

Resolution of Pump and Leak Components of Sodium and Potassium Ion Transport in Human Erythrocytes

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ABSTRACT Further support for the pump-leak concept was obtained. Net transport was resolved into pump and leak components with the cardiac glycoside, ouabain. The specificity of ouabain as a pump inhibitor was demonstrated by its ineffectiveness when the pump was already inhibited by lack of one of the three pump substrates, sodium ion, potassium ion, or adenosine triphosphate. In the presence of ouabain the rates of passive transport of sodium and potassium ions changed almost in proportion to changes in their extracellular concentrations when one ion was exchanged for the other. In the presence of ouabain and at the extracellular concentrations which produced zero net transport, the ratio of potassium ions to sodium ions was 1.2-fold higher inside the cells than outside. This finding was attributed to a residual pump activity of less than 2% of capacity. The permeability to potassium ions was 10% greater than the permeability to sodium ions. A test was made of the independence of pump and leak. Conditions were chosen to change the rate through each pathway separately or in combination. When both pathways were active, net transport was the sum of the rates observed when each acted separately. A ratio of three sodium ions pumped outward per two potassium ions pumped inward was confirmed.

Active transport is unambiguously defined as net movement of a solute across a membrane up a gradient of electrochemical potential. Passive transport accordingly would be net movement down such a gradient, regardless of mechanism. Active transport requires a source of energy and implies a special mechanism, or pump (1). A physiological steady disequilibrium of cell contents may be maintained by a balance between transport through a membrane pump and an equal and opposite passive transport through another

pathway (2, 3). This pathway may be an unspecialized leak or a system specialized for excitation or secretion (4, 5). To interpret sodium and potassium transport kinetics in human erythrocytes Harris proposed a model consisting of a linked metabolic pump and two linear leaks (6). With similar models Shaw (7), Glynn (8), and Tosteson and Hoffman (9) also interpreted kinetics of radioactive Na^+ and K^+ fluxes across erythrocyte membranes. In a recent review Passow (10) concluded, "Although the 'pump-leak' concept was very helpful in recent attempts to elucidate the mechanism of active transport, one ought to be aware that it is not self-evident and still needs support from additional and independent experimental evidence." The present effort to provide this support arose from the experience of Post and Jolly (11). In estimating the stoichiometry of the pump in human erythrocytes these authors minimized leak transport with low cation gradients across the membrane. In the presence of large gradients they made corrections for leak transport by control measurements on cells whose pump was inhibited by a cardiac glycoside. They found a ratio of 3 Na^+ pumped out per 2 K^+ pumped in. McConaghey and Maizels (12) criticized these estimations as follows: (a) that the passive permeabilities of Na^+ and K^+ might be influenced by interchanging Na^+ and K^+ in the medium, (b) that equilibrium conditions might change significantly during active transport, and (c) that high potassium ion concentrations in the medium might reverse the inhibitory action of cardiac glycosides. They concluded that "evidence against a 1:1 linkage of active Na and K fluxes is inconclusive." It was desirable, therefore, to clarify the resolution of cation transport into pump and leak components.

METHODS

Human blood was collected by venipuncture in 1/10 volume of 0.1 M HNa_3EDTA and the erythrocytes were stored in a sodium ion solution at 4° to fill them with sodium ion and deplete them of potassium ion (11). The composition of the solution was usually 110 mM NaCl , 25 mM Na_2HPO_4 , 2 mM HCl , 10 mM glucose, and 3 mM adenosine. Alternatively they were incubated overnight in a potassium ion solution at 37 or 40° to deplete them of sodium ion. The composition of this solution was the same except for the replacement of Na^+ by K^+ and the omission of glucose and adenosine. Cold favors the entrance of sodium ion and warmth favors its loss. Before use the cells were passed through a coarse sintered glass filter to remove denatured protein and small clots. Aliquots were taken only of well-mixed dilute cell suspensions to avoid the inhomogeneity associated with pipetting packed cells. For analysis of cation contents the cells were first washed twice with 100 volumes of 0.13 M MgCl_2 which had been brought close to pH 7 with about 0.05 g of MgO per liter. The packed cells were hemolyzed with 100 volumes of a solution containing concentrated ammonia, 2 ml per liter, and a nonionic detergent, "cutscum," from Fisher Scientific Company (Pittsburgh, Penn.), 0.2 ml per liter. The hemolysate was analyzed

for Na^+ and K^+ by flame photometry and for hemoglobin, Hb, by spectrophotometry of cyanmethemoglobin (13). The signal from the flame photometer was averaged electronically over a 20 sec interval to minimize random fluctuations.

RESULTS

Specificity of Cardiac Glycosides for the Pump

Jardetzky (14) has emphasized the difficulty of distinguishing transport through an "active" pathway from transport through a "passive" pathway. It is easy to inhibit active transport with metabolic inhibitors but difficult to demonstrate that inhibition is specific. The specificity of an inhibitory condition can be tested, however, by its failure to act on passive transport when active transport is already blocked by some other condition, which may be specific or not. In the human erythrocyte active cation transport can be inhibited by removing one of three substrates for the pump, namely ATP, intracellular Na^+ , or extracellular K^+ , by cooling or by adding a cardiac glycoside such as ouabain or strophanthin (15). We tested the effect of each of these inhibitory conditions on passive transport taking place in the presence of another. Only addition of cardiac glycosides was specific for the pump and convenient for further study. In 1953 Schatzmann (16) showed in one experiment that strophanthin had no effect on passive movements with the pump inhibited by cold at 4° . We tested the specificity of ouabain. The pump was inhibited first by reducing the cell content of sodium ion to one-fourth of the normal level and by replacing extracellular sodium ion with choline ion. Intracellular potassium ion was allowed to leak out. The cells were supplied with glucose. The addition of ouabain to this system had no effect on passive transport of potassium ion (Table I). In a converse experiment we allowed sodium ion to leak out of potassium-poor cells into a choline ion solution. Ouabain addition had no effect on passive transport of sodium ion (Table II). Next, the pump was blocked by preliminary depletion of ATP. Tests showed that 10–24 hr without glucose at 40° depleted the cells of intracellular ATP but did not injure them seriously in other respects. In particular the rate of passive transport was 30–100% greater than that in fed control cells treated with ouabain and active transport could be restored by addition of adenosine. The increase in passive transport showed incidentally that starvation did not act specifically on the pump. Addition of ouabain to such starved cells had no effect on the rate of passive transport of sodium and potassium ions and had no effect on hemolysis, which reached 17% after 38 hr of starvation at 40° (Table III). It was desirable to repeat the test with reversed gradients. After enrichment with Na^+ by cold storage, erythrocytes hemolyzed excessively during starvation at 40° . Therefore, the experiment was done without preliminary starvation, but with the addition of 2-deoxyglucose instead. 2-Deoxyglucose depletes cells of ATP by sub-

stitution for glucose in the hexokinase reaction, but not in later steps (17). To test the effectiveness of 2-deoxyglucose in stopping the pump one aliquot of sodium-rich cells was incubated with low concentration gradients of cations across the membrane. Pump activity would appear as net transport in this case. Net transport ceased after the first 4 hr (see *pump test* in Fig. 1). Therefore the pump was assumed to be blocked in the other cells after this time. The rest of the cells were incubated in a potassium ion solution with

TABLE I
INEFFECTIVENESS OF OUABAIN ON POTASSIUM ION
TRANSPORT IN CELLS WITH A REDUCED CONTENT
OF SODIUM ION AND IN THE PRESENCE OF
METABOLISM

Fresh erythrocytes were incubated in a potassium ion solution with glucose for 10 hr at 40° to reduce the sodium content to 3.1 mmoles (5 mmoles Hb)⁻¹. They were transferred to a solution containing 141 mM choline chloride, 2.4 mM K₂HPO₄, 9.2 mM glycylglycine, 4.6 mM KOH, 0.8 mM MgSO₄, 2.8 mM glucose, 0.14 mM inosine, and 0.37 mM adenine. The pH was 7.8 and the hematocrit was 16%. The cell suspension was divided into two parts and to one part ouabain was added. After further incubation at 40° the cells were analyzed for Na⁺, K⁺, and Hb. After 16 hr the sodium content was 1.6 mmoles (5 mmoles Hb)⁻¹ in the control and 2.2 mmoles (5 mmoles Hb)⁻¹ with ouabain.

Time	K ⁺ content	
	Control	Ouabain, μ 2.7×10^{-5}
<i>hr</i>	<i>mmoles (5 mmoles Hb)⁻¹</i>	<i>mmoles (5 mmoles Hb)⁻¹</i>
0	120	120
4	113	111
8	106	106
12	101	98
16	94	94

and without ouabain. After 4 hr ouabain had no effect on the rate of loss of sodium ion and gain of potassium ion (Fig. 1).

These four experiments confirmed Schatzmann's original demonstration of glycoside specificity for the pump and made it reasonably certain that ouabain does not influence transport of sodium and potassium ion in human erythrocytes when the pump is already blocked. The occurrence of passive transport in the presence of ouabain is evidence for a separate leak pathway.

Independence of Pump and Leak

If the pump and leak are parallel and independent pathways, it might be possible to find experimental conditions under which net transport is the sum of independent pump and leak components. The experimental conditions

should block each pathway selectively or both together. Ouabain clearly blocks the pump selectively and equilibrium across the membrane necessarily stops net transport through the leak. To adjust conditions to obtain cation equilibrium it was assumed that intracellular water dissolves all intracellular salts and proteins and that the Donnan equation characterizes equilibrium. At equilibrium the concentration of an uncharged extracellular solute per liter of medium is higher than the concentration of the solute per liter of cell

TABLE II
INEFFECTIVENESS OF OUABAIN ON SODIUM ION
TRANSPORT IN THE ABSENCE OF EXTRACELLULAR
POTASSIUM ION AND IN THE PRESENCE OF
METABOLISM

Erythrocytes were kept at 4° for 54 days in a sodium ion solution. They contained 3.2 mmoles (5 mmoles Hb)⁻¹ of potassium ion. They were suspended in a solution of 160 mM choline chloride, 7 mM glycylcine, 3 mM Na₂PO₄, 0.8 mM MgSO₄, 2.2 mM glucose, 3.7 mM inosine, 0.74 mM adenine, and 1 g (liter)⁻¹ of bovine serum albumin. The hematocrit was 10%. The suspension was divided into two parts and to one part ouabain was added. The suspension without ouabain was subdivided into two parts and to one part 10 mM KCl was added to test for active transport capability. After incubation at 40° the cells were analyzed for Na⁺, K⁺, and Hb. At 16 hr the K⁺ content was 2.3 mmoles (5 mmoles Hb)⁻¹ in the absence of added KCl. At 16 hr the cells with KCl and no ouabain contained Na⁺ at 33 and K⁺ at 51 mmoles (5 mmoles Hb)⁻¹.

Time	Na ⁺ content	
	Control	Ouabain, μ 2.7×10^{-4}
<i>hr</i>	<i>mmoles (5 mmoles Hb)⁻¹</i>	<i>mmoles (5 mmoles Hb)⁻¹</i>
0	172	175
4	154	155
8	141	141
12	124	125
16	110	113

volume because of the displacement of intracellular water by hemoglobin. On the other hand the concentration of cations per liter of water is higher inside the cell than outside because of the electrical attraction of impermeable intracellular anions. To a first approximation the displacement of intracellular water by hemoglobin compensates for the electrical attraction of the impermeable anions so that monovalent cations are close to equilibrium when the concentration per volume of cells equals the concentration per volume of medium. It only remained to employ a range of cation concentrations which would produce large changes in leak transport without affecting the rate of the pump. Previous experiments (11) indicated that any external solution con-

taining at least 20 mM extracellular potassium ion should almost saturate the pump. Isotonic solutions contain about 150 mM NaCl or KCl. A range of 130 mM is thus available which can be filled with either Na⁺ or K⁺ to change the leak rate. Intracellularly the pump rate was insensitive to changes in sodium ion content provided that this was greater than 50 mmoles per 5 mmoles of hemoglobin (11, 18). (Normal erythrocytes contain about 5.2 mmoles of hemoglobin, Hb, per liter of cells.) Accordingly cells were prepared with a

TABLE III
INEFFECTIVENESS OF OUABAIN ON NET
TRANSPORT IN STARVED CELLS

Fresh erythrocytes were depleted of ATP by preliminary incubation in a potassium ion solution at 36–40° for 17 hr without substrate. (Preliminary experiments showed that active cation transport failed to respond to restoration of glucose after 10 hr at 40° without substrate.) Then they were transferred to a solution of 150 mM NaCl, 5 mM Na₂HPO (brought to pH 7.7 with HCl), 1 mM MgSO₄, 11 mM glucose, 1 g (liter)⁻¹ of bovine serum albumin, and 0.1 g (liter)⁻¹ each of penicillin, streptomycin, and chloramphenicol. The hematocrit was 2.4%. The suspension was divided into two parts and to one part ouabain was added. After further incubation at 40° the cells were analyzed for Na⁺, K⁺ and Hb.

Time	Cation content				Hemolysis
	Na ⁺		K ⁺		
	Control	Ouabain, μ 1.3×10^{-6}	Control	Ouabain, μ 1.3×10^{-6}	
<i>hr</i>	<i>mmoles (5 mmoles Hb)⁻¹</i>		<i>mmoles (5 mmoles Hb)⁻¹</i>		<i>%</i>
0	7	7	100	98	0
4	17	17	91	91	3
8	27	25	81	83	9
12	39	37	69	73	13
21	65	64	46	47	17

high sodium ion content and were incubated in isotonic solutions containing at least 20 mM K⁺. The variables were first, the exchange of 130 mM Na⁺ for K⁺ in the external solution and second, the presence or absence of ouabain. Table IV shows the best out of five experiments. Cells in the sodium ion solution with ouabain showed little net movement and the cations were therefore close to equilibrium. Removing ouabain allowed the pump, (*P*), to act. On the other hand, replacing external Na⁺ with K⁺ in the presence of ouabain allowed the leak, (*L*), to act. Removing ouabain from the last solution allowed both pump and leak to act together (*B*). The sum of (*P*) and (*L*) equaled (*B*) for both sodium and potassium ions. Net transport was thus the sum of separate pump and leak components.

The Linearity of the Leak and Estimation of Concentrations at Cation Equilibrium

In order to resolve net transport more rigorously into pump and leak components it was necessary to characterize the leak. We tested the effect of interchanging extracellular Na^+ and K^+ . These changes would not affect anion concentrations, which presumably determine the membrane potential (10), and would not change the tonicity or ionic strength. Erythrocytes were prepared containing approximately equal amounts of sodium and potassium ions. The cells were incubated with ouabain in six solutions with interchanges of sodium and potassium ions. The time of incubation was long enough to produce measurable changes in cell contents and still maintain cation gradients. In particular, the average gradient during incubation was only 18% less than the initial value. The net transport for each ion was plotted against its external concentration. The relationships were almost linear and the slopes were almost the same for both ions (Fig. 2). The approximate linearity of the leak made it convenient to characterize leak transport by two terms, which would be almost constant during an experiment but which might vary with the preparation of the cells and the composition of the medium. The first term is the permeability, Q , that is, the ratio of change in net transport rate to change in extracellular concentration in units of millimoles (5 mmoles Hb) $^{-1}(\text{hr})^{-1}(\text{mm})^{-1}$. The second term is the extracellular equilibrium concentration, E . This term is the extracellular concentration at which passive transport is zero.

In Fig. 2 the sodium and potassium ion concentrations for zero net transport are shown by the intersections, $+$, of the transport lines with the horizontal line. If ouabain abolished the ability of the cell to distinguish between sodium and potassium ions, at equilibrium the ratios of the extracellular concentration to the cell content would be the same for both ions. In Fig. 2 it can be seen that the ratio of the extracellular concentration for zero transport, $+$, to the corresponding cation content, \uparrow or \downarrow , was higher for Na^+ than for K^+ . The ratio was greater by a factor of 1.14. This slight selectivity of the intracellular phase for potassium ion might be due to selective sorption by the cell contents (19) or to residual pump activity.

The presence of selective sorption would complicate the estimation of the ratio of extracellular concentration to cation content at equilibrium. This ratio would now vary with the cation content and the kind of ion. The following evidence tends to exclude selective sorption. Solutions of hemoglobin and intracellular organic phosphate compounds have shown no selectivity for K^+ either across artificial membranes or by titration (20–24). Whole

erythrocytes also showed no selectivity after 8 weeks of cold storage (25). On the other hand, there is evidence for residual pump activity in the presence of ouabain. Residual pump ($\text{Na}^+ + \text{K}^+$)-ATPase activity in the presence of ouabain has been observed with radioactive ATP (26). In a specific test incubating guinea pig kidney ATPase with 2.5×10^{-4} M ouabain we found 1.6% of the $\text{Na}^+ + \text{K}^+$ -sensitive activity uninhibited. For these reasons we attribute the slight selective accumulation of K^+ in these cells to residual pump activity and designate the observed concentrations for zero net transport as steady-state concentrations, S .

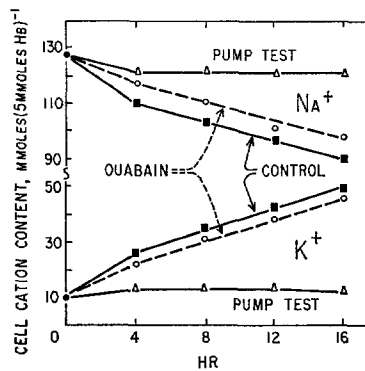


FIGURE 1. Insensitivity of passive transport to ouabain in cells depleted of ATP by 2-deoxyglucose. Erythrocytes, previously enriched in Na^+ by cold storage, were incubated in three media at 40° . The control medium contained 180 mM KCl, ■—■. To an otherwise identical medium, 2.7×10^{-5} M ouabain was added, ○- - -○. The third medium contained 175 mM NaCl, 5 mM KCl, and no ouabain to test for active transport, Δ — Δ . All media contained 5.5 mM 2-deoxyglucose, 1 g per liter of bovine serum albumin, and 10 mM glycylglycine brought to pH 7.9 with 2 mM MgO. The hematocrit was 7%.

The residual pump rate which would account for the selectivity was estimated as follows. In a steady state of zero net transport for either ion the product of the pump rate, P , in cycles per hour, and the stoichiometry, N , in number of ions pumped outward per cycle, equals the leak rate in the opposite direction. The leak rate is the product of the permeability, Q , and the difference between the extracellular steady-state concentration, S , and the extracellular equilibrium concentration, E . Solving for E :

$$E = S - (P \times N/Q).$$

In the absence of selective sorption the ratio of extracellular concentration at equilibrium to the cell content, R_e , is the same for sodium and potassium ions. Therefore the ratio of the equilibrium concentrations, $E_{\text{Na}}/E_{\text{K}}$, is the same as the ratio R_i of the sodium ion content to the potassium ion content

of the cell. R_i is determined by chemical analysis of the cells. It follows that:

$$R_i = \frac{E_{Na}}{E_K} = \frac{S_{Na} - PN_{Na}(Q_{Na})^{-1}}{S_K - PN_K(Q_K)^{-1}}$$

$N_{Na} = 3$ and $N_K = -2$ from previous estimates of the pump stoichiometry (11). Q is found graphically from the slope of the leak line. Solving for the pump rate:

TABLE IV
INDEPENDENCE OF PUMP AND LEAK

Erythrocytes were kept at 4° for 19 days in a sodium ion solution fortified with glucose and adenosine. They contained 102 mmoles of Na⁺ and 21 mmoles of K⁺ per 5 mmoles of hemoglobin. Before the transport test they were incubated at 40° for 15 min in an isotonic solution containing 0.37 mM adenosine to stimulate metabolism. Then they were incubated at 40° for 5.2 hr at a hematocrit of 2% in a sodium ion medium, "Na⁺," or a potassium ion medium, "K⁺," with or without ouabain. "Na⁺" contained 0.15 M NaCl and "K⁺" contained 0.15 M KCl. In addition both solutions contained 15 mM K₂HPO₄, 3 mM HCl, and 5.5 mM fructose. The pH during incubation was 7.3. After incubation the cells were analyzed for Na⁺, K⁺, and Hb. The letters, *P*, *L*, and *B* refer to "pump," "leak," and "both," respectively.

	Net transport			
	Na ⁺ transport		K ⁺ transport	
	Ouabain, M 2.7 × 10 ⁻⁵	Control	Ouabain, M 2.7 × 10 ⁻⁵	Control
	<i>mmoles (5 mmoles Hb)⁻¹ (5.2 hr)⁻¹</i>		<i>mmoles (5 mmoles Hb)⁻¹ (5.2 hr)⁻¹</i>	
"Na ⁺ " Medium	+1	-33 (<i>P</i>)	0	+22 (<i>P</i>)
"K ⁺ " Medium	-17 (<i>L</i>)	-51 (<i>B</i>)	+22 (<i>L</i>)	+42 (<i>B</i>)

$$P = \frac{S_{Na} - S_K R_i}{(3/Q_{Na}) + (2R_i/Q_K)}$$

With the data from Fig. 2 the rate was 0.031 pump cycles (hr)⁻¹. One pump cycle is defined as the transport of 3 Na⁺ outward and 2 K⁺ inward by the pump in units of millimoles (5 mmoles Hb)⁻¹. In eleven experiments of this sort the mean rate was 0.033 ± 0.008 SE pump cycles (hr)⁻¹. This rate is 1.3% of a normal pump capacity of about 2.5 cycles (hr)⁻¹ (18). Its magnitude was close to that of residual (Na⁺ + K⁺)-ATPase activity in the presence of ouabain and was compatible with the assumption of residual pump activity as the cause of the slight intracellular selectivity for potassium ion.

Once the residual pump activity had been calculated, as in the example above, it was then inserted in the equation for the equilibrium concentration, E , for either sodium or potassium ion. From the ratio of E to the corresponding cell content the equilibrium ratio, R_e , was determined. In Fig. 2 the

equilibrium ratio came out as 0.91. It was assumed that the equilibrium ratio did not vary with concentration when one monovalent cation was exchanged for another. Accordingly the scale of extracellular concentrations in Fig. 2 was divided by this factor to obtain the scale of corresponding cell contents

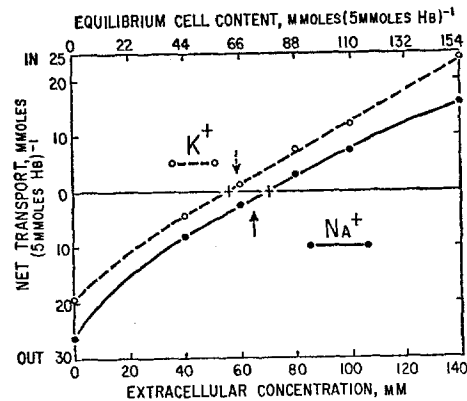


FIGURE 2. Linearity of leak of sodium and potassium ions with respect to changes in external concentration in the presence of ouabain. Cold-stored erythrocytes containing 71 mmoles of Na^+ and 65 mmoles of K^+ per 5 mmoles of Hb were incubated at 40° and pH 7.6 ± 0.2 for 16 hr in six media containing 140 mM of sodium plus potassium chloride in the following proportions respectively: 140 and 0, 100 and 40, 80 and 60, 60 and 80, 40 and 100, and 0 and 140. All media contained 2.7×10^{-5} M ouabain, 5.5 mM glucose, and 11 mM glycylglycine brought to pH 7.8 with tetraethyl ammonium. The hematocrit was 2%. After incubation the cells were analyzed for sodium, potassium, and hemoglobin, Hb. From the slopes of the straight portions of the experimental lines the permeabilities, Q , were calculated. Q_{Na} was 0.017 and Q_{K} was 0.018 mmole $(5 \text{ mmoles Hb})^{-1} (\text{hr})^{-1} (\text{mM})^{-1}$. The crosses, +, show the extracellular concentrations in a steady state with the initial cell contents. The scale at the top of the figure shows the cell cation content estimated to be in equilibrium with the corresponding extracellular concentration perpendicularly below it on the scale at the bottom of the figure. The text gives the reasoning and calculation used in determining the ratio of the scales. The vertical arrows indicate the initial cell contents of Na^+ , \uparrow , and K^+ , \downarrow , with respect to the scale of cell contents. The discrepancy between the estimated equilibrium concentrations and the steady-state concentrations was attributed to residual pump activity in the presence of ouabain as discussed in the text.

at equilibrium. It was now possible to predict the extracellular cation concentration required for equilibrium with any cell content. In effect these calculations replaced the usual estimation of an equilibrium ratio from determinations of cell water and chloride contents (9, 10). Since 20% of cell water does not act like solvent water (27), such estimations may be less accurate. In each experiment the equilibrium ratio was determined separately. In five experiments at $0.307 \text{ osmols (liter)}^{-1}$ the equilibrium ratio was $0.88 \pm 0.02 \text{ SE}$ and in five experiments at $0.405 \text{ osmols (liter)}^{-1}$ it was $1.16 \pm 0.07 \text{ SE}$.

The difference is significant, $P < 0.01$. The equilibrium ratio was proportional to the osmolarity, consistent with dilution of a simple Donnan system with water.

Before estimating leak transport in the presence of pump transport it was necessary to test the possibility that each ion might change the permeability of the other as suggested by McConaghey and Maizels (12). Erythrocytes were prepared by cold storage to contain predominantly sodium or potassium ions. They were incubated with ouabain and glucose for 16 hr at 37° in isotonic solutions of choline ions and either sodium or potassium ions in various proportions. Net movements were proportional to extracellular concentrations as in Fig. 2 and the permeabilities were similar except that loss of cations was 10–20% faster into 100% choline ion solutions than would have been predicted by extrapolation from the mixtures. (The choline solutions may have been hypotonic because of moisture in the choline chloride when it was weighed.) The loss of potassium ion into sodium and choline ion mixtures otherwise was unaffected by the proportions. The loss of sodium ion into potassium and choline ion mixtures increased 10%/100 mM decrease in extracellular potassium ion concentration. Sodium and potassium ions apparently had only minor effects on each other's permeabilities.

In the course of eleven experiments similar to that in Fig. 2 the near equality of permeabilities appeared consistently (Fig. 3). Between pH 7.2 and 7.8 the permeabilities varied twofold without a consistent relationship to the pH. The variability may be that of a normal population. With large changes the permeability varied directly with pH as Murphy (28) and Passow (10) have observed. In early experiments a contaminant in one lot of K_3PO_4 produced much larger but still equal changes in sodium and potassium ion permeability. The increase was less after addition of EDTA. Contamination of this sort may account for the single experiment in Fig. 3 with an increased permeability at normal pH. The permeability did not correlate with osmolarity in these experiments.

Estimation of Leak Transport in the Presence of Pump Transport

Since the leak was practically linear, measurement of the rate at two widely separated external concentrations would be sufficient for estimation of the permeability and the equilibrium ratio. To estimate these terms in pumping cells it is necessary that the action of the pump should not change the equilibrium ratio. Reasons for believing that this effect did not occur are given in the Discussion. Accordingly erythrocytes were allowed to pump and leak simultaneously in a solution containing predominately sodium ion and to leak in two solutions in the presence of ouabain. One solution for leaking was otherwise the same as the solution for pumping. The other solution for leaking was the same except for a substitution of most of the sodium ion by

potassium ion. The leaks were plotted against the extracellular concentrations. The permeabilities and equilibrium ratio were calculated as in Fig. 2. In the pumping cells the average content of sodium ion was lower, and of potassium ion higher, than in the leaking cells but the permeabilities were presumably the same. The product of the average content of each ion and the equilibrium ratio gave the extracellular concentration in equilibrium with these contents. These extracellular concentrations were plotted as points on the line for zero leak. Lines were drawn through these points with slopes (permeabilities)

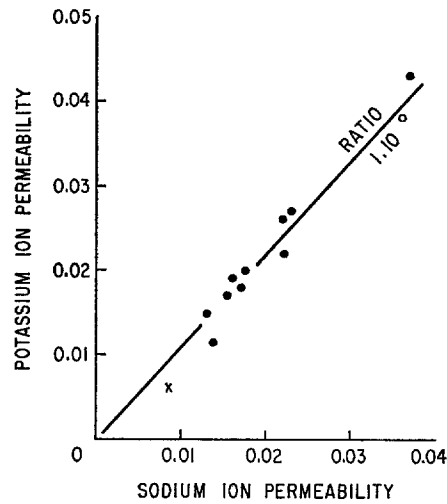


FIGURE 3. The relationship of the permeability of potassium ion to that of sodium ion. The experiments were conducted under conditions similar to those of Figs. 2 and 4 and are referred to in other respects in various figures and tables. The pH of the medium was between 7.2 and 7.8 in the case of the solid circles, ●. In the case of the cross, X, it was 6.6 and in the case of the open circle, ○, it was 8.6. The units are millimoles $(5 \text{ mmoles Hb})^{-1}(\text{hr})^{-1}(\text{mM})^{-1}$.

which were the same as those of the corresponding lines for the cells treated with ouabain. The lines were extended to the extracellular concentrations in which the cells were pumping. The vertical coordinates of the ends of the lines showed the estimated average leak of each ion in the pumping cells. Fig. 4 and Table V show the data. It can be seen that the leak of sodium ion was 1.9-fold larger in the pumping cells than in their counterparts treated with ouabain. The increase was due to a larger gradient across the membrane. But, because of the low permeability, the leak rate was only 12% of the pump rate. For potassium ions the pumping cells had an outward leak in place of the small inward leak in their counterparts. Because potassium ions were much closer to equilibrium, their leak rate was only 6% of their pump rate.

Comparison of Pump Activity and Uncorrected Net Transport under Conditions Close to Equilibrium

Earlier studies of pump kinetics (11) were conducted with cells and media adjusted so that the actual extracellular concentrations were estimated to be less than 60 mM away from concentrations in equilibrium with the cell contents; in addition, the metabolism of the cold-stored cells was stimulated with nucleosides. It was estimated that the maximal discrepancy between net transport and pump activity under these conditions was 20%. It was desirable to make a direct test of this estimate. Five experiments were conducted under these conditions and net transport was plotted against the pump activity calculated according to the procedure in Fig. 4. The data are shown in Fig. 5. It can be seen that the maximum error was -27%, but that this occurred in only two cases out of ten. It is likely, therefore, that the earlier studies were essentially correct.

The Accuracy of Leak Corrections

The experiment described in Table IV and its four less successful precursors provide data for a test of the method. On the basis of earlier studies of pump kinetics in solutions close to equilibrium (11), pump rates should have been the same in the "Na" and "K" solutions, but the leak rates should have been different. In the "Na" or "equilibrium medium" there should have been little leak. In the "K" or "disequilibrium medium" there should have been a large leak. Identity of the calculated pump rates in the two media would therefore support the accuracy of the method. Furthermore, the test is sensitive because in each experiment the pump was partially inhibited by substituting fructose for glucose, or the leak was enhanced at alkaline pH. Because of these conditions the magnitudes of the leak and the pump were similar. The equilibrium ratio and cation permeabilities were estimated from the transport data of the cells treated with ouabain according to the procedure in Figs. 2 and 4. From these data the leak rates in the pumping cells were calculated. The pump rates were determined from the difference between the net rate and the leak. Finally, for each ion the pump rate of the cells in the equilibrium medium was plotted against the pump rate in the disequilibrium medium. In Fig. 6 it can be seen that the pump rates were the same. For comparison the corresponding net transport rates are also shown in order to demonstrate the relatively large magnitude of the corrections. The correspondence of the pump rates provides evidence that this procedure for resolving net transport into pump and leak components is probably correct.

In retrospect, the experiment in Table IV was now seen to have succeeded

because the following conditions were met: (a) the cells were initially close to equilibrium with the "Na⁺" equilibrium solution and (b) the reduction of the leak in one direction as one aliquot of pumping cells came closer to equilibrium with the "K⁺" disequilibrium solution was almost equal to the in-

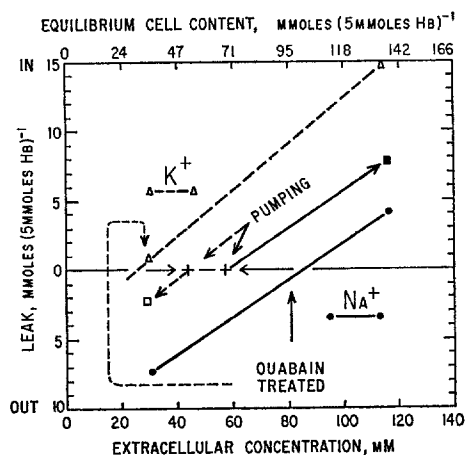


FIGURE 4. Estimation of leak transport in pumping cells. Cold-stored erythrocytes were incubated at 37° for 8.5 hr in three solutions. The pumping cells were incubated in 115 mM NaCl, 20 mM KCl, 5 mM K₂HPO₄, 10 mM imidazole, 10 mM glycylglycine, 1 mM sodium pyruvate, 10 mM glucose, and 5 mM adenosine. This was the "Na⁺" solution. The leaking cells were incubated in two solutions with 2.5×10^{-5} M ouabain. One was otherwise the same as the "Na⁺" solution. In the other solution sodium salts were replaced by potassium salts and vice versa except for the sodium pyruvate. This was the "K⁺" solution. The hematocrit was about 2%. After incubation and analysis net transports in the leaking cells, ● for Na⁺ and for Δ K⁺, were plotted against extracellular concentrations. The permeabilities, residual pump activity, and equilibrium ratio were calculated as in Fig. 2. All data and calculations are given in Table V. With reference to the scale at the top of the figure the initial cell cation contents are shown by the vertical arrows, ↑ and ↓, which extend from the legend *ouabain treated*. On the same scale the average cation contents of the pumping cells are shown by the pair of crosses, +. With reference to the scale at the bottom of the figure the crosses show the extracellular concentrations which would be in equilibrium with these contents. To estimate leak transport in these cells, lines were extended from these points with the same slopes (permeabilities) as those of the corresponding lines of the cells treated with ouabain. The lines terminate in squares, ■ and □, at the actual concentrations in the medium. The vertical coordinate of each square shows the estimated leak.

crease in the leak in the opposite direction as the other aliquot of pumping cells moved away from equilibrium with the "Na⁺" equilibrium solution. In Table IV the pump rate was estimated as 2.1 cycles (hr)⁻¹; by the procedure of Fig. 4 it was 2.4 cycles (hr)⁻¹, a difference of 14%.

A common procedure for leak corrections (9, 29) was also evaluated. The

TABLE V
CALCULATIONS TO RESOLVE PUMP AND LEAK TRANSPORT
IN THE EXPERIMENT OF FIG. 4

The extracellular concentrations are in mM. The cell cation contents are in millimoles (5 mmoles Hb)⁻¹. The permeability is in millimoles (5 mmoles Hb)⁻¹ (hr)⁻¹ (mM)⁻¹. The pump rate is in cycles of a (3 Na⁺ outward) : (2 K⁺ inward) pump expressed as millimoles (5 mmoles Hb)⁻¹ (hr)⁻¹. The capital letters refer to symbols in equations in the text. The number 8.5 refers to the hours of incubation.

	Na ⁺ data		K ⁺ data	
	"Na ⁺ " soln	"K ⁺ " soln	"Na ⁺ " soln	"K ⁺ " soln
Extracellular concentration	116	31	30	115
Initial cell content		95.9		32.8
Ratio of initial contents, R_i				2.91
Final content with ouabain	100.0	88.6	33.6	47.6
Net transport with ouabain	+4.1	-7.3	+0.8	+14.8
Permeability, Q	$\frac{4.1+7.3}{(116-31)8.5} = 0.016$		$\frac{14.8-0.8}{(115-30)8.5} = 0.019$	
Steady-state concentration, S (from Fig. 4)		85.0		25.5
Pump rate with ouabain, P	$\frac{85.0 - (25.5 \times 2.91)}{(3/0.016) + (2 \times 2.91/0.019)} = 0.022$			
Equilibrium concentrations, E		80.8		27.8
Equilibrium ratio of extracellular concentration to content, R_e		0.845		0.845
Final content without ouabain	40.0		70.1	
Net transport without ouabain	-55.9		+37.3	
Average content without ouabain	68.0		51.5	
Extracellular equilibrium concn.	57.5		43.5	
Leak transport (from Fig. 4)	+7.8		-2.2	
Pump transport	-63.7		+39.5	
Pump rate	$(63.7+39.5) \div ((3+2) \times 8.5) = 2.43$			

procedure consists of incubating one lot of cells and medium with ouabain and one lot without. The differences between the cation contents of the two lots of cells are taken as a measure of pump activity. In 30 determinations this simple procedure gave values which were from 0 to 14% less than those estimated by the more elaborate procedure of Fig. 4, with two exceptions. In the exceptions the cells were unusually permeable (see Fig. 3 for the actual magnitudes). In 13 of 15 experiments the stoichiometry of the pump was evaluated. After this simple leak correction the ratio of Na⁺ transport to K⁺

transport was 1.52 ± 0.05 SE. After complete leak correction the ratio was 1.50 ± 0.06 SE. The difference is not significant. In the two experiments which were excluded the ratio was 1.44 and 1.24. In these experiments the rate was less than 0.4 cycle $(\text{hr})^{-1}$, and was considered too low for an accurate estimation. In the other experiments the rate was between 0.7 and 2.9 cycles $(\text{hr})^{-1}$.

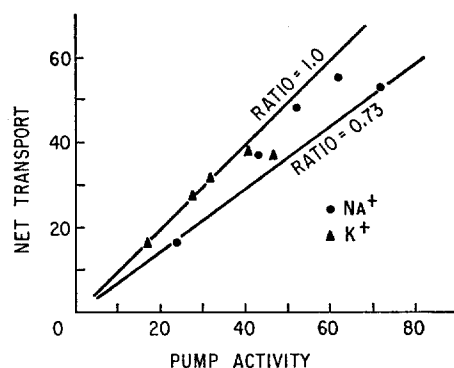


FIGURE 5. Near-equality of uncorrected net transport and pump activity under conditions close to equilibrium. The experiments were similar to that in Fig. 4. The units are millimoles $(5 \text{ mmoles Hb})^{-1}$. The difference between the extracellular cation concentration in mM and the cell content of the same ion expressed as millimoles $(5 \text{ mmoles Hb})^{-1}$ was less than 60. The ratio of net transport to pump activity for both Na^+ , ●, and K^+ , ▲, lies between 0.73 and 1.0.

DISCUSSION

These experiments have extended Schatzmann's original limited demonstration (16) that cardiac glycosides were specific inhibitors of active cation transport in human erythrocytes. In their presence transport was always passive and its rate varied proportionately to changes in extracellular concentration. In their absence subtraction of transport which would have taken place in their presence left rates of pump transport expected from earlier studies on net transport with small gradients across the membrane. The results support further the proposals of Harris (6), Shaw (7), Glynn (8), and Tosteson and Hoffman (9) that cation transport goes through two parallel pathways, an almost linear leak and a stoichiometric pump. The leak is symmetrical with respect to the sides of the membrane, distinguishes poorly between Na^+ and K^+ , and allows independent ion movements. It is glycoside insensitive. The pump, on the other hand, is oriented and stoichiometric. It transports 3 Na^+ outward, 2 K^+ inward, and hydrolyzes per cycle one terminal phosphate bond of intracellular ATP in the presence of intracellular Mg^{++} , (18, 28-32). It is inhibited by cardiac glycosides.

The resolution of net transport into pump and leak components is based on the constellation of major differences in characteristics given above. At low pump rates, the distinction between pump and leak may become unclear because of deviant behavior on the part of the pump. For example, a residue of pump activity in the presence of cardiac glycosides was invoked in the ex-

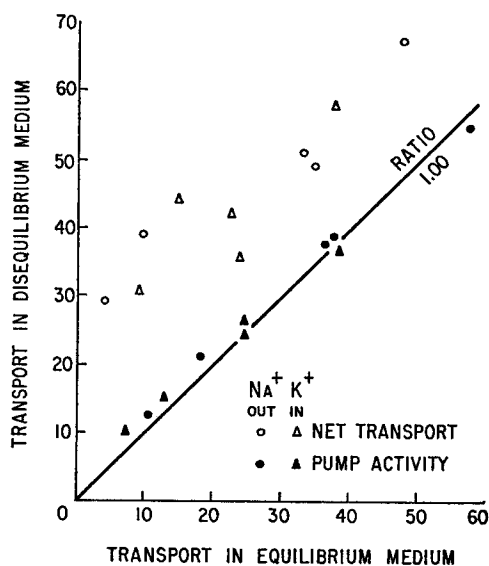


FIGURE 6. Comparison of uncorrected net transport and estimated pump activity in a medium in approximate cation equilibrium with the cells and in a medium out of equilibrium. The cell sodium ion content and medium potassium ion concentration were above levels required to saturate the pump in an equilibrium medium. The ratio of the corrected pump activity in the disequilibrium medium to that in the equilibrium medium was 1.00. Net transport, in contrast, was higher in the disequilibrium, "K⁺", medium than the equilibrium, "Na⁺", medium. The procedure was that of Table IV with the following changes. Cold storage lasted from 16 to 70 days. Incubation was from 5 to 12 hr at 30–40°. The pH was between 7.2 and 7.8. Fructose was twice replaced by glucose, and adenosine or inosine with adenine. The preincubation at 40° to stimulate metabolism was omitted twice. The permeability was between 0.008 and 0.027 mmole (5 mmole Hb)⁻¹(hr)⁻¹(mm)⁻¹. Pump activity was estimated by correcting net transport for leak transport by the procedure of Fig. 4 and Table V. The pump rate was between 0.3 and 2.6 cycles (hr)⁻¹. Pump cycles are defined in the legend to Table V.

periments of Figs. 2 and 4. As another example, reversal of the normal direction of the pump might occur in the presence of cation gradients requiring free energy beyond that in the terminal phosphate bond of ATP (33). Again, uncoupling of translocation from phosphorylation, i.e. free wheeling, might occur in the absence of intracellular ATP. The lack of effect of glycosides in starved cells (Table III and Fig. 1) makes this possibility unlikely. Finally, another situation may allow uncoupling of net transport from ATP splitting.

In human erythrocytes deprived of Na^+ a small K^+ exchange flux required inorganic phosphate and was inhibited by ouabain (18). The K^+ carrier may translocate reversibly. In this case the shuttling carrier should exchange passively all ions which ride on this form, namely Li^+ , NH_4^+ , K^+ , Rb^+ , and Cs^+ (2). The low rate will make difficult a demonstration of such an effect.

The leak pathway, as a linear system, would be expected to show the Ussing relationship (34) for the ratio of tracer fluxes through it, at least approximately. Glynn (8), however, estimated that the linear component of K^+ influx in human erythrocytes was less than one-half as large as would be expected by comparison with the efflux. It is likely that the leak is more complicated than a simple diffusion pathway.

These experiments have not answered two criticisms of McConaghey and Maizels (12). The first concerns the influence of the pump on the equilibrium across the membrane. The experiment in Fig. 4 and Table V showed the greatest change in cation contents during pumping. Here there was a decline of 15% in the content of sodium plus potassium ion. The effect of this change in cation content on the equilibrium can be estimated approximately with a model as follows. Imagine a classical red cell (10) with a flexible membrane permeable to water and monovalent anions but impermeable (for periods of minutes at least) to polyvalent anions, cations, or hemoglobin. Let B be the monovalent cation content, A be the monovalent anion content, X be the content of other osmotically active material, and let W be the content of solvent water. For convenience let O be the total amount of osmotically active material within the cell: $O = B + A + X$. Let Y be the total charge of the impermeant anions inside the cell. As for the outside, let $[A]_o$ be the concentration of permeant monovalent anions and $[C]_o$ be the concentration of osmotically active material. The extracellular concentration of monovalent cations which would be at equilibrium with the intracellular cation is $[B]_o$, and is defined by the Donnan equation as follows: $[B]_o [A]_o = BA/W^2$. The equilibrium ratio, R_e , is equal to $[B]_o/B$. Osmotic equilibrium requires that $O/W = C_o$. Intracellular electroneutrality requires that $B = A + Y$. Solving the simultaneous equations and differentiating R_e with respect to B yields the following result:

$$\frac{dR_e/R_e}{dB/B} = \frac{B}{A} - \frac{4B}{O}$$

The term on the left is the ratio of the fractional change in R_e relative to a fractional change in the impermeant cation content of the cells. The first term on the right is positive and indicates that a decrease in cation content decreases the equilibrium ratio by reducing the amount of positive charge in the cell. The second term on the right is negative and indicates that a decrease in cation content increases the equilibrium ratio by decreasing the volume of

the cell. In human erythrocytes B/A is about 2 and B/O is about 0.6. The fractional sensitivity of R_e to B would therefore be 2–2.4 or -0.4 . Assume that the ratio of small differences equals the ratio of the differentials. A 15% decrease in the impermeant cation content of erythrocytes (Table V) accordingly would induce a 6% increase in the equilibrium ratio. Recalculation of the data in Table V on this basis changed the sodium ion leak from +7.8 to +7.4 mmoles (5 mmoles Hb)⁻¹. These changes were considered insignificant and it was concluded that the change in equilibrium induced by the pump was not likely to have been important in these experiments.

With respect to the criticism that high concentrations of potassium ion may reverse inhibition by cardiac glycosides (12) it may be pointed out that this competitive effect has been observed only at low glycoside concentrations (15, 35). At the high glycoside concentrations used here 2 m K⁺ might be required for a competitive effect. This level is more than 10-fold greater than that used. Furthermore, in Fig. 2 a loss of glycoside inhibition at high K⁺ concentrations would have increased inward transport of K⁺ and would have led to a curve of transport vs. concentration which was concave upward, not downward as was actually observed. It is not likely therefore, that glycoside inhibition was significantly affected by extracellular K⁺.

The authors are indebted to Joel Feigenbaum for determining the time required starve out fresh human erythrocytes at various temperatures.

This work was supported by a grant 5 RO1 HE-01974 from the National Heart Institute, United States Public Health Service.

Received for publication 18 August 1966.

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