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PERMEABILITY OF HUMAN ERYTHROCYTE MEMBRANE VESICLES TO ALKALI CATIONS

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Summary

The permeability of inside-out and right-side-out vesicles from erythrocyte membranes to inorganic cations was determined quantitatively. Using ^{86}Rb as a K analog, we have measured the rate constant of ^{86}Rb efflux from vesicles under equilibrium exchange conditions, using a dialysis procedure. The permeability coefficients of the vesicles to Rb are only about an order of magnitude greater than that of whole erythrocytes. Furthermore, we have measured many of the specialized transport systems known to exist in erythrocytes and have shown that glucose, sulfate, ATP-dependent Ca and ATP-dependent Na transport activities are retained by the vesicle membranes. These results suggest that inside-out and right-side-out vesicles can be used effectively to study transport properties of erythrocyte membranes.

Inside-out and right-side-out vesicles of erythrocyte membranes prepared by the method of Steck and Kant [1] offer many advantages in studies of alkali cation transport. Steck and Kant showed that these vesicles are impermeable to substrates of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) and acetylcholinesterase (EC 3.1.1.7). They are also relatively impermeable to Na [2,3], K [3] and Ca [4—6].

These vesicles are very valuable for studying the sidedness of the enzymes associated with cation transport and have been used for this purpose by Fossel and Solomon [7] and Blostein and Chu [3]. In order to determine what the vesiculation process does to the vesicle transport processes, we have studied the K transport system, using ^{86}Rb as a tracer and have showed that the permeability coefficients of inside-out and right-side-out vesicles are only about an order of magnitude greater than that of whole enythrocytes. We have also shown that the vesicles retain, in large measure, the anion and glucose transport systems, as well as the ATP-dependent Na and Ca transport systems.

Materials and Methods

Preparation of ghosts and vesicles

Resealed ghosts and inside-out and right-side-out vesicles from human erythrocytes were prepared according to the methods of Schwoch and Passow [8] and Steck and Kant [1] with some modification. Blood was drawn from young adults (20 USP units heparin/ml whole blood) and preparation of vesicles was begun within 1 h. For purposes of clarity, the procedure is described in detail.

(a) Resealed ghosts. Two types of resealed ghosts were prepared: pink ghosts and white ghosts. Fresh human blood was washed 3 times in 150 mM NaCl/5 mM sodium-phosphate, pH 8.0, and then hemolyzed in 20 vols. of 5 mM sodium-phosphate, pH 8.0. All procedures were carried out at 4°C unless otherwise specified. Pink ghosts were prepared by adding a reversal solution to the suspension of hemolysed cells, followed by resealing at 37°C for 1 h. Since these resealed ghosts still retain some hemoglobin, they are red, though they are customarily known as pink ghosts. If the hemolyzed cells are washed in 5 mM sodium-phosphate, pH 8, three times and then resealed by reversal and incubation at 37°C, the resealed ghosts appear creamy white and are thus termed white ghosts. Usually, 3.0 ml of 3.3 M KCl and 1 ml 0.1 M $MgSO_4$ of reversal solution is added to 100 ml of 5 mM sodium-phosphate, pH 8, containing ghosts from 5 ml packed cells to give a final solution of 100 mM KCl, 1 mM $MgSO_4$ in 5 mM sodium phosphate, pH 8.

(b) Vesicles. Inside-out and right-side-out vesicles were prepared essentially as described by Steck and Kant [1]. Usually 40 ml of packed erythrocytes were used to prepare vesicles from fresh erythrocytes which were washed and lysed as described above. All procedures were carried out at 4°C unless otherwise specified. The ghosts were washed three times with 5 mM sodium-phosphate, pH 8, until creamy white. The ghost pellet was then suspended in 20 vols. of the sealing medium, 0.5 mM sodium-phosphate, pH 8, for 20 min. Mg (final concentration, 0.1 mM) was added to aliquots, each containing ghosts from 10 ml packed cells (10-ml aliquots), that were to be made into right-side-out vesicles. Separate 10 ml-aliquots to be made into inside-out vesicles received no Mg. After centrifugation, the supernatant was aspirated and the pellet was incubated overnight at 4°C.

After the overnight incubation, the pellets from each 10-ml aliquot were resuspended in 0.5 mM sodium-phosphate, pH 8, to a volume of approx. 7.0 ml and homogenized by passage 6 times through a 27 gauge hypodermic needle. The suspensions were then diluted in 0.5 mM sodium-phosphate, pH 8, and layered on to a Dextran T 70 shelf of 1.03 g/ml. Usually, a membrane suspension from 10 ml packed cells was diluted to 11.0 ml, layered on a 25 ml Dextran shelf and centrifuged in a Beckman SW 27 rotor at 25 000 rev./min for 3 h. Sealed inside-out vesicles and right-side-out vesicles do not penetrate the Dextran shelf and were removed by pipet. The vesicle preparations were washed in 100 vols. of 2.5 mM Tris-HCl buffer, pH 7.4.

The impermeability of each vesicle preparation to macromolecules and the vesicle sidedness were determined by acetylcholinesterase and glyceraldehyde 3-phosphate dehydrogenase activity [1] before the experiments. Usually inside-out vesicle preparations were 79.7 ± 5.6% (S.D.) inacessible to acetylcholines-

terase activity and right-side-out vesicle preparations were 94.8 ± 5.1% inaccessible to glyceraldehyde 3-phosphate dehydrogenase activity.

Spectrin-depleted vesicles were prepared by incubating the washed ghost pellet in 20 vols. 0.1 mM EDTA-NaOH at pH 8.5 for 40 min at 37°C [9]. After the incubation, the vesicles were pelleted and suspended in 0.5 mM sodium-phosphate, pH 8, and purified on a Dextran shelf as in normal vesicle preparation. Acetylcholinesterase estimates of sidedness showed that 77% of these vesicles were inside out. Further treatment with 0.1 mM Mg did not cause any reversal, yielding inside-out vesicles that were 75% inside out.

Protein determination and analysis

Membrane proteins were determined according to the method of Lowry et al. [10] after solubilization with 0.2% sodium dodecyl sulfate. The final sodium dodecyl sulfate concentration in the reaction tube was never more than 0.005%. The presence of an equivalent amount of sodium dodecyl sulfate in standard bovine serum albumin had no effect on the assay.

Protein components were analysed by gel electrophoresis according to the procedure of Fairbanks et al. [9] with minor modifications [4]. Gels were stained with Coomassie Blue and scanned on a microdensitometer (Joyce Loebl) at 515 nm.

Materials

All chemicals were reagent grade. ATP-Na_2 (presumably containing vanadate), ADP-Na_2, and ouabain were obtained from Sigma Chemical Company. Phloretin was purchased from Mann Research Laboratories. Dextran T-70 came from Pharmacia Fine Chemicals and the dialysis tubing was obtained from Arthur H. Thomas Company. ^{86}Rb, ^{22}Na, $^{35}SO_4$, ^{45}Ca, [^{14}C]glucose, and [^{14}C]-inulin were all from New England Nuclear. Scintillation fluid consisted of 2,5 diphenyloxazole (PPO, Fisher Scientific Company), and 1,4 di[2-(5-phenyloxazolyl)]-benzene (POPOP, Calbiochem) in toluene (Fisher Scientific Company) and Triton X-100 (Sigma Chemical Company) according to Patterson and Greene [11].

Vesicle volume measurements

(a) Light scattering. Volume changes in sealed vesicles were determined by a 90° light scattering apparatus built in this laboratory. Control experiments (Wiseman, personal communication) have confirmed that the scattered light is a single valued function of cell volume; the apparatus has been used successfully to measure kinetics of erythrocyte and ghost shrinking and swelling. The response is fast enough that events occurring with half times of 3—4 s can be measured.

Normal erythrocyte vesicles suspended in 10 mM Tris-HCl buffer, at pH 7.4, scatter enough light to be measured readily. The measurements are made after the vesicles reach their equilibrium volume. When the concentration of the medium was increased by 10 mM NaCl or KCl, the detector voltage increased by 0.05 V from 0.20 V to 0.25 V immediately after introduction of the salt. Further addition to raise the salt concentration by 10 mM increased the voltage by an additional 0.025 V (Fig. 1a).

The amount of light scattered was linearly proportional to the concentration of vesicles since an increase of vesicle concentration from 0.07 units of acetylcholinesterase/ml to 0.14 units/ml caused the voltage to increase from 0.05 V to 0.10 V when the light scattering signal at 0.07 units acetylcholinesterase/ml was normalized to 0.05 V.

(b) Relative vesicle volume. The relative change in volume of the vesicles in the presence of various concentrations of salts was also determined using [^{14}C]-inulin which has a molecular weight of 5000 and does not permeate the ghost membrane [12]. Non-specific binding of labelled inulin to membranes or centrifuge tubes was minimized by the presence of non-radioactive inulin which was 250 times more concentrated than carrier inulin. Tubes containing 1, 10, 25 and 50 mM KCl made up in 10 mM Tris-HCl, pH 7.4, were mixed thoroughly with a known quantity of vesicles and 10 mg/ml of [^{14}C]inulin (0.005 μCi/mg inulin). An aliquot was removed for counting and an equal aliquot of the supernatant was taken following centrifugation at 34 000 $\times g$ for 20 min.

Ghost and vesicle size determination

Samples of inside-out vesicles and right-side-out vesicles in 2.5 mM Tris-HCl, pH 7.4, buffer were examined under a phase microscope. The diameter of the vesicles ranged from about 0.2 to 2.0 μm with the majority being around 0.7 μm.

Since the magnification of a phase microscope is only about 1000×, vesicles were observed under the electron microscope at a magnification of 3000—14 000×. Vesicles diluted to 0.1 mg/ml membrane protein in 2.5 mM Tris-HCl, pH 7.4, were fixed in 0.1% glutaraldehyde and negatively stained in 2% uranyl acetate. From electron micrographs that showed a field of vesicles, an average diameter could be determined. The vesicle diameters ranged from 0.5 to 1.7 μm with a mean of 0.93 ± 0.23 μm. Since the fixed vesicles were air dried and flattened on the grid surface, it is reasonable to assume that the observed diameter seen under the electron microscope is slightly larger than the diameter of vesicles seen under the phase microscope. Therefore, we have taken the mean of the vesicle diameter to be 0.8 μm and assumed the vesicles to be perfect spheres.

The mean diameter of ghosts, examined under the phase microscope, with a micrometer, was 6.7 ± 0.8 μm. Though there was no significant difference in size between pink and white ghosts, white ghosts were somewhat smaller, with a diameter of 6.0 ± 0.9 μm. If the ghost membrane area is taken to be the same as that for a red cell ($1.35 \cdot 10^{-6}$ cm^2) [13], sealed ghosts have a volume/area ratio of $0.65 \cdot 10^{-4}$ cm, taking the average volume of ghosts as determined by a Coulter counter to be $0.88 \cdot 10^{-10}$ cm^3 [14].

Tracer efflux

To measure tracer efflux, ghosts or vesicles preloaded with a tracer are put in a dialysis bag and the amount of tracer that appears outside the dialysis bag is measured as a function of time, following the procedure of Bangham et al. [15] and Johnson and Bangham [16]. Tracers are introduced into ghosts or vesicles by sealing them, as described above, in the presence of ^{86}Rb. We have used Rb as tracer for K because its flux in the human erythrocyte is virtually

equal to that of K. ^{86}Rb has a half-life of 18.7 days and is much easier to use than ^{42}K which has a half-life of 12.4 h.

Ghosts preloaded with ^{86}Rb were prepared by adding ^{86}Rb (0.25 μCi/mmol) in the sealing medium. After incubation in the resealing medium for 1 h at 37°C as described above, the loaded ghosts were pelleted and washed in 40 vols. of the same non-radioactive medium. 2 ml resealed ghosts, resuspended to about 10% hematocrit, were put into a dialysis bag (8 × 100 mm). The bag was placed in a test tube with 20 ml of 100 mM KCl and 5 mM sodium-phosphate, pH 8. The solution was stirred vigorously by a magnetic stirrer. At various time intervals, the bag was removed, and placed serially into fresh buffer solutions from which aliquots were taken for determination of radioactivity after the dialysis bag had been removed.

^{86}Rb-containing inside-out and right-side-out vesicles were prepared by including 0.1 mM KCl (^{86}Rb) in the 0.5 mM sodium-phosphate, pH 8, vesiculation buffer, followed by the standard procedure for the preparation of inside-out and right-side-out vesicles. Since the vesicles will subsequently contain 0.1 mM KCl and 0.5 mM sodium-phosphate, pH 8, (plus 0.1 mM Mg in right-side-out vesicles), the dialysis buffer also contained the same solution. Ion efflux from inside-out and right-side-out vesicles was measured by the same dialysis procedure used for ghosts. The vesicle concentration was usually 2 acetylcholinesterase units/ml or 0.8 mg membrane protein/ml. One unit of acetylcholinesterase activity is defined as that amount of enzyme which hydrolyses 1 μmol acetylcholine per min.

Uptake assays

The solutions used to measure the uptake of various solutes were as follows:

(i) The glucose uptake reaction medium contained 0.1 mM glucose (^{14}C 10 μCi/mmol), 3.0 mM Tris-HCl, pH 7.4, 1 mM $HgCl_2$, and inside-out or right-side-out vesicles in buffer. Reaction tubes were incubated at 24°C and the reaction was stopped with a cold (4°C) wash medium containing 3.0 mM Tris-HCl, pH 7.4, and 1 mM $HgCl_2$.

(ii) The sulfate self-exchange reaction medium consisted of 1 mM Na_2SO_4 (^{35}S 2 μCi/μmol), 10 mM Tris-HCl, pH 8.0, 0.1% ethanol with or without 50 μM phloretin, with inside-out or right-side-out vesicles preincubated in non-radioactive 1 mM Na_2SO_4 and 10 mM Tris-HCl, pH 8.0. Reactions were run at 25°C and stopped with a 4°C wash medium containing 10 mM Tris-HCl, pH 8.0, and 50 μM phloretin.

(iii) The reaction mixture for ATP dependent Na influx into inside-out vesicles included 30 mM NaCl (^{22}Na 0.035 μCi/μmol), 10 mM KCl, 2 mM $MgSO_4$, 2.5 mM Tris-HCl, pH 7.4, 2 mM ADP or ATP, and inside-out vesicles sealed in buffer with or without 10^{-4} M ouabain. The reaction was run at 37°C, stopped and the vesicles were washed with a 4°C buffer containing 30 mM NaCl, 10 mM KCl, 2 mM $MgSO_4$ and 2.5 mM Tris-HCl, pH 7.4.

(iv) The Ca-stimulated K flux assay mixture included 10 mM KCl (^{86}Rb 0.1 μCi/μmol), 2.5 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$ or $CaCl_2$, and inside-out vesicles. The reaction was run at 37°C, then stopped and the vesicles were washed with a 4°C solution containing 10 mM KCl and 2.5 mM Tris-HCl, pH 7.4.

(v) ATP-dependent Ca influx into inside-out vesicles was measured in a reaction medium containing 0.2 mM $^{45}CaCl_2$ (5 μCi/μmol), 10 mM KCl, 2.5 mM Tris-HCl, pH 7.4, 1.0 mM $MgCl_2$ and 1 mM ADP or ATP at 37°C. Inside-out vesicles had been preincubated in 10 mM KCl and 2.5 mM Tris-HCl, pH 7.4, overnight. The reaction was stopped and the vesicles were washed with ice-cold 10 mM KCl in 2.5 mM Tris-HCl, pH 7.4.

The uptake of radioactivity into the vesicles was measured by Millipore filtration, using a method to be described (Sze and Solomon, to be published).

Results and Discussion

In order to see whether the vesicles were sealed to cations, their osmotic behavior was first studied by the light-scattering method in two experiments. The volume determination was made when osmotic equilibrium had been reached, within a few seconds after the establishment of the osmotic gradient. Fig. 1 shows that the relative vesicle volume decreases with increasing NaCl and KCl concentration. The theoretical curves are those for a perfect osmometer, with no intracellular protein, neglecting Donnan effects on membrane proteins.

A quantitative determination of the permeability coefficient, P_s, for K was carried out using ^{86}Rb as a tracer as already described. The time course of the efflux is characterized by two slopes, the faster one arising from diffusion across the dialysis bag (Fig. 2), and the slower one characteristic of diffusion across the ghost (Fig. 2) or vesicle membrane (Fig. 3). The results were anal-

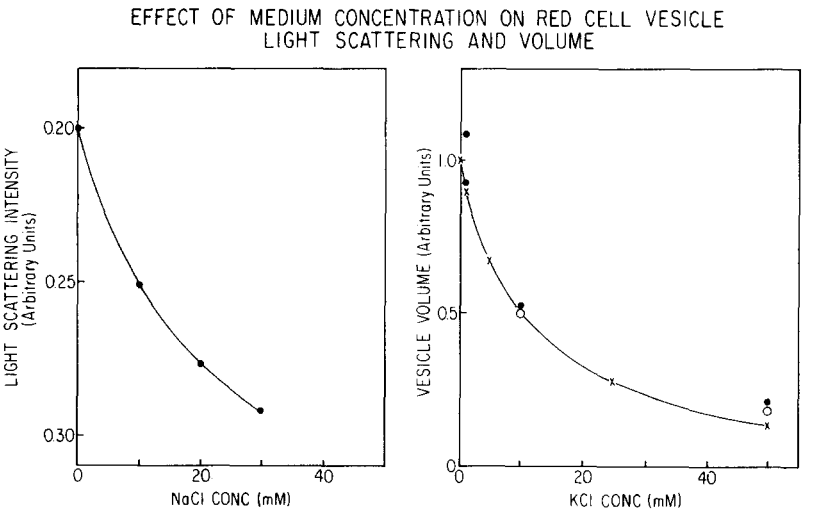

Fig. 1. Effect of medium concentration on vesicle light scattering and volume. a (left), effect of NaCl concentration on light scattering of inside-out vesicles. Vesicles were suspended in 10 mM Tris-HCl, pH 7.4, at a concentration of 0.28 units acetylcholinesterase/ml, to which aliquots of 3.3 M NaCl were added. The curve has been drawn by eye to connect the points. b (right), effect of external KCl concentration on inside-out and right-side-out vesicle volume. Vesicles were suspended in 10 mM Tris-HCl, pH 7.4, at a concentration of 7—10 units acetylcholinesterase/ml in various concentrations of KCl. •, experimental inside-out vesicle volume; ○, experimental right-side-out vesicle volume; X, theoretical volume for perfect osmotic behavior as described in the text.

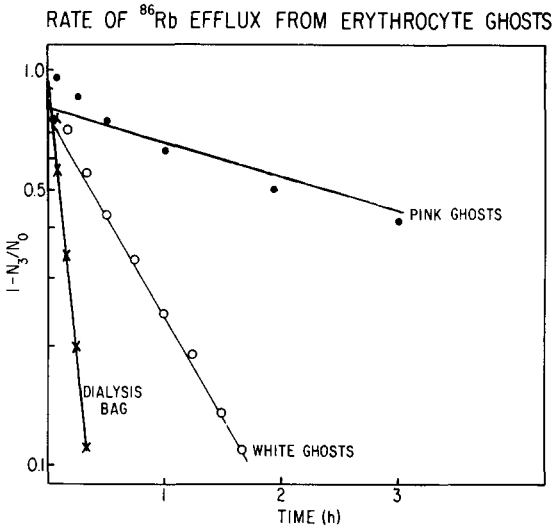

Fig. 2. The rate of ^{86}Rb efflux from ghosts contained in a dialysis bag at 24°C. Ghosts (1 unit acetylcholinesterase/ml) sealed in 5 mM sodium-phosphate, pH 8, 100 mM KCl (^{86}Rb) and 1 mM $MgSO_4$ were dialyzed against the same nonradioactive medium. $(1 - N_3/N_0)$ is a measure of radioactivity remaining in the dialysis bag, as described in the text. The straight line is the first term in the equation, whose slope gives the rate constant, k_1. Although the k_1 exponential line for the pink ghosts does not go through the points, it is the least squares fit. If the k_2 exponential term were included, the line would curve upward and go to 1.0 at zero time passing near, but not through, the early time points. The fit is no better for the other pink ghost experiment which may signify that the pink ghost population is heterogeneous, rather than homogeneous as assumed in the kinetic model. These reservations do not affect the conclusion that the pink ghosts are very much less permeable than the white ghosts, which is apparent from a qualitative examination of Fig. 2.

ysed using the kinetic expressions developed for two irreversible chemical reactions in series.

Kinetics of permeation

The flow of a radioactive tracer of component i out of a compartment of volume v_1, with surface area, A_1, into an infinite reservoir is given by

$$dN_1/dt = -k_{12}n_1^*$$

in which N_1 is the counts per minute (cpm) in compartment 1 and n_1^* is the specific activity in cpm/mol. If the system is in the steady state, both with respect to the volume of compartment 1 and the concentration of the unlabeled species in compartment 1, denoted by $[S_1]$, the equation may be integrated after substitution of $N_1/[S_1]v_1$ for n_1^*, to which it is equal, giving the equation

$$dN_1/dt = -k_1N_1$$

in which $k_1 = k_{12}/[S_1]v_1$. The permeability coefficient, P_s, may be defined as $J_1/[S_1]$ provided there is no net movement of any species except the tracer for component 1. The flux of unlabeled species per unit area is J_1 which is expressed in mol/cm^2 per min; $J_1 = (1/n_1^*A_1)(dN_1/dt)$ so that $P_s = k_1v_1/A_1$. The subscripts for v and A refer to the compartment from which the flux originates.

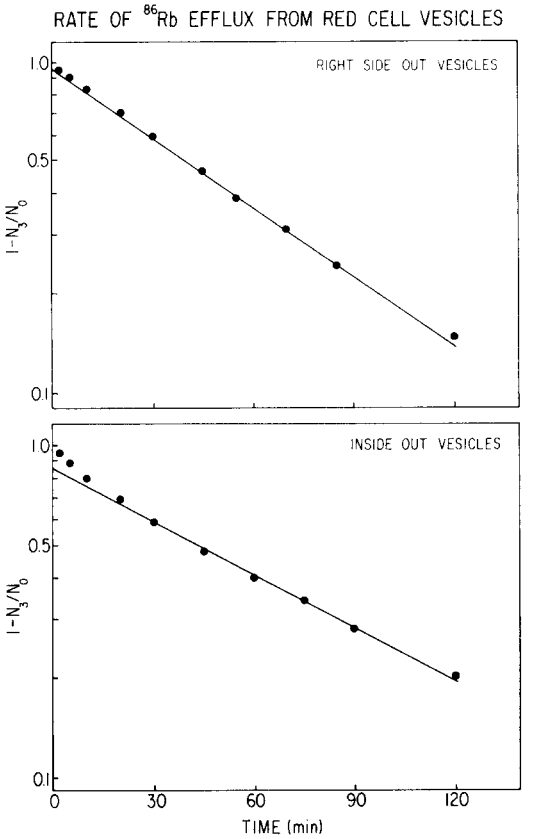

Fig. 3. The rate of ^{86}Rb efflux from vesicles contained in a dialysis bag at 24°C. Right-side-out (top) and inside-out (bottom) vesicles (2 units acetylcholinesterase/ml) sealed in 0.5 mM sodium-phosphate, pH 8, and 0.1 mM KCl (^{86}Rb) plus 0.1 mM $MgSO_4$ for right-side-out vesicles were dialysed against the same non-radioactive solution.

Using these conventions and under steady-state conditions of compartment volume and concentration of all components, the flux in the vesicle experiments can be described formally by the equations developed for two homogeneous irreversible chemical reactions in series, such that

$$N_1 \overset{k_1}{\rightarrow} N_2 \overset{k_2}{\rightarrow} N_3$$

in which $N_1 + N_2 + N_3 = N_0$ and $N_1 = a_1 N_0$; $N_2 = a_2 N_0$ (where $a_1 + a_2 = 1$); $N_3 = 0$ at $t = 0$. Compartment 1 is the vesicle compartment and compartment 2 is the dialysis bag which contains the vesicles. Compartment 3 is the series of bathing solutions to which the dialysis bag was transferred serially; this compartment can be considered an infinite reservoir, since the amount of radioactivity was never significant compared to that in the dialysis bag, and a fresh compartment 3 was used after each point was determined. The back flux from compartment 2 to compartment 1 may also be neglected as discussed in the following paragraph. The steady-state conditions required for integration of the equations are maintained by having the buffer in the dialysis bag of identical composition

to that within the vesicle, except for the presence of tracer ^{86}Rb in the vesicle contents.

Under these conditions, the solutions to the kinetic equations are:

$$N_1/N_0 = a_1 \, e^{-k_1 t}$$

$$N_2/N_0 = [k_1 a_1/(k_2 - k_1)] \, e^{-k_1 t} + [(k_2 a_2 - k_1 a_1 - k_1 a_2)/(k_2 - k_1)] \, e^{-k_2 t}$$

$$1 - (N_3/N_0) = [k_2 a_1/(k_2 - k_1)] \, e^{-k_1 t} + [(k_2 a_2 - k_1 a_1 - k_1 a_2)/(k_2 - k_1)] \, e^{-k_2 t}$$

These equations contain three unknowns: the a_1/a_2 ratio, k_1, and k_2. Since the preparation of vesicles inevitably includes some broken vesicles which are leaky to the tracer and since some radioactivity will have permeated out of the vesicles after washing, but before they are sealed in the dialysis bag and the experiment is begun, the a_1/a_2 ratio must be determined for each experiment as part of the fitting procedure. However, k_2 can be determined experimentally from control experiments with dialysis bags alone: in five experiments the average k_2 was found to be 0.122 ± 0.007 min^{-1}.

The experimental data was fitted using the method of least squares to determine the a_1/a_2 ratio and k_1 using the value of k_2 given above. Fig. 4 shows how well the fitted data agree with the experimental points for N_3 for inside-out vesicles and also shows the time course of N_1 and N_2. In this example, the value of N_2/N_0 at the end of the experiment was 0.02. When this figure is coupled with a value of about 10% for v_1/v_2, it can be seen that the back flux would only amount to about 1.2% of the forward flux at the end of the experiment, and a lesser amount earlier. Furthermore, the very good fit of the data to the theoretical curve indicates that it is reasonable to assume that the reactions may be treated as irreversible ones.

Permeability properties of vesicles and ghosts

The rate constants and permeability coefficients for ghosts and vesicles are

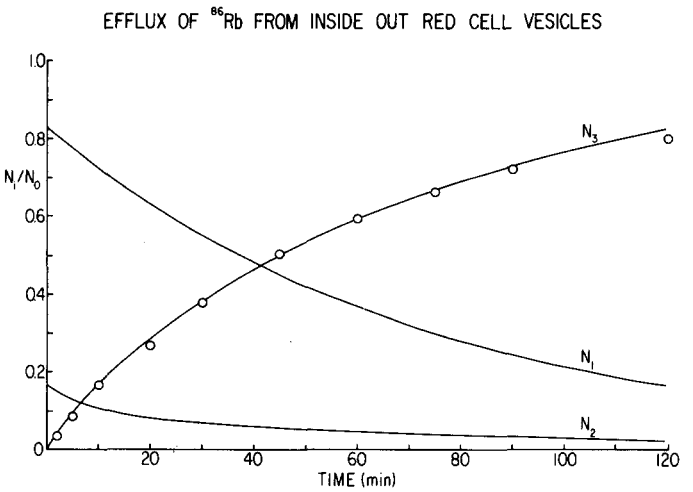

Fig. 4. Time-course of the amount of radioactive Rb contained in compartments 1, 2 and 3 during Rb efflux from inside-out vesicles. Points indicate experimentally determined values for N_3.

given in Table I. It is a well documented observation that the technique used for ghost preparation is critical for the integrity of membrane sealing and the preservation of transport functions [17]. Our determinations of permeability coefficients confirm previous observations that unwashed pink ghosts seal more tightly than washed white ghosts.

Usually, washed white ghosts cannot be sealed to small molecules, such as Na or even mannitol [12]. However, Table I shows that the K permeability coefficient of white ghosts is only one order of magnitude larger than that of pink ghosts. This discrepancy is due to the fact that we are measuring ^{86}Rb efflux only from that fraction of ghosts that retained the tracer. The population of white ghosts that retained the tracer is actually only about 10% of the population of resealed pink ghosts, as determined from the amount of tracer retained in white ghosts at the start of the efflux experiment. Thus, the permeability coefficient of white ghosts is representative of a small part of the whole ghost population, the rest being very leaky to K.

Many of the conditions needed to prepare vesicles are exactly those that have proved detrimental to the preparation of sealed ghosts, primarily the requirements for: (i) successive washing of unsealed ghosts, (ii) very low ionic strength buffers, (iii) cold temperatures, and (iv) basic pH. Under these circumstances, it might be expected that inside-out and right-side-out vesicles would not be sealed tightly to small inorganic ions. Nonetheless, we have found that inside-out and right-side-out vesicles are actually quite well sealed. Both inside-out and right-side-out vesicles are more permeable to K than intact erythrocytes by only one order of magnitude, whereas resealed white ghosts are about two orders of magnitude more permeable than intact cells. Previous reports that these vesicles were relatively impermeable to Na [2] and Ca [4] were based on qualitative studies which gave no indications as to how impermeable they were compared to sealed ghosts or intact cells. Table I shows that the vesicle preparations are well sealed with a permeability coefficient essentially the same as that of pink ghosts.

The permeability coefficient of spectrin-depleted vesicles was also determined. These depleted inside-out vesicles have lost approximately 50% of Bands 1, 2, and 5, as shown in Fig. 5 (nomenclature according to Steck [18]),

TABLE I

COMPARISON OF THE PERMEABILITY COEFFICIENTS OF ERYTHROCYTES, GHOSTS, AND VESICLES TO K UNDER EXCHANGE CONDITIONS

Errors are S.D.

Preparation	Number of experiments	Rate constant k (min^{-1})	Permeability coefficient ($cm \cdot s^{-1}$)
Dialysis bag	5	0.122	$3.2\ (\pm 0.3) \cdot 10^{-4}$
Whole cells	1	0.0031	$2.8 \cdot 10^{-10}$
Pink ghosts	2	0.0040	$4.3\ (\pm 1.2) \cdot 10^{-9}$
White ghosts	5	0.0229	$2.4\ (\pm 0.9) \cdot 10^{-8}$
Right-side-out vesicles	2	0.0129	$2.9\ (\pm 1.0) \cdot 10^{-9}$
Inside-out vesicles	5	0.0151	$3.4\ (\pm 0.9) \cdot 10^{-9}$
Spectrin-depleted vesicles	3	0.0095	$2.1\ (\pm 0.2) \cdot 10^{-9}$

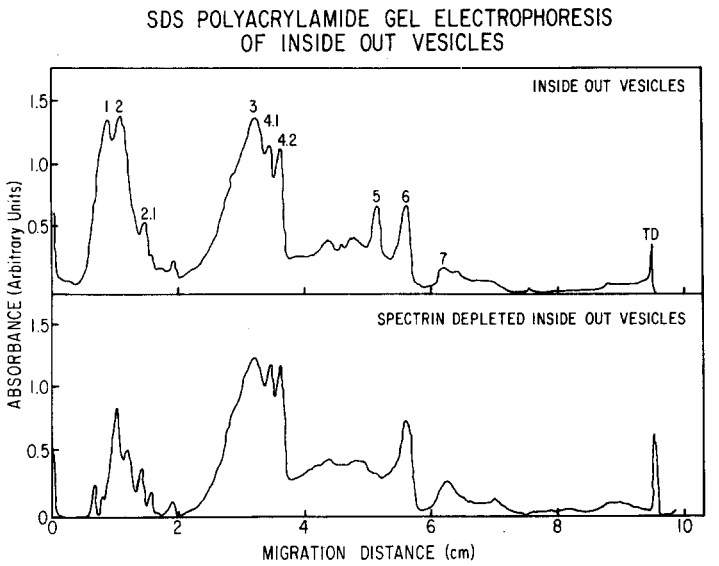

Fig. 5. SDS polyacrylamide gel electrophoresis of (top) inside-out vesicles and (bottom) spectrin-depleted inside-out vesicles. The gels contained about 60 μg protein. They were stained with Coomassie Blue and scanned at 515 nm. Bands are identified according to Steck [18]. TD, tracking dye.

yet they are also capable of resealing tightly and are characterized by a K permeability coefficient (Table I) similar to that of non-depleted vesicles. These experiments show that the extrinsic proteins, mainly Bands 1, 2 and 5, are not required to seal the vesicles to K.

Transport properties of vesicles

In order to examine the functional state of the membrane proteins in vesicles, we also studied the transport systems for sugars, inorganic anions and cations, using a Millipore filtration procedure. As Table II indicates, all these systems were functional. The vesicle membranes could transport glucose in either direction across the membrane and Hg inhibited the process. Anion transport was demonstrated by showing phloretin inhibitable sulfate self-exchange. Inside-out vesicles showed ATP-dependent Na influx inhibitable by ouabain, and ATP-dependent Ca flux. There was also a Ca-stimulated passive K flux into inside-out vesicles.

The half time for glucose permeation into vesicles can be estimated from the permeability coefficients in the literature [19] for erythrocytes, $P = 2 \cdot 10^{-5}$ cm $\cdot$ s^{-1} and, for ghosts $P = 2 \cdot 10^{-4}$ cm $\cdot$ s^{-1}. Based on an average vesicle diameter of 0.8 μm, the half-time for glucose influx into vesicles would therefore lie in the range 46—460 ms at 24°C. By the time of our earliest measurement, at 5 s, glucose distribution across inside-out or right-side-out vesicle membranes has already reached equilibrium, as would be expected. In a comparable vesicle system, Zoccoli and Lienhard [20] have studied the entry of sorbose which is sufficiently slow for them to measure. In inside-out vesicles depleted of peripheral proteins, the initial rate of sorbose uptake was 58% of that in erythrocytes.

If we assume that glucose influx into vesicles in the presence of 1 mM Hg is a

TABLE II

MEMBRANE TRANSPORT IN SEALED ERYTHROCYTE VESICLES

Substrate	Concn. (mM)	Type of vesicle	Number of experiments	Conditions	Uptake (nmol/unit ACE)	Time (min)
Glucose	0.1	IOV	3	Control	0.67	0.17
				Hg	0.03	
		ROV	2	Control	0.81	0.17
				Hg	0.03	
Sulfate	1.0	IOV	1	Control	2.66	30
				Phloretin	1.06	
		ROV	1	Control	1.92	30
				Phloretin	0.70	
Sodium	30.0	IOV	2	ADP	45.3	120
				ATP	83.5	
				ATP + ouabain	47.9	
Calcium	0.2	IOV	5	ADP	12.4	60
				ATP	67.5	
Potassium	10.0	IOV	2	Mg	3.4	1.0
				Ca	15.4	

Concentrations: $HgCl_2$, 1 mM; phloretin, 50 μM; ADP and ATP, 2 mM in the Na experiment and 1 mM in the Ca experiments; ouabain, 10^{-4} M; Mg and Ca, 1 mM in the K experiments. IOV, inside-out vesicle; ROV, right-side-out vesicle. ACE, acetylcholinesterase. The values of rates and fluxes used in the text for comparative purposes are obtained by making a linear assumption and dividing the uptake by the time in minutes.

non-specific diffusion, and that uptake is linear over the 0.17 min period observed, we can compare our results with those obtained by Jung [19] on ghosts. Our half-time for diffusion is about 100 s which corresponds to $P = 0.9 \cdot 10^{-7}$ cm $\cdot$ s^{-1} for vesicles of 0.8 μm diameter. This permeability coefficient is somewhat higher than Jung's value for D-mannitol of $0.2 \cdot 10^{-7}$ cm $\cdot$ s^{-1} in ghosts. D-Mannitol is not transported by the specific sugar carrier and the mesured value of D-mannitol equilibration across the erythrocyte membrane agrees well with the predicted value for non-specific permeation of glucose [19].

In order to measure anion exchange in vesicles we studied sulfate which enters erythrocytes by self-exchange almost five orders of magnitude more slowly [21] than chloride self-exchange at 37°C [22]. We also chose other conditions to slow the rate further including high pH, and low temperature, both of which reduce sulfate self-exchange in human erythrocytes [21] and erythrocyte ghosts [23]. The half-times of sulfate influx for inside-out and right-side-out vesicles were 15 and 20 min in two experiments. Taking an average half-time of 17.5 min and a diameter of 0.8 μm, P_{SO_4} was computed to be about $0.9 \cdot 10^{-8}$ cm $\cdot$ s^{-1}. The rate constant for sulfate exchange from red cells is about 0.004 min^{-1} at 25°C and pH 8.0 [21], which corresponds to $P_{SO_4} = 0.7 \cdot 10^{-8}$ cm $\cdot$ s^{-1}. Since P_{SO_4} is of the same order of magnitude in vesicles as in erythrocytes and since 50 μM phloretin can inhibit anion exchange by about 60%, it appears that the anion transport system in vesicles has been relatively well maintained.

That fraction of the Na uptake which is dependent upon ATP and inhibited by ouabain may be considered to be dependent upon the ($Na^+ + K^+$)-ATPase.

The rates of the ATP-dependent Na uptake were 28 and 36 nmol/unit acetylcholinesterase per h in two experiments with inside-out vesicles. Using the value of 10 units acetylcholinesterase/ml erythrocytes [4] to convert vesicle fluxes to erythrocyte fluxes, this would be equivalent to a flux of 0.28—0.36 mmol Na/l cells per h which is virtually the same as the flux of 0.44 mmol Na/l cells per h in inside-out vesicles measured by Blostein and Chu [3]. The Na flux into inside-out vesicles determined in this study is about 25% of the flux of Na observed in ghosts at 25 mM Na and 10 mM K [24]. The relative magnitude of these fluxes shows that the Na pump is functionally retained in the vesicles, which agrees with the conclusion based on the stoichiometric ratio of 1.5 Na taken up per ATP hydrolysed, as determined by Blostein and Chu [3].

The ATP-dependent Ca flux of 0.55 mmol Ca/l cells per h is also of the same order of magnitude as the flux of 0.96 mmol Ca/l cells per h in erythrocytes observed by Sarkadi et al. [25] at 0.2 mM Ca. The stoichiometry for the number of mol Ca pumped per mol ATP hydrolysed, however, is still unresolved because of the complexities concerning the determination of that portion of the Ca-stimulated ATPase activity actually involved in Ca pumping. Values of 1 and 2 Ca/ATP have been published [25—27]. If we take Quist and Roufogalis' [26] value of 0.23 μmol P_i/mg protein per h as the figure of Ca ATPase activity due to Ca flux alone, and combine this value for ATP consumption with our value for ATP-dependent Ca flux of approximately 0.14 μmol Ca/mg protein per h (Table II), the stoichiometry would be about 0.6 Ca/ATP. This is smaller than, but comparable with, the value found by others [25—27].

Our results show that it is possible to prepare a population of inside-out or right-side-out vesicles which are sealed to an inorganic monovalent cation, like K. We have also shown that many of the specialized transport systems known to exist in red cells retain most of their activity in vesicles. Thus, inside-out and right-side-out vesicle preparations seem to be useful tools for studying the transport properties of erythrocyte membranes, especially in relation to membrane asymmetry.

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Our value for sulfate flux in vesicles is comparable with that of ref. 28.

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