters (7 μm pore size) (Rostgaard & Christensen, 1975). The capsules were placed in 10 mM phosphate buffer, which was then slowly replaced by distilled water by a procedure of continuous washout. The medium in the capsules was then changed to 100% acetone through a similar procedure of continuously raising the concentration. This procedure was chosen to minimize shrinkage during dehydration. The dehydrated capsules were transferred to liquid CO_2 and dried in a critical point drying apparatus (Polaron, Ltd., England). The dried ghosts adhering to the nylon filter discs were coated with carbon followed by Au-Pd in an Edwards evaporator. Cracking of ghosts for exposure of their interior was achieved by gentle scraping of dried preparations before coating. Specimens were examined at 10–20 kV in a JEOL JSM-U3 scanning electron microscope.

Results

Morphology and Protein Chemistry

The resealed white ghosts were examined by scanning- and by transmission electron microscopy. Figure 1 A shows a scanning electron micrograph of the hemoglobin-depleted resealed ghosts at their hemolytic vol-

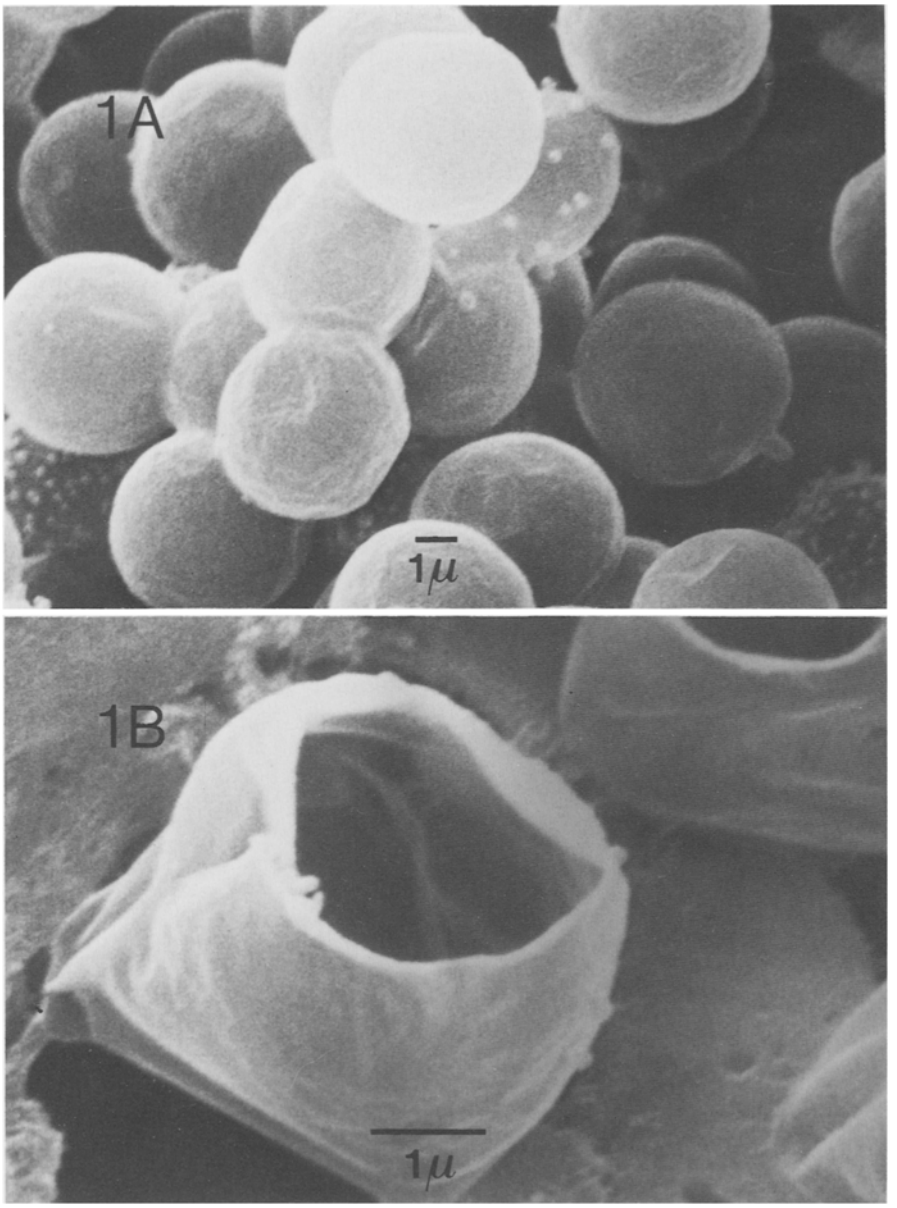

Fig. 1. Scanning electron micrographs of human erythrocyte ghosts. More than 99.96% of the hemoglobin has been removed by repetitive osmotic hemolysis. (*A*): Resealed ghosts (×600). (*B*): Resealed ghosts ruptured mechanically after fixation (×17,000)

ume. Most of the ghosts are spherocytes, but a few echinocytes can be seen. The formation of echinocytes could be inhibited by adding ATP (2 mM) to the suspension before resealing. By phase contrast light

microscopy it was observed that this inhibition of echinocyte formation lasted for about 30 min at room temperature. Similarly, ghosts prepared with a smaller volume by reversal (adding of KCl) were smooth biconcave discs or smooth stomatocytes when ATP was present. Figure 1 *B* shows a population of ghosts, in which the membranes have been ruptured by mechanical means. These ghosts appear as empty open sacks.

The structure of the resealed white ghost membranes was further examined after negative staining or shadowing of air-dried membrane fragments. The membranes were examined on hydrophilic as well as hydrophobic grid surfaces. Examination of unfixed membranes stained with uranyl acetate on hydrophilic grid surfaces revealed a very faint negatively stained filamentous network associated with the membrane (Fig. 2 *A*). Homogeneous staining of membrane on hydrophilic grid surfaces was difficult to achieve and great variations were seen in different specimens. When ghosts were similarly treated on hydrophobic grid surfaces advantage was taken of two effects. Firstly, upon blotting the grid the staining solution glanced off and the liquid front sweeping along the surface caused extensive rupture of the ghosts, thus facilitating observations on single membrane layers. Secondly, due to the hydrophobic surface the residual stain present after blotting concentrated in hydrophilic structures on the grid, resulting in a positive staining of the filamentous network. This effect was observed with unfixed (Fig. 2 *B*), glutaraldehyde-fixed (Fig. 2 *C*), and OsO_4-fixed membrane (Fig. 2 *D*). OsO_4-fixed membrane flakes on hydrophobic grids in particular showed remarkably uniform and reproducible staining of the network, compared to ordinary negative staining. Glutaraldehyde-fixed membranes dried on hydrophobic grid surfaces showed incidentally that the network structure was spread probably as a result of surface tension. This spreading makes observation of the network easier (Fig. 2C).

The fibrous nature of the network was further revealed by low-angle shadowing of unfixed membranes. Fragments of ruptured membranes were found to be joined by thin filaments (Fig. 2 *E*). It seems therefore likely that the network, which forms a reticulum with meshholes of 300–600 Å in diameter, is identical to the spectrin network found by Hainfield and Steck (1977).

A comparison of the protein composition of hemoglobin-depleted ghosts prepared by the methods of Dodge *et al.* (1963) (Fig. 3 *A*) and by the present method (Fig. 3 *B*) was performed. The membrane proteins were examined by crossed immunoelectrophoresis as described by Bjerrum and Bøg-Hansen (1976).

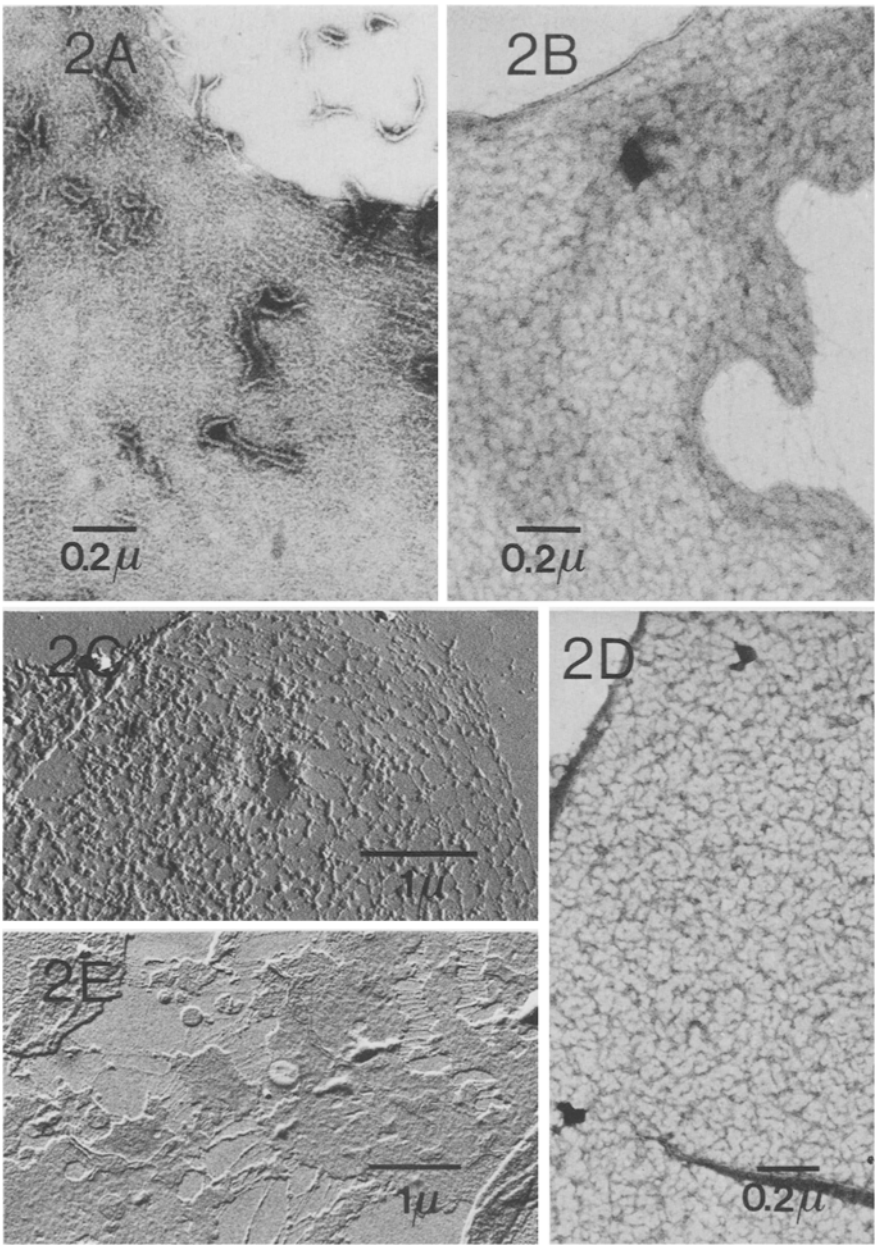

Fig. 2. Unfixed membrane fragments on (*A*) hydrophilic- and (*B*) hydrophobic grid surfaces stained with uranyl acetate (×46,200). A faint *negatively* stained network (spectrin) associated with the membrane can be seen in (*A*). The positively stained network (*B*) is continuous with long fibers visisble along the torn edge of the membrane fragment (for details, *see* text). (*E*): The fibers are seen more clearly after low angle shadowing (×14,200). (*C*): The shadowed membrane network after fixation with 1% glutaraldehyde, which fixes the proteins but not the lipids (×17,400). An expanded network associated with particles is seen. (*D*): The network also persists after fixation with osmium tetraoxide (×46,200)

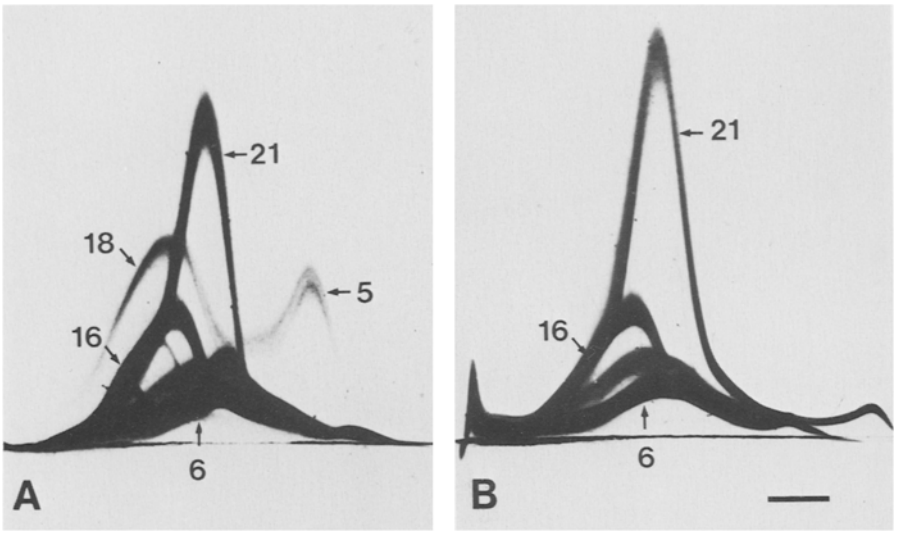

Fig. 3. Crossed immunoelectrophoresis of human erythrocyte membrane proteins from (*A*) ghosts prepared as described by Dodge *et al.* (1963); (*B*) ghosts prepared as described in the methods section. The various major precipitates were identified according to Bjerrum and Bøg-Hansen (1976). Precipitate 6 contains spectrin(s), precipitate 16 and 18 the major "intrinsic" protein (the band III protein) and precipitate 21 the MN-glycoprotein. Precipitate 5, *see* text. (Numbering of the Coomasie Brilliant Blue stained precipitates after Bjerrum and Bøg-Hansen (1976)). The same relative proportions of the various major erythrocyte membrane proteins, *viz* spectrin, major "intrinsic" protein and MN-glycoprotein, were observed in the two ghost preparations. The differences between *A* and *B* are discussed in the text. The bar represents 1 cm

The protein precipitates of crossed immunoelectrophoresis have been correlated to the major protein bands found by SDS-polyacrylamid-gel-electrophoresis (Norrild, Bjerrum & Vestergaard, 1977). The protein precipitates of the two different ghost preparations showed the same relative proportions of the predominant membrane proteins: spectrin, major "intrinsic" protein (band III protein), and the major MN-glycoprotein (glycophorin). Protein precipitate number 16, which contains the so-called Band III protein believed to be involved in anion transport (Cabantchik & Rothstein, 1974), is seen with both preparations. However, after preparation of white gosts by the methods of Dodge *et al.*, two more precipitates appear (precipitates number 5 and 18). The precipitate (18) which is not found with the resealed white ghost preparations may possibly arise from dissociation of band III protein from a "peptide complex" in precipitate 16 (Norrild *et al.*, 1977). Precipitate 5 shows partial identity with precipitate 18, and disappears after treatment of

the membranes with divalent cations (Ca^{++}) (Bjerrum & Bøg-Hansen, 1976).

Characterization of the Ghost Population

The amount of extracellular inulin trapped between the packed white ghosts was found to be 22.7% (wt/wt) (Table 1). This value agrees closely with the value of 22.1% (wt/wt) calculated from the kinetics of ^{36}Cl exchange (Table 6), as discussed later. It can therefore be concluded that the space which is inaccessible to inulin is the true intracellular space. Further characterizing data for the resealed ghost preparations are shown in Table 2. The average ghost membrane weight was 10^{-12} g.

The hemoglobin content of the resealed white ghost preparations was determined by analysis of the iron content of the ghosts (Table 3). The results show that the ghosts were as depleted for hemoglobin as the ghosts prepared for biochemical analysis by the methods of Dodge *et al.* (1963). Hemoglobin constituted approximately 0.9% (wt/wt) of the membrane material corresponding to approximately 8×10^4 molecules per ghost.

The volume distributions of pink- and white ghost preparations are shown in Fig. 4. Apart from the fact that the hemoglobin-depleted white ghosts have significantly smaller mean cell volume, the two ghost preparations display a similar volume distribution.

Transport Functions

A number of passive transport processes were examined in order to characterize the permeability properties of the resealed hemoglobin

Table 1. Trapping of inulin between packed hemoglobin-depleted ghosts % (wt/wt)

Exp. No.	1	2	3	4	5	6	7	8	mean	SD
MCV_{uncorr}	59.6	87.2	59.9	56.1	55.4	58.5	59.4	59.9	62.0	10.3
Inulin space %	21.3	21.9	21.8	22.3	23.5	25.4	22.8	22.8	22.7	1.3
$MCV_{cor\ \mu m^3}$	46.9	67.9	46.7	43.6	42.4	43.6	46.3	46.7	48.0	8.2

The MCV_{uncorr} are the mean ghost volumes calculated from the ghost count (Coulter Counter model DN) and the cytocrit without correcting the latter for the amount of medium trapped between the packed ghosts.

Table 2. The membrane dry wt of resealed, hemoglobin-depleted ghosts

No.	Water g/kg ghosts	Solids g/kg ghosts	Membrane material/ g/kg ghosts	$10^{-13}\times$ number of ghosts/kg	$10^{12}\times$ membrane material g/ghost
1	966.9	33.1	21.0	1.68	1.25
2	961.7	38.3	26.2	1.71	1.53
3	968.8	31.2	19.1	1.55	1.23
4	975.3	24.7	12.6	1.50	0.84
5	973.6	26.4	14.3	1.62	0.88
6	975.4	24.6	12.5	1.53	0.82
7	974.1	25.9	13.8	1.49	0.92
8	969.5	30.5	18.4	1.87	0.98
9	975.0	25.0	12.9	1.75	0.74
10	977.2	22.8	10.7	1.32	0.81
11	968.9	31.1	19.0	1.42	1.34
12	970.1	29.9	17.8	1.53	1.17
Mean	971.3 (SD 4.3)	28.6 (SD 4.5)	16.5 (SD 4.5)	1.58 (SD 0.15)	1.04 (SD 0.29)

"kg ghosts" refer to kg packed ghosts (packed by centrifugation) and the values shown have not been corrected for the 22.7% extracellular fluid volume measured as inulin space (*see* Table 1). The salt content of the packed ghosts (165 mM KCl) was 12.1 g/kg packed ghosts. The mean value of the mean cellular volume (MCV) for all 12 experiments was 48.2 (SD 4.5) μm^3.

Table 3. Hemoglobin content of the hemoglobin depleted "white" ghosts

Preparation No.	$Fe^{++}\times 10^{-17}$ g/ghosts	$Hgb\times 10^{-14}$ g/ghosts	% of erythrocyte hemoglobin wt/wt	% of erythrocyte membrane dry wt (wt/wt)
1 P.B.	2.13	0.64	0.019	0.62
2 P.B.	1.73	0.52	0.015	0.50
3 J.B.	2.51	0.75	0.022	0.72
4 J.S.	2.73	0.82	0.024	0.79
5 P.B.	3.15	0.95	0.028	0.91
6 P.B.	3.23	0.97	0.028	0.93
7 J.B.	2.94	0.79	0.023	0.76
8 J.B.	3.24	0.82	0.024	0.79
9 N.P.	4.51	1.35	0.039	1.30
10 P.B.	2.45	0.74	0.022	0.71
11 J.B.	3.23	0.97	0.028	0.93
12 P.B.	4.46	1.34	0.039	1.29
Mean	3.03 (SD 0.83)	0.89 (SD 0.25)	0.026 (SD 0.007	0.85 (SD 0.24)

The % (wt/wt of erythrocyte hemoglobin for white ghosts was calculated by using a value for hemoglobin in intact erythrocytes of 3.42×10^{-11} g/RBC (Dodge *et al.*, 1963).

The % (wt/wt) of the erythrocyte membrane dry wt was calculated using the membrane material weight value of 10.4×10^{-13} g/ghosts (*see* Table 2).

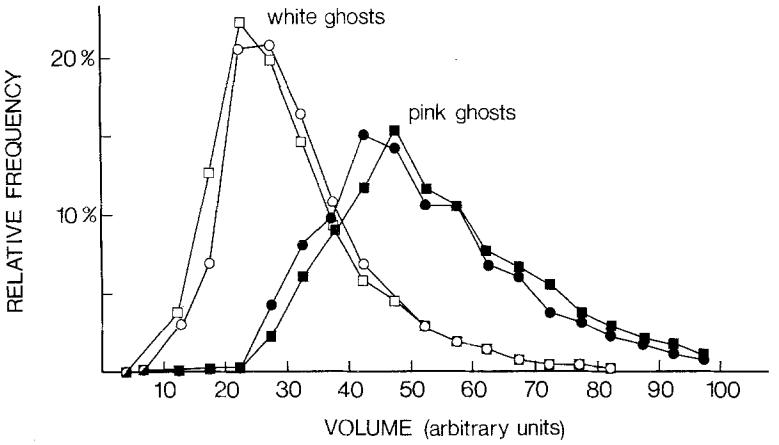

Fig. 4. Volume distribution of resealed pink and white ghosts, supended in a medium containing KCl (165 mM) and tris (2 mM). The abscissa shows the volume in arbitrary units (1 div = 1.65 μm^3). The ordinate of the graph is relative number of ghosts N/N_o found over 5 discrimination units. The current setting of the coulter counter was adjusted so that all ghosts were found within the range of 100 arbitrary volume units. The skewness of the size distribution is a counting artefact due to coincidence of two or three particles (Brecher, Jacobek & Schneiderman, 1962). The mean cellular volume (determined from the total number of ghosts and the cytocrit corrected for the amount of inulin trapped extracellularly was 80.5 μm^3 for pink ghosts (●, ■) and 49.5 μm^3 for white ghosts (○, □). The standard deviation of the size distribution for the white and pink ghosts (measured by plotting the graph on probability paper) was 28 μm^3 (35%) for pink ghosts and 17 μm^3 (34%) for white ghosts

Table 4. Passive mannitol permeability of white ghosts

Temp. °C	MCV μm^3	$k \times 10^5$ sec^{-1}	$P_{app_{Ma}} \times 10^{10}$ cm/sec^{-1}
0	48.9	0.16	0.54
	48.9	0.14	0.47
20	53.3	0.53	1.99
25	48.9	0.87	2.99
	48.9	0.66	2.27
	43.5	0.85	2.60
38	48.9	1.26	4.33
	48.9	1.51	5.20

The rate coefficients (sec^{-1}) has been corrected for the rupture of ghost caused by stirring. The efflux of tracer was followed over at least an 18 hr period. The Arrhenius activation energy for the transport process is about 10–12 kcal mol^{-1}.

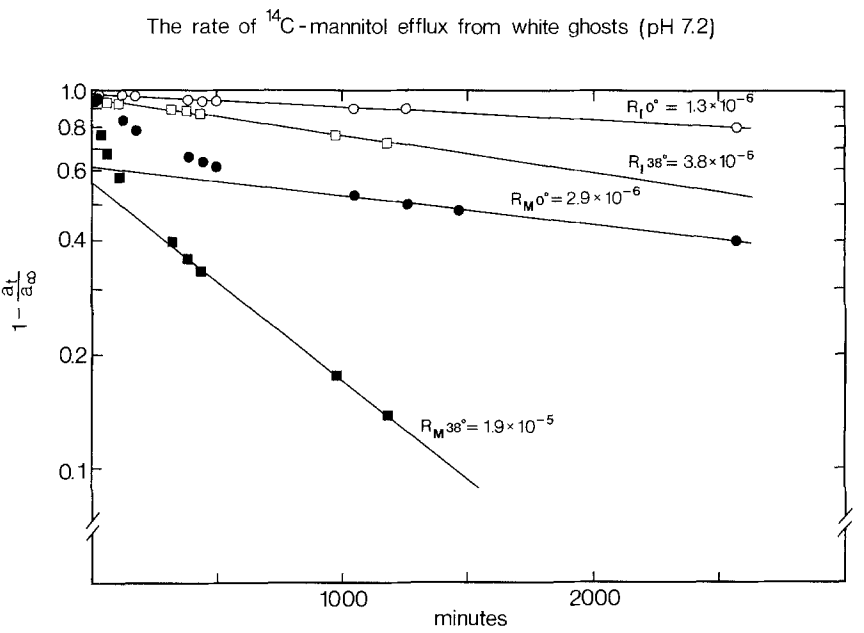

Fig. 5. The rate of ^{14}C-mannitol (●, ■) and ^{3}H-inulin (○, □) release from white ghosts at 0 and at 38 °C (pH 7.2). The ordinate has a logarithmic scale. The ^{3}H-inulin release was measured to correct for rupture of ghosts during the long incubation. The numbers indicate the rate coefficients (sec^{-1}) for the straight lines determined by linear regression analysis. The mannitol-tight fraction of the resealed ghosts used was estimated to be 52% at 0 °C and 49% at 38 °C. The values have been calculated from the intercepts of the graphs, about 0.6, corrected for the amount of tracer lost, 14%, by washing at 0 °C before the efflux experiments were performed

depleted ghost: The rather slow transport of mannitol and the rapid transport of water, together with the function of the specific anion exchange transport system and the "tightness" of the membrane to potassium and sodium ions.

Mannitol Permeability. The permeability of intact red cell membranes to mannitol is extremely low (3.8×10^{-10} cm sec^{-1}, 39 °C; Bowman & Levitt, 1977). The results shown in Table 4 indicate that the mannitol permeability of white ghosts is of the same order of magnitude. The Arrhenius activation energy for the transport process is about 10–12 kcal mol^{-1}. This low permeability was found only for about 50–60% of the ghost population. As shown in the "washout" curve in Fig. 5, The white ghost preparation was found to be heterogeneous with respect to mannitol permeability, consisting of at least two popula-

Table 5. ^3H_2O permeability of white ghost. Inhibition with *p*CMB (25 °C)

Inhibitor	MCV μm^3	k sec^{-1}	$P_{app} \times 10^3$ cm sec^{-1}	Inhibition %
none	52.5	52.5	1.90	0
none	37.8	73.6	1.91	0
*p*CMB	43.0	20.7	0.61	68

The ghosts were treated with 1 mM *p*CMB for 90 min at 25 °C. The efflux of ^3H_2O was determined by the flow-tube technique (Brahm, 1977) in a medium containing 165 mM KCl+2 mM tris, pH 7.4.

tions of ghosts—one with a high and one with a low permeability to mannitol.

Water permeability. Table 5 shows the permeability of white ghost membranes to tritiated water before and after treatment with *p*CMB. *p*CMB has been found to lower the water permeability by interaction with SH-groups in the membrane (Macey, Karan & Farmer, 1972).

The white ghost preparation was found after *p*CMB-treatment to have a water permeability of 6×10^{-4} cm sec^{-1}. This result indicates that proteinaceous channels are still present in the untreated hemoglobin-depleted ghosts and that the permeability after inhibition with mercurials can be reduced to a level which is as low as that of the "tightest" bimolecular lipid membranes (Cass & Finkelstein, 1967).

Anion exchange. The permeability of the white ghosts to ^{36}Cl was characterized in a series of experiments. Figure 6 shows the rate of self-exchange of chloride in white and pink ghosts. The rate of self-exchange of chloride from white ghosts ($k = 5.01 \times 10^{-2}$ sec^{-1}) was about twice the rate of exchange from the pink ghosts ($k = 2.54 \times 10^{-2}$ sec^{-1}). The MCV for pink ghosts was 88.9 μm^3 and for white ghosts 44.7 μm^3, and the self-exchange flux was therefore almost the same in the two sets of experiments shown in the figure, *viz.* pink ghosts: 2.64×10^{-10} mole cm^{-2} sec^{-1}; white ghosts: 2.59×10^{-10} mole cm^{-2} sec^{-1}. The intercept at the ordinate was 0.92 for pink ghosts and 0.82 for the white ghosts.

Table 6 shows data for chloride self-exchange in 11 different preparations of resealed white ghosts. The chloride permeability was 1.6×10^{-6} cm sec^{-1} at 0 °C and a chloride concentration of 165 mM corresponding to a self-exchange flux 2.6×10^{-10} mole cm^{-2} sec^{-1}. Fun-

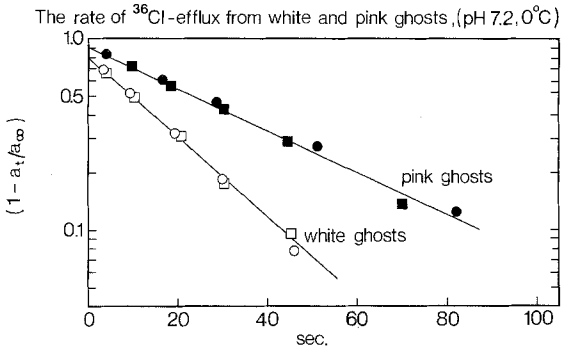

Fig. 6. The rate of ^{36}Cl self-exchange efflux from white and pink ghosts at pH 7.2 (0 °C). The ordinate is logarithmic: a_t is activity of ^{36}Cl in the medium at the time of sampling and a_∞ is the activity of ^{36}Cl after isotopic equilibrium has been attained

Table 6. The chloride self-exchange flux in hemoglobin-depleted resealed human erythrocyte ghosts (pH 7.2, 0 °C)

No.	Rate coefficient k sec^{-1}	MCV mean volume μm^3	Water content g/kg ghosts	Chloride distribution ration Cl_i/Cl_o	Volume of extracellular trapped ^{36}Cl % (1-intercept) × 100	Permeability $P_{app} \times 10^6$ cm sec^{-1}	Chloride self-exchange flux $J \times 10^{10}$ mole cm^{-2} sec^{-1}
1	0.0447	54.3	977	1.001	18.4	1.69	2.78
2	0.0382	57.4	977	1.014	19.4	1.54	2.55
3	0.0422	58.6	965	1.031	13.0	1.74	2.87
4	0.0423	53.3	969	0.997	11.3	1.54	2.55
5	0.0423	41.9	971	0.990	29.4	1.72	2.83
6	0.0417	51.0	954	1.028	24.4	1.51	2.48
7	0.0329	55.1	975	1.016	26.3	1.28	2.10
8	0.0452	44.7	972	0.987	23.9	1.38	2.27
9	0.0616	43.4	975	0.985	19.2	1.82	3.01
10	0.0520	45.0	972	0.986	28.0	1.59	2.63
11	0.0393	49.5	975	0.969	29.8	1.31	2.15
Mean	0.0438	50.4	971	1.000	22.1	1.56	2.57
SD	0.0075	5.9	7	0.020	6.3	0.18	0.30

der and Wieth (1976) found a value for pink ghosts of 3.2×10^{-10} mole cm^{-2} sec^{-1}.

It should be noted in Table 6 that the chloride distribution ratio (Cl_i/Cl_o) is one, and that the value for extracellular fluid volume obtained by the intercept of the efflux rate plot (Fig. 5) was found to be 22.1%, in agreement with the value determined using inulin.

Table 7. DIDS inhibition of chloride self-exchange in white ghosts 0 °C

No.	Inhibitor	MCV μm^3	$k \times 10^2$ sec^{-1}	$P_{app} \times 10^6$ cm sec^{-1}	$J \times 10^{10}$ mole cm^{-2} sec^{-1}	Inhibition %
1	none	34.8	6.51	1.57	2.59	0
	DIDS	34.9	0.0117	0.00284	0.00467	99.82
2	none	105.7	1.87	1.39	2.30	0
	DIDS	97.7	0.0158	0.0105	0.0174	99.24

Ghosts (10 ml, 50% suspension) were treated for 45 min at 38 °C with 0.1 ml 10^{-2} M DIDS. Hemoglobin depleted ghosts with a volume of about 100 μm^3 (expt. 2) could be prepared using hemolysis medium *A* and ghost washing medium *B* containing 40 mM sucrose. Because KCl permeates the unsealed membrane more rapidly than sucrose, the ghosts take up KCl and swell osmotically during the subsequent exposure to the sucrose-free washing medium (medium *D*). The ultimate ghost preparation contains 165 mM KCl and no sucrose.

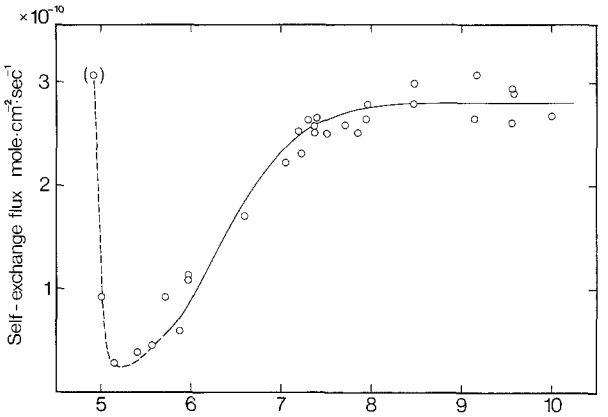

Fig. 7. The chloride self-exchange flux in resealed white ghosts as a function of pH at 0 °C. It can be seen that the chloride transport is constant at pH values over 7.2 but decreases between pH 7.2 and 5.1. The relation between permeability and pH can be described by the equation:

$$J = J_{max}/(1+(10^{-pH}/K)) \text{ mole cm}^{-2} \text{ sec}^{-1}$$

where K is the dissociation constant for a functional group in the transport system binding a single proton. The continous curve shows the theoretical relation for $K = 10^{-6.3}$ and $J_{max} = 2.8 \times 10^{10}$ mole cm^{-2} sec^{-1}. The curve for chloride transport at pH values below 5.5–5.7 is dotted since the rate was found to increase with time in this pH region

Table 7 shows that >99% inhibition of chloride self-exchange was produced by the amino-reagent DIDS, which is believed to be a specific inhibitor of anion exchange in red cells (Ship *et al.*, 1977; Lepke *et al.*, 1976).

The pH-dependence of chloride self-exchange at 0° was examined (Fig. 7). The results agree with those of Funder and Wieth (1976). It

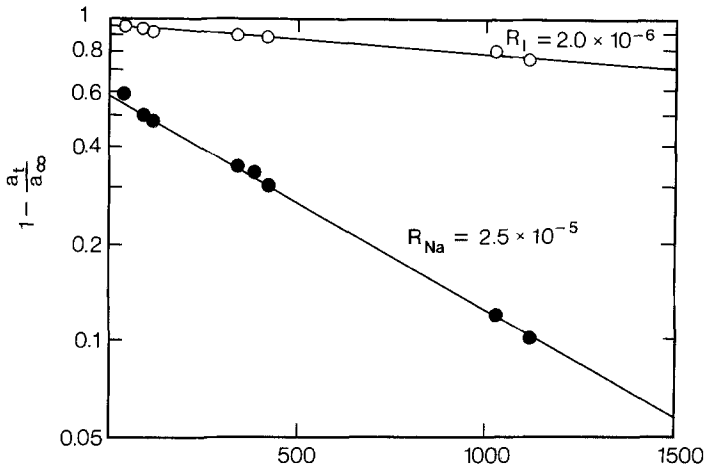

Fig. 8. The rate of ^{22}Na (●) and ^{3}H-inulin (○) release from resealed white ghosts at 38 °C and pH 7.2. The ^{3}H-inulin release was measured to correct for rupture of ghosts during the incubation. The numbers indicate the rate coefficients (sec^{-1}) calculated by the least squares technique. The cation-tight fraction of the resealed ghosts was found to be 43%. The value was calculated from the intercept of the graph, about 0.6 corrected for the amount of tracer lost (26%) from the loaded ghosts by washing twice at 0 °C shortly before the efflux experiment was performed (*see Materials and Methods*)

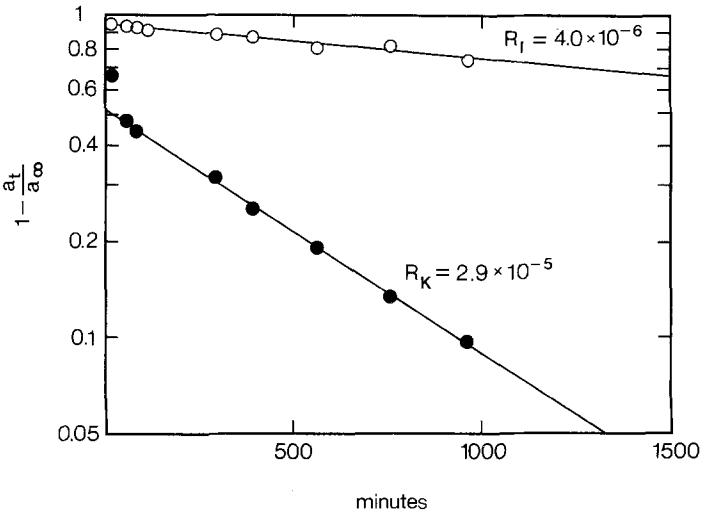

Fig. 9. The rate of ^{42}K (●) and ^{3}H-inulin (○) release from resealed white ghosts at 38 °C and pH 7.2. The ^{3}H-inulin release was measured to correct for rupture of ghosts during the incubation. The numbers indicate the rate coefficients (sec^{-1}) determined as the slope of the straight lines. The cation-tight fraction of the resealed ghosts was estimated to be 32%. The value was calculated from the intercept of the graph about 0.5 corrected for the amount of tracer lost (37%) by washing (*see Materials and Methods*)

Table 8. Passive sodium ion permeability of white ghosts

Temp. °C	MCV μm^3	$k \times 10^5$ sec^{-1}	P_{app} Na $\times 10^{10}$ cm sec^{-1}
25	39.6	1.40	3.90
	39.6	1.04	2.90
	38.5	1.17	3.17
	38.5	0.96	2.61
38	39.6	2.18	6.09
	39.6	2.55	7.11
	38.5	3.04	8.06

The rate coefficients (sec^{-1}) determined by regression analysis from at least an 18-hr period, were corrected for the rupture of ghosts caused by stirring.

Table 9. Passive potassium ion permeability of white ghosts[a]

Temp. °C	MCV μm^3	$k \times 10^5$ sec^{-1}	$P_{app} \times 10^{10}$ cm sec^{-1}
0°	46.0	1.07	3.46
	50.2	0.78	2.77
	51.8	0.74	2.70
25	46.0	1.38	4.48
	46.6	1.66	5.46
	46.0	1.29	4.17
	44.9	1.89	6.00
38	46.0	2.46	7.96
	46.6	2.75	9.02
	44.9	2.83	8.95
	51.0	2.14	7.67

The rate of ^{42}K efflux, followed over at least an 18 hr period, were corrected for the rupture of ghosts caused by stirring.

[a] The ghosts were prepared in the presence of 0.5 mM EGTA.

should be noted that there is no decrease in the flux when the pH is increased from 7.2–10. Below pH 7 the flux decreases as if it depends upon the presence of a single proton-binding group with a pK of 6.3. The flux increases very steeply with falling pH below pH 5.2. This increase in flux is time dependent, as noted by Gunn, Wieth and Tosteson (1975). The correlation of this time-dependent increase in chloride permeability with morphological changes in the membrane has been discussed in a preliminary communication (Bjerrum & Tranum-Jensen, 1977).

Passive permeability to sodium and potassium. Bodemann and Passow (1972) have found that only a fraction of pink ghosts (the type II ghosts), prepared at 0 °C, reseal to sodium and potassium under optimal conditions. This finding was confirmed for white ghosts (Figs. 8 and 9). The figures shows that both sodium and potassium exchange with a single low rate coefficient after a lapse of about 1 hr. During the initial period of about 1 hr the ghosts lose sodium and potassium rather rapidly, indicating that a fraction of the ghosts have a higher permeability to sodium and potassium than the rest of the ghosts. The cation tight fraction of the ghosts was calculated to range from 30 to 50% in the different preparations and was found to have a permeability of $6\text{–}8 \times 10^{-10}$ cm sec^{-1} for sodium and $8\text{–}9 \times 10^{-10}$ cm sec^{-1} for potassium at 38 °C (*see* Tables 8 and 9).

The ghosts used for the potassium experiments were prepared in the presence of 0.5 mM EGTA to exclude traces of calcium. When 0.4 mM calcium was present together with 0.5 mM EGTA the permeability increased to about 10^{-7} cm sec^{-1} (38 °C), indicating that the passive potassium permeability of the white ghost membrane was sensitive to calcium, reminiscent of the calcium dependent "Gardos effect" (Gardos, 1958).

Discussion

As shown by Johnson (1975), resealing is a very slow process at 0 °C. This fact can be exploited to wash the membranes free of hemoglobin before they are resealed by heating. The present study shows that it is possible to prepare a population of resealed hemoglobin-depleted erythrocyte ghosts by repetitive osmotic hemolysis followed by subsequent washout of hemoglobin in the open state. The ghosts prepared were found to have a permeability towards mannitol, water, chloride, and sodium and potassium which is quantitatively almost identical to that of erythrocytes.

Morphology

The morphology of the erythrocyte membranes has been examined previously by transmission electron microscopy on air-dried erythrocyte membrane flakes (Hillier & Hoffman, 1953; Hoogeveen *et al.*, 1970).

Little evidence was obtained in these studies for the existence of the spectrin network which has been found in Triton-x-100-extracted membranes (Steck, 1974). The existence of the spectrin network has later been confirmed by high resolution scanning electron microscopy on ghosts, which had been ruptured (Hainfield & Steck, 1977). The present method of visualizing the spectrin network in resealed ghosts, by transmission electron microscopy on membrane fragments, is easy to perform and makes it possible to carry out routine experiments which correlate changes in membrane transport function with changes in morphology, as described by Bjerrum and Tranum-Jensen (1977).

Structural Composition

The hemoglobin content of the hemoglobin-depleted ghosts was found to be reduced to 0.02–0.04% of the "normal" content, corresponding to 50,000–120,000 hemoglobin molecules per ghost. This value is as low as that for ghosts prepared by the method of Dodge *et al.* (1963).

The dry weight of the prepared white ghost membranes was found to be 1.04×10^{-12} g per ghost. This value is in agreement within the standard deviation of the ghost membrane weight with that found by Dodge *et al.*, *viz.* 1.1×10^{-12} g per ghost, and with a value of 1.07×10^{-12} g per ghost calculated from the work of Weed, Reed and Berg (1963). It is therefore unlikely that a substantial amount of the membrane proteins is lost during preparation of the ghosts.

The protein composition of the hemoglobin-depleted resealed ghosts was compared to that of the hemoglobin-depleted ghosts commonly used in biochemical studies (Dodge *et al.*, 1963). Crossed immunoelectrophoresis of the membrane proteins performed after solubilization of membranes in nonionic detergent (a very mild procedure with respect to effects on protein conformation and denaturation (Helenius & Simons, 1975), shows that the two ghost preparations have almost the same relative amount of the major protein constituents of the membrane, *viz.* the spectrins, the major integral proteins (band III protein), and the major MN-glycoprotein, glycophorin.

The only differences between the two ghost preparations were another two precipitates (numbers 5 and 18, Fig. 3) found with ghosts prepared according to Dodge *et al.* (1963). Both precipitates seem to be dissociation products from the "peptide complex" in precipitate 16 (Bjerrum & Bøg-Hansen, 1976). The apparent "integrity" of the "peptide complex" in

precipitate 16 (band III protein) of resealed ghosts, (probably stabilized by the presence of divalent cations (Mg^{++})) was further supported by studies of the functional properties of the chloride transport system, which are an even more sensitive indicator of the state of the membrane proteins.

Transport Functions

The chloride transport system of the erythrocyte is related to the function of an integral membrane protein with a mol wt of 95,000 (Cabantchik & Rothstein, 1974; Zaki *et al.*, 1975). Quantitative agreement was found between the chloride transport properties of hemoglobin-depleted ghosts and that determined previously (Funder & Wieth, 1976) for pink ghosts and erythrocytes, as regards the transport capacity, the pH-dependence of chloride self-exchange, and the response to the inhibitor DIDS. The pH-dependence of chloride transport for resealed white ghosts showed the same difference relative to that for red cells (Gunn *et al.*, 1973) as found previously for pink ghosts (Funder & Wieth, 1976). As described in great detail by Funder & Wieth (1976), this difference is possibly not a property of the transport system, but rather reflects the different effects of pH on intracellular chloride content and membrane potential in cells and ghosts, respectively.

The restoration of the membrane function in the hemoglobin-depleted resealed ghosts was further demonstrated by measuring the permeability of two polar molecules, *viz.* mannitol which has a low permeability and water which has a high permeability. About 50% of the white ghosts were found to reseal completely to mannitol. The permeability of this fraction (38 °C) was found to be $4–5 \times 10^{-10}$ cm sec^{-1}, in agreement with the permeability of intact red cells. The water permeability of white ghosts was found to be about 2×10^{-3} cm sec^{-1}. By inhibiting the water flux by the use of *p*CMB we found a residual permeability as low as that of the tightest bimolecular lipid membrane (Cass & Finkelstein, 1967). It seems therefore unlikely that the ghost preparation procedure has induced water leak channels in the membrane to any great extent.

When passive cation permeabilities were examined it was found that about half of the ghosts (30–50%) reseal completely to cations. This result is in agreement with the work of Bodemann and Passow (1972) (their type II ghosts). The sodium and potassium permeabilities of this

tight fraction were found to be $6–8 \times 10^{-10}$ cm sec^{-1} and $8–9 \times 10^{-10}$ cm sec^{-1} (38°), respectively. These permeabilities are of the same order of magnitude as those of intact erythrocytes, calculated from the results of Funder and Wieth (1967).

In conclusion, the present results indicate that it is possible to prepare almost hemoglobin-free erythrocyte ghosts which can be used for biochemical, morphological and transport studies. The preparation of white ghosts is time consuming and more laborious than preparation of the classical pink ghosts. Their field of application is, therefore, limited to situations where the complete removal of hemoglobin and other intracellular macromolecules are necessary. Hemoglobin removal is necessary in comparative studies of changes in membrane morphology and membrane permeability as reported in a preliminary communication (Bjerrum & Tranum-Jensen, 1977). Moreover, the method has turned out to be useful in experimental situation where the complete removal of intracellular enzymes (as, e.g., carbonic anhydrase and the activator protein of active calcium transport) is critical.

Hazlewood, Beall and Singer (1978) have recently pointed out that many ghost preparations contain a considerable fraction of "solid, shrunken cells." This problem is completely avoided by the present method, which emphasizes the importance of preparing the ghosts at low temperature, whereby spontaneous resealing can be avoided.

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